Investigating the role of D-alanine and D-serine in the gut and brain, an *in vivo* and *in vitro* study**.**



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ABSTRACT

D-alanine co-localization to corticotroph cells of the anterior pituitary inspired the research in this thesis, as this co-localization may imply a relationship. Investigations into effects that D-alanine have on the anterior pituitary, via AtT20 cells found that D-alanine does in fact have an inhibitory relationship on ACTH secretion in AtT20 cells, and this was robustly proven. Additionally, D-serine was investigated for comparison, and this D-amino acid had similar effects. To ascertain inhibitory hormone effects by D-amino acids, an *in vivo* study using CD1 mice found that animals treated with D-alanine or D-serine had lower levels of corticosterone in their plasma. This supported the finding that these D-amino acids have an inhibitory effect on HPA axis hormones.

In order to manipulate this regulation for potential therapeutic benefits the mechanism behind this inhibition was sought, and I found that this may be via a combination of Asc-1 and ASCT2 amino acid transporters and the SGK-1-POMC system. NMDARs and GlyRs were explored but the data did not support the direct involvement of these receptors in inhibition of HPA axis hormones: ACTH and corticosterone. Asc-1 involvement was determined by transfecting AtT20 cells with Asc-1 and observing a more pronounced inhibition in cells overexpressed with this amino acid transporter and treated with D-alanine.

To support the *in vitro* data, 2 *in vivo* studies were performed – a model that increased D-alanine by feeding it to CD1 mice and a germ free model, which would lack D-alanine due to the lack of gut microbiota, the source of mammalian D-alanine. However the ‘D-alanine knockout model’ of germ-free mice did not have opposing effects on SGK1 and POMC in the cortex – though this may be due to presence of D-serine in these animals, as D-serine and D-alanine was found to exert similar effects on HPA axis hormones and SGK1.

Determining that D-amino acids may have a therapeutic benefit of HPA axis regulation, the effects these molecules exert on the site of entry, the gut, was investigated in colorectal Human carcinoma cells Caco-2, and it was found that D-serine had pro-inflammatory effects on these gut cells, but not D-alanine, however the mechanism behind this inflammation was not determined. However, this was an interesting find as it supports the use of D-alanine over D-serine as a potential stress axis regulator, as this D-amino acid does not promote gut inflammation.

The robust inhibition of ACTH in the anterior pituitary by D-alanine, and D-serine (Chapter 2), is not directly linked to NMDA receptor or GlyRs activity, but may involve the SGK1 system, which interacts with POMC, precursor to ACTH, and the amino acid transporter Asc-1 (Chapter 3). However, ingestion of D-serine supplements may have a pro-inflammatory effect in the gut, as observed in Caco-2 colorectal cells in Chapter 4. The inhibition of HPA axis hormones (Corticosterone) by D-alanine and D-serine was also observed in CD1 mice (Chapter 5).

Keywords: D-alanine, D-serine, AtT20 cells, Caco-2 cells, Asc-1

DECLARATION

This thesis is the product of my own work and includes nothing that is an outcome of collaborative research except when specified in the text.

I state that no substantial part of my dissertation has already been submitted, or is being concurrently submitted for any such degree, diploma or other qualification at the University of Oxford or any other University or similar institution.

In accordance with guidelines, this thesis does not exceed 50,000 words and it contains less than 150 figures.

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DPhil Candidate in Psychiatry

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ABBREVIATIONS

1. Asc-1 - Alanine Serine Cysteine – 1 (Asc-1)
2. ASCT2 - Alanine Serine Cysteine Transporter 2
3. NMDARs - N-methyl-D-aspartate receptors
4. GlyRs - glycine receptors
5. HPA - hypothalamic pituitary adrenal
6. CRH - corticotropin releasing hormone
7. ACTH - adrenocorticotropic hormone
8. CORT - cortisol/corticosterone
9. PVN - paraventricular nucleus
10. CRHR1 - CRH receptor 1
11. CRHR2 - CRH receptor 2
12. CRHBP - CRH binding protein
13. cAMP - cyclic adenosine monophosphate
14. PKA - protein kinase A
15. CREB - cAMP response element binding
16. PC1 - proprotein convertase 1
17. MC2R - melanocortin 2 receptor
18. GR - glucocorticoid receptors
19. POMC – proopiomelanocortin
20. α-MSH - alpha-melanocyte stimulating hormone
21. GCs – glucocorticoids
22. AVP - arginine vasopressin
23. IAD - isolated adrenocorticotrophic hormone deficiency

24. EGFR - epidermal growth factor receptor

25. NFκβ - Nuclear Factor Kappa Beta

26. HSL - hormone-sensitive lipase

27. StAR - steroidogenic acute regulatory protein

28. MRAP - melanocortin receptor accessory protein

29. MR - mineralocorticoid receptors

30. CBG - corticosteroid binding globulin

31. GREs - glucocorticoid response elements

32. NGFI-B - Nerve growth factor IB

33. LIF - leukaemia inhibitory factor

34. DOPA – dihydroxyphenylalanines

35. GABA - Gamma aminobutyric acid

36. SGK1 - Serum glucocorticoid kinase 1

37. PI3K - phosphoinositide 3 kinase

38. PTSD - posttraumatic stress disorder

39. IBS - irritable bowel syndrome

40. BDNF - brain derived neurotrophic factor

41. DAO - D-amino acid Oxidase

42. HAT - heterodimer amino acid transporter

43. HP1 – hairpin 1

44. HP2 – hairpin 2

45. PKB – Protein Kinase B

46. ALS - Amyotrophic Lateral Sclerosis

47. GFAP - Glial fibrillary acid protein

48. mGlu - metabotropic glutamate receptors

49. GMS - glycine modulatory site

50. ENS - enteric nervous system

51. NO - nitric oxide

52. CCI - cortical impact model

53. LTA – lipoteichoic acid

54. TNF-α - Tumour Necrosis Factor alpha

55. IFN-γ - Interferon gamma

56. IL-10 - interleukin 10

57. IL-1β - interleukin 1β

58. TJ – tight junction

59. GLP-2 - Glucagon-like-peptide 2

60. TGF-β - transforming growth factor β

61. IBD - Irritable Bowel Disorder

62. GLRA1 - Glycine Receptor Alpha 1 subunit

63. CNS - central nervous system

64. ELISA - enzyme linked immunosorbent assay

65. DMEM - Dulbecco's Modified Eagle's Media

66. FBS - Fetal Bovine Serum

67. PBS - phosphate buffer saline

68. PVDF - polyvinylidene difluoride

69. ECL - enhanced chemiluminescence

70. NF water - nuclease free water

71. RT - reverse transcriptase

72. Ct - cycle threshold

73. VTA - ventral tegmental area

74. CSF - cerebrospinal fluid

75. PTTG1 - pituitary tumour transforming gene 1

76. GFP - green fluorescent protein

77. GlyT1- glycine transporter 1

78. GlyT2 - glycine transporter 2

79. GluN1 - N-methyl D-aspartate receptor type 1

80. TJ - Tight junctions

81. Aβ - Amyloid Beta

82. LPS - lipopolysaccharide

83. BDNF - brain derived neurotrophic factor

84. GLP-1 - glucagon like peptide 1

85. GLP1R - glucagon like peptide -1 receptor

86. GI - gastro-intestinal

87. LB - lysogeny broth

88. TNBS - trinitrobenzene sulfonic acid

89. GF - germ-free

90. SPF - specific pathogen free

91. JAK2 - Janus kinase 2

92. STAT - signal transducer and activator of transcription

This Thesis is dedicated to

No. 47 Trisula Avenue, Arena Park, Chatsworth –

To all those who have thought of this place as home,

And there were many –

Ubuntu,

I am because you are.

CHAPTER ONE: INTRODUCTION

In this chapter I will cover the mechanistic principles that support this thesis – consisting of a thorough literature review of concepts addressed later on, so it is understood why I am investigating these ideas, where the respective field of research is currently, and subsequently how this thesis could contribute to these fields. The core question of this thesis is how D-alanine affects ACTH signalling in the anterior pituitary gland. I explain the relevance of this question by firstly addressing the importance of ACTH regulation in terms of the stress axis of the body, followed by the relevance of this axis to gut microbiota – which mammalian D-alanine is a product of. Subsequently, the function of D-amino acids is explored, and their roles in pathological states in the body, with special emphasis to stress-related psychiatric disorders. I then address how D-alanine derived from the gut can exert effects on the brain by delving into its transporters – Alanine Serine Cysteine –1 (Asc-1) and Alanine Serine Cysteine Transporter 2 (ASCT2). Next, I address what could be responsible for D-alanine derived effects observed by better understanding the receptors that D-alanine binds to, as well as using other stronger agonists as comparison (D-serine for N-methyl-D-aspartate receptors (NMDARs) and taurine for Glycine receptors (GlyRs)). Lastly, I elucidate the relevance of the gut in this project, by reviewing the literature on the intestinal inflammatory effects of gut microbiota, which will provide a supporting layer that this thesis will build on with regard to the colonic inflammatory effects of D-amino acids, as well as looking at amino acid transporter presence in the gut, to better understand how these D-amino acids exert their effects throughout the body.

1.1. The hypothalamic pituitary adrenal (HPA) axis

The HPA axis' primary role is to respond to stress by modulating GC hormones (cortisol in humans, corticosterone in rodents) in blood circulation. It is the major neuroendocrine axis that regulates homeostasis in mammals, and it executes this job predominantly by GC effect management. GCs are responsible for the regulation of metabolic processes, the immune system, reproduction, behaviour and cognitive functions (Gjerstad et al., 2018, Macfarlane et al., 2008, de Kloet, 2000). The axis features three key hormones – corticotropin releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and cortisol/corticosterone (CORT), which interact with each other to influence downstream processes based on the body's need (whether in basal, or stressful circumstances), and the levels of these hormones are controlled by a negative feedback mechanism, which I will delve into deeper later on in this chapter.

1.1.1. CRH: Management of levels by negative feedback and effects through receptor binding

CRH is a 41 amino acid peptide that is synthesized in the paraventricular nucleus (PVN) of the hypothalamus and stored in neurosecretory vesicles of axon terminals in the external zone of the median eminence. Afferent neural pathway activation(such as signals from the amygdala responsible for fear processing) causes a sudden calcium influx in CRH neurons that influence the merger of CRH-containing vesicles to the cell membrane, and the subsequent release of CRH into the pituitary portal vasculature (Antoni, 1986).

CRH binds to either CRH receptor 1 (CRHR1) or CRH receptor 2 (CRHR2) in the pituitary, which stimulates the release of ACTH, which subsequently sets off the stress cycle by stimulating the release of GCs (Smith and Vale, 2006). CRHR1 has higher expression in the

brain and pituitary, whilst CRHR2 has higher levels in the periphery (Potter et al., 1994). Within the same protein family of CRH are urocortins, peptides which bind to *either* CRH receptors with *equal* affinity, whereas CRH is partial to CRHR1, and therefore urocortin may be the preferred agonist for CRHR2 (Dedic et al., 2018). The availability of CRH to act at these receptors is dependent on CRH binding protein (CRHBP), which is found in the hippocampus, amygdala, lateral septum and liver, and in general 65 – 90% of CRH in the body is bound to CRHBP, and when CRH synthesis increases due to stress, there is a homeostatic increase of CRHBP (Dunlop and Wong, 2019). This binding protein facilitates the sequestration of CRH, which assists in negative feedback mechanisms on CRHR1. CRH binding to the relevant receptor in the pituitary stimulates the production of adenylate cyclase, which then sets off the cyclic adenosine monophosphate (cAMP) pathway, eventually leading to the release of ACTH from the pituitary. Therefore, CRH is an established potent stimulator of ACTH (Perrin and Vale, 1999).

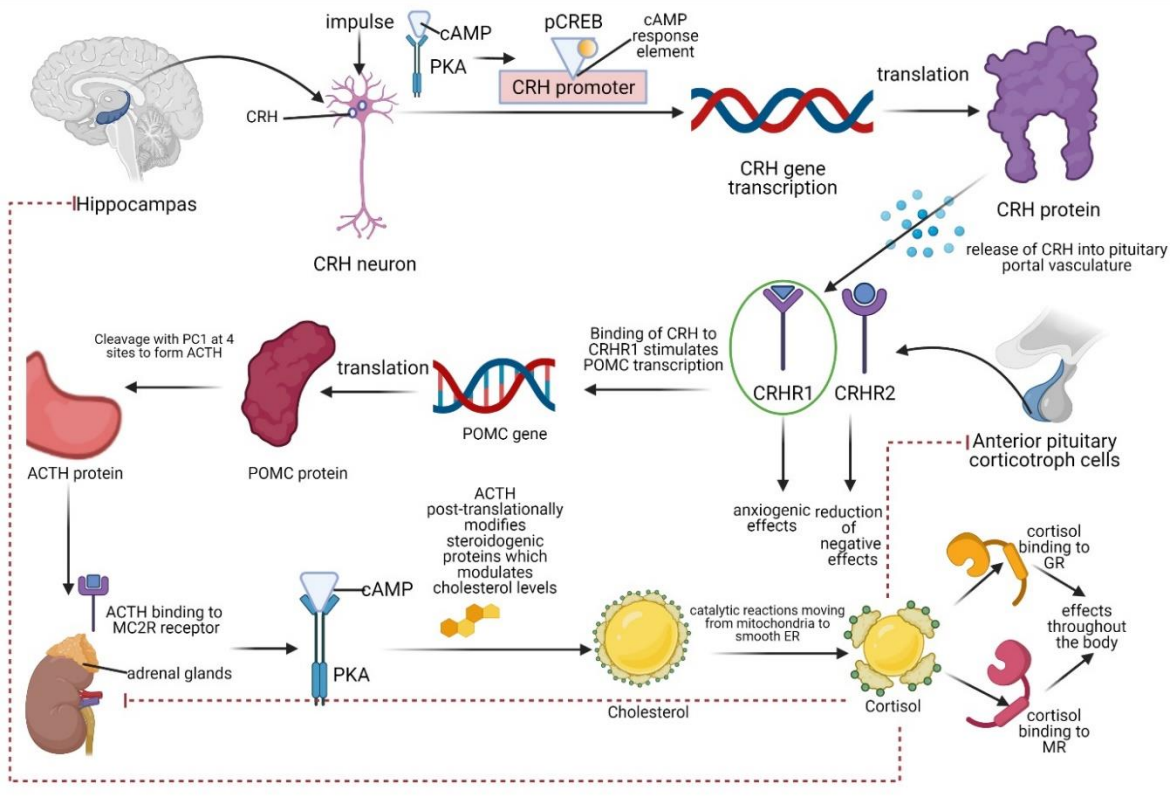


Figure 25.1 - The HPA axis with the negative feedback loop in red dotted line. Green circle denotes preferred receptor. PKA - protein kinase A, cAMP - cyclic AMP, CREB - cAMP response element binding, PC1 - proprotein convertase 1, MC2R - melanocortin 2 receptor. Diagram drawn using Biorender.com, information from Dunlop and Wong, 2019.

De novo synthesis of CRH is explained in the diagram above (**Figure 1.1**). Consistent production of CRH is important as excess CRH is stored away, and made available when CRH levels become depleted, so that the HPA axis can respond with rapidity when necessary (Watts, 2005). This production and secretion are negatively regulated by CORT, where an increase in CRH expression in the PVN (both in basal and stressful conditions) is shown in rodents that have had their adrenals removed (Lightman and Young, 1988). In mice with disrupted glucocorticoid receptors (GR) in the PVN, there is a genetic overexpression of CRH, as well as increases in ACTH and CORT (Laryea et al., 2013). This negative feedback by CORT is achieved by the presence of GR in the PVN, so that feedback can be exerted directly, as well as by inhibition of neuronal pathways afferent to the PVN (Gjerstad et al., 2018).

1.1.2. ACTH: regulation in the body

Following the central Dogma in endocrinology of hormone derivation from a larger precursor, ACTH is a product of the larger precursor - proopiomelanocortin (POMC) (Stevens and White, 2009). POMC is produced in various tissues (brain, lymphocytes and placenta) but production in the anterior pituitary gland has been the most thoroughly investigated, as this is where ACTH is also produced as a member of the HPA axis (Bicknell, 2008).

However, a study by Gibson et al (1994) showed that POMC processing in the pituitary is not wholly efficient, and significant amounts of viable precursor is released into circulation,

where it could potentially travel to different organs and serve as precursor for hormone production. This hormone production is tissue specific, as POMC in the anterior lobe of the pituitary predominantly produces ACTH (by cleaving four out of the eight cleavage sites which all contain lysine-arginine residues), whilst in the intermediate lobe of the pituitary further cleavage occurs (at all eight cleavage sites of POMC) to produce alpha-melanocyte stimulating hormone (α-MSH) and β–endorphins (Bicknell, 2008; Raffin-Sanson et al., 2003), which is important to note as POMC levels as a marker for its products are dependent on where production occurs. Aside from location, Pax7 is a transcriptional factor which determines whether POMC will be cleaved to form ACTH or β-lipoprotein (Drouin, 2016). Understanding the importance of POMC in relation to ACTH production begs the question of what factors affect the production of POMC, as this will subsequently affect ACTH formation.

There is basal production of POMC independent of stimulation (Lugo and Pintar, 1996), but there are elements in the POMC gene that respond to regulation by CRH, glucocorticoids (GCs) and arginine vasopressin (AVP – anterior diuretic hormone produced in the anterior pituitary gland) (Stevens and White, 2009). There are strong links between POMC and the HPA axis, as it is a central regulator of this system, which is emphasized via Tpit. Tpit is a cell-specific transcription factor for the expression of POMC. It is expressed solely in the pituitary of POMC-expressing lineages; the corticotropes and the melanotrophs (Pulichino et al., 2003). The inactivation of Tpit in humans results in isolated adrenocorticotrophic hormone deficiency (IAD), a lethal condition that has presented in a small number of children (Lamolet et al., 2004). Additionally, a mutual pathway of signalling factors have a role to play in how POMC and ACTH interact. Stimulation of epidermal growth factor receptor (EGFR) likely increases the expression of the POMC gene, which may contribute to upregulation of ACTH secretion (Reincke et al., 2015). Nuclear Factor Kappa Beta

(NFκβ) is a transcriptional factor responsible for transcription of DNA, production of cytokines, cell survival, and induction of POMC transcription (Takayasu et al., 2010).

The secretion of ACTH from the pituitary is pulsatile (high frequency and low amplitude pulses) that depend on calcium, but not on hypothalamus stimulation – implying an intrinsic pulse generating mechanism in the pituitary, and is subject to the circadian rhythm (Gambacciani et al., 1987). Short term stimulation by CRH (3 minutes) *in vivo* affects ACTH secretion, but not POMC, suggesting that these two proteins are stored in different secretory granules (Stevens and White, 2009). Stimulation by CRH *in vitro* (AtT20 cells for 2 hours) resulted in an increase in both intracellular and secreted ACTH levels, but no change in POMC secretion. Longer term stimulation by CRH (72 hours) *in vitro* showed a 1.2-fold increase in POMC and 1.3-fold increase in ACTH present in the cell medium, but no changes were detected intracellularly. This implies that CRH stimulation can result in ACTH release from cells in a non-genomic manner, but POMC is secreted constitutively and can only be regulated genomically (Stevens and White, 2009).

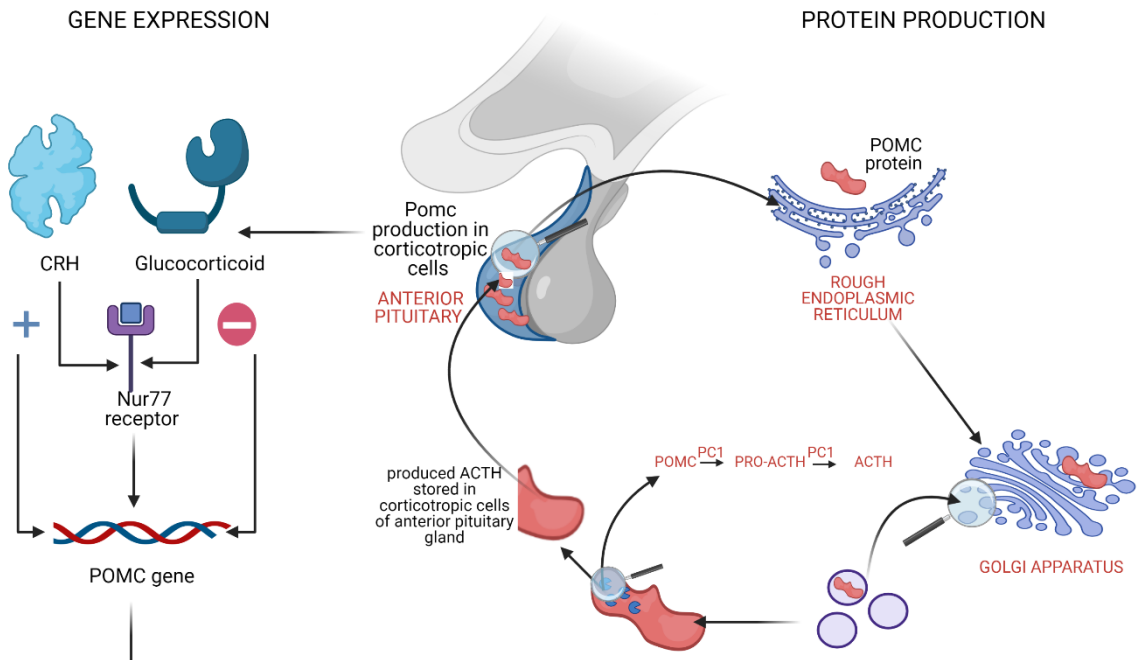


Figure 1.26 - ACTH production from POMC including production and storage sites. Made on Biorender.com based on information from Steven and White, 2009 and Raffin - Sanson et al., 2003. PC1 = prohormone convertase 1

As mentioned in **Figure 1.2** above, POMC gene expression and ACTH secretion occurs in corticotroph cells of the anterior pituitary, which comprise 5 – 20% of the adult pituitary cell population (Levy, 2002). The main effect of ACTH is the stimulation of GCs such as CORT, which occurs via the binding of circulating ACTH to MC2R, which are located on the surface of adrenal cortical cells. The adrenal cortex consists of three different regions which are characterized based on the hormones they produce. From the outermost to the innermost they are the zona glomerulosa, the zona fasciculata and the zona reticularis. The zona glomerulosa predominantly produces aldosterone, the middle zona fasciculata produces CORT, whilst the outer zona reticularis produces androgens. Stimulation of these hormones are achieved by respective upstream proteins, with angiotensin II and potassium stimulating aldosterone production, whilst ACTH stimulates CORT and androgens (Markou et al., 2015).

1.1.3. GCs: Downstream effects

As mentioned above, ACTH released into the body's circulatory system stimulates CORT synthesis by activating its receptor MC2R, which lies in the steroidogenic cells of the zona fasciculata of the adrenal cortex. ACTH binding to MC2R sets off an increase in cAMP levels, which activates the PKA pathway (as seen in **Figure 1.1**). CORT is a lipophilic molecule, and therefore cannot be stored in vesicles but is quickly synthesized and released following ACTH stimulation. ACTH modulates CORT in many ways. It modulates CORT non-genomically when it post-translationally modifies steroidogenic proteins, such as phosphorylation of proteins linked to cholesterol metabolism, specifically hormone-sensitive lipase (HSL) and steroidogenic acute regulatory protein (StAR), which modulates the levels of intracellular cholesterol and its transport within the mitochondrial matrix

(location of steroidogenic process initiation). This cholesterol then undergoes a series of catalytic reactions (in mitochondria and smooth endoplasmic reticulum), finally leading to synthesis and secretion of CORT (Gjerstad et al., 2018, **Figure 1.2**). ACTH modulation also happens genomically, with regards to the transcription of genes encoded for steroidogenic proteins, as well as regulating the expression of MCR2 and melanocortin receptor accessory protein (MRAP – which regulates activity of MCR2 at cell surface and therefore cell's responsivity to ACTH) (Gjerstad et al., 2018, Metherell et al., 2005). The production of CORT occurs in a pulsatile way as the adrenal glands responds quickly to physiological pulses of ACTH (Gjerstad et al., 2018).

CORT can bind both to GRs and mineralocorticoid receptors (MR – found predominantly in limbic regions of the brain). Dexamethasone, a synthetic GC binds to GR with a greater affinity, and therefore has been very useful for GR specific studies. CORT binds to MRs with 6-10-fold greater affinity than GRs, and in low stress states MR is the primary receptor for CORT, and its activity may be related to threat evaluation and immediate stress response. As stress increases, GR then becomes the primary receptor for CORT and assists in mediation of the primary stress response (Dunlop and Wong, 2019). GRs are found in the adrenal cortex which implies that along with its effect in the pituitary and hypothalamus, CORT can affect its self-synthesis by a local feedback mechanism in the adrenal glands themself (Spiga et al., 2017). CORT secretion follows a diurnal pattern, with highest amount detected within an hour of wakening and lowest in the middle hours of sleep. Healthy adults produce 10 mg of cortisol daily from adrenal glands, but periods of intense stress can produce up to 400 mg a day (Dunlop and Wong, 2019). Plasma CORT (95%) is bound to plasma protein (corticosteroid binding globulin CBG), and only the remaining free, unbound CORT has biological activity (hence measuring salivary cortisol as a biological measure for

stress activity, as it is unbound and active). During stressful periods free CORT rises drastically due to oversaturation of CBG (Moison et al., 2014). GRs are found ubiquitously in the body, and in the brain, where they assist in brain energetics and synaptic physiology (Myers et al., 2014).

GRs at the cellular level reside in the cytosol, where they process the slow, genomic effects of GCs. When CORT binds to GRs, the resultant complex is conformationally altered and stimulates transactivation, which involves homodimerization and translocation to the nucleus. At the nucleus it binds to glucocorticoid response elements (GREs) to activate transcription of specific genes (Grad and Picard, 2007).

1.1.4. Negative feedback loop of the HPA axis

As seen in **Figure 1.1** above, POMC gene expression is regulated positively by CRH and negatively by GCs like cortisol, and this is how ACTH production can be regulated in this negative feedback loop. This loop consists of GCs inhibiting its production by acting on the adrenal gland where it is produced and the anterior pituitary gland and hypothalamus, to regulate ACTH and CRH production respectively. Central to this loop is nerve growth factor IB (NGFI-B or Nur77), a receptor which is upregulated by the presence of CRH (mediated through CREB), which stimulates transcription of POMC, but also inhibits POMC transcription when interacting with the GR (Stevens and White, 2009; Martens et al., 2008).

ACTH is also directly involved in this negative feedback loop, where its stress related release is mediated by the presence of hypothalamic CRH and augmented by AVP, and stress related release can be limited by the presence of GCs, therefore exerting feedback inhibition on ACTH secretion (Stevens and White, 2009). This GC inhibition of ACTH

secretion occurs at short/rapid and longer timeframes by non-genomic and genomic mechanisms respectively (Panettieri et al., 2019). It potentially exerts its suppression via blocking calcium signalling which CRH is dependent on (Antoni, 1996). Whilst CRH and GCs remain to be the most potent regulator of ACTH secretion, other factors have been shown to influence secretion, such as leukaemia inhibitory factor (LIF – secreted by corticotroph cells) which suppresses GCs (Auernhammer et al., 1998), catecholamines, dihydroxyphenylalanines (DOPA), serotonin, and Gamma aminobutyric acid (GABA), which are all achieved either directly or through CRH (Stevens and White, 2009).

1.2. How does serum glucocorticoid kinase 1 (SGK1) affect POMC expression?

A member of the serine/threonine kinase family, SGK1 is universally expressed in various tissues, especially in corticotrophs of the anterior pituitary gland (Deng et al., 2018, Reiter et al., 2011). As shown in **Figure 1.3** below, SGK1 exhibits its functions following activation of various receptors – GRs, retinoid X receptor and peroxisome proliferator – activated receptor γ, as well as NFκβ activation (Lang et al., 2009). These functions include regulation of hypertension, epithelial sodium channel activity, insulin sensitivity and secretion, and hippocampal neurogenesis (Deng et al., 2018). SGK1 is responsible for the upregulation of various plasma membrane ion channels and regulates the activity of transcription factors (Lang et al., 2006).

Its most significant function is the mediation of Na^+ reabsorption (stimulated by mineralocorticoids) and K^+ elimination in the renal epithelium, which contributes to the regulation of arterial blood pressure (Soundararajan et al., 1999). Classically, GCs display a proapoptotic effect, but despite being an early target of GCs, SGK1 increases cell survival by regulating transcription factors that govern apoptosis (Brunet et al., 2001). SGK1 is transcriptionally upregulated in the presence of serum, GC, and mineralocorticoids (Chen et

al., 2004, Itani et al., 2002), as well as activated post-translationally by phosphoinositide 3 kinase (PI3K)-dependent phosphorylation (Kobayashi and Cohen, 1999).

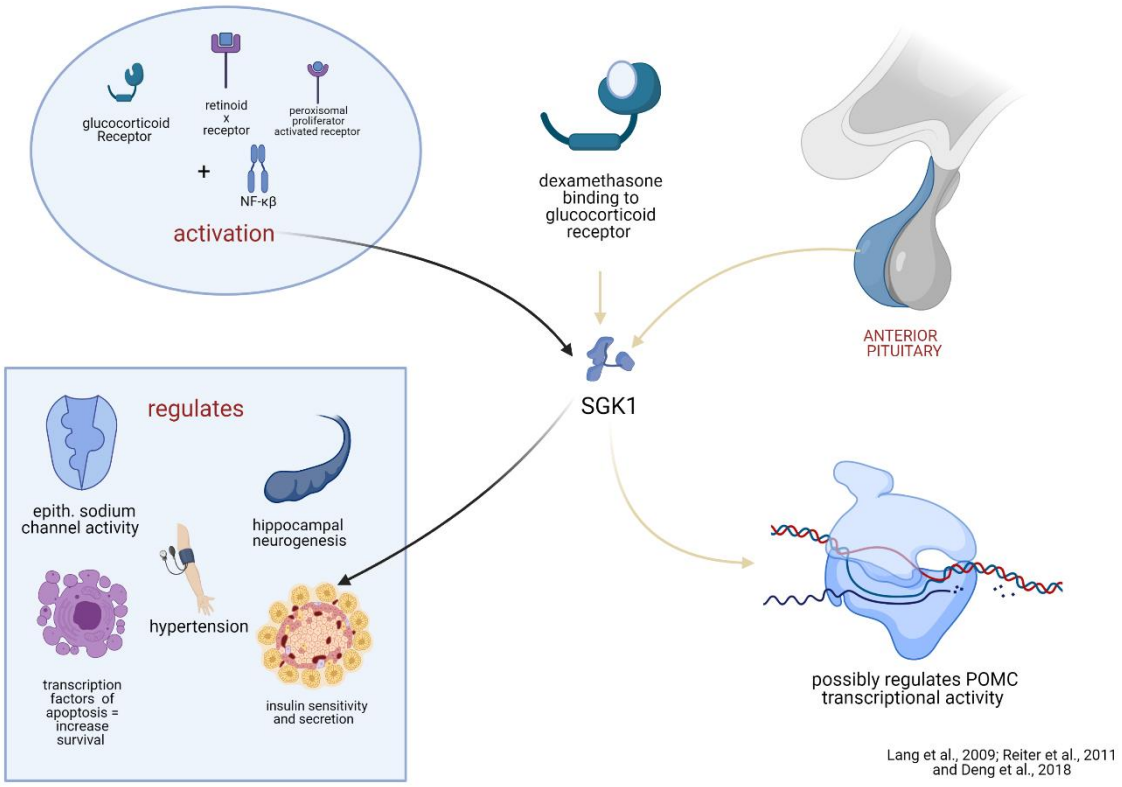


Figure 1.27 - Figure 3.1. The role of the SGK1 system, exhibiting what is necessary for its activation, and what measures in the body it regulates. Based on information from Lang et al., 2009, Reiter et al., 2011 and Deng et al., 2018. Drawn using Biorender.com

Dexamethasone is often used in HPA axis studies (Spitzer et al., 2018, Schinke et al., 2017 and Helmreich et al., 2008) and stimulates a rapid surge in SGK1 in AtT20 cells as early as 30 minutes post-treatment. This stimulation occurs due to GR activation and is diminished following treatment with PI3K inhibitor LY-294002 (Reiter et al., 2011). SGK1 is an early target of Dexamethasone, and Reiter et al (2011) hypothesized that SGK1 regulates POMC transcriptional activity in pituitary corticotrophs. Overexpressing SGK1 via cell transfection resulted in an increase in POMC promoter activity and ACTH release from AtT20 cells, and conversely the knockdown of SGK1 by SiRNA resulted in diminished POMC promoter

activity, without affecting ACTH secretion, implying that basal POMC transcription is dependent on SGK1 (Reiter et al., 2011). This is interesting as this is contradictory to the classical inhibitory effects of GCs on the HPA axis (Laryea et al., 2015).

1.3. The HPA Axis influence on stress-related psychiatric disorders

The HPA axis is linked to the nervous system as many of its hormones originate from the brain, and therefore may have a role to play in psychopathology, stressing the importance of externally regulating this axis. A psychological consequence of severe trauma (near death experiences, sexual assault etc.) may result in post-traumatic stress disorder (PTSD), which presents with stress-related symptoms evoked from re-experiencing the traumatic event, hyperarousal and negative cognitions and mood (Schumacher et al., 2019). Depression from PTSD shows an increase in negative feedback of the HPA axis, which is similar to what is seen in some of the studies on atypical depression (Juruena et al., 2018, Gold, 2015). Understandably, investigation into the HPA axis to find treatment for PTSD is sensible, as this axis is our main response to stress, and it is theorized that individuals experiencing PTSD fail to achieve recovery and restitution of physiological homeostasis following traumatic events (Yehuda and LeDoux, 2007). Schumacher et al (2019) found that whilst the amount of studies in HPA axis and PTSD was significant, most effect estimates (based on the suppression rate or on post-dexamethasone cortisol comparisons between groups, without taking into consideration the initial cortisol levels of each group prior to dexamethasone treatment) in their meta-analysis was non-significant, with the exception of one effect estimate – all significant effect size pointed to lower cortisol levels for PTSD patients compared to controls. They found that their hypothesis of enhanced cortisol suppression in PTSD individuals was refuted. Another systematic review by Dunlop and Wong (2019) reached similar conclusions, that whilst this area is well studied, the findings are fairly inconsistent, which they state is predominantly due to small sample sizes in studies

and lack of longitudinal data within an individual. Inconsistencies in the literature could also be a result of dexamethasone being used, as it is characteristically distinct from cortisol, even though they are both GCs, there is a lack of translatability between rodent and human studies in this case as the impact of stressors applied at different development ages may not translate in the rodent and human timeline.

1.4. The HPA axis: common regulator of gut microbiota and psychiatric illnesses?

Growing evidence cements the theory that there is a bidirectional communication between the major neuroendocrine system, the HPA axis, and gut microbiota (community of microorganisms that live in the intestinal tract of mammals). Aside from its relationship with the HPA axis, there is evidence that there is a strong link between the gut microbiota and brain function, with the gut microbiota formation at the start of life shown to influence certain aspects of brain function and behaviour, such as the HPA axis response to stress (O'Mahony et al., 2017; Dinan and Cryan, 2012). Stress exposure may compromise the integrity of the gastrointestinal barrier, increasing gut permeability and stimulating an autoimmune response. The blood brain barrier, a line of defence protecting the brain from potentially harmful infiltrating substances, depends on intestinal microbiota to maintain its integrity, and therefore changes to the microbiota can be linked to blood brain barrier impairment (Geng et al., 2020; Spadoni et al.,2015).

The link between these two systems are further implied by the disorders that are associated with disruptions in respective systems, e.g. depression and irritable bowel syndrome (IBS). Hyperactivity of the HPA axis has been noted in both patients with depression, and IBS (however the results are more inconsistent in the latter case, but most studies report hyperactivity) (Juruena et al., 2017; Videlock et al., 2016). Dysregulation of the HPA axis

has also been linked to psychotic and mood disorders via chronic stress exposure, which also results in inflammation and decreased levels of brain derived neurotrophic factor (BDNF), epigenetic dysregulation and metabolic disruptions (Misiak et al., 2020; Baumeister et al., 2016; Theleritis et al., 2014). Heim et al (2000) investigated women with a history of childhood physical and sexual abuse, and found that hyperactivity of the HPA axis, noted by persistently high cortisol levels, may contribute to adulthood psychopharmacological conditions, whilst Kelly et al (2015) showed that the HPA axis influenced the composition of the gut microbiota and increased permeability of the intestines. A common symptom of both IBS and depression, inflammation, is caused by dysbiosis of the gut microbiota, where the shift in bacterial composition results in bacterial translocation, which leads to bacteria leaking out of the gut and triggering the immune system nearby (Slyepchenko et al., 2107; Camilleri et al., 2012).These studies consistently present the HPA axis as a common regulator of the gut microbiome and psychological diseases.

1.5. Components of the Gut Microbiome – significance of D-amino acids?

The gut microbiome consists of bacteria, viruses, yeast etc. This thesis focuses specifically on bacteria, as I am interested in the effects gut bacterial content may have on the body, following its digestion in the GIT. This led me to investigate the D-amino acids as they are sourced from the gut microbiota, with special interest into D-alanine as it is not endogenously produced in the body.

1.5.1. What are D-amino acids?

Cells and cellular components are made up of proteins, which are the most abundant biological macromolecules. Proteins are composed of 20 amino acids, made unique by covalently linked peptide bonds in various combinations and sequences to produce

different products (hormones, enzymes, transporters etc.) The standard 20 amino acids are α-amino acids. α-amino acids have the universal structure including a carboxyl group and an amino group, both bonded to the α-carbon atom. This α-carbon is linked both to a hydrogen and to a side chain group, and this side chain group is what makes amino acids distinctive from each other. These α-amino acids can be mirror images of each other, which we refer to as the stereochemistry of an amino acid. α-amino acids that contain substances that rotate clockwise to the plane of polarized light is dextrorotatory (Latin for right), and those that rotate counter-clockwise are laevorotatory (Latin for left) (Genchi, 2017). Therefore D-amino acids are α-amino acids in the D-configuration in terms of stereochemistry.

1.5.2. Where does D-alanine, D-serine and taurine in the body originate from?

There have been contradictory opinions on the origin of mammalian D-alanine. Nagata et al (1991) found that free D-amino acid content in the serum, kidney, liver, brain and small intestine of GF mice was no different to SPF mice, suggesting that the free D-amino acids in those tissue are not of microbial origin. However, their study ascertained D-amino acid content by measuring the enzyme responsible for its breakdown, D-amino acid oxidase (DAO) and not actual content of D-alanine, and this is not enough to support their argument that mammalian D-alanine is not of bacterial origin. Their findings contradicted those of Hoeprich (1965) who suggested that D-alanine in the body is derived from microflora and only a negligible amount of D-alanine exists in GF mice. Another supporter of D-alanine derivation from intestinal bacteria was Konno et al (1990), who showed that following oral administration of the antibiotic amoxicillin, there was a lower level of D-alanine in the urine of mice. However, since the D-alanine synthesising enzyme, alanine racemase, has not been detected in mammalian tissues but is present in bacteria, the

literature is more consistent with the notion that D-alanine in the body is of microbial origin (Sasabe and Suzuki, 2018).

L-serine is a non-essential amino acid, which means that the body can synthesise it using various enzymes (phosphoglycerate dehydrogenase, phosphoserine aminotransferase and phosphoserine phosphatase), and then can be converted to D-serine by naturally occurring serine racemase (El- Hattab, 2016). Serine in both its stereochemistries is neutral amino acids (in terms of overall charge of side chains) that have a significant part to play in signalling, energetic and anabolic functions that affect neuronal function and survival in the central nervous system. L-serine is imperative in neuronal sphingolipid synthesis, whilst D-serine is an endogenous co-agonist of NMDARs which in turn regulate synaptic activity and plasticity, influence neuronal migration and display a direct role on excitotoxicity (Gliddon et al., 2009). D-amino acids like D-serine have a significant responsibility in the body, and this is alternate to the idea that L-amino acids are the sole effectors on physiology. Therefore, it is important to elucidate the purpose D-alanine has in the body, with regards to potential mediation of the HPA axis due to its localization.

Unlike the above mentioned α-amino acids, taurine is a β-amino acid and is found ubiquitously throughout the body, but especially in excitable tissue (Schaffer and Kim, 2018). Taurine is best known for its cytoprotective functions, where it can greatly change the nutritional and health status of various species, hence its now widespread use in infant formula and energy drinks. Taurine is now being investigated for its therapeutic capabilities, where it has been found to have protective effects against ischemic reperfusion injury (Ginguay et al., 2016). Taurine is an essential amino acid in humans, as it is derived predominantly from diet, and is of special interest in this thesis as it is an agonist of the glycine receptor and has the ability to activate these inhibitory receptors in the brain even

with their basal concentration at less than 1mM (Schaffer and Kim, 2018), and I therefore utilize it as a positive control for the glycine receptor activity later on.

1.5.3. The distribution of D-amino acids in mammals

A study by Nagata et al (1992) showed that 2% of amino acids in the plasma of healthy subjects were of D-isomer formation with regards to alanine, serine and proline (Wolosker et al., 1999). Whilst not synthesized in mammals, D-alanine was discovered by Hoeprich in 1965, making it the inaugural essential D-amino acid discovered in mammals. D-alanine is prominently found in the anterior pituitary gland of the brain (Etoh et al., 2009), but is also found in concentrated quantities in the pancreas (Ota et al., 2014). In the anterior pituitary of Sprague Dawley rats, there was 9.49 nmol/g tissue of D-alanine, whilst in the pancreas there was 309 nmol/g concentration of D-alanine in islet tissue (Ota et al., 2014).

In 1992, Hashimoto et al. found a high concentration of D-serine in the mammalian brain in various regions. In the forebrain, D-serine is present at 400 nmol/g wet weight in mammals but was considerably lower in the cerebellum and brain stem. The grey and white matter of the cerebrum of rodents were compared for levels of free D-serine, and it was found that there was a higher D-serine/L-serine ratio in grey matter than white matter (Nagata et al., 1994). Studies have shown that in healthy individuals, D-serine and serine racemase can also be found in neurons as well as astrocytes. A study by Balu et al (2014) conclusively found that majority of D-serine is both synthesized and stored in neurons. They achieved this using immunohistochemistry techniques, where they found that serine racemase is expressed predominantly in forebrain glutamatergic neurons, and the remainder in GABAergic interneurons.

1.5.4. The purpose of D-amino acids in mammals

The presence of D-amino acids in mammals was initially thought to be unusual, but there is now compelling evidence for their functional role in peripheral and central systems (Fuchs et al., 2005). The transient increase of D-aspartic acid during postnatal development is concurrent with the maturation of corresponding tissues, which implies that D-aspartic acid may have a role to play in tissue development processes (Hashimoto et al., 1995), as well as secretion of hormones melatonin and testosterone (Takigawa et al., 1998, D'Aniello et al., 1996).

Regional distribution of D-serine corresponds with presence of NMDARs. This is sensible as D-serine is a co-agonist of this receptor and potentiates the glutamic acid-elicited response of this receptor (Hamase et al., 1997). D-serine is the intrinsic regulator of this receptor in mammals (Morikawa et al., 2001). With regards to pathology, Danysz and Parsons (2012) showed that via NMDAR-mediated neurotoxic insults, D-serine may have a role to play in the development of Alzheimer's disease. A comprehensive study by Lin et al (2019) showed that increased D-serine levels predicted worse cognitive function in patients with Alzheimer's disease and mild cognitive impairment, especially with regards to word recall tasks, orientation, comprehension and word-finding difficulty, whilst increased levels of D-alanine were connected to behavioural symptoms. This supports the finding of Fisher et al (1991) of an increase in free D-alanine levels in the grey matter of Alzheimer brains compared to normal brains. Additionally, with regards to pathophysiology, increases in D-Aspartate, D-serine and D-alanine in serum positively correlate with the progression of renal failure (Fukushima et al., 1995; Brückner and Hausch, 1993).

Interestingly, mammalian plasma D-alanine levels display a circadian rhythm, where it is higher in the daytime than the night-time. This implies that D-alanine may play a role in the mammalian circadian clock (Morikawa et al., 2008). An agonist of the NMDAR, D-alanine also regulates the NMDAR mediated neurotransmission similarly to D-serine and mediates normal neurodevelopment and maintenance of healthy cognitive function. (Sakata et al., 1999). D-alanine is stored in the beta cells of the rat pancreas in a high concentration and therefore maybe involved in the modulation of blood glucose levels via insulin modulation (Morikawa et al., 2007). D-alanine is stored in significant levels in the anterior pituitary gland, in the same cells that store ACTH, which implies it may have a role to play in the secretion of these hormone (Etoh et al., 2009), which I look at in detail in this thesis.

1.6. D-amino acids distribution in mammals – Amino Acid transporters

D-alanine and D-serine are derived from microbial sources in the gut but go on to be stored in various organs of the body, and this is achieved by various transporters, which will be discussed further below.

1.6.1. The mechanism of amino acid transporters

Changes of intracellular amino acid content is a danger to cell survival, therefore a complex network of enzymes and membrane transporters work together to maintain homeostasis and homeorhesis – regulating the flow of amino acids, which is thought to be more important than keeping amino acids at constant concentrations (Taylor, 2014; Carroll et al., 2015). There are 4 systems responsible for amino acid transport in the central nervous system: system ‘Gly’ which specializes in uptake of glycine, system ‘A’ specializing in the uptake of alanine, system ‘L’ which specializes in the uptake of leucine, and ‘ASC’, which

specializes in uptake for alanine, serine and cysteine. System 'ASC' has two subsets – ASCT1 and ASCT2 (Christensen, 1990, Hashimoto and Oka, 1997).

The most notable difference between ASCT1/2 and Asc-1 are their dependency on Na^+. ASCT1/2 are Na^+-dependent systems, which are able to move the substrate *against* its concentration gradient, whilst Asc-1 Na^+- independent systems allow amino-acid flux *in either direction* according to its concentration gradient (Christensen, 1990).

1.6.1.1. Asc-1

It was thought that Asc-1 in humans is predominantly present in neuronal presynaptic complexes in the brain, to assist with D-serine clearance which may have profound physiological consequences (Helboe et al., 2003, Danysz and Parsons, 1998). 4F2hc is an ancillary subunit of the Asc-1 transporter and assists with bidirectional transport of D-serine coupled with counter-transport of small neutral amino acids. This process is called the exchange mode (Fukusawa et al., 2000). Rosenberg et al (2013) showed that Asc-1 facilitates the release of neutral amino acids by passive transport through facilitated diffusion. This was shown utilizing D-isoleucine stimulation of D-serine exchange in acute hippocampal slices. This resulted in Asc-1 mediated D-serine release increasing NMDAR synaptic activity at the hippocampal CA1. This is interesting as initially Asc-1 was only thought to be involved in the uptake of D-serine, and not its release (Fukusawa et al., 2000). Sason et al (2017) indicate that the Asc-1 exchange mode may contribute to NMDAR activation. Acute inhibition of Asc-1 resulted in downregulation of D-serine release that changes the NMDAR activation of forebrain neurons, implying that Asc-1 inhibitors may be useful to prevent overactivation of NMDARs in neurodegenerative conditions (Sason et al., 2017).

The **Na^+-independent system**, Asc-1, or SLC7A10, belongs to the light chain of heterodimer amino acid transporter (HAT), and has been shown to have a greater affinity for D-serine than **Na^+-dependent** ASCT1/ASCT2 (Rosenberg et al., 2013, Helboe et al., 2003) as it is thought to be less stereospecific (Fukusawa at el., 2000, Nakauchi et al., 2000). Rutter et al (2007) discovered that Asc-1 is the main D-serine transporter in the brain by using Asc-1 knockout mice. Mice lacking Asc-1 displayed a 70-80% reduction in synaptosomal uptake of D-serine, as well as seizures that resulted in early postnatal death (Xie et al., 2005). Asc-1 has a high affinity for D-alanine (Fukasawa et al., 2000).

1.6.1.2. ASCT2

Biochemical pathways are compartmentalized in eukaryotes; therefore, transporters are required either on the plasma membrane or on membranes of intracellular organelles. ASCT2 is a plasma membrane transporter (Scalise et al., 2018). The physiological mechanism of ASCT2 lies on its antiport mode of transport coupled to a sodium gradient that regulates pools of amino acids in intra- and extra-cellular spaces (Scalise et al., 2018). With regards to the antiport mechanism of ASCT2, an amino acid capable of inducing efflux of another substrate is considered a substrate as well. Therefore, cysteine is able to induce efflux of radiolabelled substrates like alanine or glutamine. However, experiments measuring radiolabelled cysteine transport show very low transport of this amino acid (Utsunomiya- Tate et al., 1996). Substrates transfer from one membrane side to the other via an elevator mechanism in recombinant human ASCT2. Elevator movement refers to the way Na^+ ions move from one side of the membrane to the other in exchange for protons (Lee et al., 2013). The elevator consists of a domain containing two membrane hairpins labelled HP1 and HP2. This mobile part is anchored to a fixed domain of the protein embedded in

the membrane, and the sliding of the elevator domain allows for substrate translocation (Garaeva et al., 2018, Lee et al., 2013).

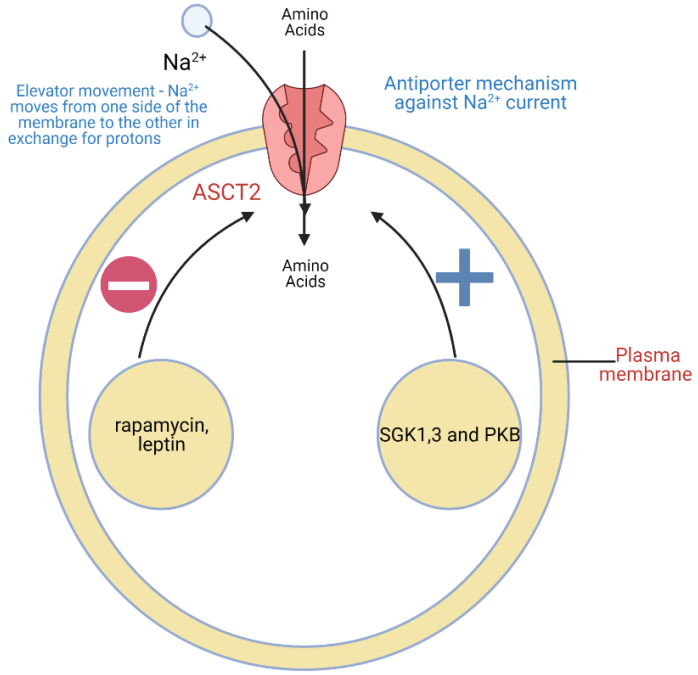


Figure 1.28 - ASCT2 transporter mechanisms adapted from Scalise et al., 2018. Plus sign = increase, minus sign = decrease. Image made with biorender.com.

The Na^{+}-dependent ASC-family of transporters has a preference for L-amino acids over D-amino acids, shown by a preference to [^{3}H] L-serine transport into the synaptosomes of the rat brain (Arriza et al., 1993, Utsunomiya-Tate et al., 1996) and it has the greatest affinity for L-alanine, and whilst not as strong, a high affinity for L-cysteine and L-serine (Helboe et al., 2003). Gliddon et al (2009) found that ASCT1 and ASCT2 both display a high affinity for alanine, serine and cysteine, but ASCT2 **also** accepts L-glutamine and L-asparagine as high affinity substrates and D-serine, L-methionine and L-leucine as low affinity substrates.

There has been much investigation in which of these two transporters, ASCT1 or ASCT2, are predominantly used to take up D-serine. Using knockout mice for ASCT1 and ASCT2, Kaplan et al (2018) found that ASCT1, rather than ASCT2, was a physiological regulator of D-serine metabolism. ASCT1 is a key component for astrocytic D-serine uptake and can also export L-serine through hetero-exchange, supplying neurons with the substrate for synthesis of D-serine. So the research is quite conflicting as to which transporter subtype is more responsible for D-serine regulation.

1.6.2. Regulation of Amino Acid Transporters

It is important we regulate amino acid transporters as this will in turn assist in homeorhesis of amino acids in the body. The interaction between ASCT2 and D-serine is one of low-affinity, which implies that the function of D-serine transport by ASCT2 is not its primary function (Gliddon et al., 2009). mTOR is the chief regulator of detecting nutrient viability in cells, and in doing so modulates numerous pathways. It was reported that mTOR may modulate ASCT2 expression as rapamycin (an inhibitor of mTOR) caused a reduction in ASCT2 expression (Fuchs et al., 2007). Palmada et al (2005) reported that SGK1, 3 and protein kinase B (PKB) kinases stimulate an increase in ASCT2 abundance in plasma membrane, whilst leptin inhibited ASCT2 expression in the rat intestine (Ducroc et al., 2010).

The phosphorylation motif in the sequence of ASCT2 has not been determined, therefore the positive effect of kinase activity on Vmax of ASCT2 is attributed to phosphorylation of ASCT2 in a non-canonical site, or to the effect of an unknown protein undergoing phosphorylation and in turn regulating ASCT2 activity. ASCT2 is stabilized in cells via ubiquitination of 7 lysine residues. Most ASCT2 regulatory mechanisms have been studied

in tumour cells. The benefit of such experiments is the allowance of reproducibility, whereas a disadvantage is that with the remodelling of cancer cells there may be a difference in molecular mechanisms in comparison to normal tissue (Scalise et al., 2018).

1.6.3. The distribution of amino acid transporters

1.6.3.1. Asc-1

Asc-1 has been detected in the lung and small intestine (Chairoungdua et al., 2001). Of the small neutral amino acid transporters only Asc-1 is found in significant amounts in the brain (Wagner et al., 2001), and Helboe et al (2003) determined the specific distribution in the brain by raising polyclonal antibodies specific for murine Asc-1. They found that it was widely distributed in the brain but limited to neuronal structures, especially their terminal ends. Prominent levels of D-serine are present in the cerebral cortex, hippocampus, striatum, and in lesser amounts, the limbic forebrain, diencephalon and brainstem. Similarly, Asc-1 is highly expressed in these areas. However, some areas that had a low presence of D-serine had a high presence of Asc-1 such as the hypothalamus, brainstem and cerebellum (Helboe et al., 2003). These areas are rich in glycine and L-alanine (Schell et al., 1997). This corroborates findings by Helboe et al (2003) and Fukusawa at el al (2000) that Asc-1 transports L-alanine and glycine as well.

Seeing as Asc-1 distribution in the brain coincided with the presence of D-serine in those same areas (Helboe et al., 2003), this co-localization may imply that Asc-1 may modulate the endogenous levels of the co-agonists of the NMDARs, as Asc-1 may assist in the accumulation of D-serine in neurons, which may lead to swift clearance from the synaptic cleft of this co-transmitter (Baranano et al., 2001). D-serine fluxes are paired to L-serine

countermovement, implying that D-serine transport is facilitated by an antiporter mechanism (Ribeiro et al., 2002, Rothman et al., 1999).

1.6.3.2. ASCT2

ASCT2 is widely expressed in the lung, kidney (where it is abundant), skeletal muscle, testis, T-cells, large intestine, adipose tissue and brain (Poffenberger and Jones, 2014, Pinho et al., 2007; Utsonimiya – Tate el al., 1996). Of particular interest, ASCT2 expression was noted in cancers derived from human tissues in which normally this transporter is not present (Scalise et al., 2018). ASCT2's presence in the gut is well documented, with Ducroc et al (2010) stating that ASCT2 expression is inhibited by the gut hormone leptin in the large intestine of rats. ASCT2 is also the key modulator of D-serine levels in the brain. This is important as D-serine can be potentially neurotoxic and has been linked to neurological disorders such as Amyotrophic Lateral Sclerosis (ALS) (Scalise et al., 2018).

In the CNS, ASCT1 has been detected in astrocytes and neurons (Yamamoto et al., 2003, 2004). Immunochemical data for ASCT1 in rodent brains display a strong glial localization (Weiss et al., 2001, Yamamoto et al., 2003). There are no selective inhibitors to differentiate between ASCT1 and ASCT2, therefore functional evidence for ASCT2 in the CNS is based predominantly on identification of uptake of or uptake inhibition by ASCT2 selective amino acids – L-glutamine, L-asparagine or D-serine. Utilizing this mechanism, ASCT2 has been detected in cultured astrocytes, the rat glial C6 cells and the retinal glial cells - Müller glia (Gliddon et al., 2009).

Whilst there is evidence of ASCT2 activity in neurons as Su et al (1997) observed ASC-like glutamine uptake by cerebellar granule cell and cortical neuron cultures, this evidence is

weaker than that of ASCT2 functional data in astrocytes. Synaptosomes were also proven to be a region where D-serine is taken up by ASCT2. The data regarding ASCT2 expression in neurons is conflicting, with some studies detecting the transporter, and others not (Gliddon et al., 2009). Tetsuka et al (2003) detected ASCT in mouse brain homogenates using western blot, as well as in non-Glial fibrillary acid protein (GFAP) positive cells surrounding brain capillaries. Evidence for ASCT2 in the brain was limited, and immunohistochemical detection was required to support its presence in the brain, which Gliddon et al (2009) successfully achieved. They detected its presence in the cortex, striatum and hippocampal CA1 and dentate gyrus regions and found that it co-localized with MAP2 (a marker for dendrites and neuronal perikarya) immunoreactivity, but not neuronal cell bodies or astrocytes. This co-localization suggests ASCT2 expression is limited to dendrites in the brain. This finding of ASCT2 on dendrites was supported with functional data that showed ASCT2-like low affinity for Na^+-dependent D-serine and glutamine uptake in P2 synaptosomal membrane fractions. In the cerebellum, ASCT2 was also detected in the purkinje cell bodies, as well as dendrites. ASCT2 immunoreactivity was surprisingly absent in astrocytes of all brain regions explored, however it was detected by western blot in astrocyte cultures. ASCT2 was not detected in cell regions that expressed neuronal nuclear marker, NeuN, which implies that ASCT2 is not expressed in neuronal cell bodies, with the exception of its presence in purkinje cells in the cerebellum (Gliddon et al., 2009).

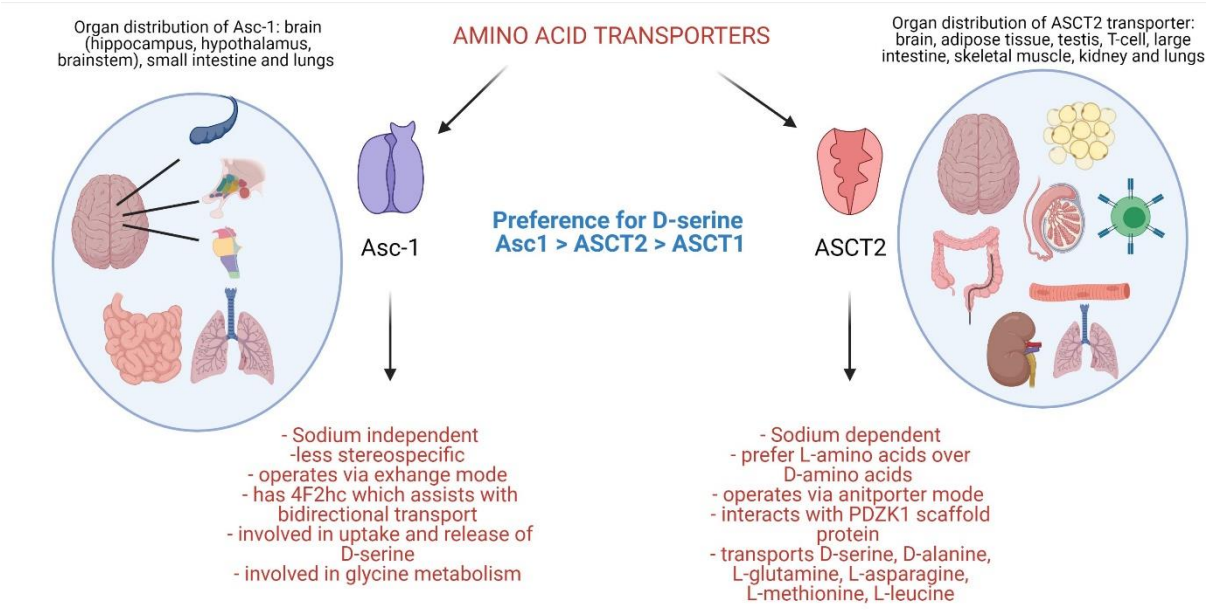


Figure 1.29 – Summary of amino acid transporter organ distribution, differences between transporters and relative preference for D-serine. Made with Biorender.com.

1.7. D-amino acid receptors

D-amino acids predominantly exert their effects by binding to receptors that influence various processes in the body, however these range from direct effects to indirect effects. It is important to know the difference because we can then manipulate this system by interfering with the upstream mechanisms of the effect, and we should know what level the disruption should occur for it to be effective.

1.7.1. NMDARs – brain and gut distribution

The brain was shown to contain high quantities of endogenous D-serine by Hashimoto et al (1993). D-serine is a physiological regulator of NMDARs, and therefore implied to be involved in physiological and pathological NMDAR dependent processes (synaptic plasticity, neurotoxicity, cell migration) (Wolosker et al., 2008). The overactivation of NMDARs may be a contributing factor to several pathological conditions (stroke and neurodegenerative diseases) (Hashimoto et al., 1993). There is a noted correlation between areas rich with NMDARs also presenting with high levels of D-serine (Wolosker et al.,

2008). Glutamate is the most prevalent amino acid neurotransmitter in the brain. It activates both metabotropic glutamate receptors (mGlu) and ionotropic receptors – NMDAR, α-amino-3-hydroxy-5-methylisoxaole-4-propionate receptors and kainite receptors. NMDARs are enriched in the corticolimbic regions of the brain (Yota and Coyle, 2019).

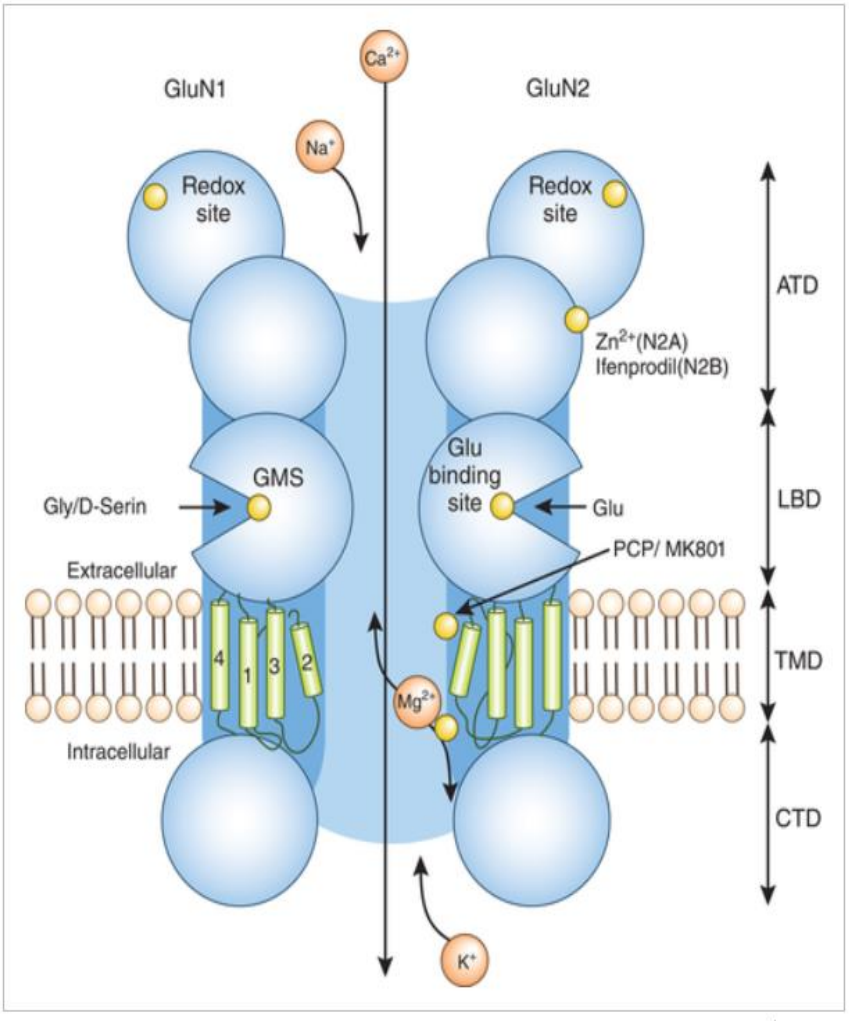


Figure 1.30 – conventional NMDAR with 2 GluN1 subunits and 2 GluN2 subunits (Yota and Coyle, 2019).

As seen in the **figure1.6** above, NMDAR is comprised of 2 essential GluN1 subunits, and either 2 GluN2 subunits or a mix of GluN2 (4 variants A-D encoded by *GRIN2A-D*) and GluN3 (2 variants A-B encoded by *GRIN3A-B*) subunits. Each subunit has a similar structure consisting of 2 clamshell-like extracellular domains (containing a ligand binding domain),

a transmembrane domain and carboxyl terminal domain (involved in receptor tracking and signalling). The extracellular amino terminal domain and carboxyl terminal domain have the most divergence, which allows for alteration in function for NMDARs (Paoletti et al., 2013). D-serine and glycine are ligands of the GluN1 and GluN3 subunit at the glycine modulatory site (GMS), whilst glutamate binds to GluN2 at the glutamine binding site (Yao et al., 2013). NMDARs are activated when postsynaptic depolarization removes the magnesium block in the GluN1 channel allowing the binding of D-serine or glycine to GluN1 subunit, and binding of glutamate to its receptor on GluN2 to open the channel and allow the entry of calcium. Calcium then sets off a cascade of events that results in regulation of acute functional synaptic plasticity and alters gene expression for long term neuronal structure plasticity (Balu, 2016).

The enteric nervous system (ENS) is an extremely important aspect of the body's nervous system, as it controls for gastrointestinal function, and dysregulation of this could lead to gastrointestinal diseases which are the leading cause of morbidity (Golovynska et al., 2018). Of special interest are the factors that regulate gastric acid secretion, as it is important to maintain pH levels in the gut as hyperacidity can cause ulcers, and hypoacidity can cause dysbiosis, suppression of proteolytic enzymes and stomach cancer (Manko et al., 2014). The discovery of NMDAR mRNA in the enteric neurons of the gut is not new (Burns et al., 1994), but it was not well researched until it was proposed that NMDARs may be involved in gastric acid secretion. This finding was further supported by Tsai and colleagues (1994) as well as Liu et al (1997) confirming the presence of NMDARs in the ENS by immunohistochemical technique. NMDARs have been identified in most cells of the ENS (Zhou et al., 2006, Liu et al., 1997, Tsai et al., 1994 and Seo et al., 2014), and in the stomach they are expressed in parietal, surface cells and chief cells (Seo et al., 2014). In particular,

GluN1 is well expressed in the ENS nerve cells of the myenteric and submucosal plexus (Zhou et al., 2006, Watanabe et al., 2008 and Tsai et al., 2006). Golovynska and colleagues (2018) found that NMDARs were expressed in secretory cells of the stomach mucosa, as well as in the Meisner's plexus, and found that NMDARs had a stimulatory effect on gastric acid secretion in the Meisner's plexus, but an inhibitory effect on gastric acid secretion in parietal cells (shown in the **Figure 1.7** below). This study highlights the importance of elucidating the function of NMDARs in the gut, as we find that there is a strong association between the nervous system and the gut activities and diseases, and vice versa.

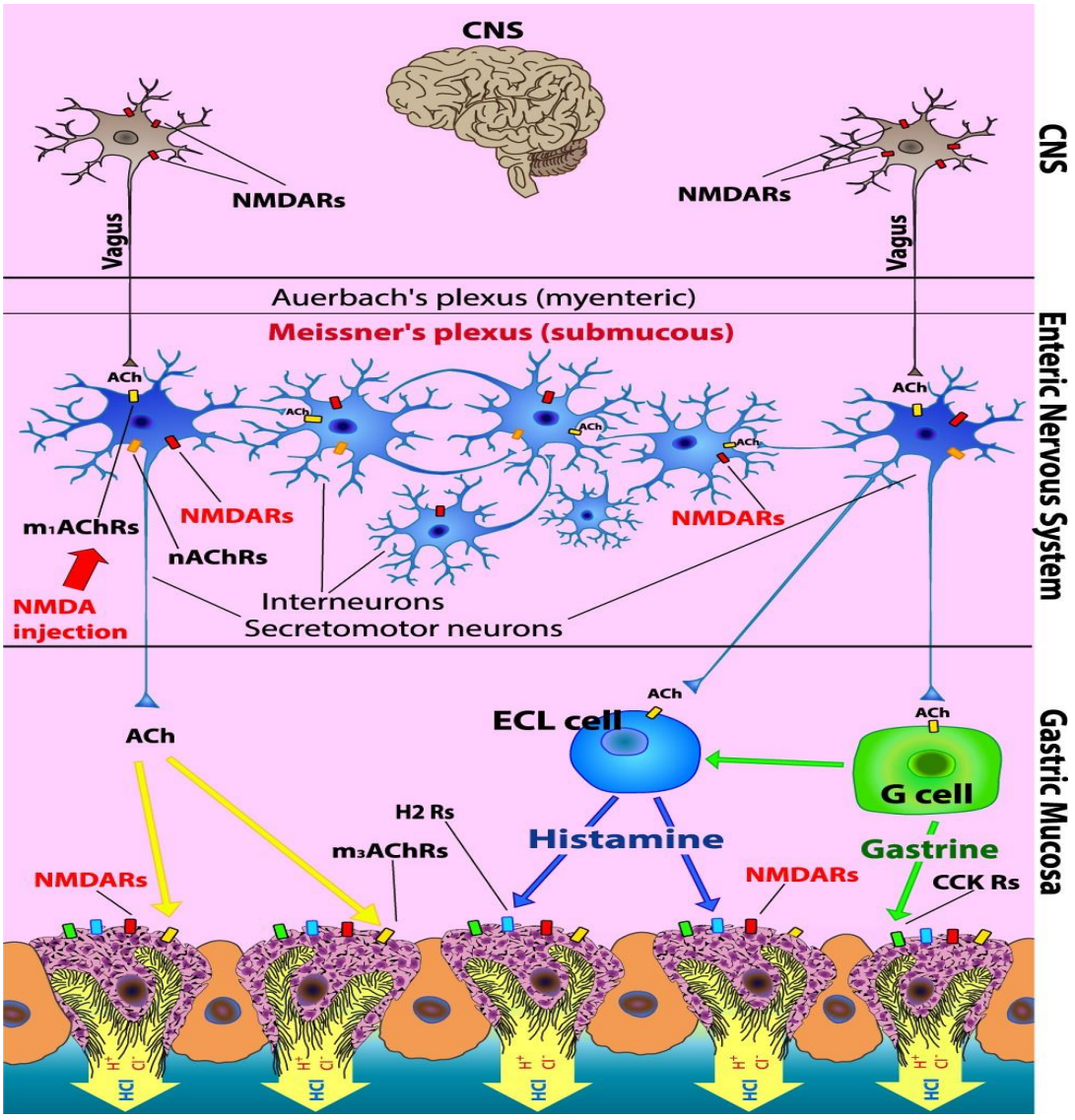


Figure1. 31 - A detailed description of NMDARs in the gut, with emphasis on potential gastric acid secretion regulation (highlighted in red) in the gut (Golovynska et al., 2018)

With regards to agonists of the NMDARs, in 1987, Johnson and Ascher discovered the importance of glycine with regards to NMDARs using cultured neurons, specifically that NMDARs required glycine to bind at a site specifically for glutamate to open the cation channel. Soon after it was discovered that D-serine is as potent an agonist for this 'glycine site' on the GluN1 subunit of NMDAR, which explained the high presence of this D-amino acid in the brain (Kleckner and Dingledine, 1987). This was confirmed by Mothet et al (2000) who cemented the role of D-serine in NMDAR function by perfusing cultured neurons and brain slices with DAO which ceased NMDAR activation (shown by intracellular recording and nitric oxide (NO) production) and this loss of function was regained by perfusion with endogenous D-serine. This was confirmed repetitively in the literature (Papouin et al., 2012, Yang et al., 2003) and D-serine was coined the endogenous co-agonist of the NMDAR.

Using a controlled cortical impact model (CCI) in mice, Perez and colleagues (2017) found that D-serine aggravated synaptic dysfunction, as D-serine released from inflammatory astrocytes, which proliferate following CCI, are responsible for hippocampal neuronal damage. This is as D-serine is released into the extracellular space and activate extrasynaptic receptors (as opposed to being released into the synapse). Extrasynaptic NMDARs have been associated with excitotoxic neuronal damage (Parsons and Raymond, 2014), whilst synaptic NMDARs are linked to functional and structural neuroplasticity (Hardingham and Bading, 2010).

D-serine's role as a co-transmitter (along with glutamate) of the NMDAR has been questioned recently, as it was confirmed that D-serine is released by facilitated transport by Asc-1 (Sason et al., 2017) from GABAergic neurons (Balu et al., 2014), rather than

glutamatergic neurons, and GABA receptors are not known to have a glycine modulatory site (Coyle et al., 2020).

McBain et al (1986) found that D-alanine is a potent and selective agonist of the glycine site of NMDARs. Cognitive decline and psychosis are linked to NMDAR hypofunction in schizophrenia (Coyle, 2006). D-alanine is found in the CNS and is metabolised by DAO, and based on findings by Tsai et al (2006) it is proposed to improve negative symptoms and cognition, but not depression, in schizophrenic patients, when administered in conjunction with antipsychotics, and importantly, its administration garnered no side effects. NMDARs are responsible for neurotoxic assaults observed in Alzheimer's patients, and these NMDARs can be activated by D-alanine, where higher levels may predict behavioural symptoms of Alzheimer's disease, where D-alanine may be a potential biomarker for the disease through its binding to NMDARs (Lin et al., 2019).

1.7.2. GlyRs – brain and gut

Presynaptic (modulate neurotransmitter release at glutamatergic and glycinergic synapses (Dutertre et al., 2012a)) and extrasynaptic (modulate neurodevelopment (Avila et al., 2013)) glycine receptors are expressed widely in the brain. Their postsynaptic versions are located in the brainstem and spinal cord where they mediate inhibition (Lynch et al., 2017). They have also been detected in enteric neurons of the gut (Neunlist et al., 2001). In humans, GlyRs consist of α1-4 and β subunits arranged either in a homomeric or heteromeric fashion (2 alphas and 3 betas or 3 betas and 2 alphas) (Durisic et al., 2012). Dysfunctions in GlyRs have been linked to hyperekplexia (startle disease - due to loss of glycinergic synaptic signalling (Bakker et al., 2006)), temporal lobe epilepsy (caused by posttranslational alterations to GlyR α3 subunit (Eichler et al., 2008)), respiratory disorders (failure of

glycinergic inhibitory transmission from the brain stem respiratory network (Busselberg et al., 2001)) and autism (disruption of GlyR α2 function (Pilorge et al., 2015)).

Each transmembrane subunit of GlyRs consists of an extracellular domain which contains the ligand binding site, a transmembrane domain and an intracellular loop domain which mediates interaction with intracellular proteins (Carland et al., 2009). Agonists that bind to the GlyR are D-alanine, L-alanine, β-alanine, taurine, L-serine and GABA (Dutertre et al., 2012b). Release of glycine from presynaptic terminals sets off the opening of glycine receptors and a flux of anions through the channel, this results in a hyperpolarization and inhibition of the postsynaptic neuron, which is an essential process in the body, especially with regards to muscle tone modulation, sensory processing and motor control (Lynch, 2009).

Strychnine is a competitive antagonist of GlyRs. The binding site for agonists of GlyRs and strychnine can be found on a pocket at the interface of adjacent subunits in the extracellular domain. The core residues that strychnine bind to are conserved across GlyRs and this accounts for strychnine's high selectivity for GlyRs over other receptors (Lynch et al., 2017).

D-alanine is found in high concentrations (1500 – 3000 nmol/g) in the insulin producing β-cells of the pancreas, an exocrine and endocrine gland that secretes various hormones and enzymes (Morikawa et al., 2003). The secretion of insulin from these β-cells is essential for glucose homeostasis, and its disruption is the underlying cause of Diabetes. Human pancreatic β-cells express the glycine receptor, which oversees depolarizing Cl^- currents which increase action potential firing, insulin secretion and Ca^{2+}-dependent exocytosis. The modulation of electrical activity and initial Ca^{2+} concentrations occurs through an autocrine

feedback loop with insulin (Yan-Do et al., 2016). Based on the co-localization of glycine receptors and D-alanine in the pancreas, there may be a link between this agonist and its receptor with regards to insulin secretion, which should be further explored.

1.8. Inflammation in the gut - are D-amino acids involved?

A reduction of D-alanine content in lipoteichoic acid (LTA) in *Lactobacillus platarum* (Dlt^-mutant) enhanced the anti-inflammatory properties in a mouse colitis model. It was found that this mutant species decreased the activation-induced release of Tumour Necrosis Factor alpha (TNF-α) and Interferon gamma (IFN-γ) from mesenteric T cells, as well as interleukin 10 (IL-10) levels in colonic tissue, whilst increasing the activation-induced secretion of anti-inflammatory IL-10 in splenocytes and mesenteric lymphocytes. Therefore, implies that D-alanine reduction decreases inflammatory responses, thereby implying that D-alanine may have a pro-inflammatory response in the gut (Duncker et al., 2008).

The inflammatory properties of D-serine have yet to be investigated in the gut, however, neurotoxic level alterations in the hippocampus of patients with Alzheimer's disease imply that D-serine has a role to play in the pathogenesis of Alzheimer's Disease, excitotoxicity and neuroinflammation (Wu et al.,2004). D-serine significantly increased TNF-α and interleukin 1β (IL-1β) levels in the cortex and hippocampus in adult Sprague-Dawley rats, implying a pro-inflammatory effect in the brain (Armagan et al., 2012).

As mentioned above, D-amino acids relay their inflammatory effects via an array of cytokines. TNF-α, a pro-inflammatory cytokine, plays a key role in intestinal inflammation of a plethora of inflammatory disorders including Crohn's disease (supported by elevations of TNF-α in intestinal tissue, serum and stool of patients with Crohn's Disease) (Ma et al., 2005). It achieves this by stimulating an increase in intestinal epithelial tight junction (TJ)

permeability, through the elevation of permeability of the epithelium and a decrease in transepithelial resistance (Ma et al., 2004). Therefore TNF-α has been proposed as one of the proinflammatory mechanisms contributing to intestinal inflammation (Ma et al., 2005).

Another protein linked to inflammation in the gut is Glucagon-like-peptide 2 (GLP-2). GLP-2 is an enteroendocrine peptide released from the distal ileal L cells in response to nutrients and mucosal injury and has a favourable effect on gut mucosal integrity. Utilizing transforming growth factor β (TGF-β), GLP-2 stimulates epithelial proliferation and improves intestinal wound healing (Bulut et al., 2004). In Caco-2 cells, GLP-2 has a positive effect on barrier function and TJ expression, and an overall protective action against the response to TNF-α (Moran et al., 2012).

Crohn's disease - a chronic and relapsing inflammatory disease of the intestine (Chen et al., 2016) and ulcerative colitis - the continuous mucosal inflammation of the colon (Ungaro et al., 2019) constitute Irritable Bowel Disorder (IBD) which is a global health concern that severely impacts quality of life (Gracie et al., 2019). Effective and targeted treatment is much needed, as inappropriate treatment contributes to the high proportion of patients with IBD with persistent gastrointestinal issues in the absence of inflammation (Gracie et al., 2019). A consistent link is clear in abdominal pain or IBS-type symptoms and psychological health, with this association persisting over time (Gracie et al., 2018, Gracie et al., 2017). 30% of patients with IBD also have diagnosed psychological disorders, which indicate gut-brain axis dysregulation as a potential cause of IBD, which have yet to be elucidated, and this theory is supported by many studies (Mikocka-Walus et al., 2016, Persoons et al., 2005, Mittermaier et al., 2004). Inflammation has also been shown to assist in the development of psychological disorders (Lix et al., 2008, Porcelli et al., 1996). Studies are investigating the potential bidirectional activity of psychological disorders and IBD, by treating the psychological disorders and investigating if this alleviates IBD symptoms, but currently the

number of studies are very limited with limitations in their designs which affect findings (Gracie et al., 2019). In murine colitis models, alterations in psychological wellbeing and behaviour are linked to gut microbial composition, where inflammation saw shifts in microbiota composition (Emge et al., 2016). These effects are thought to be orchestrated by the vagus nerve which effects inflammatory cytokines, where it exerts an inhibition on pro-inflammatory cytokines which diminishes during stressful scenarios, leading to increased inflammation (Matteoli and Boeckxstaens, 2013). Intrigued by potential effects gut microbial products, like D-alanine, may have on inflammation in the gut, which we can see in terms of IBD has considerable effects on health, I investigated the inflammatory effects D-amino acids have on human colonic cells (Caco-2), as well as investigated amino acid transporter presence in these cells, to better understand how D-amino acids may be taken up in its primary organ of existence – the gut.

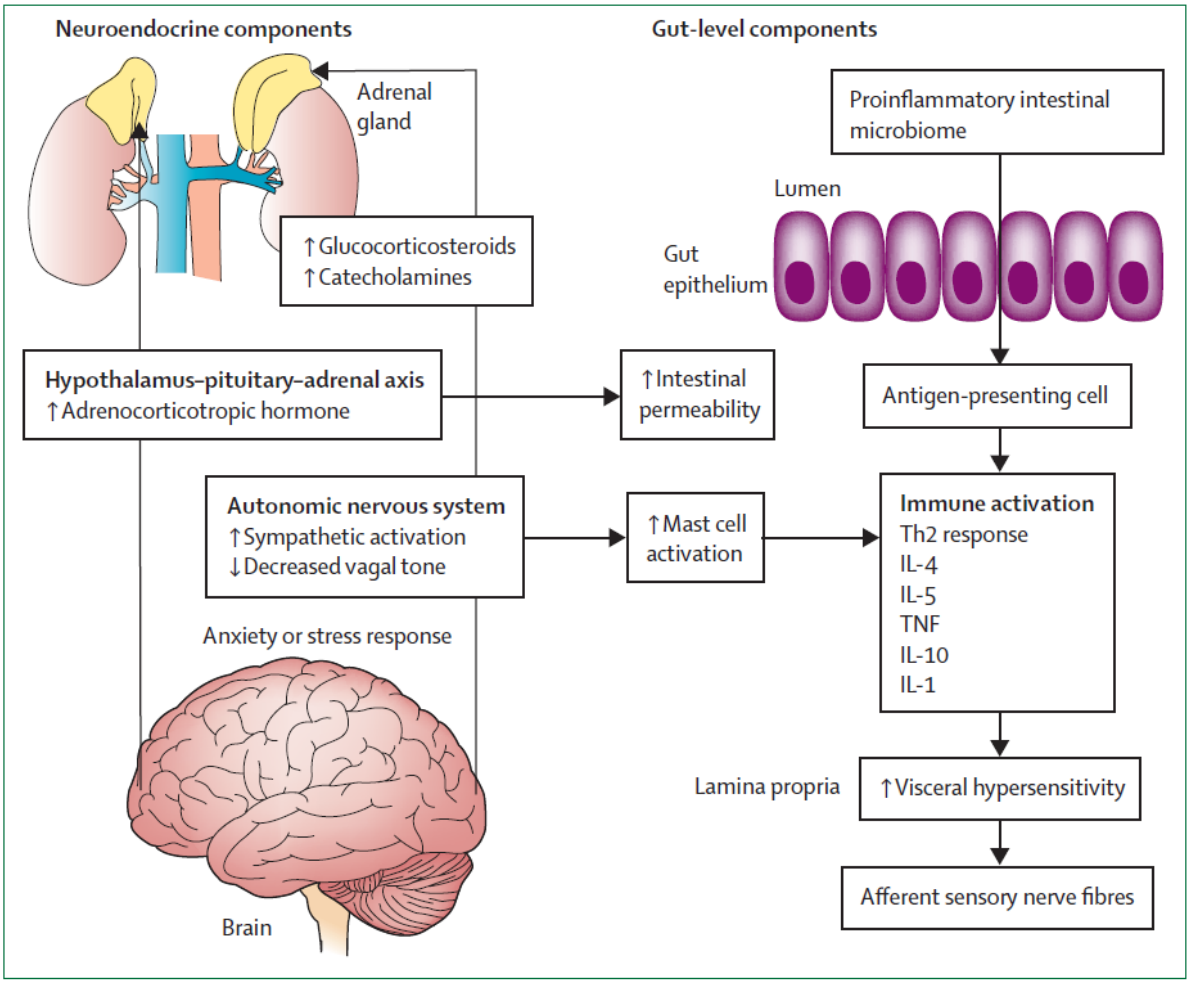


Figure 1.32 - Neuroendocrine pathways involved in the gut-brain axis (Gracie et al., 2019).

1.9 Aims of Thesis

1.9.1. Chapter Two Aims

- To determine the effect D-alanine has on ACTH in the Anterior Pituitary using AtT20 anterior pituitary corticotroph cells – both extracellularly (secreted ACTH detected by ELISA) and intracellularly (via western blots). For comparison, D-serine effects on ACTH will be measured as well.
- To confirm effective production of ACTH by these cells by using the positive stimulator of ACTH, CRH, and determining how D-amino acids effect ACTH in CRH stimulated cells.
- To investigate the Mechanism behind potential D-amino acid effects on ACTH by measuring NMDARs and GlyRs in AtT20 cells, and additionally the activity of GlyRs by using its agonist taurine as a positive control, and antagonist strychnine to confirm its role in these cells.

1.9.2. Chapter Three aims

- To determine the D-amino acid effect in activated corticotroph cells via dexamethasone on ACTH secretion, and POMC via SGK1.
- To investigate the role of Asc-1 transporter in D-amino driven effects on ACTH secretion in AtT20 cells by transfecting cells with Asc-1 transporter (human SLC7A10).
- To determine what receptors may have a role to play in overexpression of Asc-1 in AtT20 cells by measuring NMDARs and GlyRs by Western blot and qPCR.

1.9.3. Chapter Four aims

- To investigate the presence of Asc-1 and ASCT2 transporters in the gut by using human colorectal cancer cells, Caco-2.
- To determine the effects D-amino acids may have on the gut – especially with regards to receptor changes (NMDARs and GlyRs) and inflammation (cytokine measures).
- To determine if changes in cytokines are mediated by NMDARs by using MK-801 NMDAR antagonist.
- To investigate the effects of overexpression of Asc-1 on Caco-2 cells with regards to inflammation and changes in receptor expression, both in proteins and mRNA.

1.9.4. Chapter Five Aims

- Confirm if observed *in vitro* effects of D-amino acids on ACTH secretion in AtT20 cells translate to hormonal effects *in vivo* using CD1 mice.
- To determine the effects of D-amino acids on mRNA expression of POMC, D-amino acid transporters and receptors in various brain regions in CD1 mice.
- To determine the importance of D-alanine in the brains by using Germ-Free (GF) mice on NMDAR, glycine, amino acid transporters and SGK1 expression.

CHAPTER TWO: THE EFFECTS THE D-AMINO ACIDS, D-ALANINE AND D-SERINE, HAVE ON MOUSE CORTICOTROPH CELLS, AtT20.

2.1. Introduction

In this chapter I look at *in vitro* effects of D-alanine, which is found in the same cells that secrete ACTH in the anterior pituitary gland. This co-localization may imply a relationship between D-alanine and ACTH. Comparisons will be made with D-serine as it is also a co-agonist of the NMDARs, but unlike D-alanine is endogenously produced in the body and does not rely on gut microbiota for entry into mammals. The mouse corticotroph AtT20 cells are used as a model for the anterior pituitary, which is the primary organ of interest for this thesis as a whole.

2.1.1. The relationship between D-amino acids and ACTH

In the rat, D-alanine is highly concentrated in the ACTH containing cells of the anterior pituitary, suggesting that it may be involved in the modulation of ACTH secretion and/or metabolism, and therefore the activity of the HPA axis (Etoh et al., 2009). Precedents for D-alanine displaying a potential endocrine function arise from studies that show it is concentrated in, and can be secreted from, pancreatic insulin-containing cells (Ota et al., 2014 and Morikawa et al., 2007). Therefore, this may also hold true for D-alanine in the pituitary where, in this instance, it would modulate ACTH release. However, at present there is no information on whether GlyRs, are expressed in the anterior pituitary.

2.1.2. The presence of D-alanine in the anterior pituitary – how do they get there?

As previously mentioned, Etoh et al (2009) determined the high presence of D-alanine in the same cells that secrete ACTH in the anterior pituitary. They hypothesized that the presence of this D-alanine may be due to amino acid transporters, such as Asc-1, which is why I investigated transporter presence in AtT20 cells.

2.1.3. Receptor mediated effects of D-amino acids

A distinctive feature of NMDARs is that they require more than one agonist to be activated. Glutamate, the primary NMDAR agonist, fails to activate the receptor unless a co-agonist binding site is occupied in the GluN1 subunit of the receptor. This co-agonist can be glycine (which has a 10-fold higher presence than D-serine in the brain (Toyoda et al., 2014)), D-serine (which is up to threefold higher affinity for binding to NMDAR than Glycine due to the fact that D-serine makes three additional hydrogen bonds and displaces a water molecule from the pocket), or D-alanine (Rosenberg et al., 2013 and Sakai at el., 2003). Therefore, the high presence of D-alanine in the anterior pituitary may allude to high presence of its relevant receptors in this area as well. Hence, I investigated the Glycine Receptor Alpha 1 subunit (GLRA1), in addition to NMDA receptor subunits.

2.1.4 ACTH signalling in AtT20 cells

POMC is a precursor protein that is responsible for the synthesis of ACTH (Gjerstad et al., 2018). SGK1 is produced in the same cells that secrete ACTH in the anterior pituitary and is relevant for the upregulation of a plethora of membrane ion channels (Reiter et al., 2011). Reiter and co (2011) showed that the established inhibitory negative feedback effect of GCs on POMC is negated in the presence of SGK1, where SGK1 instead caused a

stimulation of POMC in AtT20 cells. Therefore, there seems to be a link between SGK1 and POMC synthesis, and potentially ACTH release.

2.1.5. Positive stimulation of ACTH by CRH

During a stressful event, hypothalamic CRH releases ACTH from the pituitary, which then circulates to the adrenal glands and stimulates the release of CORT (Herman et al., 2016). CRH binding to the relevant receptor in the pituitary stimulates the production of adenylate cyclase, which then sets off cAMP pathway, eventually leading to the release of ACTH from the pituitary. Therefore, CRH is an established potent stimulator of ACTH (Perrin and Vale, 1999), hence my investigation into how D-amino acids presence may alter the usual effects of CRH on ACTH secretion.

2.1.6. Stimulation of GlyRs by Taurine

One of the key propellers of inhibition in the central nervous system (CNS) is glycine, where it exerts this ability by binding to chloride channelled GlyRs, however little is known about GlyR presence and HPA axis control, which is why I sought to investigate this in the anterior pituitary. Taurine is an amino acid that endogenously binds to the GlyR and serves the function of volume regulation and neurotransmission in the CNS (Albrecht and Schousboe, 2005).The alkaloid strychnine antagonizes glycine binding with high affinity, and therefore serves as an inhibitor of the GlyR (Vandenberg et al., 1992). It is therefore a robust model for determining mechanistic involvement of GlyR agonists downstream effects. Further information about glycine receptors can be found in Chapter 1: Introduction.

2.1.8. Aims of Chapter

1. The primary aim of this chapter is to determine the effects D-alanine and D-serine have on ACTH secretion and the molecular mechanisms that regulate ACTH signalling in AtT20 cells. This has been achieved by analysing the culture medium of AtT20 cells and detecting the presence and quantity of ACTH by enzyme linked immunosorbent assay (ELISA), as well as the protein content of ACTH in the cells itself by western blot analysis.

2. In order to elaborate further on the ACTH signalling mechanism, I also investigated the effects of genes and proteins that have been known to affect ACTH signalling, such as POMC, the precursor to ACTH, gene expression, alongside SGK1, which has been linked to POMC expression.

3. ASCT2 and Asc-1 gene expression was also analysed, to determine the presence of the transporters in these cells to ensure that the D-amino acids are sufficiently taken up.

4. NMDARs and GlyRs presence in these cells were investigated by the use of agonists and antagonists. NMDAR subunits GluN1 and GluN3A, as well as GlyR subunit, GLRA1, have been detected by protein and gene expression. This was so I could have a better understanding of the receptor mediated effects of these D-amino acids, which are known agonists, to the above-mentioned subunits.

2.2. Methodology

2.2.1. Cell Culture

The AtT-20/D16v-F2 mouse anterior pituitary cell-line was purchased from ATCC (CRL 1795). Cells were defrosted and grown in initial plating media (Appendix**)**. Initial plating

media consisted of Dulbecco's Modified Eagle's Media (DMEM) (D5546, Sigma Aldrich, United Kingdom), containing 17% Fetal Bovine Serum (FBS) (12103C-500ML, Sigma Aldrich, United Kingdom) and 4 mM L-glutamine (G7513-20ML, Sigma Aldrich, United Kingdom). The cells were initially grown in a T25 flask (83.3910.002, Sarstedt, Germany) in 5 mL of initial plating medium. The following day, another 5 mL of initial plating medium was added, bringing up the volume to 10 ml. The initial plating media was used for the first week, and when the cells reached confluence, the concentration of FBS was dropped to 10%. This 10% FBS DMEM was the growth medium. Cells were maintained at 300 000 cells/ml growth media, and once confluent, were transferred to a T75 flask (Sarstedt, 83.3911.002). Cells were then passaged every 2-3 days.

2.2.2 Protocols for Treatment of AtT20 cells

In order to investigate the effects of the agonists, D-alanine and D-serine, on the anterior pituitary, AtT20 cells were exposed to D-alanine, D-serine and Taurine at specific concentrations and times, outlined below (**Figure 2.1** and **Figure 2.3)**. As a positive control for ACTH secretion, CRH effects were also observed in this cell line, detailed below (**Figure 2.2)**.

2.2.2.1. The effect that D-amino acids have on AtT20 cells

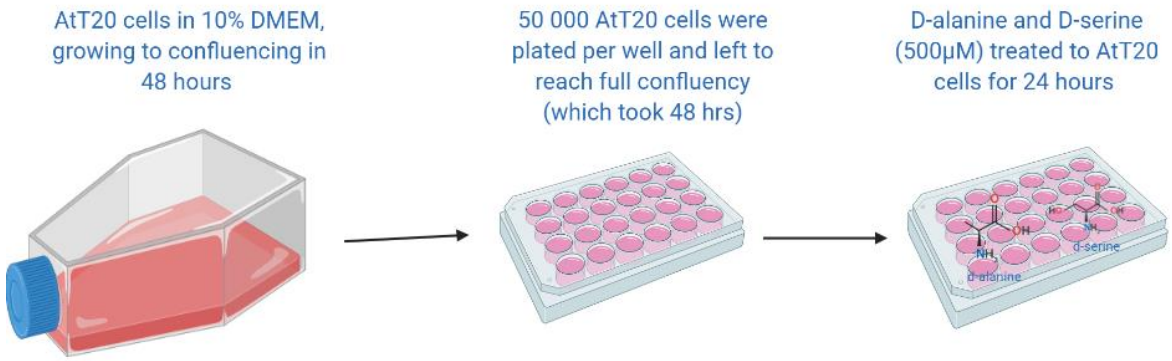


Figure 2.33 – Diagrammatic representation of treatment schedule for AtT20 cells treated with D-amino acids. 50 000 cells were plated into each well of a 24-well plate, cells were then left for 48 hours to reach confluency. Individual wells then received 500 µM of D-alanine or D-serine for 24 hours, or phosphate buffer saline (PBS) as a control. The concentration of D-amino acids was determined by dose-dependent preliminary studies. In order to achieve a sample size of 6 replicates, 3 X 24-well plates were used, with 3 wells pooled together to constitute one sample. Image made with Biorender.com

2.2.2.2. Stimulation of ACTH secretion by CRH

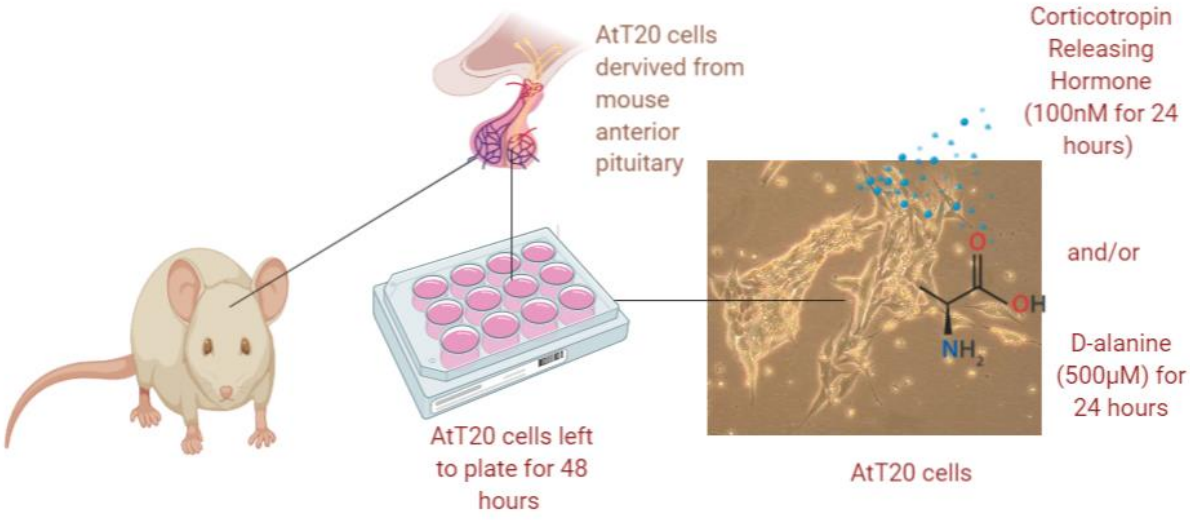


Figure 2.34 - Diagrammatic representation of treatment schedule for AtT20 cells treated with CRH in the presence, or absence of D-alanine. As explained in the previous set of experiments, 50 000 AtT20 cells/ well were left to grow to confluency in a 24-well plate, which took 48 hours. These corticotrope cells were then treated with 100 nM of CRH, in the presence, or absence, of D-alanine (500 µM) for 24-hours. The concentration of CRH used and treatment times were based on literature

2.2.2.3 Stimulation of GlyRs by taurine/D-alanine or Inhibition of GlyRs with strychnine

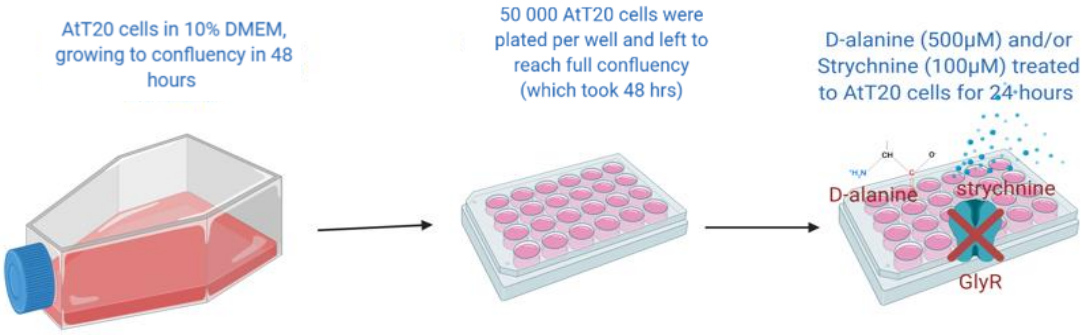


Figure 2.35 - Diagram of treatment schedule of AtT20 cells treated with Taurine/D-alanine or strychnine. 50 000 AtT20 cells were left to reach confluency over 48 hours, and then treated with 500 µM of Taurine/D-alanine presence or absence of a glycine antagonist, strychnine (100 µM) for 24 hours. N = 6, consisting of 3 wells of cells pooled together as 1 sample. PBS was used as the control. Concentration of strychnine was determined based on the published literature (Vandenberg et al., 1992).

2.2.3. Experimental Procedures

To determine the molecular effects of the above described treatment protocols, western blot, ELISA, and qPCR analysis, were utilized. Further details of these protocols are described below.

2.2.3.1. ACTH ELISA protocol

Following the treatment schedules, culture medium of cells were collected for analysis by ELISA assay. This involved adding 50 µl of ACTH standard (4034845.0500, Bachem, Switzerland) or sample (cell culture medium of treated cells) into each well of a 96 well plate, as well as 2µl of glutaraldehyde (G6257, Sigma Aldrich, United Kingdom).

This solution was then left in the incubator (UY-79520-16, Cole Parmer, United Kingdom) at 37°C for 45 minutes. The plate was then washed four times with PBS, and 200 µl/well

0.05% goat serum (G9023, Sigma Aldrich, United Kingdom) was used for blocking. This lasted 30 minutes at 37°C, whilst shaking slowly.

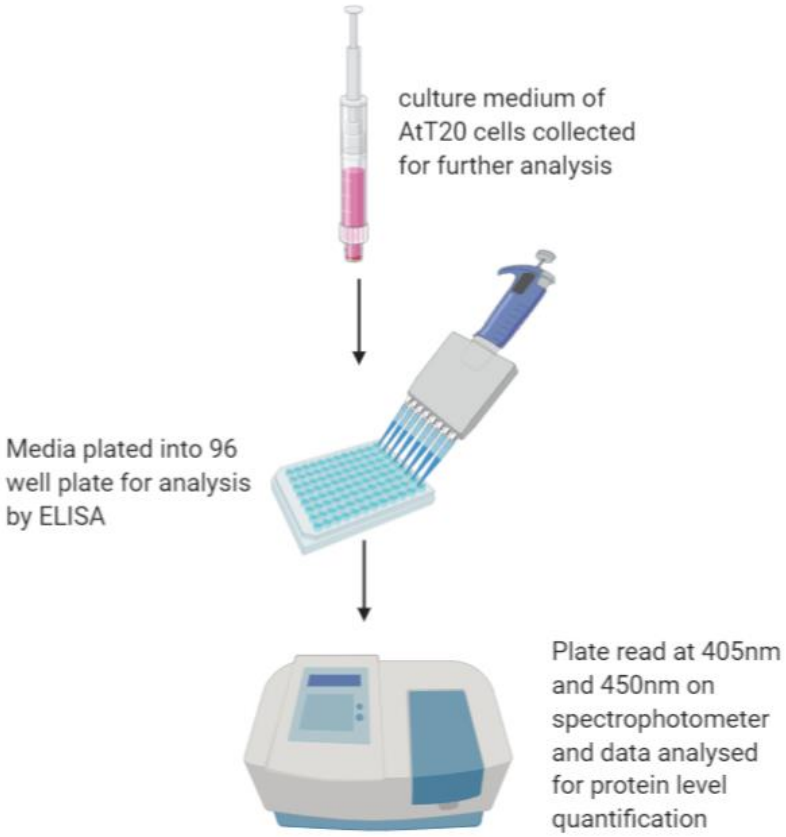


Figure 2.36 - Diagrammatic summary of an ELISA assay. Image made with Biorender.com

Blocking solution was discarded and the ACTH primary antibody (200µl, #701293, Invitrogen, United Kingdom) was added at a concentration of 1:200 for 1 hour. The plate was washed four times, followed by incubation with secondary goat antirabbit antibody (200µl, #1706515, Bio-Rad, United Kingdom) for 1 hour. The plate was then washed four times and then a chromogenic substrate (A3219-100mL, 3- ethylbenzothiazoline-6-sulfonic acid, Sigma Aldrich, United Kingdom) was added to each well at 100 µl for 2 minutes. As shown in **Figure 2.4** above the plate was then read on the Spectramax190 spectrophotometer (DS849B, Beckman Coulter, California USA) at wavelengths of 405nm and 450nm. ELISA

results were determined drawing up a standard curve on Microsoft Excel and quantifying unknown variables.

2.2.3.2. Western Blot Protocol

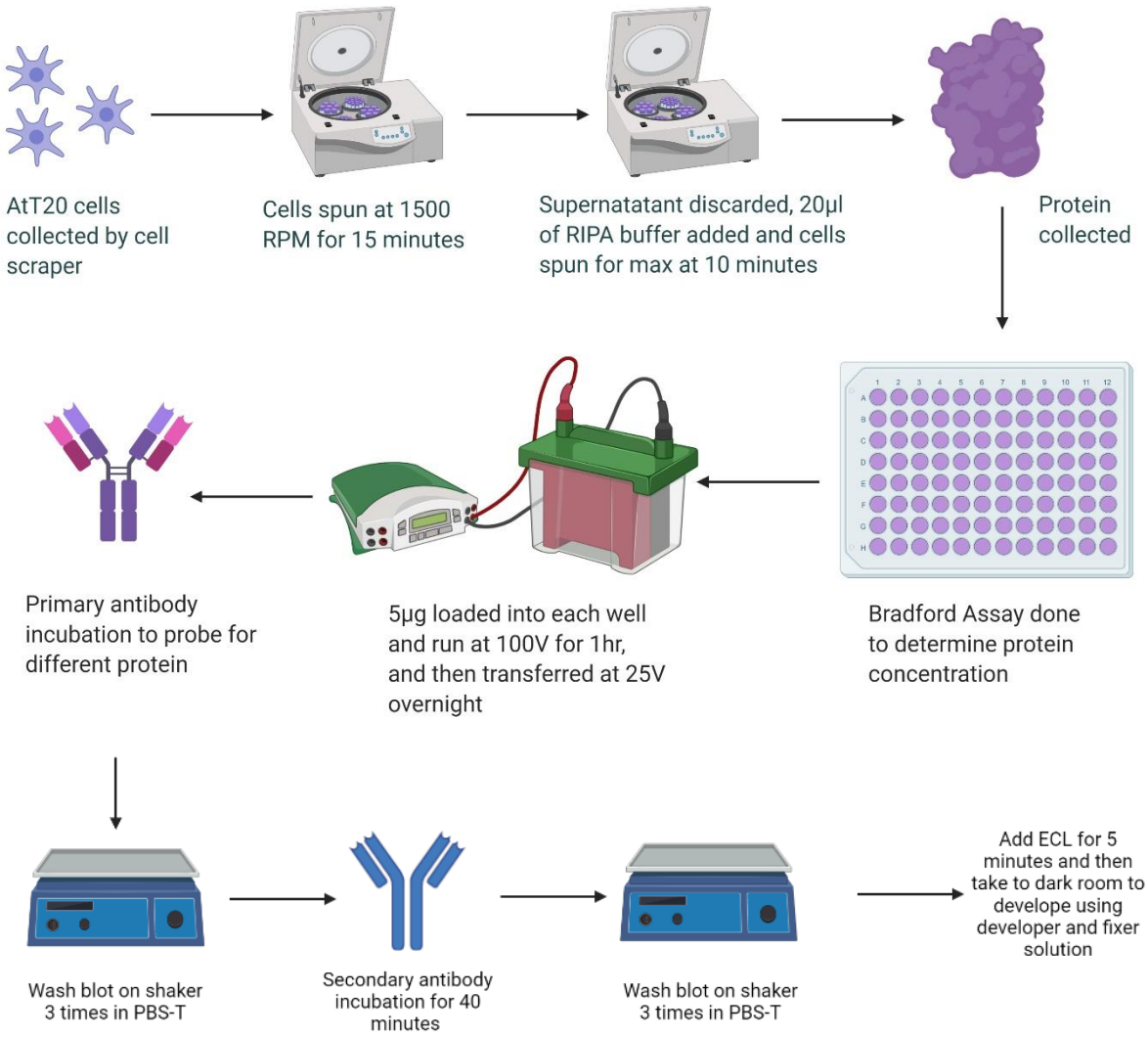


Figure 2.37 - Diagrammatic representation of western blot analysis. Image made with Biorender.com

Following the respective treatments, cells were collected using a cell scraper (#83.1832, Sarstedt, Germany) and collected in PBS (D8662, Sigma Aldrich, United Kingdom), and centrifuged at 1500 RPM for 5 minutes. The pellets were then resuspended in 20 µl of RIPA (R0278, Sigma Aldrich, United Kingdom), and the mixture was centrifuged at max speed for 10 minutes. The lysed protein (2 µl of) was added to 18 µl of distilled water for a 1:10

dilution. Aforementioned diluted protein samples (10 µl) were added to the wells of a 96 well plate (82.1581. Sarstedt, Germany), and then 200 µl of Bradford reagent assay (#500205, Bio-Rad, United Kingdom) was added to these diluted protein samples. The product of this combination was left to sit for 5 minutes and then analysed on the spectrophotometer at 590nm and 450nm.

These readings were then analysed using Microsoft Excel to determine protein levels of each sample. Calculations to determine how much sample is needed for 5 µg of protein per sample were completed, and then each sample was topped up to 18 µl with RIPA buffer. Samples containing RIPA and lysed protein at 5 µg had 4.5 µl Laemmli buffer (#1610747, Bio-Rad, United Kingdom) added to it. Samples were then boiled for 10 minutes. Prior to boiling, samples were transferred to screw capped 1.5 mL Eppendorf to prevent evaporation during heating.

As seen in **Figure 2.5** above, each sample (15ul) was loaded onto a well of the western gel (4-20% Mini-PROTEAN TGX precast protein gels, #4561096, Bio-Rad, United Kingdom), and the gel was run at 100 V for 1.5 hours. The gel was then transferred onto a western blot polyvinylidene difluoride (PVDF) membrane (Immobilon-P membrane, Merch Millipore, Germany) by transferring it overnight at 25 V.

The resultant blot was probed for protein by initially blocking it in 5% blocking buffer (dry non-fat milk in distilled water) for 1 hour, shaking at room temperature.

All antibodies were diluted in 2% blocking buffer (dry non-fat milk in distilled water). Primary antibody at various concentrations (2 µg/mL for **ACTH**, 1:800 for **GLRA1**, 1:1000 for **GluN1** and 1:1000 for **GluN3A**) were added to the blot for 1 hour.

The blot was then washed 3 times for 10 minutes with wash buffer.

Secondary antibody (anti-rabbit for **ACTH, GLRA1** and **GluN3A** at 1:10 000 and anti-mouse for **GluN1** at 1:5000) was added for 1 hour (with shaking).

The blot was washed three times with PBS-T and then once with PBS for 5 minutes. The blot was then activated with enhanced chemiluminescence (ECL) (RPN2236, GE Healthcare, United Kingdom) and carried over to the dark room where it was developed using developer and fixer reagent, and film (Appendix**).**

The resultant film showed the binding of the primary antibody to the respective protein of interest on the blot. A molecular ladder (#1610374, Precision Plus Protein Dual Colour Standards, Bio-Rad, United Kingdom) was loaded into the first well of the gel, so that it can be used as a marker for protein bands in experiments. Quantification was achieved using densitometry measurements on AlphaImager 3400, Alpha Innotech, California, USA. The densitometry measures for each band was measured and normalized to housekeeping protein (conjugated β-actin (ab4900, Abcam, United Kingdom)), and then normalization to control to eventually have treatments are presented in relation to control.

2.2.3.3. qPCR Protocol

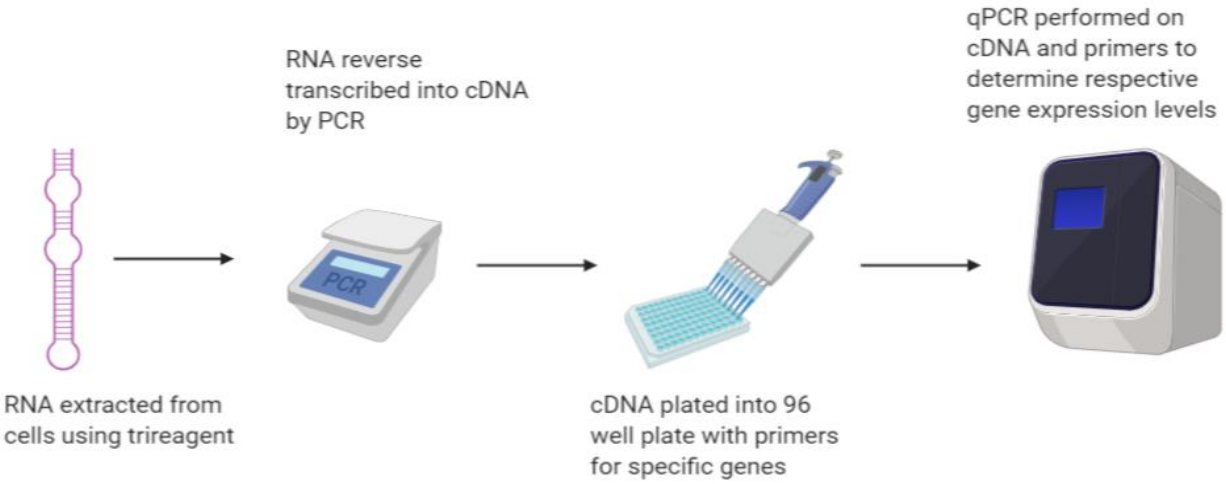


Figure 2.38 - Diagrammatic breakdown of qPCR protocol. Image made with Biorender.com

Following treatment for the allotted time and respective concentrations of amino acids, cells were collected for mRNA analysis by adding 200 µl of Tri-reagent (93289-25mL, Sigma Aldrich, United Kingdom) to each sample. Two treatment samples were combined for a result of 400 µl of cells in Tri-reagent.

Chloroform (#288306-2L, Sigma Aldrich, United Kingdom, 80 µl) was added to each sample, and they were spun down at max in the centrifuge for 5 minutes. Supernatant was collected, and 300 µl of isopropanol (I9516-1L, Sigma Aldrich, United Kingdom) was added to each sample. Samples were left on ice for 20 minutes in isopropanol, and then spun at max in the centrifuge for 10 minutes.

The pellet was collected and resuspended in 500 µl 70% ethanol (#1042867, Fisher Scientific, United Kingdom), and then centrifuged at maximum speed for 5 minutes. The supernatant was discarded and the pellet was left to air dry for 40 minutes. The dried pellet was then resuspended in 10 µl nuclease free (NF) water (#4387936, Thermofisher Scientific,

United Kingdom). The concentration of RNA was measured by spectrophotometry (NanoDrop^{TM} 1000 Spectrophotometer, Thermo Fisher Scientific, United Kingdom), at a wavelength of 260nm.

Prior to reverse transcription, contaminating DNA was removed by adding 2 µl of DNAse (M6101, Promega, United Kingdom) to 2000ng of each sample in a total volume of 10ul made up with NF water, and incubated in a Thermal Cycler (Veriti Thermal Cycler, Applied Biosystems, Thermo Fisher Scientific, United Kingdom) at 37oC for 20 min, and then 70°C for 5 min, to deactivate DNAse. A total of 5ul of these samples were then pipetted into tubes containing 5ul of a reverse transcription reaction mix (Appendix). Negative controls were achieved by replacing reverse transcriptase (RT) buffer with NF water. This was placed in the Thermal Cycler for 1 hour and the resultant cDNA diluted to 50 ng/µl.

Each sample was further diluted to 3 ng/µl, and 12 ng of cDNA was added to each well of a 96 well plate. Along with this, the respective primer for gene of interest (**Appendix**) was added to each well, at a ratio of 1 forward primer: 1 reverse primer: 6 SYBR reagent. There was 4 µl of cDNA and 8 µl of reaction mix in each well. The plate was then placed in the qPCR machine (Quant Studio 6 Flex, Thermofisher, United Kingdom) for 90 minutes. With regards to qPCR, cycle threshold (Ct) values were extracted from the qPCR machine and processed on Microsoft excel, and then normalized to housekeeping gene, and then subjected to POWER analysis followed by normalization to control so that treatments are presented in relation to control.

2.2.4. Statistical Analysis

Data initially processed on Microsoft Excel was then transferred onto SPSS statistics (Version 26) were initial statistics of One-Way ANOVA was run, and if significant, post-hoc analysis via Tukey's Test was done to determine significant differences between

treatment groups. Graphs were then drawn using GraphPad Prism (Version 9). Results are presented as mean ± SEM.

2.3. Results

2.3.1. The effect of D-amino Acids on ACTH secretion in AtT20 cells

To determine the D-amino acid driven effects in corticotrope mouse cells, my initial question delved into the effects D-alanine and D-serine had on ACTH signalling. As previously described (**Figure 2.4**), ELISA assay was utilized to analyse the cell culture medium of cells treated with D-alanine, and D-serine. I found that there was a significantly lower level of ACTH ($F_{2,51}$ = 6.38, p = 0.03, **Figure 2.7**) in the cultured treatment medium of cells treated with D-alanine (p = 0.004, -48%) and D-serine (p = 0.025, - 38%) when compared to PBS control.

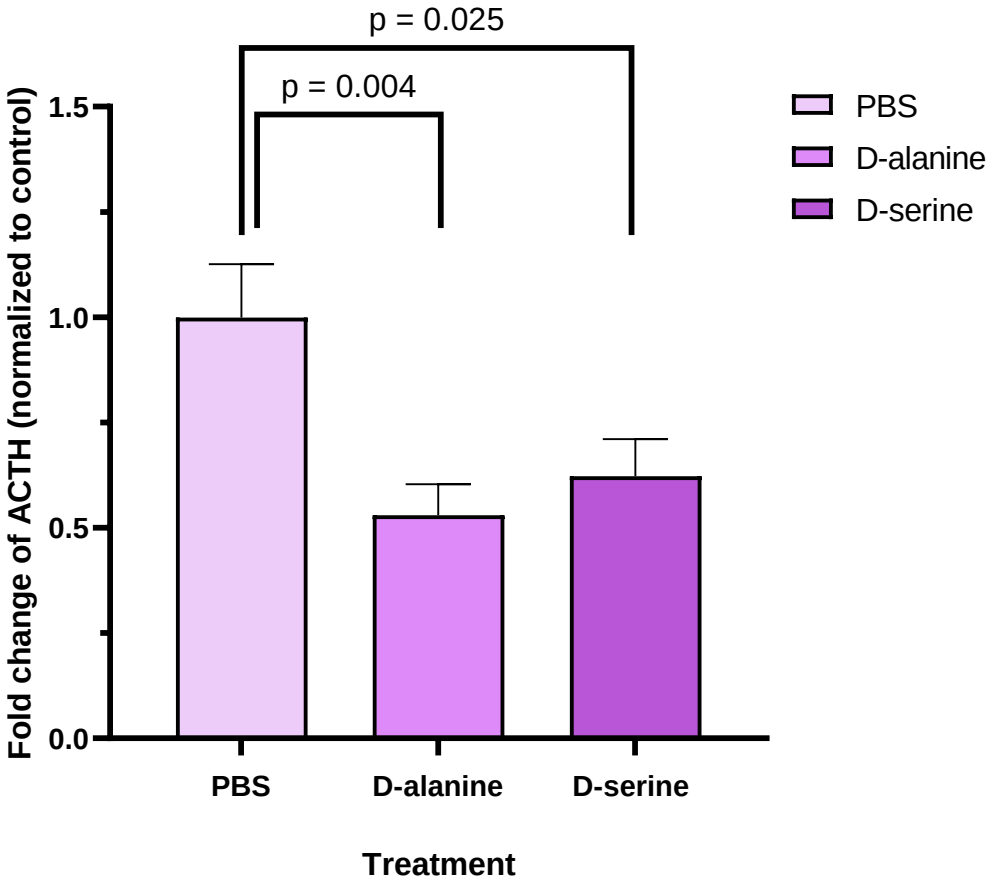


Figure 2.39 - Fold change of ACTH concentrations in the culture medium of 150 000 AtT20 cells treated with D-alanine or D-serine (500 µM), with PBS as a control, for 24 hours. Cells treated with D-alanine or D-serine had significantly lower levels of ACTH in their culture medium ($p = 0.004$, -48% and $p = 0.025$, -38% respectively) when compared to control. N=12.

2.3.2. D-amino acid effects on NMDAR and GlyR in AtT20 cells

Following the observation of a suppressive effect on ACTH secretion in anterior pituitary cells, I sought to determine what drives these effects, and first looked at the respective receptors that these agonists bind to – GlyRs and NMDARs.

There was a significant effect in GLRA1 protein expression ($F_{3,17} = 3.67$, $p = 0.033$, **Figure 2.8b)** in cells treated with D-alanine ($p = 0.023$, +65%) when compared to controls, but not D-serine. However, there was a D-serine effect ($F_{2,9} = 5.63$, $p = 0.026$, **Figure 2.8c)** on GLRA1 mRNA, but there was no effect of D-alanine on GLRA1 gene expression levels in the same cells. D-serine treated cells had lower GLRA1 levels compared to controls ($p = 0.039$, - 66%).

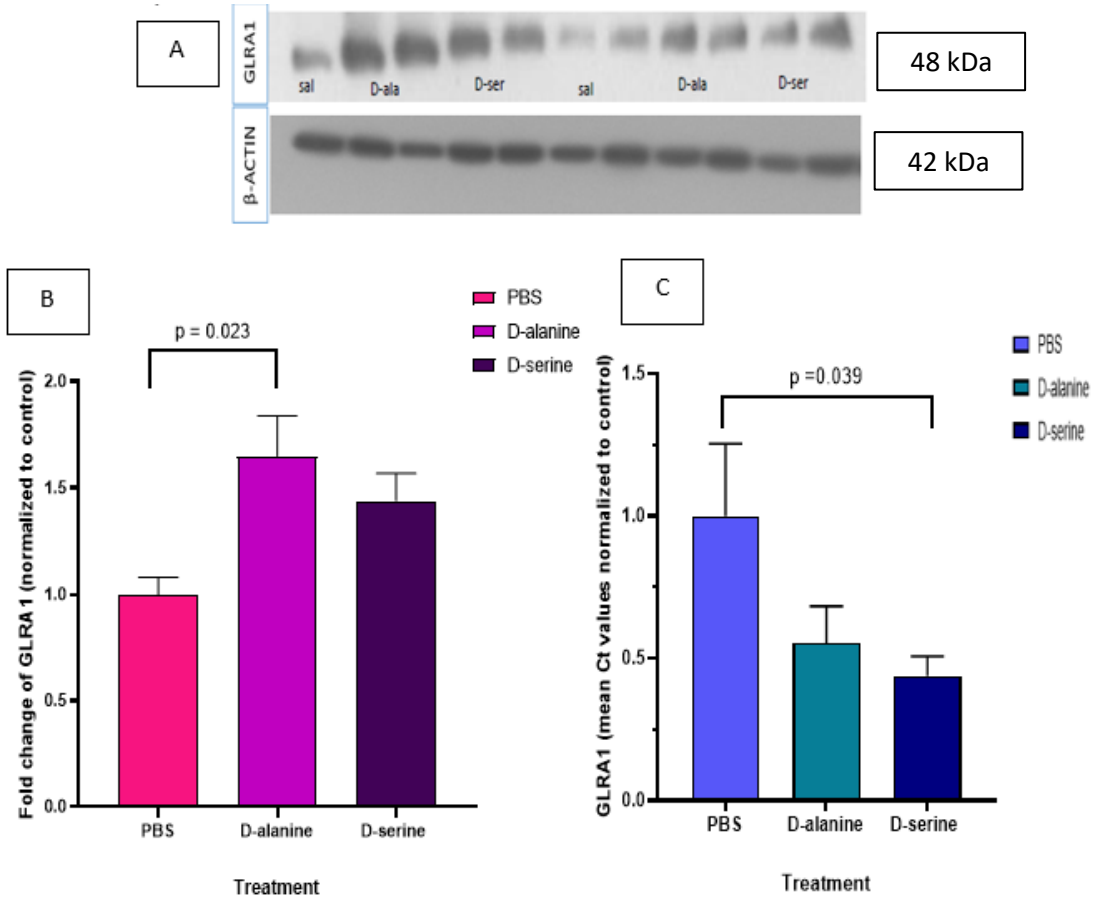


Figure 2.40 - Fold change of GLRA1 immunoreactivity measured by western blot (A and B) and mRNA abundance by qPCR (C) in 150 000 AtT20 cells treated with D-alanine or D-serine (500 µM), with PBS as a control, for 24 hours. B) Cells treated with D-alanine had significantly greater levels of GLRA1 ($p = 0.023$, +65%) when compared to PBS. C)There was a significant decrease in GLRA1 gene expression between cells exposed to D-serine compared to PBS ($p = 0.039$, -66%). Data is the mean of 6 replicates.

There were no changes in GluN3A (**Figure 2.9)** or GluN1 (**Figure 2.10)** protein or gene expression levels in anterior pituitary cells exposed to D-alanine or D-serine.

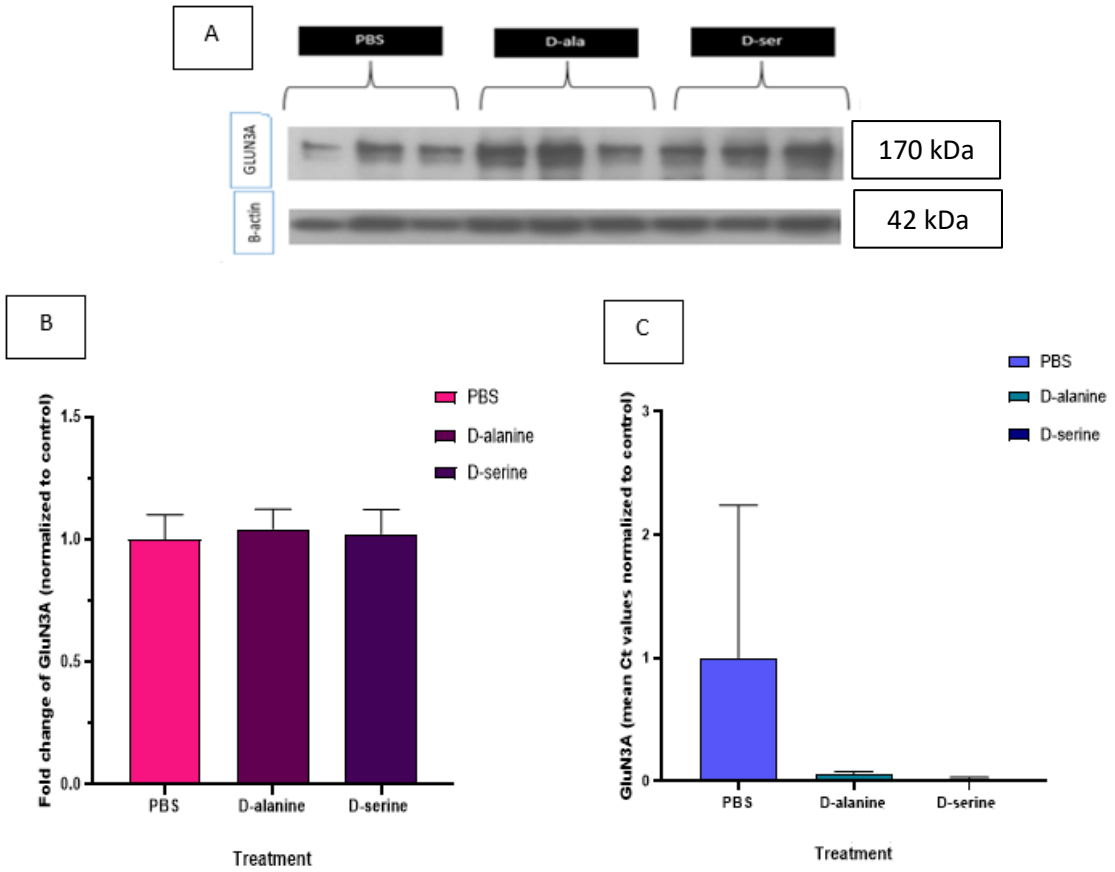


Figure 2.41 - GluN3A protein levels where measured (A and B) and gene expression quantified (C). This was achieved by treating 150 000 AtT20 cells with D-alanine and D-serine (500 µM), with PBS as a control, for 24 hours. There were no significant differences in GluN3A gene or protein expression between the different treatment groups. N= 6.

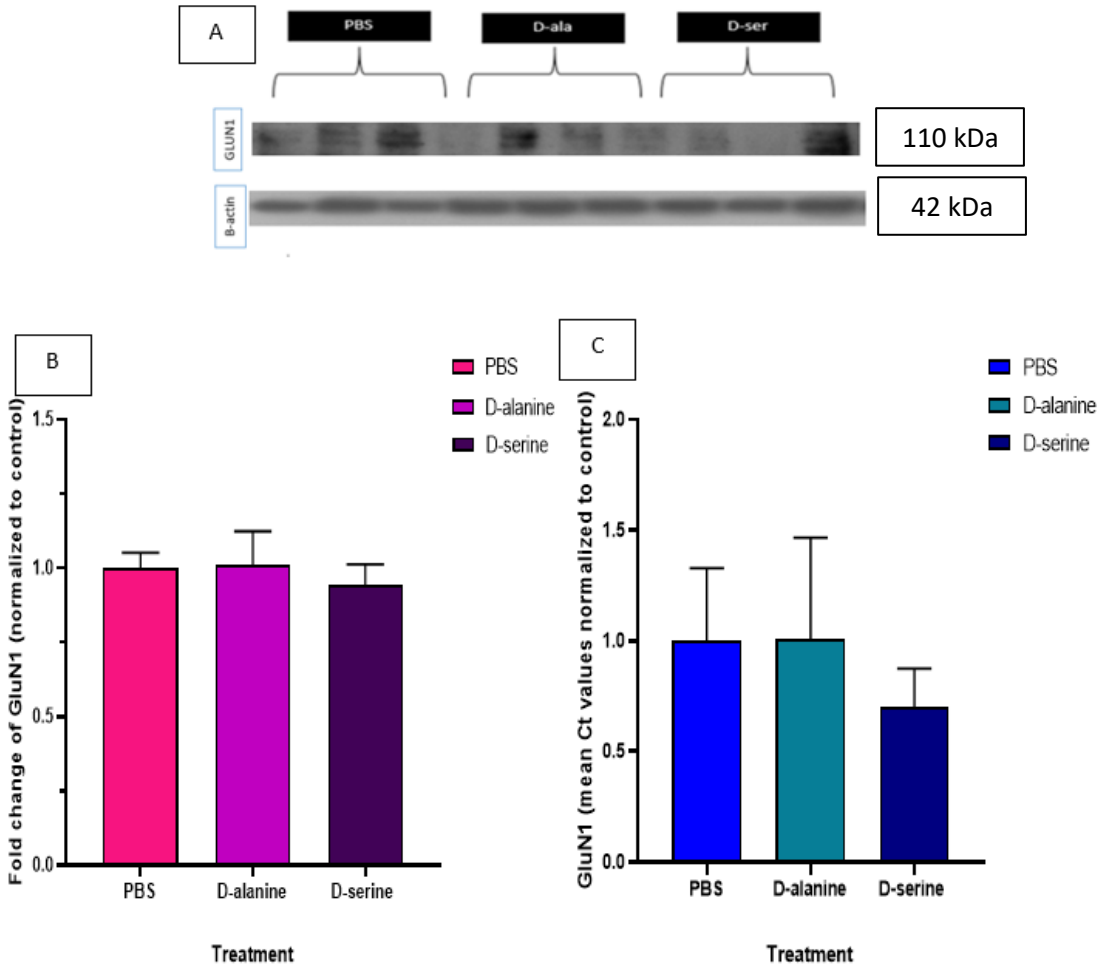


Figure 2.42 - GluN1 levels were measured for protein expression (A and B) and mRNA abundance (C). This was achieved by treating 150 000 AtT20 cells with D-alanine and D-serine (500µM), with PBS as a control, for 24 hours. There were no significant differences in GluN1 gene or protein expression between the different treatment groups. N= 6.

2.3.3. D-amino acid effects on the SGK1-POMC system

Based on the inhibition observed in ACTH secretion, I sought to understand where this modulation took place in the ACTH signalling process, and therefore looked at the upstream ACTH regulator, POMC, which is a precursor of ACTH, and also interacts with the SGK1 system (Reiter et al., 2011). Both D-amino acids affected the SGK1 system

($F_{3,18}$ = 19.06, p = 0.000, **Figure 2.11a**) There was a significant increase in SGK1 mRNA (**Figure 2.11a**) levels in AtT20 cells treated with both D-alanine and D-serine (p = 0.005, +316% and p = 0.007, +268% respectively) when compared to control.

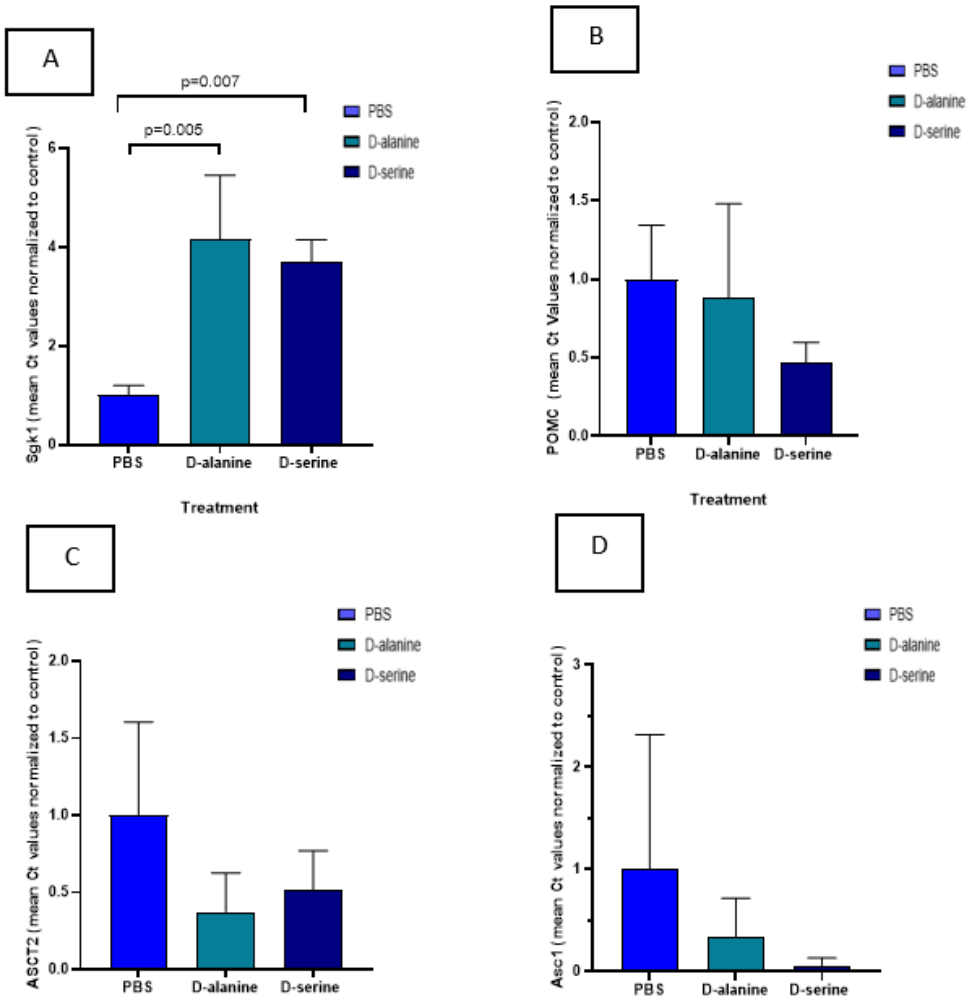


Figure 2.43 - Gene expression analysis was done on AtT20 cells (150 000 cells) treated with 500 µM D-alanine or D-serine for 24 hours, with PBS as a control. There were 6 replicates, and gene expression was achieved by qPCR. A) SGK1 levels were significantly higher in cell treated with D-alanine (p = 0.005, + 316%) and D-serine (p = 0.007, +269%) when compared to PBS treated cells (the control). There were no significant differences in POMC (B), ASCT2 (C) and Asc1 (D) gene expression between the different treatment groups.

2.3.4 D-amino acid effects on their transporters, Asc-1 and ASCT2

Since there was an effect on both D-alanine and D-serine, I investigated if their transporters may somehow be linked to this suppressive effect, since they both carry D-alanine and D-serine. Whilst detecting the presence of transporters in the anterior pituitary cell line using qPCR analysis, there were no significant alterations in the treatment groups (**Figure 2.11c and Figure 2.11d**).

2.3.5. Positive stimulation of ACTH by CRH

CRH is the natural stimulator of ACTH, so I sought to determine if the AtT20 cells were a good working model for corticotropes by observing CRH mediated effects on ACTH secretion, as well as molecular changes in AtT20 cells itself.

D-alanine in the presence of CRH affected ACTH secretion ($F_{3,20} = 3.43$, $p = 0.037$, **Figure 2.12b)** as well as intracellular ACTH levels ($F_{3.19} = 9.67$, $p = 0.00$, **Figure 2.12c).** Specifically, there was a significant reduction in ACTH levels in culture medium of cells treated with both CRH and D-alanine compared to cells treated with just D-alanine ($p = 0.046$, - 11%), whilst there was an increase in the intracellular protein expression of these same cells ($p = 0.029$, +24%). There is also a significant increase in intracellular ACTH protein levels in cells treated with CRH and D-alanine when compared to controls ($p = 0.002$, +37%) (**Figure 2.12c**).

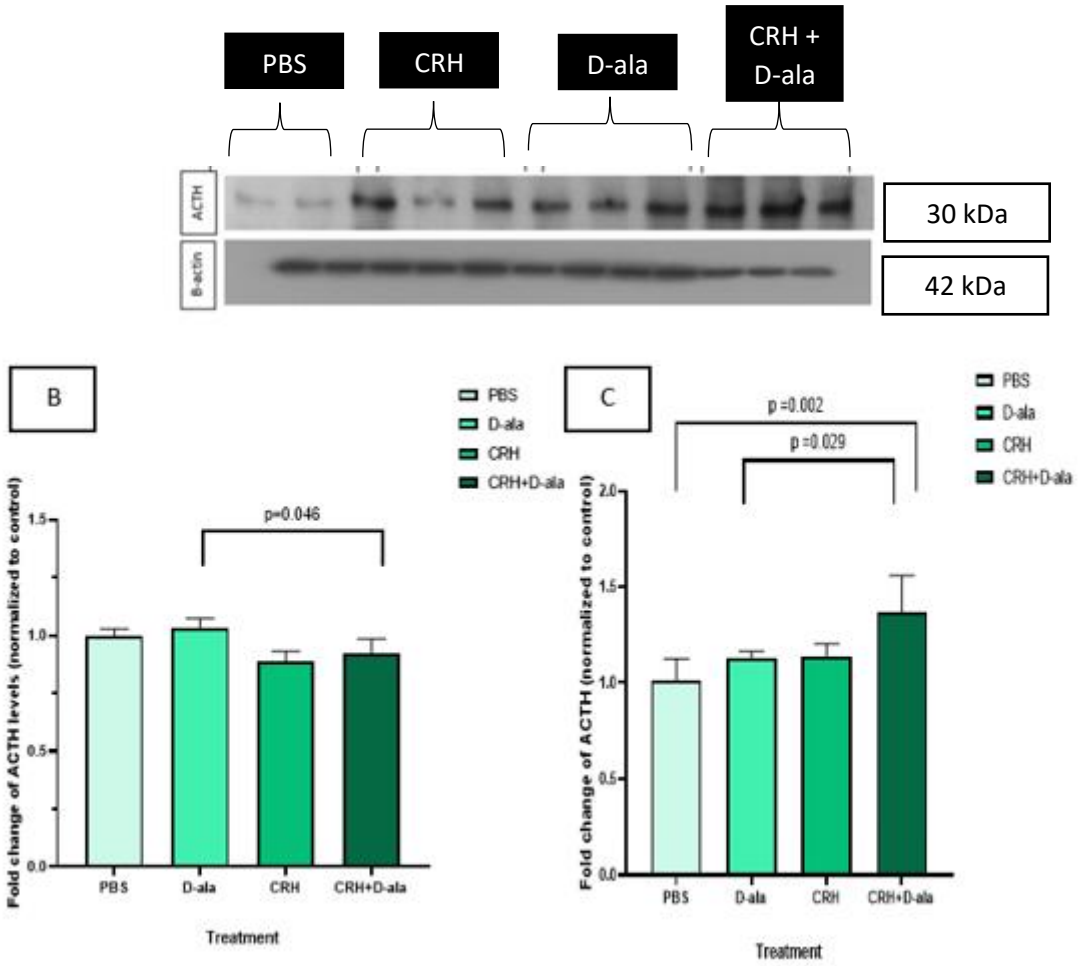


Figure 2.44 - Fold change of ACTH measured by ELISA (B) in the culture medium of 150 000 AtT20 cells treated with D-alanine (500 µM) in the presence or absence of CRH (100 nM), with PBS as a control, for 24 hours. Cells treated with D-alanine and CRH had significantly lower levels of ACTH in their culture medium ($p = 0.046$, -11%) in comparison to cells treated with just D-alanine. C) This graph depicts the intracellular protein ACTH levels in 150 000 AtT20 cells treated with 500 µM D-alanine in the presence or absence of CRH (100 nM). There was a significantly higher ACTH expression in cells treated with CRH and D-alanine compared to PBS ($p = 0.002$, +37%), as well as CRH and D-alanine compared to cells treated with just D-alanine ($p = 0.029$, +24%). Data is the mean of 6 replicates.

2.3.6 Gene expression effects of CRH in AtT20 cells in the presence or absence of D-alanine

To explain the observed ACTH effects, genetic expression of receptors (GLRA1, GluN1, GluN3A), transporters (Asc-1 and ASCT2), as well as regulators of ACTH signalling (SGK1 and POMC) were measured. There were no significant alterations between any of the treatment measures (**Figure 2.13 – Figure 2.14**)

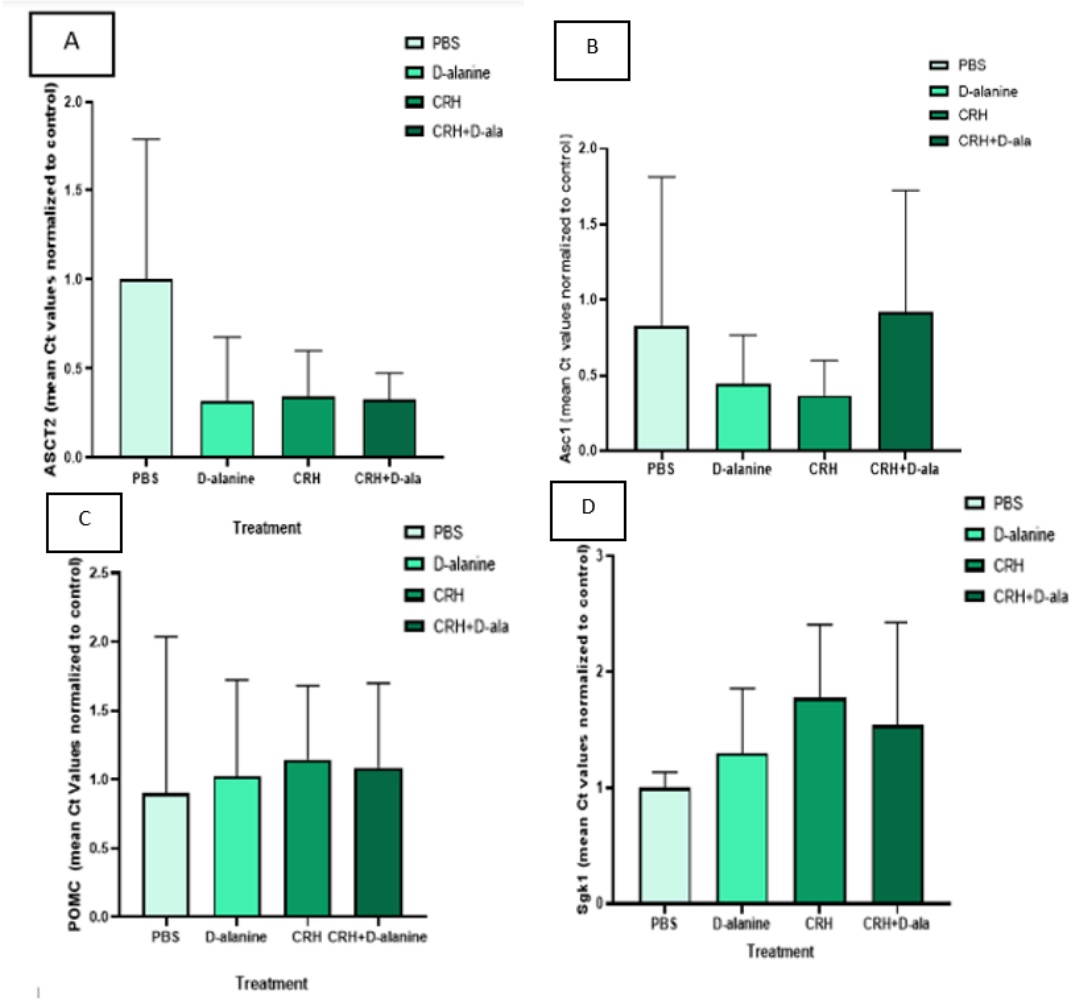


Figure 2.45 - Gene expression analysis was done on AtT20 cells (150 000 cells treated with 100 nM CRH and/or 500 µM D-alanine for 24 hours), with PBS as a control. There were 6 replicates, and gene expression was achieved by qPCR. There were no significant differences between the ASCT2 (A), Asc-1 (B), POMC (C) or Sgk1 (D) levels between any of the treatment groups.

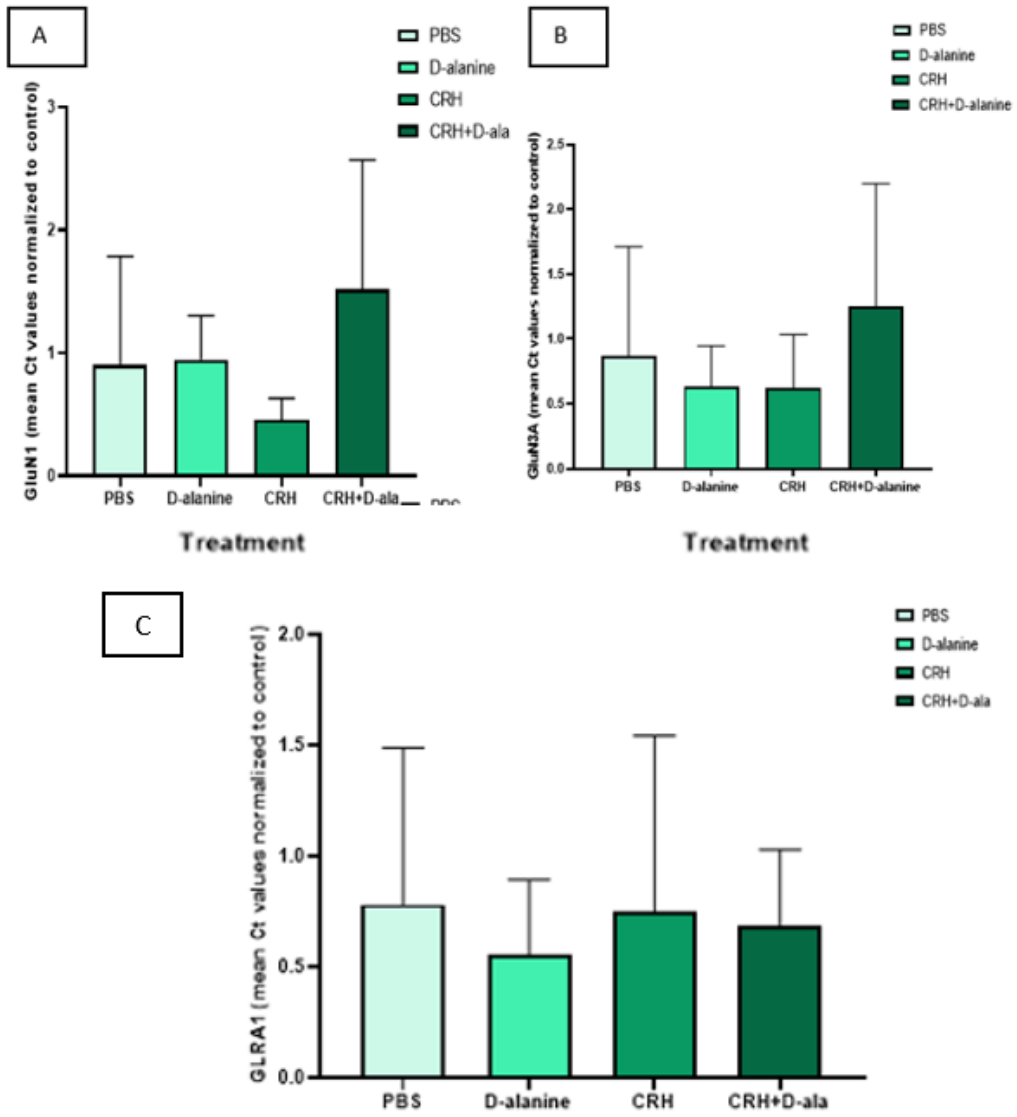


Figure 2.46 - qPCR for mRNA quantification was done on AtT20 cells treated with 100 nM CRH in the presence or absence of 500 µM D-alanine for 24 hours. There was no significant difference in gene expression levels of GluN1 (A), GluN3A (B), or GLRA1 (C). Data are mean of 6 replicates.

2.3.7. Stimulation of GlyRs by Taurine and inhibition of GlyRs by Strychnine

Based on the D-alanine effects on the GlyRs, experiments were conducted to better understand the effect of this receptor in these cells, and this was achieved using its endogenous agonist – Taurine, as well as it's antagonist – Strychnine.

Taurine significantly reduced GLRA1 protein levels when compared to PBS control (p = 0.041, - 35%), but there were no corresponding alterations in mRNA levels (**Figure 2.15**).

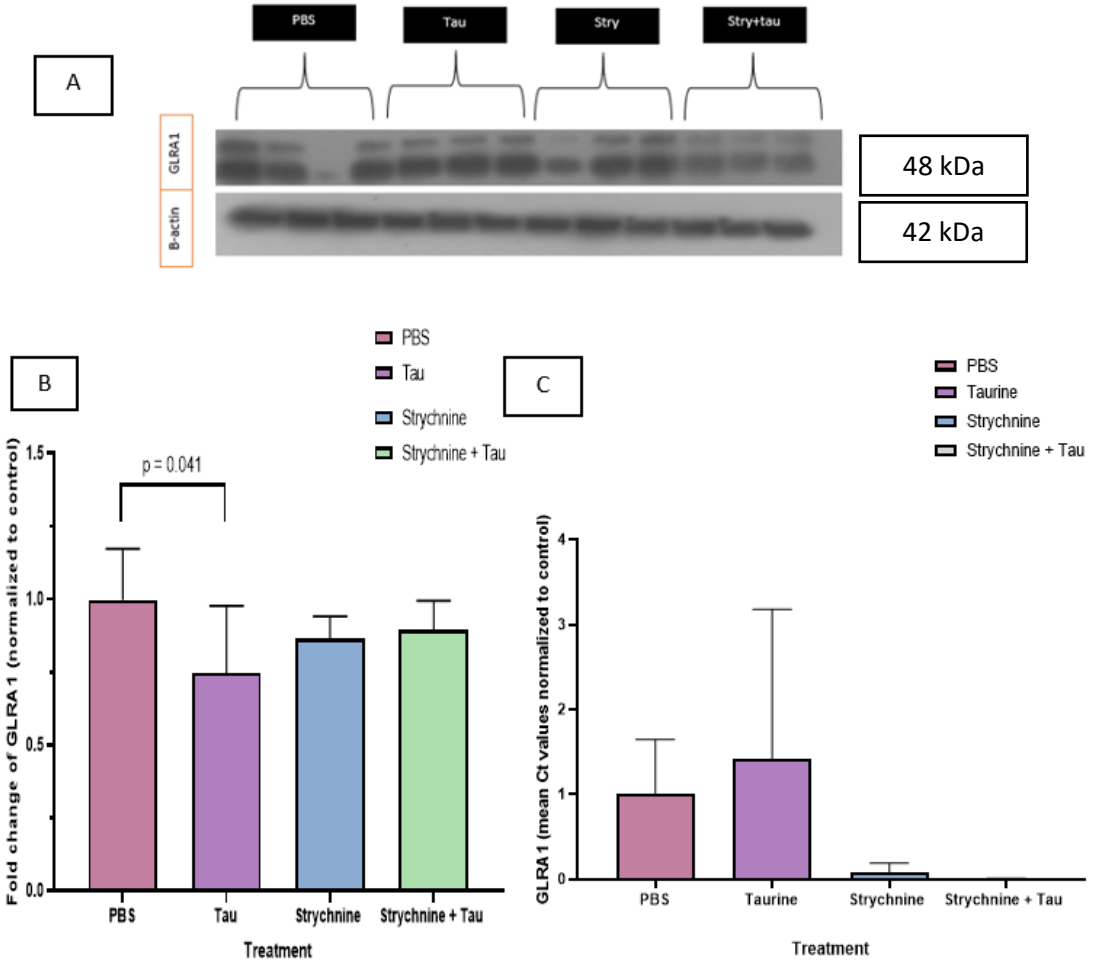


Figure 2.47 - Protein expression of GLRA1 was measured by western blot (A and B), where AtT20 cells (150 000 cells treated with 500 µM Taurine and/or 100 µM Strychnine for 24 hours) had a lower level of protein expressed in cells treated with just Taurine (p = 0.041, -35%) compared to negative control PBS. C) There was no significant change in gene expression of GLRA1 in the treatment groups. Data is the average of 6 replicates, normalized to PBS.

2.3.7. The effects of Taurine on ACTH secretion in AtT20 cells

To better understand if there is GlyR link to the ACTH suppression that was observed, Taurine was treated to AtT20 cells, with PBS as a control, and the ACTH levels were measured in the cell culture medium of treated cells.

It was found that Taurine had a stimulatory effect on ACTH secretion, compared to PBS control (p = 0.009, +43%) (**Figure 2.16**).

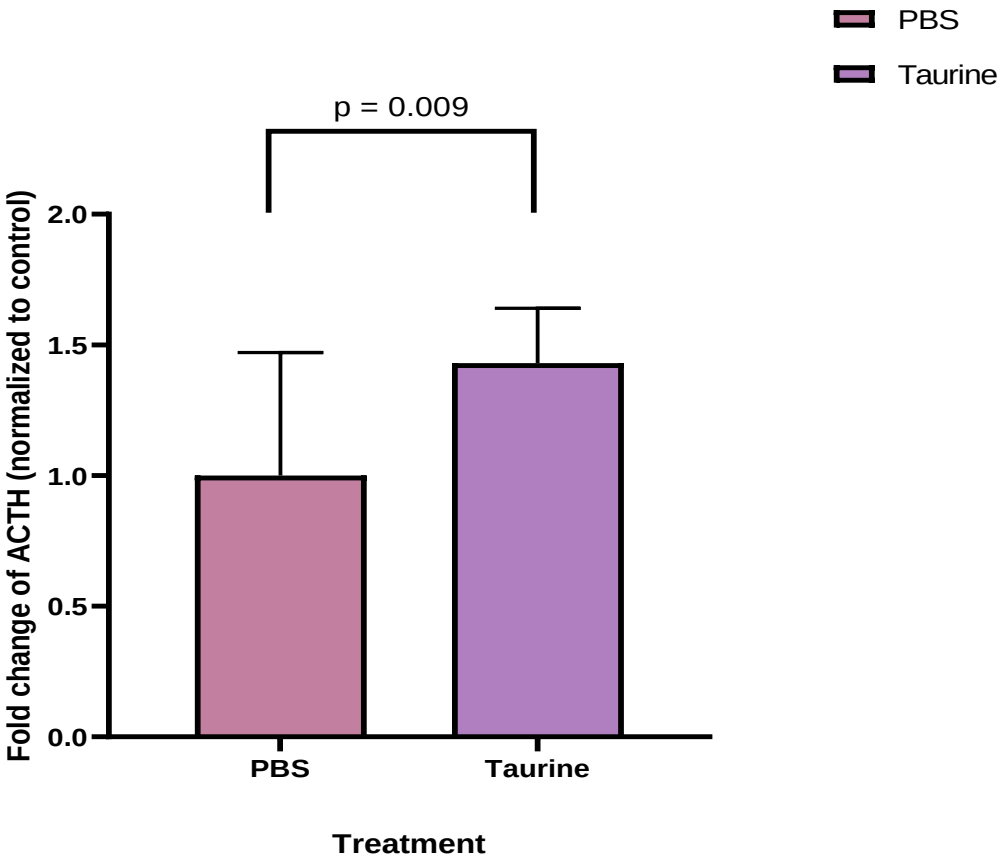


Figure 2.48 - The graph above displays the fold change of ACTH measured by ELISA in the culture medium of 150 000 AtT20 cells treated with Taurine (500 µM), with PBS as a control, for 24 hours. Cells treated with Taurine had significantly higher levels of ACTH in their culture medium (p = 0.009, +43%) when compared to normalized control. Statistical analysis was achieved by Independent Samples T-Test. Data are the mean of 6 replicates.

2.3.9 Gene Expression in AtT20 cells treated with Taurine and/or Strychnine

As shown in **Figure 2.17**, there was no significant changes in genetic expression in cells exposed to Taurine and/or Strychnine.

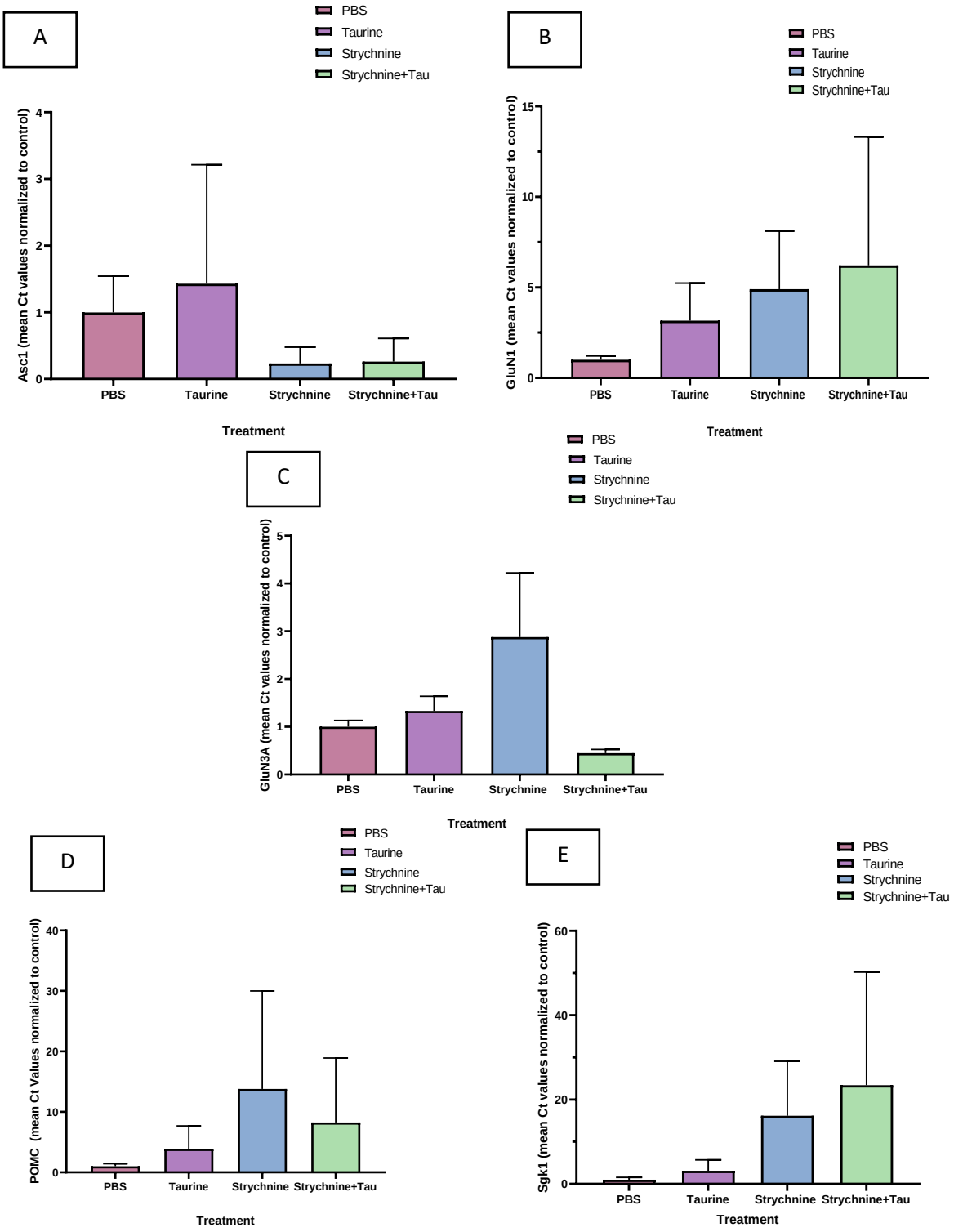


Figure 2.17. qPCR for mRNA abundance was done on AtT20 cells treated with 500 µM Taurine in the presence or absence of 100 µM Strychnine for 24 hours. There was no significant difference in gene expression levels of Asc1 (A), GluN1 (B), GluN3A (C) POMC (D) and Sgk1 (E). Data are mean of 6 replicates.

2.3.10. Glycine Receptor effects of D-alanine in the presence or absence of Glycine receptor blocker, Strychnine

To further understand the glycine receptor's role in ACTH signalling in the anterior pituitary cells, AtT20 cells were also exposed to the GLRA1 agonist, D-alanine, as well as GLRA1 antagonist, Strychnine. D-alanine had an effect on GLRA1 protein expression ($F_{3,28} = 3.81$, $p = 0.021$, **Figure 2.19b).** There was a significant increase in GLRA1 protein expression in cells treated with D-alanine compared to PBS control ($p = 0.043$, +103%) (**Figure 2.19b**).

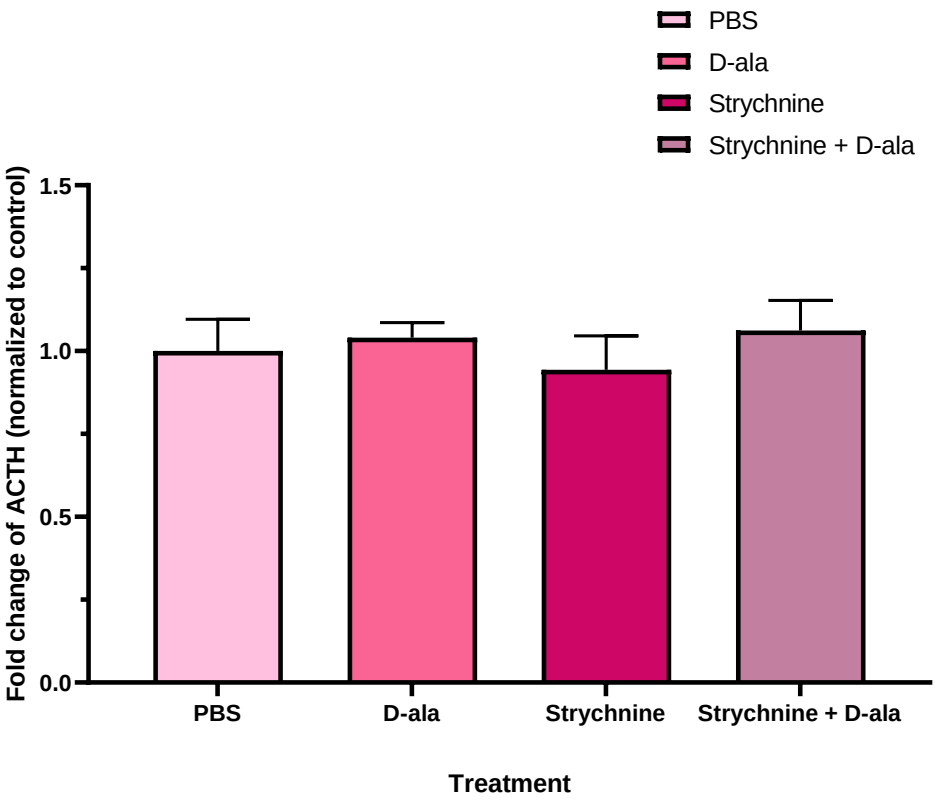


Figure 2.18. Protein expression determined by ELISA assay of 6 replicates of AtT20 cells (150 000) treated with 500 µM D-alanine in the presence and/or absence of Strychnine (100 µM) show that there are no significant differences between the treatments for the level of ACTH.

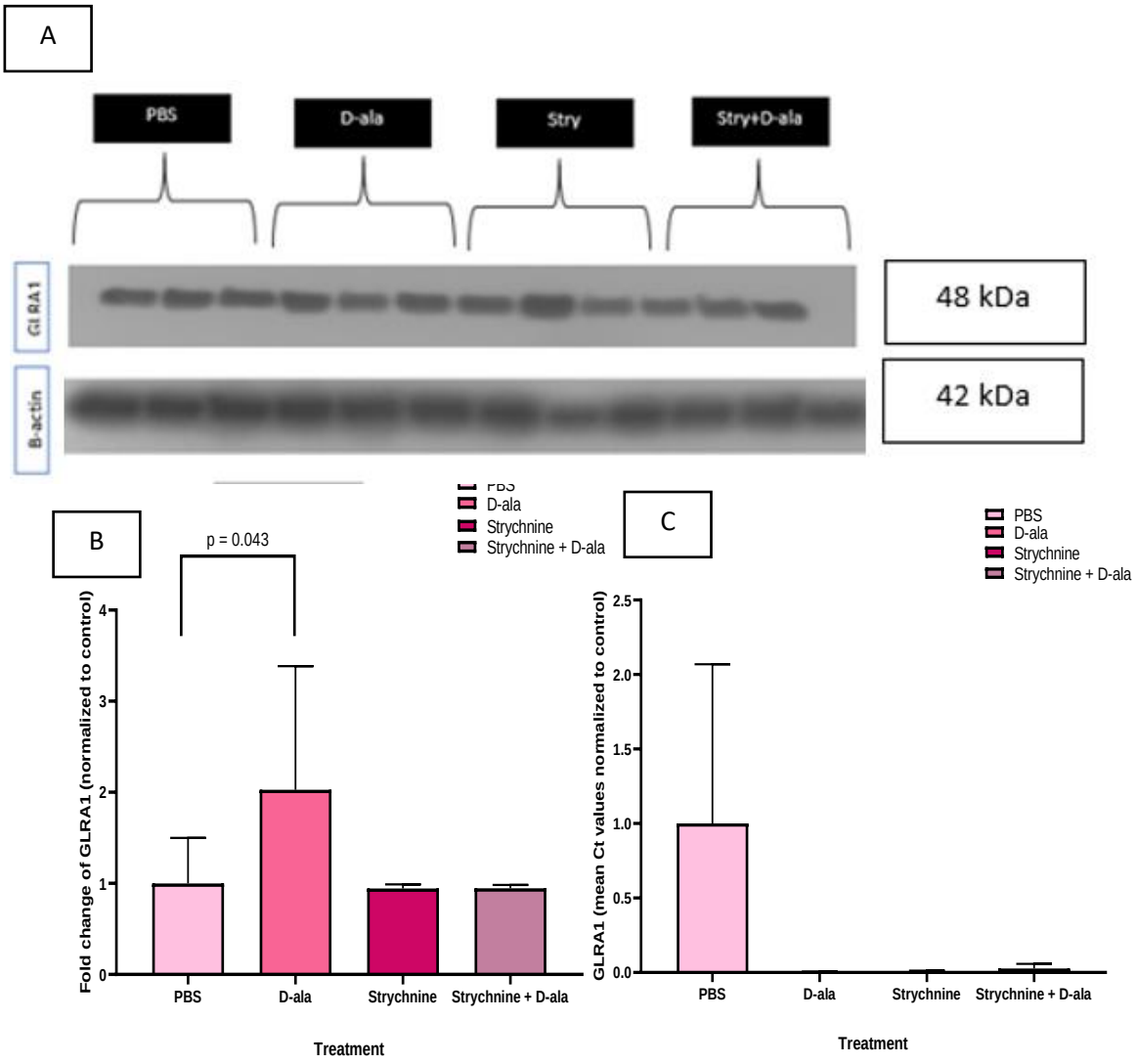


Figure 2.19 GlyR protein expression was achieved by western blot (A and B) as well as qPCR (C). There was a significantly higher level of GLRA1 in cells treated with D-alanine (500 µM) for 24 hours compared to control (p = 0.043, +103%), but no significant

differences between AtT20 cells treated with 100 µl strychnine in the presence or absence of D-alanine. Data are the mean of 6 replicates.

2.3.11. Gene expression study of AtT20 cells treated with D-alanine in the presence or absence of Strychnine

To determine what may mediate the alterations in glycine receptor activation in D-alanine treated cells, receptors, transporters and regulatory molecules, genetic expressions were investigated (**Figure 2.20**). Strychnine and/or D-alanine affected GluN3A levels in AtT20 cells ($F_{3,17} = 4.75$, $p = 0.014$, **Figure 2.20b**) whilst D-alanine affected Asc-1 in AtT20 cells ($F_{3,17} = 3.37$, $p = 0.043$, **Figure 2.20c)** and strychnine and D-alanine affected POMC levels in AtT20 cells ($F_{3,19} = 2.98$, $p = 0.57$, **Figure 2.20d).** There was a significant increase in GluN3A levels in cells treated with both Strychnine and D-alanine when compared to PBS ($p = 0.016$, +171%) and strychnine ($p = 0.041$, +70%). There were significantly lower levels of Asc-1 in cells treated with D-alanine compared to controls ($p = 0.041$, -93%). There was also a significantly lower level of POMC in cells treated with D-alanine and Strychnine ($p = 0.042$, -200%) compared to cells treated with D-alanine.

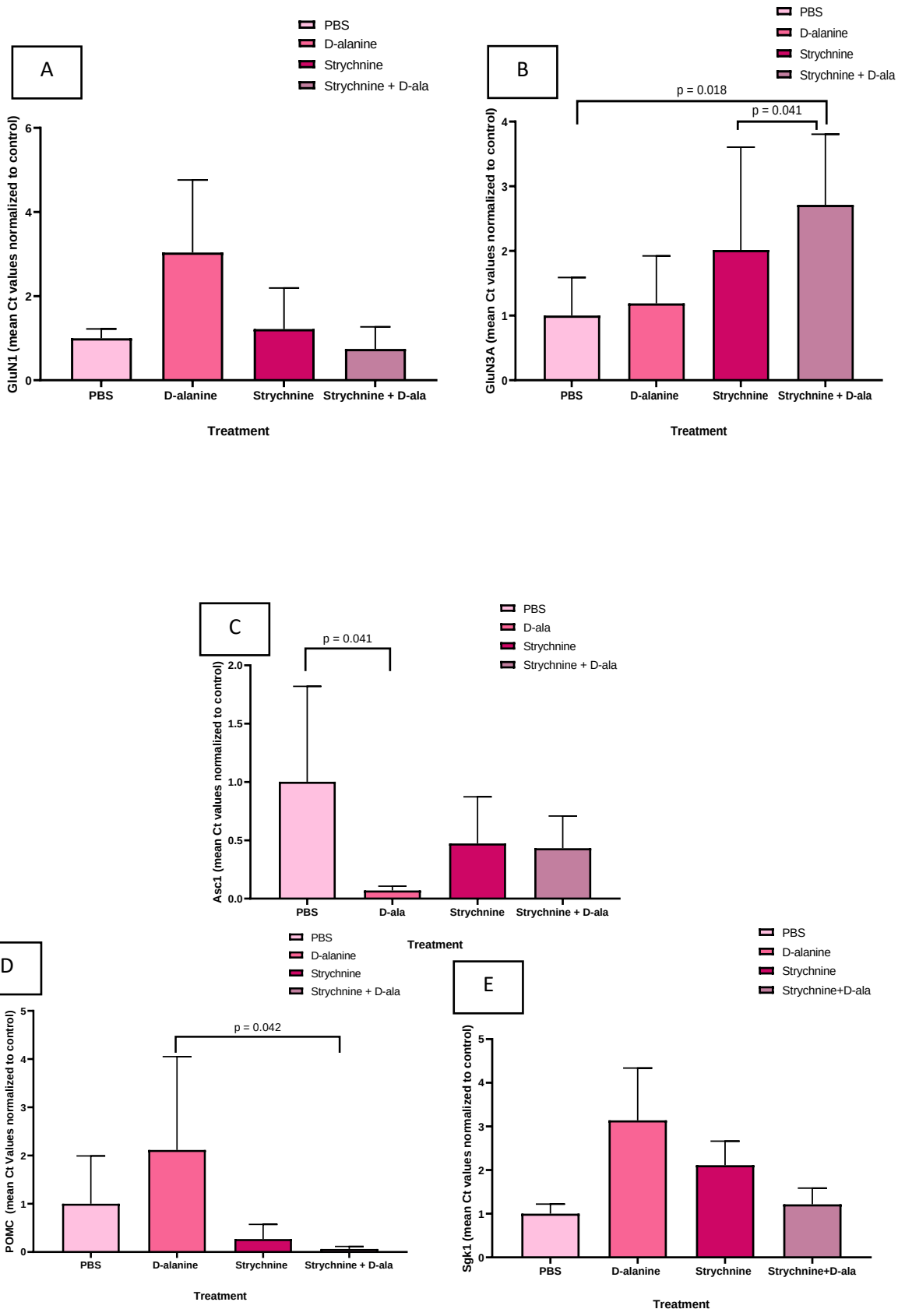

A
PBS
D-alanine
Strychnine
Strychnine + D-ala
GluN1 (mean Ct values normalized to control)
0
2
4
6
PBS
D-alanine
Strychnine
Strychnine + D-ala
Treatment
B
PBS
D-alanine
Strychnine
Strychnine + D-ala
p = 0.018
p = 0.041
GluN3A (mean Ct values normalized to control)
0
1
2
3
4
PBS
D-alanine
Strychnine
Strychnine + D-ala
Treatment
C
PBS
D-ala
Strychnine
Strychnine + D-ala
p = 0.041
Asc1 (mean Ct values normalized to control)
0.0
0.5
1.0
1.5
2.0
PBS
D-ala
Strychnine
Strychnine + D-ala
Treatment
D
PBS
D-alanine
Strychnine
Strychnine + D-ala
p = 0.042
POMC (mean Ct Values normalized to control)
0
1
2
3
4
5
PBS
D-alanine
Strychnine
Strychnine + D-ala
Treatment
E
PBS
D-alanine
Strychnine
Strychnine+D-ala
Sgk1 (mean Ct values normalized to control)
0
1
2
3
4
5
PBS
D-alanine
Strychnine
Strychnine+D-ala
Treatment

Figure 2.20. mRNA levels of GluN1 (A), GluN2 (B), Asc1 (C), POMC (D) and Sgk1 (E). Six replicates of 150 000 AtT20 cells were treated with D-alanine (500 μM) in the presence or absence of Strychnine (100 μM). There was a significant increase in GluN3A levels in cells treated with both Strychnine and D-alanine when compared to PBS ($p = 0.016$, +171%) and strychnine ($p = 0.041$, +70%). There were significantly lower levels of Asc1 in cells treated with D-alanine compared to controls ($p = 0.041$, -93%). There was a significantly lower level of POMC in cells treated with D-alanine and Strychnine ($p = 0.042$, -200%) compared to cells treated with D-alanine. Data are means of 6 replicates.

2.4. Discussion

2.4.1. The inhibition of ACTH secretion by D-alanine and D-serine

In preliminary studies, when optimizing the AtT20 cells maintenance protocol, as well as establishing treatment dose concentrations and durations (Appendix), I found that there was a significant decrease in ACTH secretion from AtT20 cells in the presence of D-amino acids depended on passage number, as early passages secreted ACTH at levels higher than the sensitivity range of the ELISA kit. AtT20 cells may require time for possible physiological development of receptors or transporters, as molecular alterations only came into effect post-passage 7 of these cells. There is evidence in the literature that supports these later passage effects of mouse cell lines, where O' Driscoll and colleagues (2007) found that there were significant changes in various mRNAs involved in regulated secretion, adhesion and proliferation in MIN-6 mouse insulinoma cells, when looking at later passages versus earlier ones. Therefore, for further experiments involving AtT20 cells, I ensured that cells were post-passage 7.

I was unable to detect GluN2 protein or mRNA in these cells, which supports what Zhou and Lapingo (2014) observed, that these cells do not express the GluN2 subunits, but managed to replicate their findings by detecting the presence of GluN1 subunits.

The significant decrease in ACTH release in the presence of D-alanine and D-serine in later passage cells of AtT20, in subsequent pooled experiments (**Figure 2.7)**, suggested that there may be NMDA receptor involvement, as both D-alanine and D-serine bind to the NMDA receptor. A study by Nasihatkon et al (2014) investigated the effects that memantine, a therapeutic drug for Alzheimer's, injected into the ventral tegmental area (VTA) had on plasma corticosterone levels. They found that male Wistar rats that were injected with memantine and exposed to stress, had a diminished stress response, reflected in corticosterone levels. They proposed that this inhibition by memantine was achieved via its inhibition of the NMDA receptors in the VTA. They based this conclusion on a study that found that adrenal cortex NMDA receptor inhibition resulted in an increase in corticosteroid release in adrenal cortical cells (Pirnik et al., 2001). Therefore, there is evidence for NMDA receptor involvement in HPA axis hormonal signalling, and it is very probable that the reduction in ACTH secretion may be due to these receptors.

2.4.2. NMDA Receptors expression in AtT20 cells

The subunits GluN1 and GluN3A were detected in AtT20 cells (**Figure 2.9** and **Figure 2.10)**, but D-alanine and D-serine failed to stimulate changes in these receptor subtypes. GluN3A was of interest, as its presence has been shown to decrease calcium influx and has been established as an inhibitory subtype of the NMDA receptor as it only has a glycine binding site (Nakanishi et al., 2009). GluN3A expression in these cells (**Figure 2.9)**, has not been shown in the literature, whilst the detection of GluN1 reinforces previous finding by Etoh et al (2009).

Despite the fact that NMDA receptors have not been significantly altered in the presence of D-alanine or D-serine in AtT20 cells, this finding doesn't negate its potential involvement in the inhibition of ACTH, especially as it has exhibited effects on corticosteroid secretion in adrenal cells (Pirnik et al., 2001), showing a previous link to HPA axis hormones. To elucidate the involvement of NMDARs in HPA axis hormone inhibition, I look at this receptor in further detail in the *in vivo* study later on in Chapter 5.

2.4.3. Stimulation of GLRA1 protein expression by D-alanine

Stojilkovic et al (2010) stated that GlyRs are not found in the anterior pituitary, but my data proves that it is abundantly expressed (**Figure 2.8)**. The significant increase of GLRA1 observed by western blot, in cells treated with D-alanine, showed that the presence of D-alanine stimulated the increase of this receptor (**Figure 2.8b)**, whilst D-serine decreased GLRA1 mRNA levels (**Figure 2.8c)** This is particularly interesting as D-serine is not an agonist of GLRA1(Dutertre et al., 2012, Schmieden and Betz, 1995), therefore it is unlikely that the observed effect is through direct action of D-serine on GLRA1. One explanation would be that the uptake of both D-amino acids into neurons is mediated by the Asc-1 transporter, which has a higher affinity for D-alanine than D-serine (Rosenberg et al., 2013), and D-alanine exhibits a stronger inhibition on ACTH secretion than D-serine (**Figure 2.7)**. Asc-1 knock out mice display a selective reduction in glycine in the brain as well as impaired glycinergic inhibitory transmission, and it was hypothesized that Asc-1 may be a regulator of glycine metabolism (Safory et al., 2017). Therefore, this effect on GLRA1 in the presence of D-alanine and D-serine may result from activation of the Asc-1 transporter affecting glycine levels in these cells and therefore GlyRs, an observation that has not been previously reported. To further determine GlyR involvement, Taurine, a more potent agonist of GlyRs, and strychnine, an antagonist of GlyRs were utilized, and this is discussed further below.

2.4.4 Amino Acid Transporters in the Anterior Pituitary

Asc-1 and ASCT2, which transport both D-alanine and D-serine, were expressed in these cells genetically, but there were no significant differences between the treatment measures (**Figure 2.11)**. These transporters have not yet been reported within these cells, though their existence is implied in the literature as they are responsible for D-alanine and D-serine uptake (Schmidt and Thompson, 2016, Maucler et al., 2013 and Etoh et al., 2009). I was unable to determine protein levels for these transporters, as the only available antibody purchased reacts with human tissue. AtT20 mouse cells were later transfected with human Asc-1 vector, and then probed for protein expression using the human antibody, to better understand the effect these transporters have in these cells, and if it may have a role to play in ACTH secretion. I discuss this further in the next chapter.

2.4.5. D-alanine and D-serine stimulation of SGK1

I found that both D-alanine and D-serine significantly increased SGK1 (**Figure 2.11a)**, but did not significantly reduce POMC, which is what one would assume based on the reduction in ACTH in the culture medium. Reiter et al (2011) showed that an increase in SGK1 has opposing effects on typical glucocorticoid feedback inhibition, where it actually stimulated POMC and ACTH release, and perhaps that is why a reduction in POMC was not observed, as D-amino acid treated cells have a high presence of SGK1 which stimulates POMC, whilst D-amino acids itself may be inhibiting POMC, and both these actions negate each other. The absence of change in POMC might also be due to suboptimum treatment duration, as some experiments report POMC changes much earlier on – at 6 hrs, therefore 24 hrs may be too long a treatment duration (Asari et al., 2019).

2.4.6. Positive stimulation of ACTH by CRH

Following the investigation of the effects of amino acids in a basal study, I investigated what may happen when the cells were stimulated by CRH and then treated with D-alanine. There was a significant decrease in ACTH levels in the culture medium of cells treated with CRH in the presence of D-alanine and cells treated with just D-alanine, and a reciprocal increase in intracellular ACTH protein (measured by western blot) in the cells undergoing the same treatments (**Figure 2.12**). These data are quite confusing, but it may be due to the fact that CRH is successfully stimulating ACTH to the point that its levels are higher than 35 000 pg/ml. Perhaps the high concentration of ACTH is a limitation of this ACTH assay, as we observed that when ACTH levels are too high, we do not notice the inhibitory effect of D-alanine, so perhaps in this stimulated system where ACTH levels are too high , treatment mediated effects cannot be detected in this assay. I was then only able to see the differences in ACTH measures when something inhibits it, such as D-alanine and D-serine, to levels that are workable in the assay itself. During the optimization of the ACTH assay, dilution factors were utilized to bring samples within expected range of the assay, but this was never achieved successfully with the CRH study. Another reason could be that this particular strain of AtT20 cells may not be sensitive to CRH, shown by a study by Fukata et al (1989), where they had to incubate cells with IL-1B and CRH to find an ACTH response.

2.4.7. Stimulation of GlyR by Taurine

Taurine, the endogenous agonist of GlyRs, failed to increase the level of GLRA1 (**Figure 2.15)**, which further supported the hypothesis that the increase of GLRA1 by D-alanine is linked with the Asc-1 transporter, and not a direct activation of the GLRA1 subtype, as taurine is not taken up by this transporter. Moreover, taurine increased ACTH secretion from AtT20 cells, in contrast to the decrease observed with D-alanine. This particular

observation is in-keeping with previous findings in the pancreas where GlyRs stimulate the release of insulin (Chen et al., 2018).

2.4.8. Strychnine effects in AtT20 cells

To further investigate the GlyRs, instead of using just its agonists – Taurine and D-alanine, I also observed the effects of an antagonist on this receptor – strychnine. The effect of D-alanine stimulating GLRA1 in protein was replicated in the experiments (**Figure 2.19b)** that had AtT20 exposed to D-alanine, in both the presence or absence of strychnine. However, in the presence of strychnine, the stimulatory effect of GLRA1 by D-alanine is negated, which shows that strychnine successfully diminished D-alanine effects on GlyR by blocking its activation (**Figure 2.19b**). An inhibitory effect on Asc-1 was detected in cells treated with just D-alanine (**Figure 2.20c)**. This could be due to Asc-1 being used to transport D-alanine, and therefore the cells have modulated for a downregulation of this overactive transporter. A curious finding of the strychnine experiments was that cells treated with strychnine had an undetermined level of ASCT2 mRNA (Appendix). This may be due to the fact that Strychnine blocked the GlyRs, which meant a high level of free glycine is present in the cell, due to its inability to bind to GLRA1. ASCT2 is known to take up glycine (Utsunomiya-Tate et al., 1996), and glycine receptor blockage by strychnine results in an increase in free extracellular glycine. ASCT2 may then become over-activated due to this increase in free glycine and subsequently severely downregulated to undetectable levels in these cells. Thus, the experiments in this first chapter suggest that the inhibition of ACTH release by D-alanine and D-serine are not mediated solely by GlyRs but may involve Asc-1 or ASCT2 transporters and the SGK1-POMC system, which is investigated further in Chapter 3.

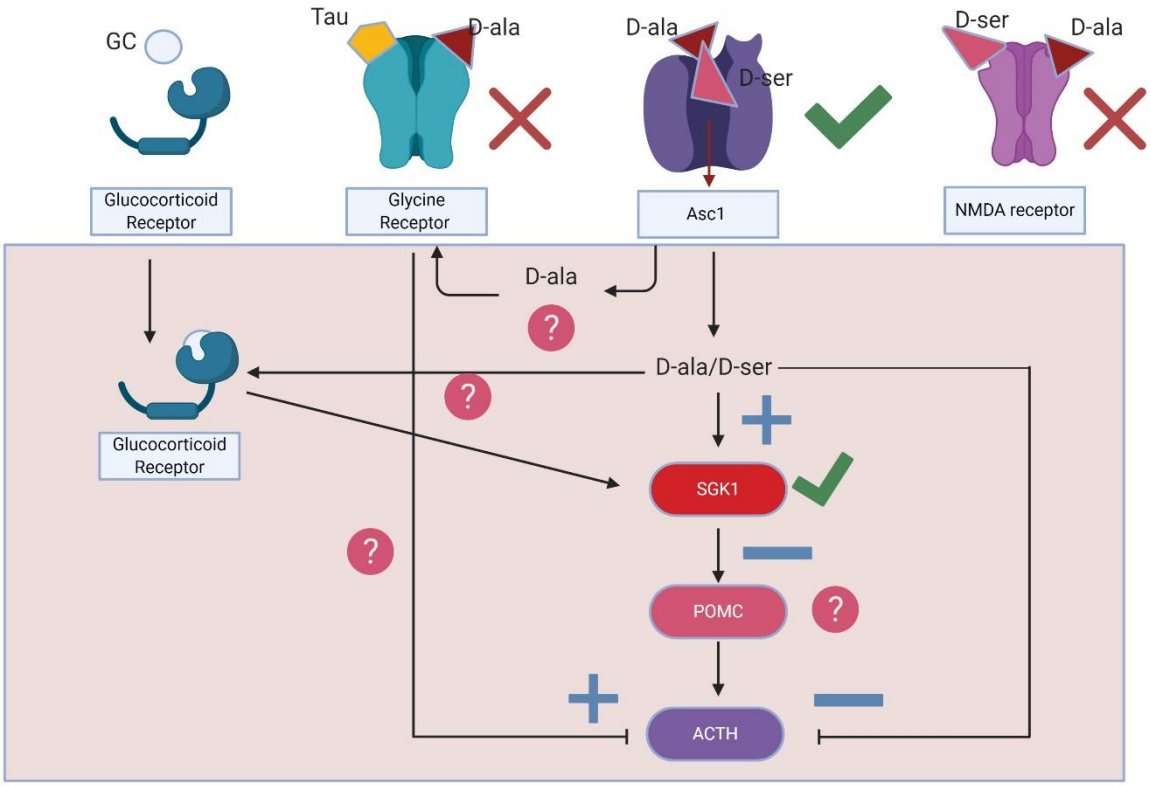


Figure 2.21 Diagrammatic representation of Potential pathways mediating the modulation of ACTH secretion by D-amino acids. Image made with Biorender.com

As depicted in **Figure 2.21** above, I have looked at SGK1 (which is linked to the GRs), GlyRs (which both D-alanine and Taurine bind to), Asc-1 transporter (which transport both D-alanine and D-serine), and NMDA receptor (which D-alanine and D-serine bind to). Red crosses demarcate pathways that this chapter has found are probably not responsible for the decrease in ACTH first detected (Glycine receptor and NMDA receptor). Green ticks show the pathways that I have proven to be involved, such as the SGK1 system and Asc-1 transporter system. Pink question marks are avenues yet to be investigated.

2.4.5. Concluding remarks, limitations and further studies

Therefore, as shown in **Figure 2.21**, D-alanine and D-serine reduced the secretion of ACTH levels. Taurine, a full agonist of GlyRs, resulted in an increase in ACTH levels.

The NMDA receptors expression did not change, which presently suggests that they are not involved in the observed ACTH effects, but this can be confirmed later *in vivo*. D-alanine caused an increase in GLRA1 protein but not D-serine, but this was not surprising as D-alanine is a GlyR agonist whilst D-serine is not. D-serine and D-alanine caused an increase in SGK1, but that didn't correlate with a decrease in POMC, which would have been expected, because POMC links the SGK1 and ACTH system. So, a decrease in POMC is expected for a decrease in ACTH. GRs may also have a role to play, now that there is strong evidence that the SGK1 system is involved, as GRs are known to regulate SGK1 (Chen et al., 2019) but these receptors have yet to be investigated. The Asc-1 transporter system has been shown to be genetically expressed in the anterior pituitary, and with such a strong presence of D-alanine in these cells, it is likely that this transporter is responsible for carrying this D-amino acid to the anterior pituitary.

Limitations to the experiments in this chapter was the lack of a western blot antibody that can react to murine samples for Asc-1. Additionally the passage dependency of AtT20 D16v cells was a limitation, as the variability in ACTH secretion between passages was retrospectively noticed following inconsistent data. I found that AtT20 cells mature around passage number 7 and following this had to replicate experiments post-passage number 7 and ensure going forward all experiments were conducted on cells in the later passage. These cells have been shown to have variability between passage numbers, and it is suggested to use D16:16 strain of AtT20 cells instead, as they displayed more consistent ACTH levels between passages (Woods et al., 1992).

Therefore, based on all our findings in this chapter, the inhibition of ACTH by D-alanine and D-serine may be due to a link between the SGK1-POMC system, and the Asc-1 transporter, which is known to transport both D-alanine and D-serine. Therefore I have investigated the effects an over-expression of Asc-1 transporter may have on D-amino

acids in AtT20 cells in Chapter 3, as well as what the activated system (with dexamethasone) does to SGK1-POMC and ACTH in Chapter 3.

CHAPTER THREE: INVESTIGATING THE MECHANISM FOR THE INHIBITION OF ADRENOCORTICOTROPIC HORMONE (ACTH) BY D-AMINO ACIDS

3.1 Introduction

In the last chapter I found that D-alanine and D-serine inhibited ACTH secretion from AtT20 mouse corticotroph cells – and this inhibition was not through direct action of the receptors they bind to – NMDARs or GlyRs. However, the SGK1 system was affected in these cells (**Figure 2.11)**, which affects POMC, the precursor of ACTH, and the Asc-1 (**Figure 2.20).** This transporter, along with ASCT2, transports both D-alanine and D-serine, so could be a potential avenue to investigate with regards to mechanism of ACTH inhibition. Additionally, Asc-1 has been shown to affect insulin resistance in adipocytes via glutathione (Jersin et al., 2021), so perhaps a similar thing is happening in the anterior pituitary with ACTH via D-amino acids. I studied the involvement of Asc-1 in this Chapter by overexpressing its gene in AtT20 cells and observing ACTH changes when these cells are exposed to D-alanine or D-serine. SGK1 involvement was investigated by activating these corticotroph cells with synthetic glucocorticoid Dexamethasone in this chapter.

3.1.1. How does dexamethasone affect ACTH secretion?

My studies so far have involved the basal secretion of ACTH, but to truly reflect the HPA axis I needed to investigate the effects D-amino acids may have on ACTH secretion when corticotroph cells are activated, and this was achieved with the synthetic GC dexamethasone. Carnes et al (1987) studied the effects of dexamethasone on ACTH. secretion in rodents and found that prolonged exposure to dexamethasone resulted in an inhibition of ACTH release into cerebrospinal fluid (CSF). A clinical trial of patients with PTSD found that dexamethasone greatly suppressed both cortisol and ACTH secretion in

these patients (Yehuda et al., 2004). Therefore the effects of dexamethasone, a known HPA axis inhibitor was also observed in this chapter.

3.1.2. Can AtT20 cells be transfected?

AtT20 cells have successfully been transfected with plasmid DNA in numerous studies (Asari et al., 2017, Reiter et al., 2011 and Pineda et al., 2004). The transfection protocol for this study was adapted from the methodology of the studies below.

Zhang et al (1999) detailed their transfection protocol of AtT20 cells, as they investigated, the oncogene, Pituitary tumour transforming gene 1 (PTTG1). PTTG1 is a signature gene expressed by pituitary tumours. Therefore PTTG1 – siRNA fragments were transfected into AtT20 cells to observe the effects it has on POMC. HiPerfect transfection reagent (Qiagen) was used to transfect AtT20 cells with siRNA. AtT20 cells were plated at 120 000 cells/well, in 1ml culture medium containing siRNA specific for PTTG1, for 48 hours, after which POMC levels were quantified via quantitative reverse-transcription PCR.

Reiter et al (2011) measured POMC levels following transfection of AtT20 cells with SGK1, as they wished to investigate the effect the regulatory protein would have on the HPA axis. They received SGK1/pcDNA expression plasmid containing nucleotides 12 to 1436 of mouse SGK1, where 1µg of plasmid DNA and 10µl of SuperFect (Qiagen) reagent was added to each well containing 300 000 cells per well to achieve successful transfection.

3.1.3. Asc-1 transfection into cells

HATs are composed of a heavy subunit (rBAT or 4F2hc) and a light subunit, which are linked by a disulphide bridge (Chillarón et al., 2001). Asc-1 (SLC7A10), a HAT, displays substrate selectivity for L- and D- small neutral amino acids such as D-serine, glycine and L-cysteine, and in humans it is expressed predominantly in the brain, placenta, kidney and

heart (Nakauchi et al., 2000). Leclerc et al (2001) hypothesized that SLC7A10 could be an addition gene for cystinuria, as it is found in the kidney and could potentially have a role in renal reabsorption for cysteine. Pineda et al (2004) then sought to investigate this further, and obtained a full-length cDNA clone of 1807 bp, encoding human Asc-1, cloned in pBluescript SK^-, and transfected into HeLa cells. Transfections involved standard calcium phosphate precipitation in 10 cm diameter plates with a mixture of DNA consisting of 2 µg of green fluorescent protein (GFP) and 18 µg of Asc-1 pCDNA3, followed by an overnight incubation with DNA-calcium phosphate coprecipitate. Asc-1 transfection of AtT20 cells in this chapter was achieved following methodology mentioned in the above experiment.

3.1.4. Does Asc-1 have a role to play in inhibition via receptors?

Ehmsen and colleagues (2016) also looked at the effect *slc7a10*-null mice may have on NMDARs and noted a decrease in glutamatergic excitatory transmission in motor neurons of these animals. NMDAR hypofunction has been postulated to play a role in schizophrenic symptoms, and therefore this receptor has been an active pharmacological target. Inhibition of glycine transporters (GLYT1 and GLYT2) have improved negative symptoms of schizophrenia (Harvey and Yee, 2013), and this inhibition of GLYT1 increases NMDAR signalling (Chen et al., 2003). Therefore, inhibitors of another glycine transporter, SLC7A10, are currently being considered as therapeutic options for treatment of schizophrenia (Brown et al., 2014), and therefore I investigated the link between SLC7A10 and NMDARs in this chapter, by overexpressing AtT20 cells with Asc-1 and observing NMDAR expression, so as to better understand transporter links with receptors.

3.1.5. Aims of the Chapter

The aim of this chapter is to determine the mechanism involved in the D-amino acid driven inhibition of ACTH we reported in our previous chapter **(Figure 2.7, Chapter 2).** I found

that GlyRs are not likely responsible for this reduction in ACTH, but inhibitory mechanisms may involve the SGK -POMC system, or the amino acid transporters, Asc-1 and ASCT2.

1. I investigated the role of the SGK1 and POMC system by utilizing dexamethasone, which should stimulate the SGK1 pathway in the anterior pituitary mouse cell line, AtT20. SGK1, POMC, N-methyl D-Aspartate receptor type 1 (GluN1) and GLRA1 were measured by qPCR. These genetic measures were conducted to better understand the interactions between SGK1 and POMC, as well as its interaction with inhibitory receptors, GLRA1 and GluN1. ACTH culture medium levels were also determined using ELISA analysis.
2. The latter half of this chapter looks at the potential effects of AtT20 cells overexpressed with the transporter, Asc-1 (SLC7A10), which takes up both D-alanine and D-serine. Cells transfected with SLC7A10 were then treated with D-alanine or D-serine. Asc-1 and ASCT2 transporter levels, GluN1, GluN3A, GLRA1 receptor levels, as well as SGK1and POMC levels were measured using qPCR analysis. GLRA1 and Asc-1 levels were further confirmed using western blot analysis. ACTH protein levels were measured using both ELISA and western blot analysis. Unfortunately, the protein measurements for GLRA1 and Asc-1 in AtT20 cells transfected with SLC7A10 and treated with D-serine were not completed due to COVID-19 pandemic restrictions.

3.2 Materials and Methods

AtT20 cells were grown as described in **Materials and Methods, Chapter 2**.

3.2.1 Dexamethasone studies

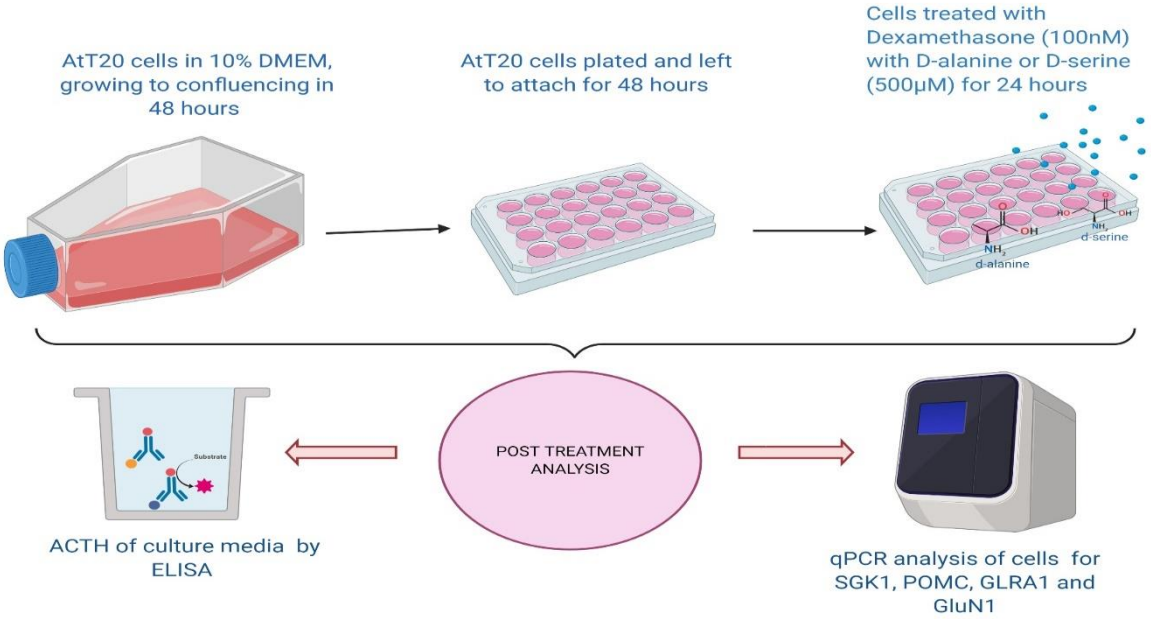


Figure 3.1 - The above is the schematic representation of the treatment protocol for the experimental procedure analysing the activation of the HPA system in AtT20 cells utilizing dexamethasone and D-amino acids. Image made with Biorender.com

To determine the effects of D-amino acids on AtT20 cells in a system that has an activated HPA axis, dexamethasone, a synthetic GC is utilized. As shown in the figure above **(Figure 3.1),** 50 000 AtT20 cells were plated into each well of a 24-well plate and left to reach confluence over the next 48 hours. Cells were then treated with 100 nM of Dexamethasone in the presence, or absence of D-alanine or D-serine, at 500 µM for 24 hours. PBS was used as a control, and two wells of treatment were merged to form 1 sample to achieve an n = 6 for each treatment measure. Cells were then analysed for POMC, SGK1, GluN1 and GLRA1 mRNA using qPCR analysis (**Materials and Methods, Chapter 2**) and ACTH levels in culture medium were detected using ELISA **(Material and Methods, Chapter 2).**

3.2.2. Overexpression of Asc-1 in AtT20 cells studies

3.2.2.1. Optimization of Cell transfection protocol with Green Fluorescent Protein (GFP)

The cytomegaloavirus -driven green fluorescent protein (CMV-GFP) plasmid construct was a generous gift from Mr Adria Dangla Valls (DPhil student, Department of Psychiatry, Oxford).

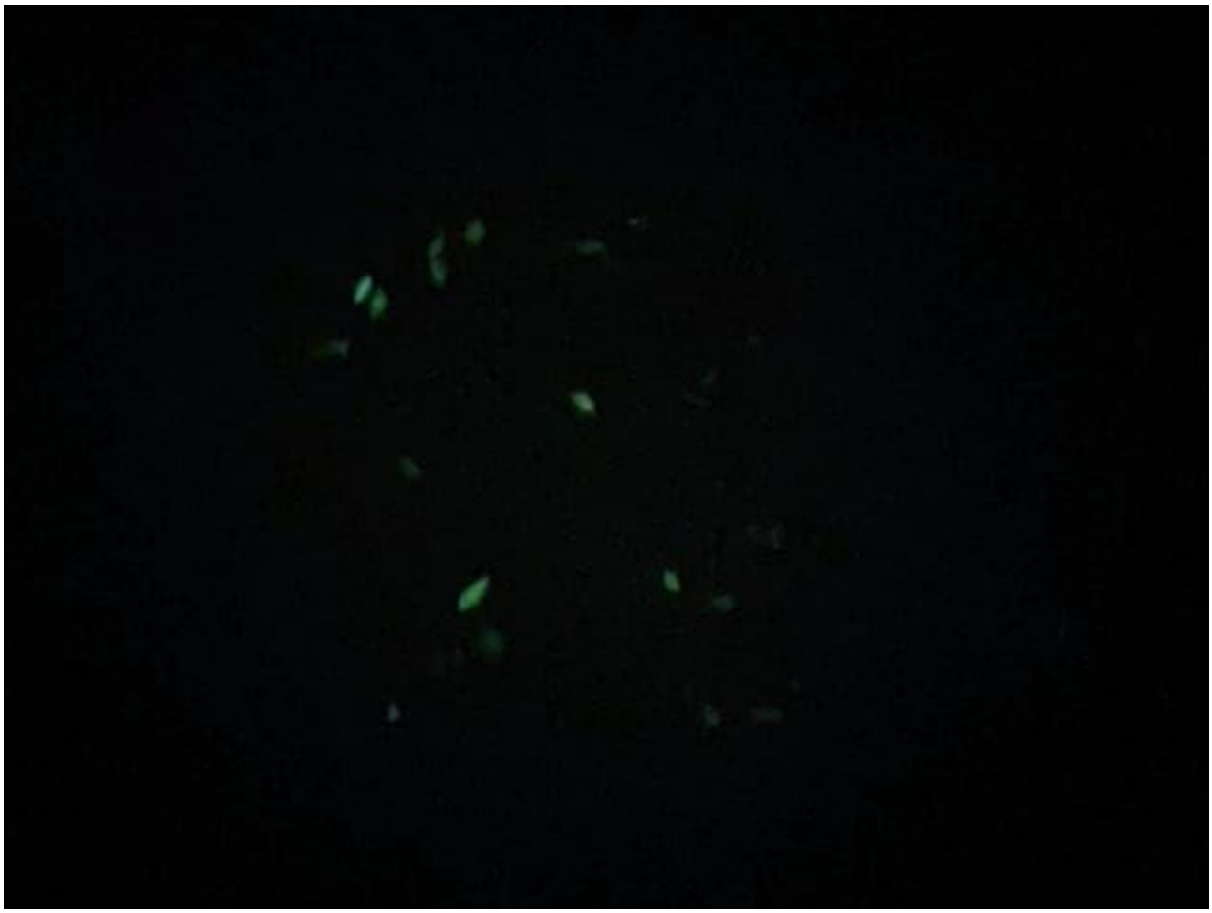


Figure 3.2. The above shows GFP successfully transfected into AtT20 cells at a rate of approximately 50% (determined by eye). The optimized protocol required 1µg of GFP vector and 2µl lipofectin in 500µl OptiMEM medium of 100 000 AtT20 cells, left overnight. Following the successful transfection of AtT20, experiments overexpressing SLC7A10 transporter were pursued. Further details in **Appendix.**

3.2.2.2. Overexpression of SLC7A10 in AtT20 cells in the presence of D-alanine

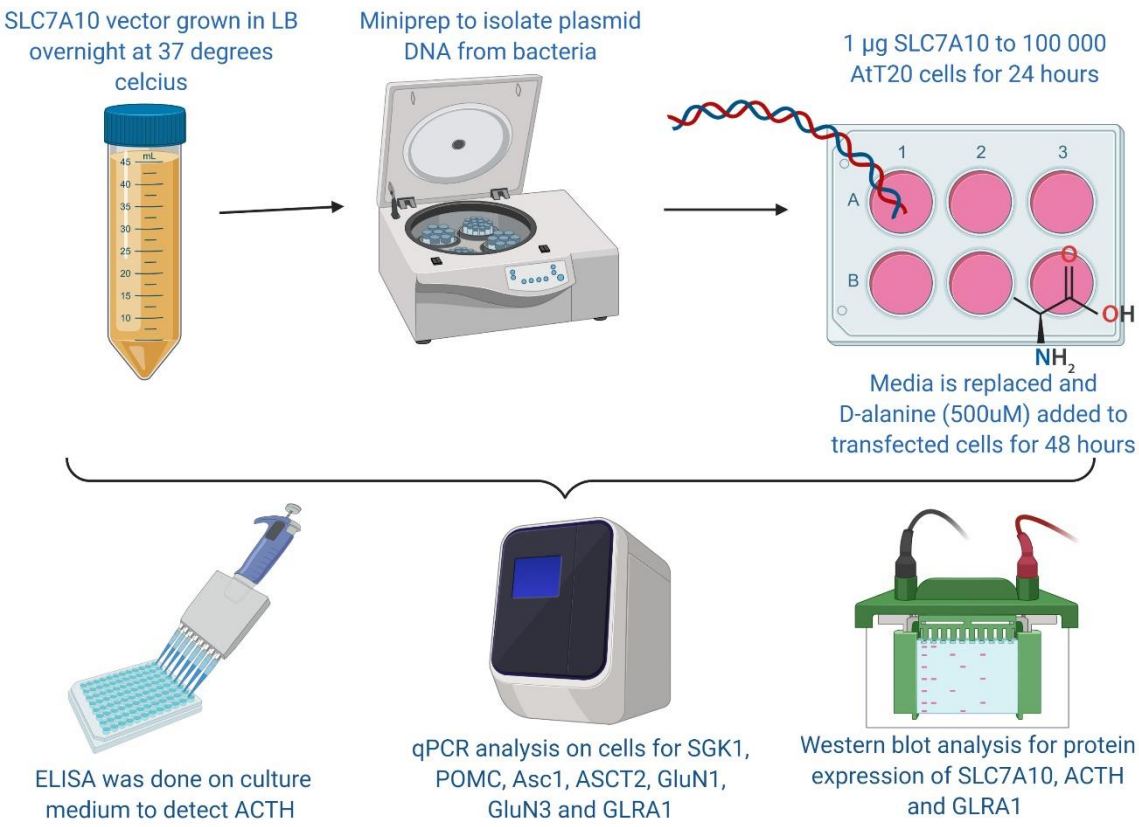


Figure 3.3 The above describes the process of transfecting AtT20 cells with human SLC7A10, and then treating both transfected and non-transfected cells with D-alanine (500µM). Image made with Biorender.com

Human SLC7A10 (ABIN3816998, Antibodies-online, Germany) was transformed using 125 µl of SOC medium (15544034, Thermo Fisher Scientific, United Kingdom) and 25 µl of human SLC7A10. This mixture was incubated for 2 hours at 37°C whilst shaking. 5 µl of the above mixture was added to 5 ml of liquid broth, with 5 µl of Kanamycin antibiotic (K0254, Sigma Aldrich, United Kingdom). The resultant mixture was left to incubate overnight at 37°C whilst shaking.

QIAprep Spin Miniprep Kit (27104, Qiagen, The Netherlands) was used to extract human SLC7A10 vector DNA from liquid broth culture as described in the kit:

1. Bacterial culture was aliquoted into 1.5 ml Eppendorf tubes and spun down at 8000 RPM for 3 minutes.
2. The resultant pellet was resuspended in 250 µl of Buffer P1 and 25 µl Buffer P2.
3. Solution was mixed thoroughly by inversion 4-6 times. The solution then turned blue.
4. 350 µl of Buffer N3 was added to the mixture and turned clear, and then centrifuged at max for 10 minutes.
5. The resultant supernatant was applied to QIAprep spin columns by decanting and spun for 60 seconds in the centrifuge.
6. 0.5 ml Buffer PB was added and spun down again in the centrifuge at max for 60 seconds.
7. 0.75 ml Buffer PE was added and washed by spinning down in centrifuge at max for 60 seconds.
8. To ensure all the residual buffer is removed, samples were spun down once more at max for 1 minute.
9. QIAprep spin column was inserted into clean 1.5ml Eppendorf, and 50µl of Buffer EB was added to the mixture and spun down at max for 1 minute to elute the DNA.
10. The resultant DNA was quantified using Nanodrop and stored at -20°C for future use.

Following optimizing of transfection protocol using GFP, 100 000 AtT20 cells were grown in 6 well plates for 48 hours. Half the wells' culture medium was then replaced with 500 µM of OptiMEM Medium (#11524456, Fischer Scientific, United Kingdom). 1 µg of human SLC7A10 and 2µl of Lipofectin (18292011, Thermoscientific, United Kingdom) were added to these wells. This was done overnight. The following day, all wells were replaced with fresh growth medium and some were treated with 500µl D-alanine or PBS for 48 hours.

Cell culture medium was then analysed for ACTH by ELISA **(Materials and Methods, Chapter 2),** and cells were collected for Western Blot analysis (**Materials and Methods, Chapter 2**) or qPCR analysis (**Materials and Methods, Chapter 2**).

3.2.2.3 Overexpression of AtT20 cells with SLC7A10 protein and exposure to D-serine

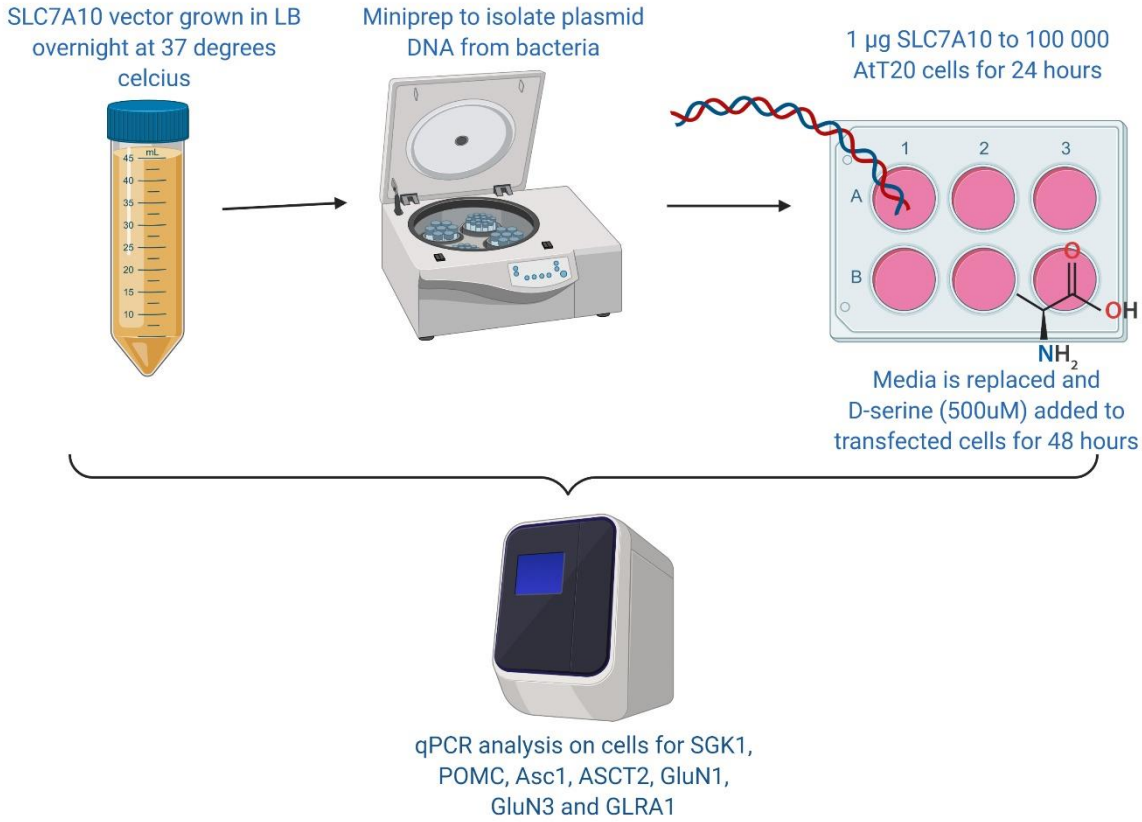


Figure 3.4 Depiction of the transfection process of AtT20 cells with SLC7A10, followed by the treatment of D-serine (500 µl). Image made with Biorender.com

AtT20 cells were again transfected with human SLC7A10, but this time treated with D-serine (500µM for 48 hours). Protocol for qPCR analysis was the same as above for D-alanine. Unfortunately due to lab restrictions as a result of the COVID-19 Pandemic, the ACTH analysis by ELISA and western blot analysis could not be complete.

3.2.3. Statistical analysis

ELISA results were determined drawing up a standard curve on Microsoft Excel and quantifying unknown variables. For Western Blot, densitometry measures for each band was measured and normalized to housekeeping protein, and then normalization to control to eventually have treatments are presented in relation to control. With regards to qPCR, Ct values were extracted from the qPCR machine and processed on Microsoft excel, and then normalized to housekeeping gene, and then subjected to POWER analysis followed by normalization to control so that treatments are presented in relation to control.

These quantities were then transferred onto SPSS statistics (Version 26) were initial statistics of One-Way ANOVA was run, and if significant ($p < 0.05$), post-hoc analysis via Tukey's Test was done to determine significant differences between treatment groups. Graphs were then drawn using GraphPad Prism (Version 9), and results are expressed as means ± SEM.

3.3. Results

3.3.1 Dexamethasone Study

Dexamethasone did not affect ACTH (**Figure 3.5)** in AtT20 cells**.** Dexamethasone, both independently and when combined with D-alanine or D-serine had increased SGK1 mRNA abundance (**Figure 3.6a**). i) Dexamethasone vs PBS ($p <0.001$ + 237%), ii) Dexamethasone and D-alanine vs PBS ($p < 0.001$, +287%), iii) Dexamethasone and D-serine vs PBS ($p < 0.001$, +325%). POMC (**Figure 3.6b**) mRNA suppression occurred in cells treated with Dexamethasone ($p = 0.015$, -52%), Dexamethasone and D-alanine ($p = 0.004$, -60%), as well as Dexamethasone and D-serine ($p = 0.005$, -59%) when compared to controls. Dexamethasone did not affect GLRA1 receptors in AtT20 cells (**Figure 3.6).**

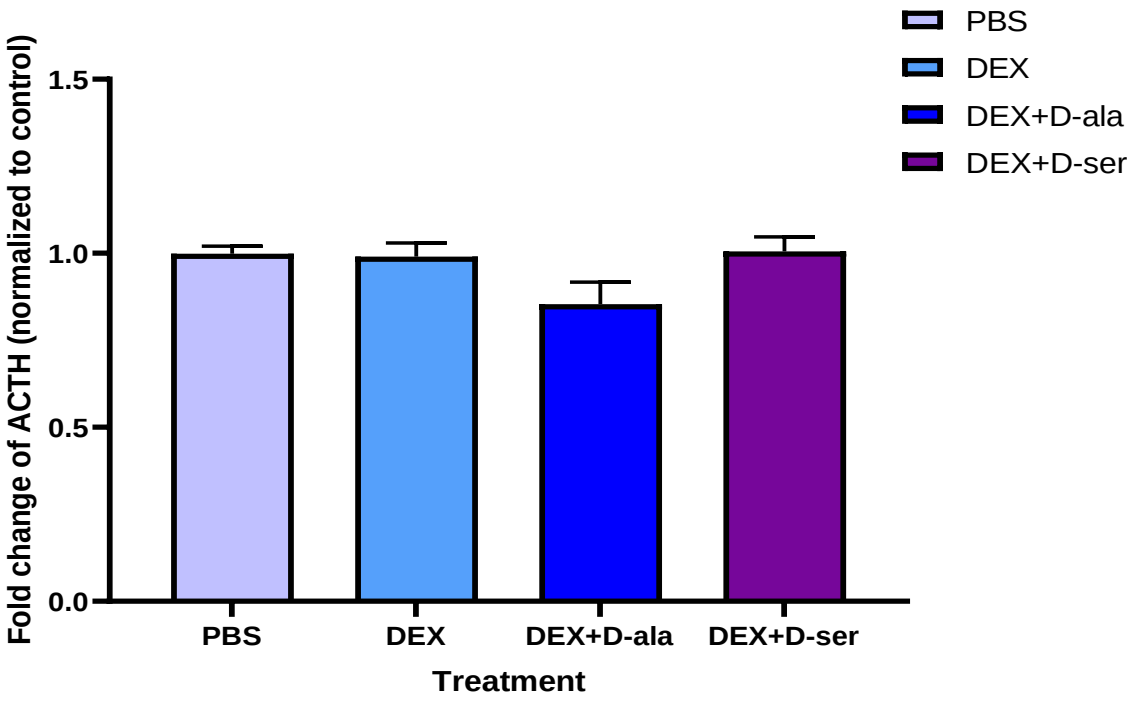


Figure 3.5. The above graph displays the levels of ACTH present in the culture medium of 50 000 AtT20 cells treated with Dexamethasone (100 nM) in the presence of absence of D-alanine or D-serine (500 µM) for 24 hours. The levels of ACTH were measured using ELISA, with the data a mean of 6 replicates. There were no significant changes between the different treatment groups.

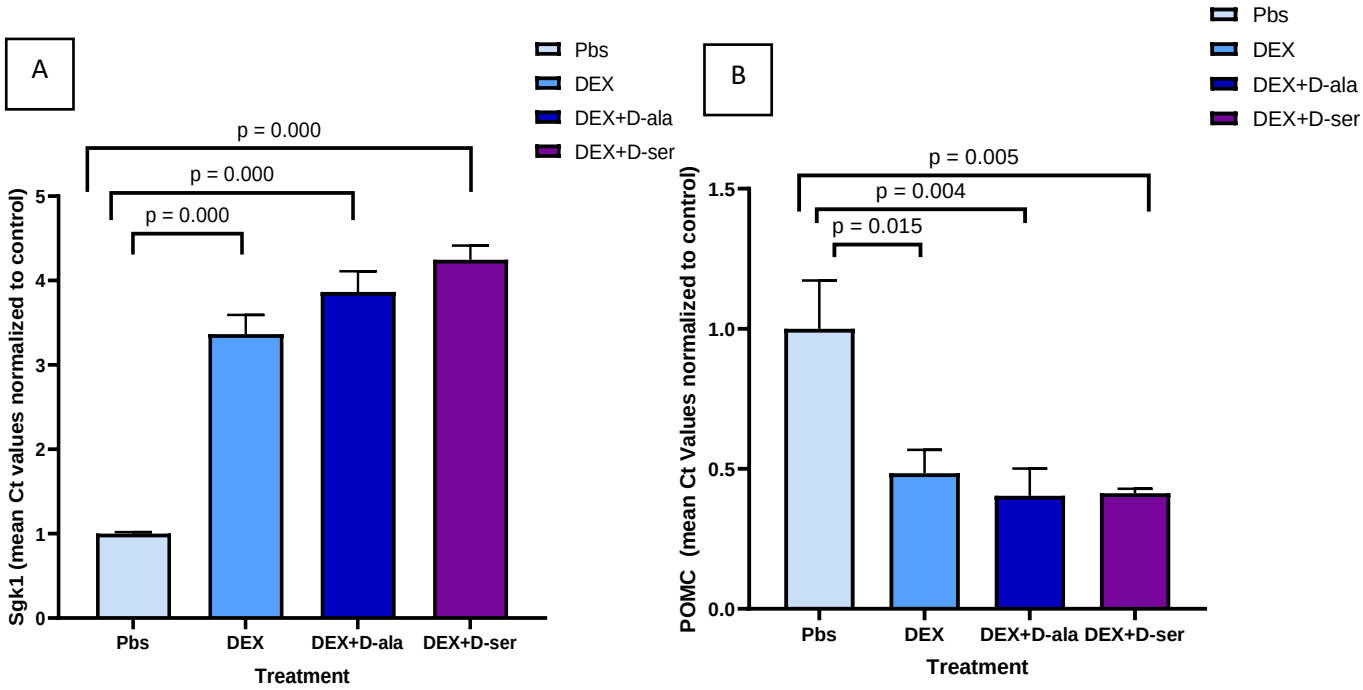


Figure 3.6. SGK1 (A) and POMC (B) mRNA levels were measured using qPCR analysis. 50 000 AtT20 cells were treated with Dexamethasone (100nM) in the presence or absence of D-serine or D-alanine (500µM) for 24 hours (n = 6, Pbs = control). SGK1 levels increased significantly in cells treated with Dexamethasone (p = 0.000, + 237%), Dexamethasone and D-alanine (p = 0.000, +287%), as well as Dexamethasone and D-serine (p = 0.000, +325%) compared to control. POMC suppression occurred in cells treated with Dexamethasone (p = 0.015, -52%), Dexamethasone and D-alanine (p = 0.004, -60%), as well as Dexamethasone and D-serine (p = 0.005, -59%), when compared to control treatment.

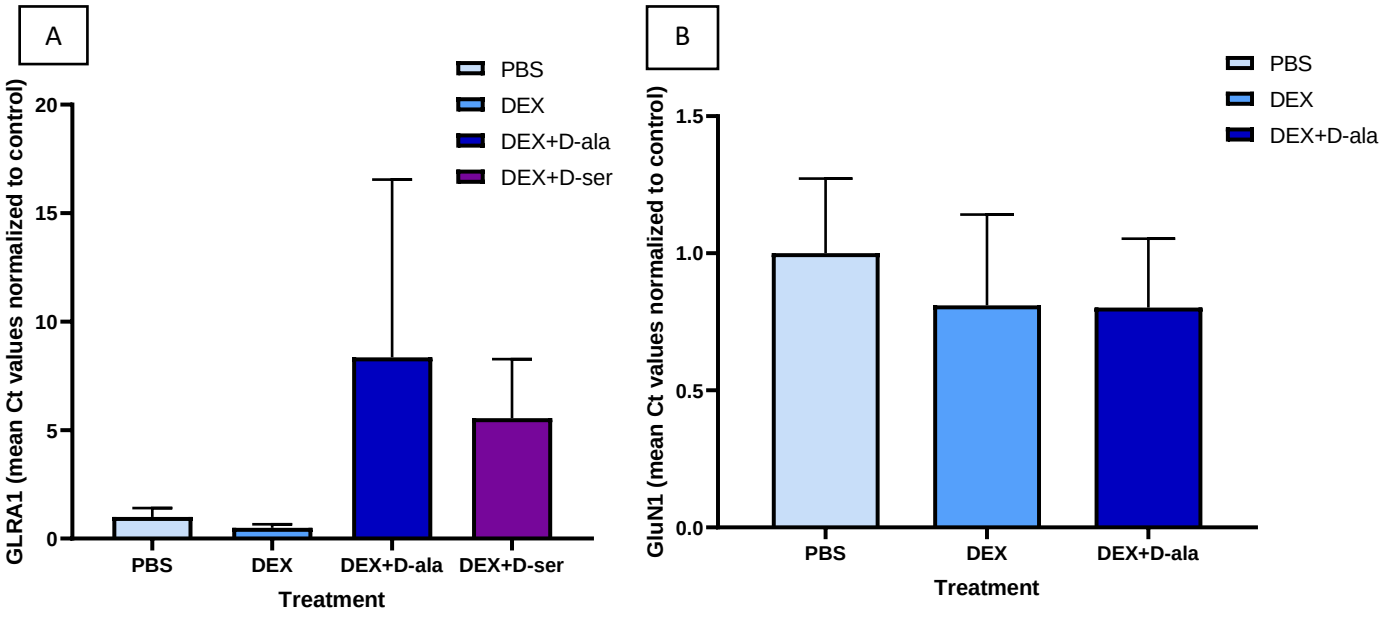


Figure 3.7. The mRNA levels of GLRA1 (A) and GluN1 (B) was measured using qPCR analysis for 50 000 AtT20 cells treated with Dexamethasone (100nM) and/or D-amino acids (500µM) for 24 hours (with PBS as a control and data the mean of six replicates). There were no significant differences between the different treatment measures.

3.3.2 Overexpression of AtT20 cells treated with D-alanine

To determine the effects of Asc-1 (SLC7A10) transporter in the suppression of ACTH by its passengers, D-alanine and D-serine, SLC7A10 was overexpressed in AtT20 cells.

Firstly, ACTH levels were measured to determine the effects transporter overexpression will have on secretion of this hormone, and it was found to have a significant effect ($F_{3,23} = 22.57$, $p = 0.000$). There was a significant reduction of ACTH levels in the cell culture medium of cells transfected with SLC7A10, and then treated with D-alanine, when compared to control, as well as cells transfected with SLC7A10, and then exposed to PBS ($p = 0.000$, -23% and $p = 0.025$, -9% respectively) **(Figure 3.8).** Significantly lower levels of ACTH were expressed in cells treated with just D-alanine compared to control ($p = 0.000$, -16%), as well as in cells transfected with transporter, and then treated with PBS, compared to control ($p = 0.000$, -14%). However, there were no changes in intracellular ACTH in AtT20 cells transfected with Asc-1 and treated with D-alanine (**Figure 3.9).**

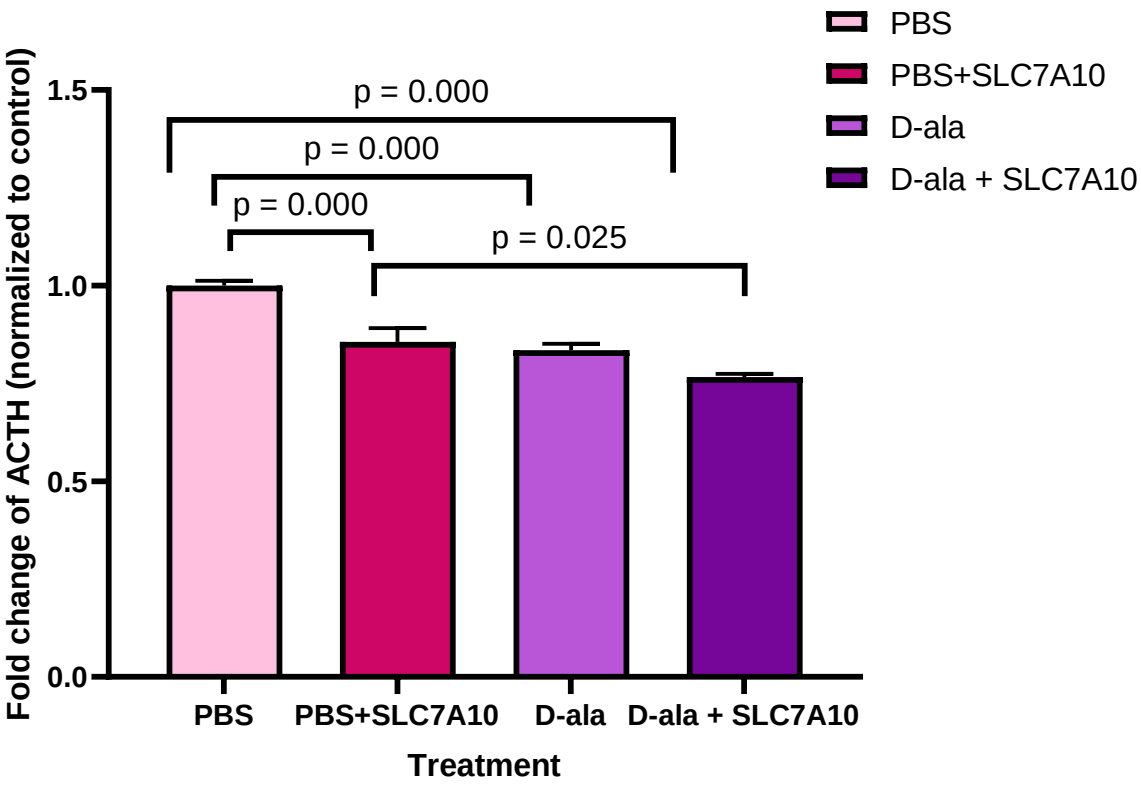


Figure 3.8. ACTH was measured using ELISA. ACTH was detected in the culture medium of 100 000 AtT20 cells transfected with SLC7A10 transporter for 24 hours, followed by a 24-hour treatment with D-alanine (500µM) or PBS. Cells that were not transfected with

transporter and exposed to PBS were used as controls. Data are a mean of six replicates. There was a significant reduction in culture medium ACTH levels in cells transfected with SLC7A10 and then treated with D-alanine when compared to i) control (p = 0.000, -23%), and ii) cells transfected with SLC7A10 and exposed to PBS (p = 0.025, -9%). Significantly lower levels of ACTH were expressed in cells treated with just D-alanine compared to control (p = 0.000, - 16%), as well as cells transfected with SLC7A10 and then treated with PBS compared to control (p = 0.000, - 14%).

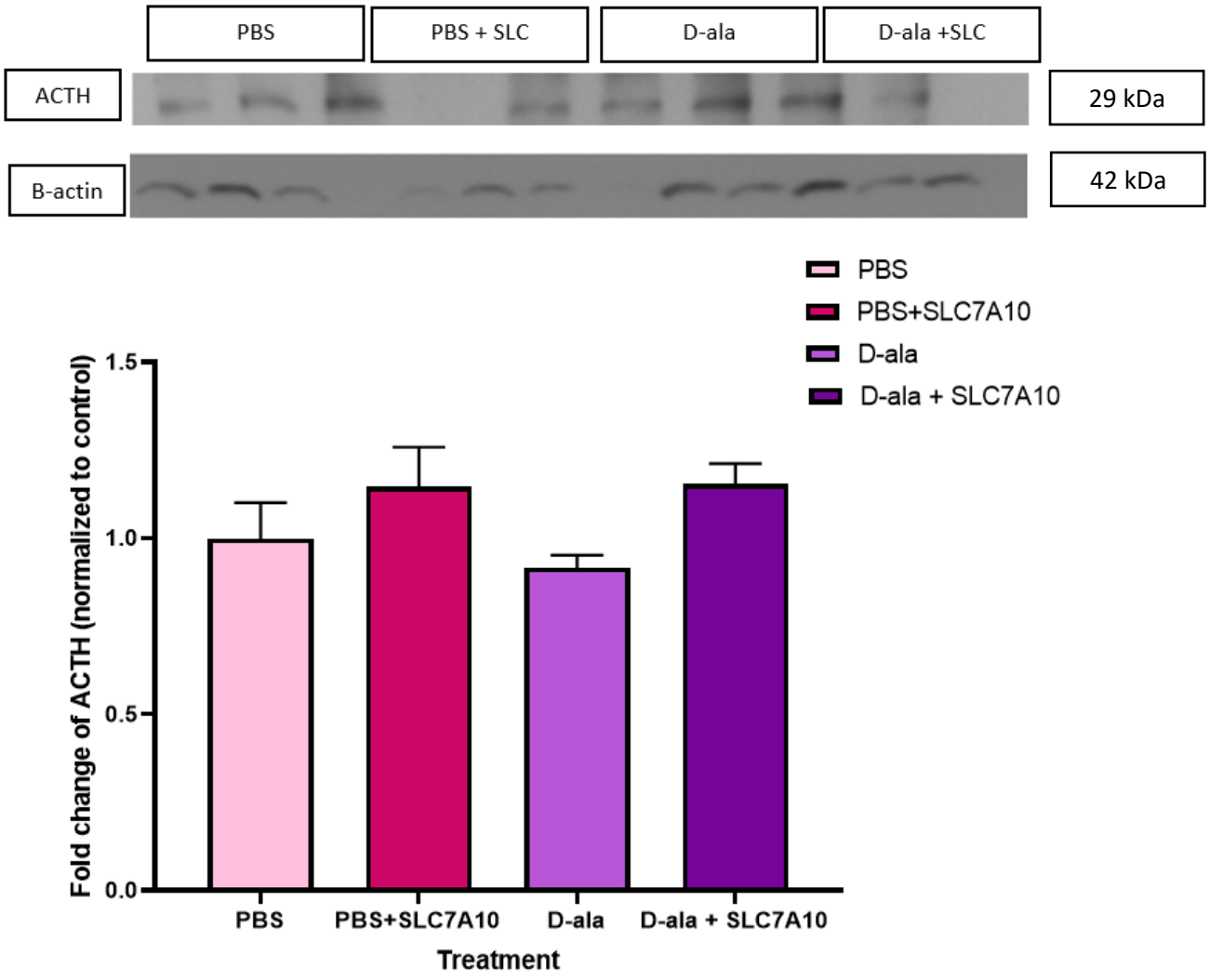


Figure 3.9. The above shows the western blot analysis of ACTH present in AtT20 cells (100 000 cells per well, n = 6), transfected with SLC7A10 transporter for 24 hours, followed by

a treatment with D-alanine at 500µM, or PBS for 24 hours. There were no significant differences between treatment groups.

Following the confirmed reduction of ACTH above, the SGK1 and POMC system was investigated to determine if it has a role to play in the inhibitory effect observed. Overexpression of Asc-1 did affect POMC mRNA levels (**Figure 3.10,** $F_{3,22} = 3.89$, $p = 0.025$). A significantly lower level of POMC mRNA was noted in cells treated with D-alanine following transfection with the transporter ($p = 0.024$, -88%) when compared to control. There was a trending decrease in these cells when compared to cells transfected with transporter and then treated with PBS ($p = 0.072$, -65%) **(Figure 3.10).**

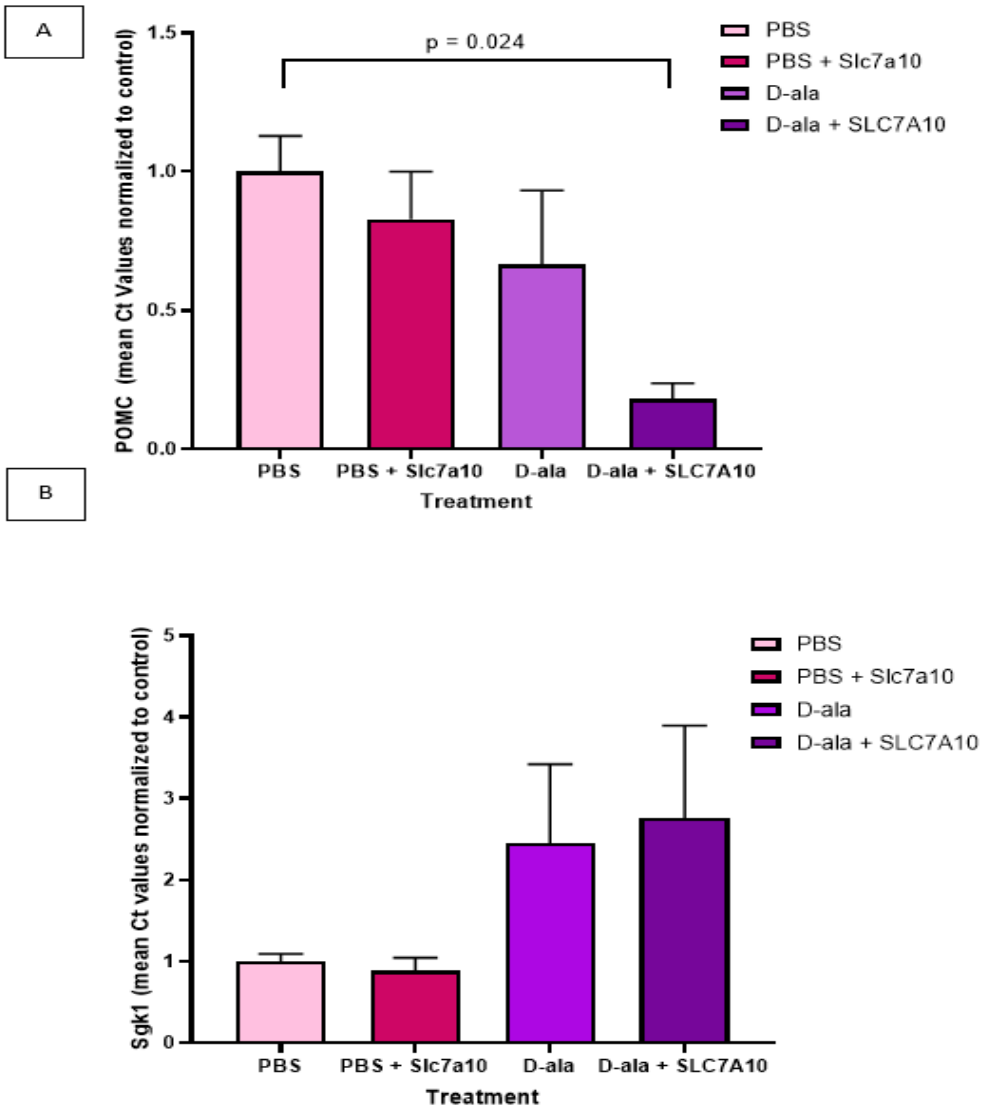


Figure 3.10. POMC (A) and Sgk1 (B) mRNA levels were measured in AtT20 cells (100 000) treated with D-alanine (500µM for 24 hours) following transfection with SLC7A10 transporter (24 hours). Results are presented as a mean of 6 replicates ± SEM and controls were treated with PBS. There was a significantly lower level of POMC in cells treated with D-alanine following transfection with the transporter ($p = 0.024$, -88%) when compared to control, as well as a trending decrease in the same cells when compared to cells transfected with transporter and then treated with PBS ($p = 0.072$, -65%). There were no significant changes in SGK1 mRNA between the groups.

To confirm the successful transfection of AtT20 cells, cells were then analysed for the presence of amino acid transporters Asc-1. SLC7A10 overexpression affected SLC7A10 intracellular protein (**Figure 3.11,** $F_{3,23} = 6.58$, $p = 0.003$). There was a significantly higher level of SLC7A10 transporter protein in cells transfected with SLC7A10, and then treated with D-alanine ($p = 0.002$, +29%), as well as cells treated with just D-alanine (500µM for 24 hours, $p = 0.044$, +19%) compared to PBS control **(Figure 3.11).**

SLC7A10 overexpression affected mRNA levels of ASc-1 and ASCT2 as well (**Figure 3.12,** $F_{3, 22} = 4.10$, $p = 0.021$ and $F_{3, 19} = 5.36$, $p = 0.010$ respectively). There was significantly lower Asc-1 and ASCT2 gene expression in cells treated with D-alanine compared to PBS control ($p = 0.013$, - 90% and $p = 0.009$, -95% respectively). There is significantly lower level of ASCT2 expression in cells transfected with SLC7A10, and then treated with D-alanine when compared to control ($p = 0.034$, -83%) (**Figure 3.12**).

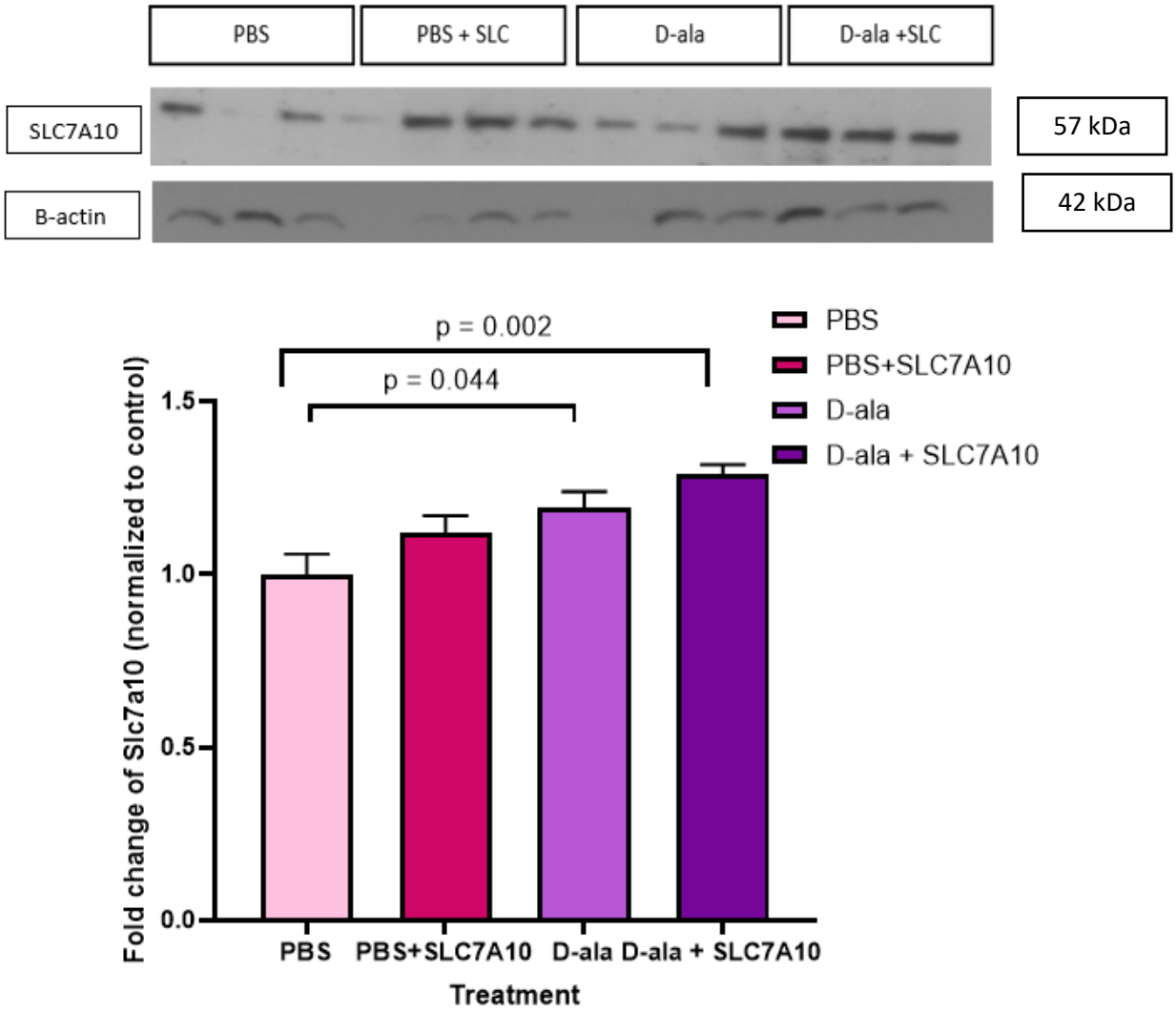


Figure 3.11. 100 000 AtT20 cells were transfected with human SLC7A10 for 24 hours, followed by a treatment with D-alanine (500µM for 24 hours), or PBS (as a control). Data are the mean of 6 replicates, where SLC7A10 protein was detected using Western Blot (bands for SLC7A10 displayed above). There was a significantly higher level of SLC7A10 transporter in cells transfected with SLC7A10 and treated with D-alanine ($p = 0.002$, +29%), as well as cells treated with just D-alanine (500µM for 24 hours, $p = 0.044$, +19%) compared to PBS control.

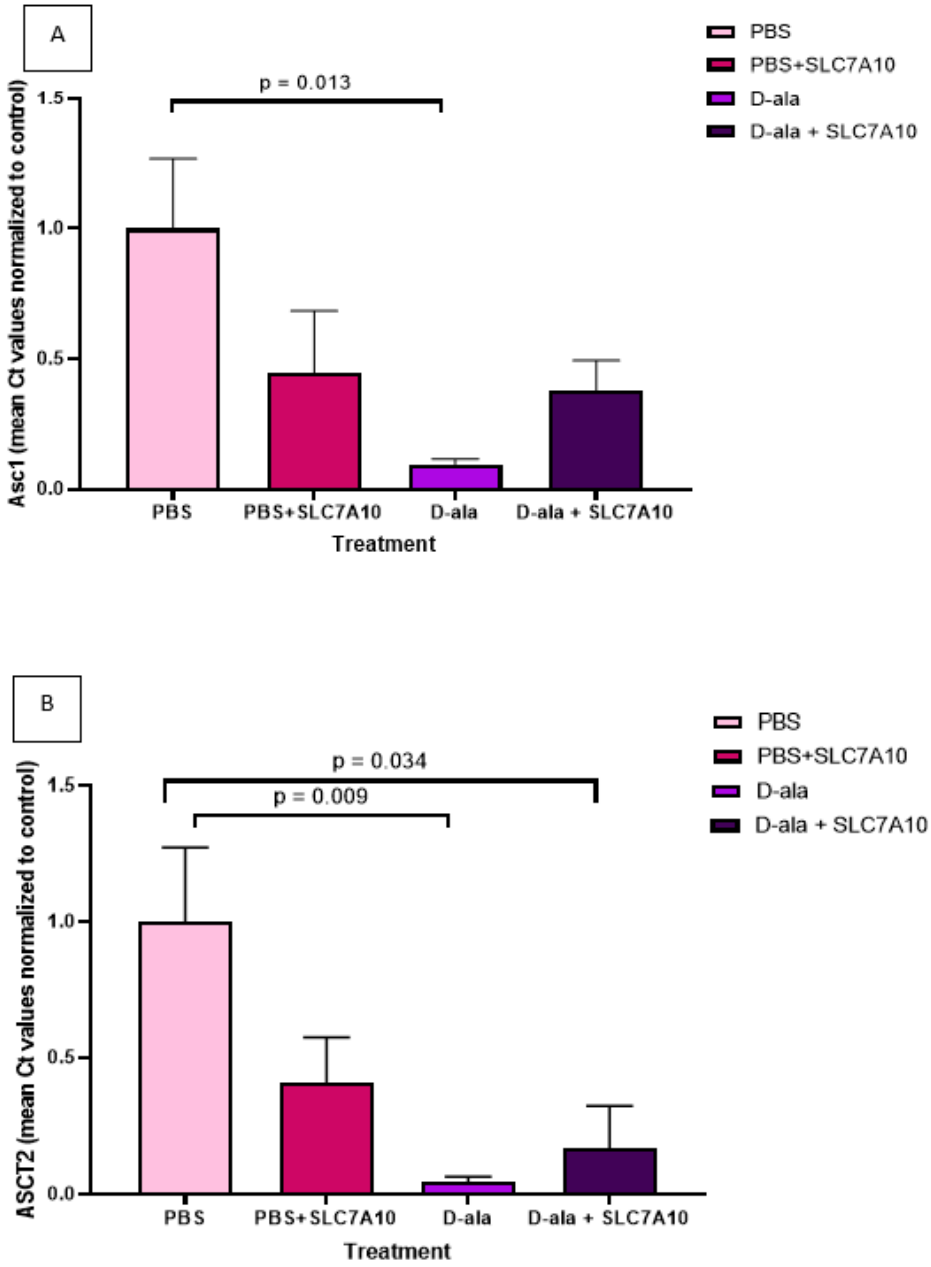


Figure 3.12. Transporter levels were further investigated using qPCR analysis. Asc1 (SLC7A10, A) and ASCT2 (B) mRNA levels were measured in AtT20 cells (100 000, n = 6, pbs = control). Cells were transfected with human SLC7A10 for 24 hours, and then treated with D-alanine (500µM for 24 hours), with PBS in the absence of transfection as a control. Some cells were only treated with 500µM D-alanine for 24 hours. There was a significantly lower Asc1 (p = 0.013, - 90%) and ASCT2 (p = 0.009, -95%) gene expression in cells treated

with D-alanine compared to PBS control. There is significantly lower level of ASCT2 expression in cells transfected with SLC7A10 and treated with D-alanine when compared to control (p = 0.034, -83%).

D-alanine and D-serine are agonists of the inhibitory GLRA1, GluN1 and GluN3 receptors, so receptor presence was also investigated, and overexpression of SLC7A10 affected GLRA1 intracellular protein **(Figure 3.13**, $F_{3, 23}$ = 4.45, p = 0.015) and GLRA1 mRNA (**Figure 3.14,** $F_{3, 19}$ = 12.25, p = 0.000). There was a significant increase in GLRA1 protein in cells transfected with SLC7A10 and then treated with D-alanine when compared to cells transfected with SLC7A10 and treated with just PBS (p = 0.037, +5%) **(Figure 3.13).** Lower levels of GLRA1 mRNA was observed in cells transfected with SLC7A10 and treated with D-alanine (p = 0.000, -98%) or PBS (p = 0.003, -71%), as well as cells not transfected with human SLC7A10 and treated with D-alanine (p = 0.001, - 90%), when compared to PBS control (**Figure 3.16**).

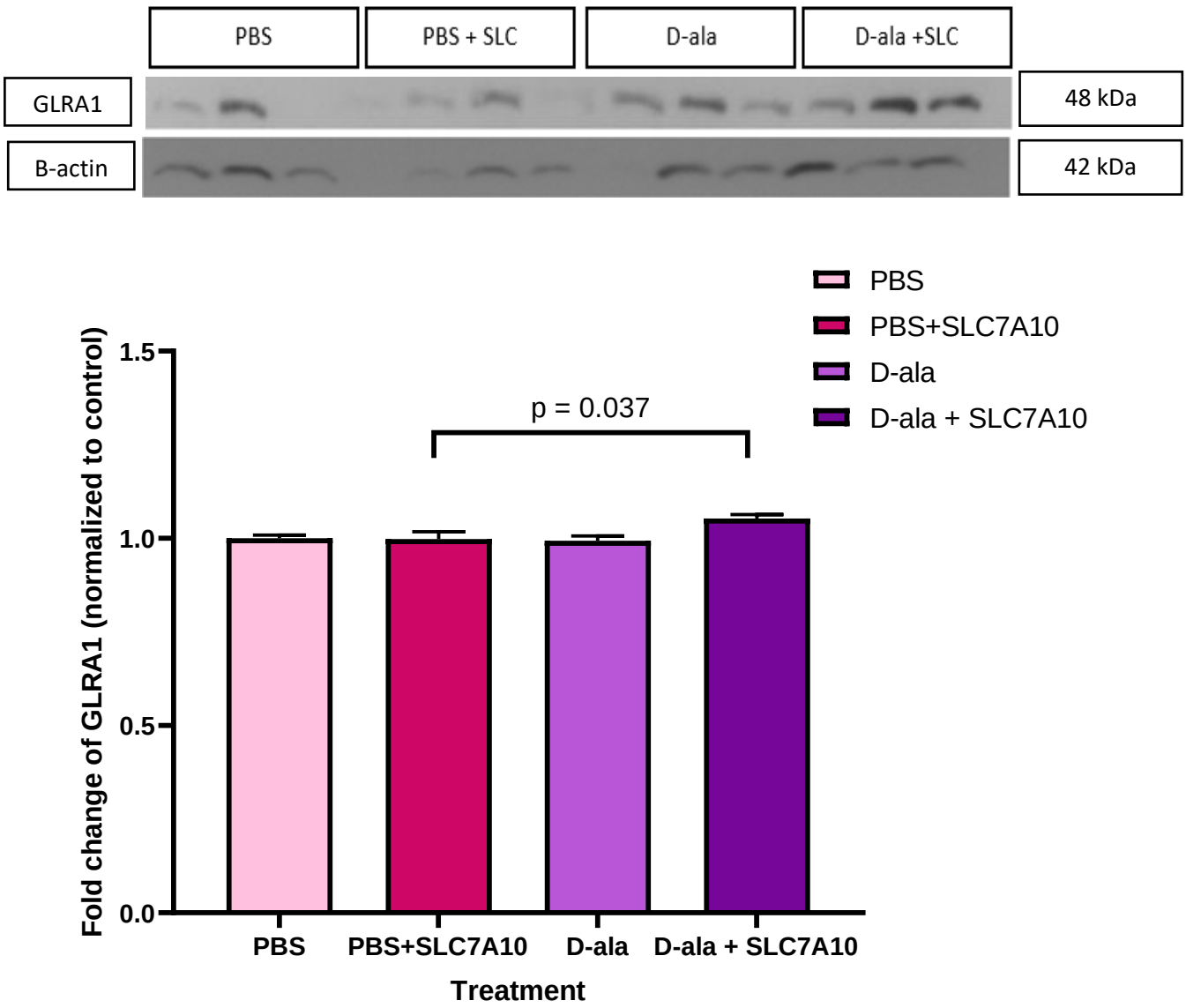


Figure 3.13. The inhibitory Glycine receptor GLRA1 protein levels were measured (bands displayed above) in AtT20 cells (100 000 cells/well) transfected with human amino acid transporter SLC7A10 for 24 hours, and then treated with D-alanine (500µM) for 24 hours, or PBS for 24 hours as a control. There was a significant increase in GLRA1 in cells transfected with SLC7A10 and then treated with D-alanine when compared to cells transfected with SLC7A10 and treated with just PBS ($p = 0.037$, +5%). Data are the mean of 6 replicates.

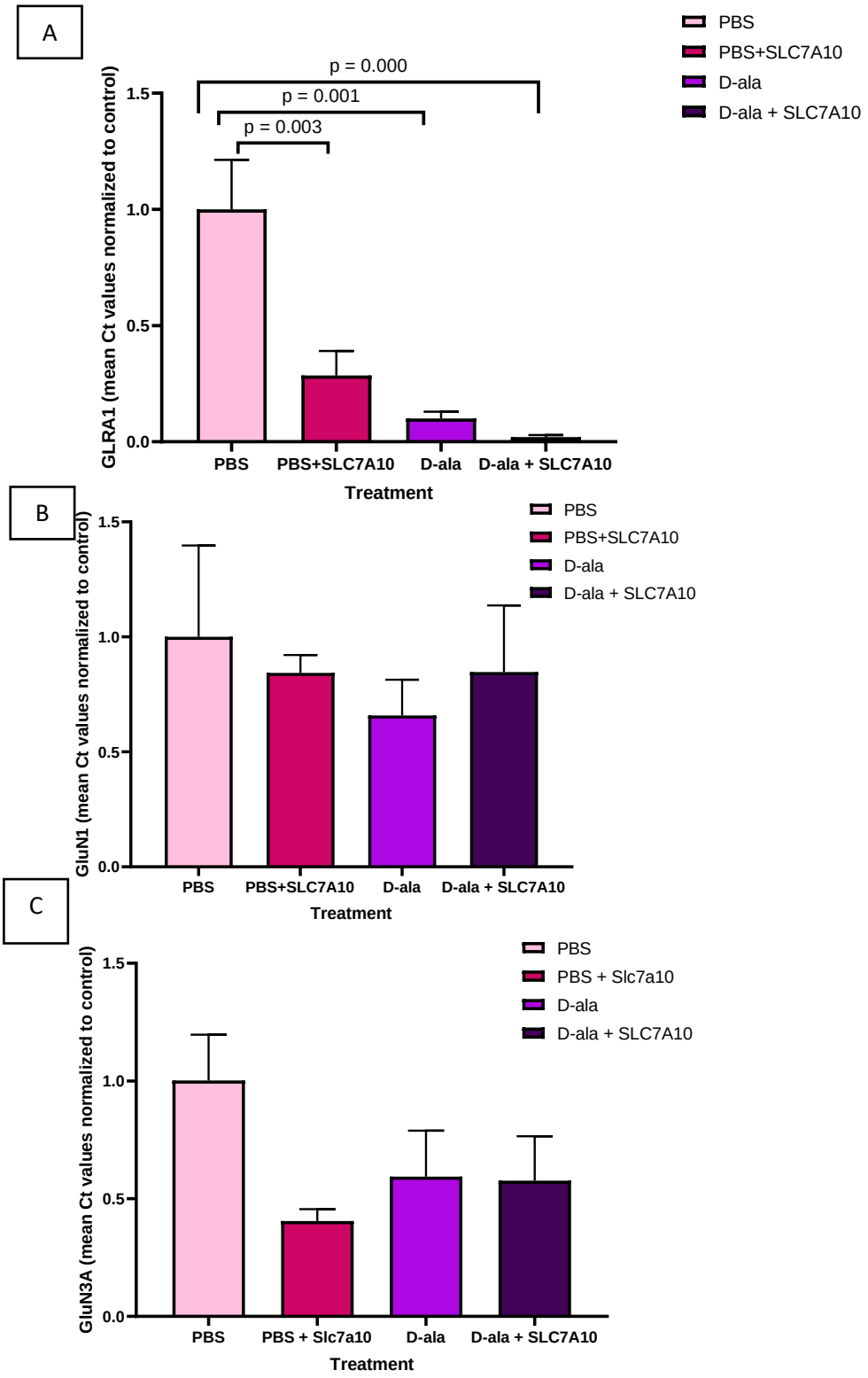


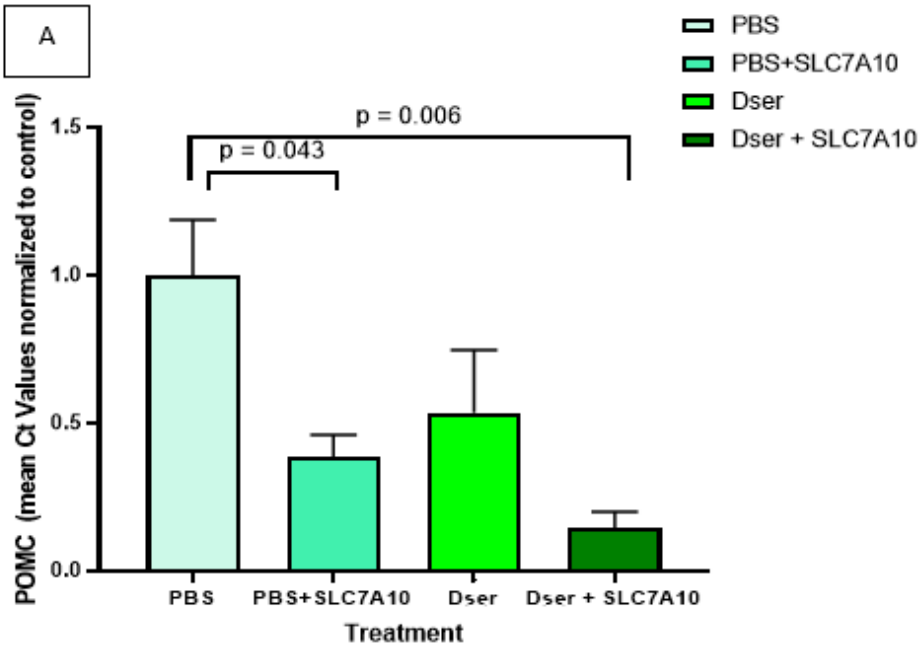
Figure 3.14. The mRNA levels of receptors GLRA1 (A), GluN1 (B) and GluN3A (C) were measured using qPCR. Data was mean of 6 replicates, with PBS as a control, where 100 000 AtT20 cells were transfected with human SLC7A10 for 24 hours, then treated with 500µM

D-alanine for 24 hours. Some cells were transfected, and received PBS has a control treatment. There were significant changes in GluN1 and GluN3A levels, but there was a significantly lower level of GLRA1 in cells treated with D-alanine that have been transfected ($p = 0.000$, -98%) and not transfected with human SLC7A10 ($p = 0.001$, - 90%), as well as cells transfected and treated with PBS ($p = 0.003$, -71%) when compared to PBS control.

3.3.3. Overexpression of SLC7A10 in AtT20 cells treated with D-serine

Given the similarities in D-alanine and D-serine activity (both inhibit ACTH secretion, and both are transported by SLC7A10), I next investigated whether D-serine would have similar effects as D-alanine on SLC7A10 transfected AtT20 cells.

I looked at POMC gene expression as POMC is a regulator of ACTH secretion, and found that overexpression of SLC7A10 affected POMC mRNA levels (**Figure 3.15,** $F_{3, 22} = 5.35$, $p = 0.008$. There was significantly lower levels of POMC mRNA in cells transfected and treated with D-serine ($p= 0.006$, -86%) and PBS ($p = 0.043$, -62%) when compared to PBS control **(Figure 3.15).**



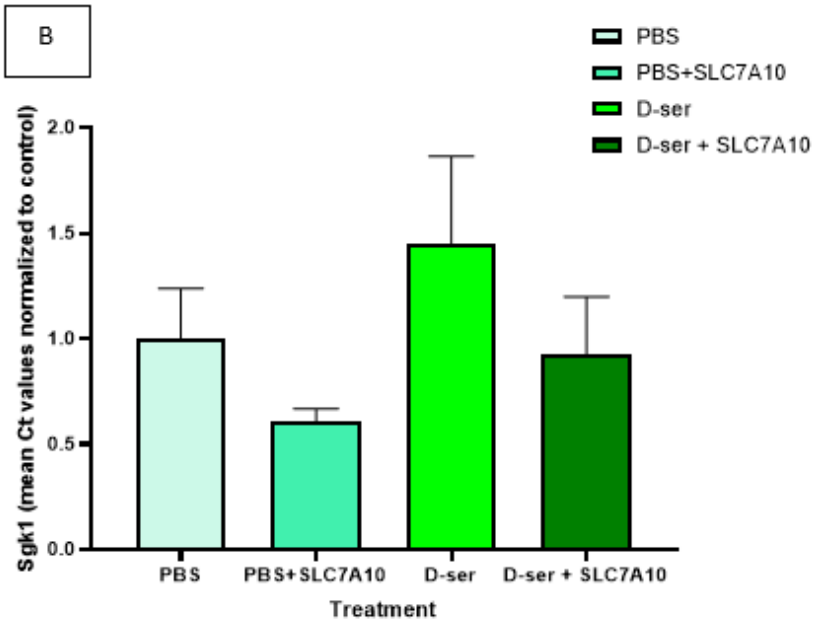


Figure 3.15. POMC (A) and Sgk1 (B) mRNA levels were measured in 100 000 AtT20 cells transfected with human SLC7A10 and treated with D-serine (500µM). The transfection lasted for 24 hours, followed by treatment for 24 hours and PBS was used as a control in the presence and absence of transfection. The data are mean of 6 replicates. There was

significantly lower level of POMC in cells transfected and treated with D-serine (p = 0.006, -86%) and cells transfected with transporter and treated with PBS (p = 0.043, -62%) when compared to PBS control. There were no significant changes in SGK1 cDNA levels between treatment groups.

The Asc-1 and ASCT2 mRNA levels were affected by overexpression of AtT20 cells with Asc-1 (**Figure 3.16,** $F_{3, 20} = 24.62$, p = 0.000 and $F_{3, 21} = 43.78$, p = 0.000 respectively). There was significantly lower Asc1 (**Figure 3.16a**) and ASCT2 (**Figure 3.16b**) gene expression in cells transfected and treated with D-serine (p = 0.024-77%, and p = 0.000, -76% respectively) when compared to controls. Cells transfected with SLC7A10 and treated with PBS had significantly lower Asc-1 and ASCT2 mRNA levels compared to controls (p = 0.002, - 95% and p = 0.000, - 90%). Cells treated with just D-serine had lower Asc-1 and ASCT levels (p = 0.002, -96% and p = 0.001, - 89% respectively) when compared to PBS controls.

There were no significant changes in GLRA1 and GluN1 mRNA levels for the respective groups, but Asc-1 overexpression affected GLUN3A mRNA ($F_{3, 20} = 15.06$, p = 0.000). There were significantly lower levels of GluN3A in cells transfected and treated with D-serine (p = 0.003, -66%) or PBS (p = 0.000, - 91%) or just D-serine (p = 0.000, -88%) when compared to PBS control (**Figure 3.17**).

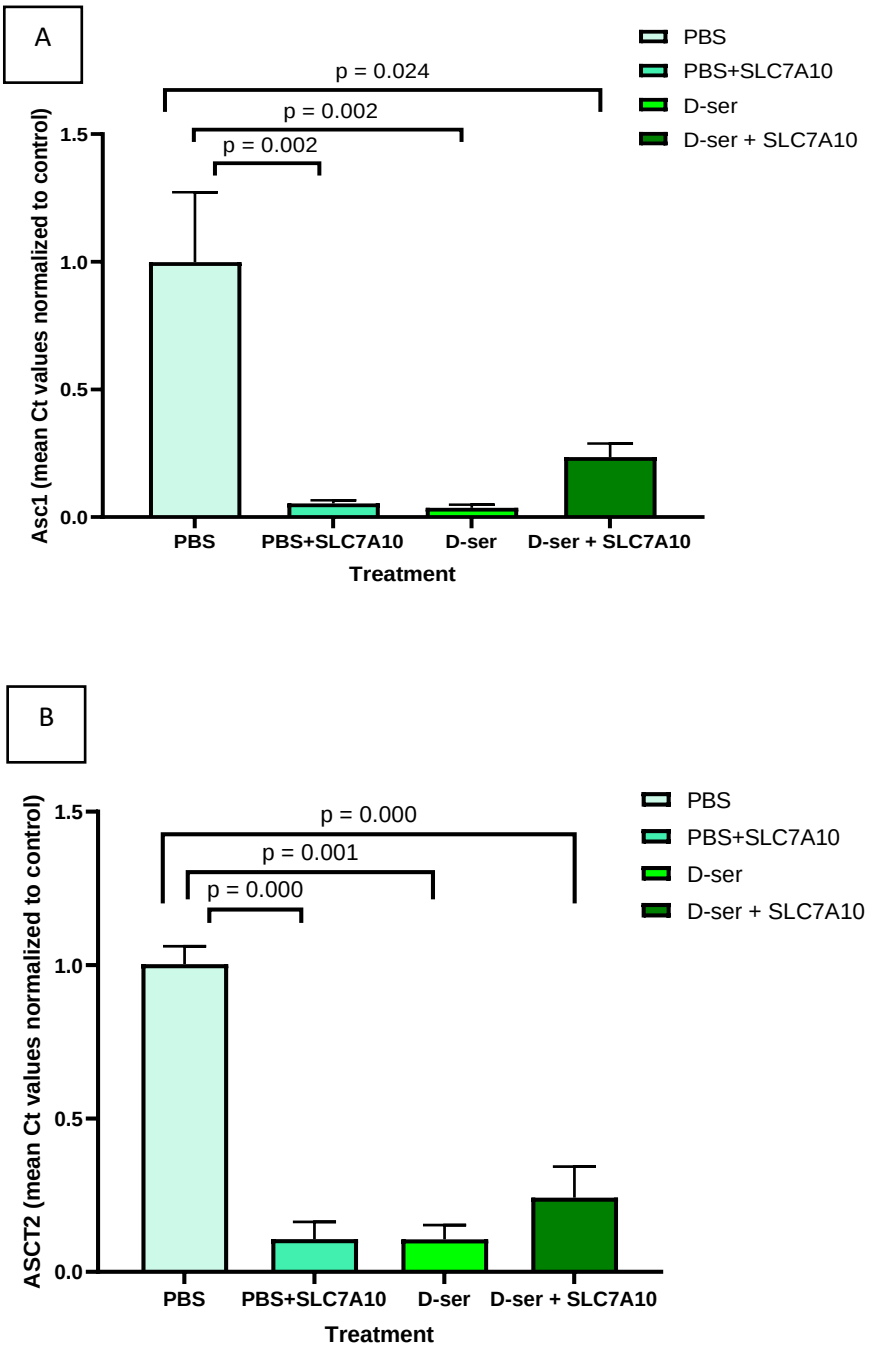


Figure 3.16. qPCR analysis of transporter levels. Asc1 (SLC7A10, A) and ASCT2 (B) mRNA levels were measured in AtT20 cells (100 000, n = 6, pbs = control). Cells were transfected with human SLC7A10 for 24 hours, and then treated with D-serine (500µM for 24 hours), with PBS, in the absence of transfection, as a control. Some cells were only treated with 500µM D-serine for 24 hours. There was significantly lower Asc1 and ASCT2 expression in cells transfected and treated with D-alanine (p = 0.024-77%, and p = 0.000, -

76% respectively), and cells transfected and treated with PBS (p = 0.002, - 95% and p = 0.000, - 90% respectively), as well as cells treated with just D-serine (p = 0.002, -96% and p = 0.001, - 89% respectively) when compared to PBS controls.

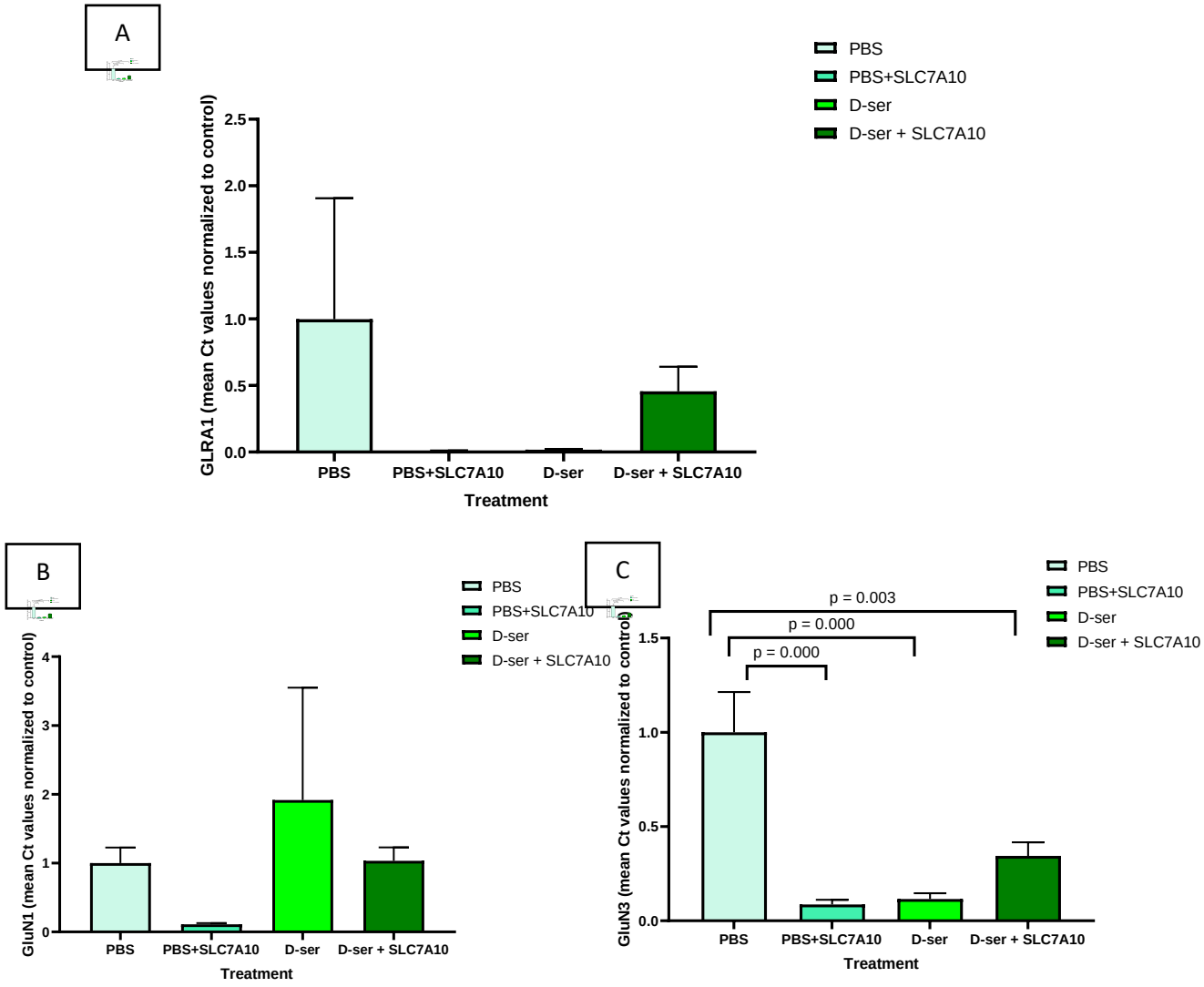


Figure 3.17. The above graph show the mRNA levels of multiple receptors in AtT20 cells transfected with human SLC7A10 vector. 100 000 cells were treated in 6 replicates, with PBS used as a control. Transfection occurred after overnight incubation with plasmid DNA. 500µM of D-serine was administered to transfected cells for 24 hours, and cells treated with just PBS was used as a control. There were no significant changes in GLRA1 and GluN1 levels in mRNA for respective groups, but there was a significantly lower level of GluN3A between cells transfected and treated with D-serine (p = 0.003, -66%) or cells transfected

and treated with PBS (p = 0.000, - 91%) or just D-serine (p = 0.000, -88%) when compared to PBS control.

3.4. Discussion

I found that Dexamethasone increased gene expression of SGK1 in AtT20 cells, whilst in contrast, decreased POMC mRNA levels (**Figure 3.6)**, and these changes were more pronounced in cells that received D-alanine or D-serine. AtT20 cells were successfully transfected with Asc-1 (**Figure 3.11)**, and affected ACTH secretion, with cells overexpressed with Asc-1 and treated with D-alanine exhibiting the most pronounced inhibition on ACTH secretion (**Figure 3.8),** and overexpression of Asc-1 in AtT20 cells treated with D-serine showing the most pronounced inhibition of POMC (**Figure 3.15).**

3.4.1. Dexamethasone abolishes the inhibitory effects of ACTH secretion in AtT20 cells

The attenuation of the suppressive effect of D-amino acids on ACTH secretion in AtT20 cells in the presence of dexamethasone is an interesting result (**Figure 3.5**). According to the literature, Dexamethasone has displayed inhibitory effects on ACTH secretion in a plethora of studies (Riebold et al., 2015, Suda et al., 1993, Gambardella et al., 1989, Hohnloser et al., 1989, and Suda et al., 1988). Cassarino et al (2016) showed that dexamethasone had diverging effects on ACTH secretion, where they showed both decreases and increases from pituitary adenomas. Seeing as there was an inhibitory effect detected in POMC mRNA levels of AtT20 cells exposed to Dexamethasone in the presence of D-amino acids (**Figure 3.6***)*, but not POMC mRNA levels in the absence of Dexamethasone (**Figure 2.11b, Chapter 2**), it is possible that this GC may assist in POMC gene expression regulation, and perhaps prolongs the transcription phase in these cells, so that downstream translation into POMC occurs later, which will affect ACTH protein production. This can be further investigated by looking at ACTH content in media of AtT20

cells treated after a longer duration, so that we have a better understanding of the dexamethasone effect on ACTH secretion.

3.4.2 D-alanine and D-serine potentiate the increase of SGK1 mRNA but inhibits POMC mRNA expression in the presence of Dexamethasone in AtT20 cells.

As observed in Chapter 2 (**Figure 2.11a, Chapter 2**), D-serine and D-alanine have a stimulatory effect on SGK1 mRNA expression when compared to the control treatment, and this effect is potentiated in the presence of the synthetic glucocorticoid, dexamethasone. This supports the finding of Reiter et al (2011), that dexamethasone stimulates SGK1 in AtT20 cells, possibly via GR activation, as they found the stimulatory effect was abolished in the presence of GR antagonist, RU-486.

D-amino acids failed to inhibit POMC mRNA expression independently (**Figure 2.11b, Chapter 2**), which was interesting as they successfully inhibited ACTH secretion, a downstream product of POMC following enzymatic cleavage. However, in the presence of dexamethasone, and in fact, with dexamethasone independently, POMC expression was supressed when compared to the control treatment (**Figure 3.6)**.

Cassarino et al (2016) investigated dexamethasone effects on POMC in 53 ACTH-secreting adenomas in humans. These adenomas were incubated with 10 nM of dexamethasone for 24 hrs, and it was found that resultant POMC genetic expression was varied, with significant increases and decreases noted in the adenomas. There was no correlation between POMC gene expression and ACTH secretion. This implies that POMC peptide processing, or transport, plays a key role in regulating ACTH secretion, rather than POMC gene expression. This may explain why I saw an earlier inhibition of ACTH (**Figure 2.7, Chapter 2**), but no corresponding inhibition of POMC mRNA (**Figure 2.11, Chapter 2**). However, my findings are supported by basal levels of steady state POMC mRNA levels that were supressed in nonadenoma cells exposed to 1 µg/dl dexamethasone for 24hrs (Suda et al., 1988), and they

further established this dexamethasone inhibitory effect on POMC mRNA levels in a later study in 10 pituitary adenomas (Suda et al., 1993).

Reiter et al (2011) found that SGK1 over expression by transfection stimulated POMC expression in AtT20 cells. I have found that dexamethasone significantly increases SGK1 expression, which should lead to a stimulation of POMC according to Reiter et al (2011), however, I observed the opposite. This could be due to methodological differences. The feedback inhibition of POMC by dexamethasone, outlined in Suda et al (1988 and 1993), may overpower the stimulatory effects of SGK1, as SGK1 levels in the current study may not be as potent as they were in Reiter et al (2011), as they had overexpressed it via transfection rather than stimulated it via dexamethasone, as I did.

3.4.3. Suppressive effects of SLC7A10 transfection, in the presence or absence of D-alanine, on ACTH secretion

The inhibitory effect of human SLC7A10 DNA transfected in AtT20 cells on ACTH secretion supports the hypothesis that the original suppressive effects of ACTH by D-alanine and D-serine **(Figure 2.7, Chapter 2).** Transfected cells in the presence and absence of D-alanine (**Figure 3.8**), significantly decreased ACTH levels when compared to controls, and our initial finding **(Figure 2.7, Chapter 2)** of a decrease in ACTH when cells were exposed to D-alanine was successfully repeated. Interestingly, there was a significantly lower level of ACTH secreted in cells that had been treated with D-alanine following transfection, when compared to transfected cells that received just PBS. This shows that the decrease in ACTH we observed may have something to do with D-alanine, as overexpression of the transporter SLC7A10 should lead to a higher presence of D-alanine in the cells, as more D-alanine should be taken up. To confirm these findings, one could look at manipulating the transporter by blocking its activity using an antagonist like Lu AE00527 (Mesuret et al., 2018) and observing if this affects ACTH release.

It would be of interest in future studies, to observe the transporter effects on ACTH levels in SLC7A10 knockout mice, as well as corticosterone levels, to determine the effects this transporter has lower down the HPA axis. Alternatively, germ free mice ACTH levels will be of interest, as germ-free mice should lack D-alanine, as mammalian D-alanine is of microbial origin, and we can determine the effects the presence of D-alanine has on ACTH secretion.

4.4.4 Inhibition of POMC mRNA in AtT20 cells transfected with SLC7A10, in the presence of either D-alanine or D-serine

The significant reduction in POMC mRNA levels in SLC7A10 transfected AtT20 cells in the presence of D-alanine in comparison to control **(Figure 3.10)** supports the reduction we see in the POMC peptide product, ACTH in the media (**Figure 3.8**). Unfortunately, due to time constraints, we were unable to analyse the ACTH levels in culture media of AtT20 cells transfected with SLC7A10 and treated with D-serine, to see if it corresponds with the decrease in POMC mRNA **(Figure 3.15)** we observed in cells overexpressed with transporter in the presence or absence of D-serine, when compared to control**.**

A possible explanation for the failure to reproduce the reduction we observe in POMC mRNA in cells transfected with SLC7A10, and then treated with PBS in the D-alanine vs. D-serine experiments (**Figure 3.10. vs Figure 3.15**) is that the cells used in the transfection experiments treated with D-serine (Passage 9) were older than the cells used in transfection experiments treated with D-alanine (Passage 7). I have previously done experiments into the passage effects of AtT20 cells, and found that cells in the later passage seemed to display D-amino acid affects more robustly, when compared to earlier passages, where cells as early as passage 3, did not display any observed effects we see in passage 7 and older cells (**Appendix**). This could be due to receptor maturation occurring in the later passage, and in this case, possible, transporter maturation. Our group has observed that older mice receiving

D-alanine treatment displayed anxiolytic effects, whilst younger mice did not, inferring that D-amino acid effects may be age-dependent (unpublished findings), and perhaps there is translatability of this *in vitro.*

In the absence of SLC7A10 overexpression, the reduction in POMC mRNA does not occur (**Figure 3.10** and **Figure 3.15**), which supports the finding in **Figure 2.12a**, that D-amino acids alone do not inhibit POMC mRNA levels.

The reduction in POMC mRNA in SLC7A10 transfected cells treated with D-alanine (**Figure 3.10**) or D-serine (**Figure 3.15**) fits well with what we see in ACTH secretion (**Figure 3.8**), and it is therefore possible that the reduction in ACTH we observe is via POMC signalling. Entry of calcium via receptor activation (potentially the binding of D-alanine or D-serine to NMDAR) may lead to activation of the ERK signalling pathway, which eventually acts on POMC expression (Bonfiglio et al., 2011). Therefore, an increase in D-alanine uptake by transporter SLC7A10, may lead to increased binding of D-alanine to inhibitory receptors, such as GlyRs, or inhibitory subunits of the NMDA receptors, which may lead to a decrease in ERK phosphorylation. This decrease means that this transcription factor affects POMC expression to a lesser extent than cells that have lower D-alanine levels (due to the lack of overexpression of the SLC7A10 transporter), which is shown in the mRNA levels of POMC in transfected cells treated with D-alanine compared to control cells.

However, the initial results of a decrease in ACTH in the culture medium of cells treated with D-alanine or D-serine (**Figure 2.7**), does not have a corresponding reduction in POMC mRNA (**Figure 2.11a**). Perhaps, what we see in the cells that are overexpressed with transporter, and therefore have higher levels of D-alanine and D-serine, is longer lasting effects on POMC mRNA signalling due to potency of the D-amino acids. Without transporter overexpression, in the time frame of 24 hrs, we observe only the downstream

effects at the ACTH level, and the POMC signalling has normalized in that time frame due to lesser exposure of D-amino acids. To confirm that POMC gene expression changes may occur earlier on, we could possibly do an experiment with a shorter time frame where AtT20 cells are treated with D-amino acids independently, or we could increase the concentration of D-amino acids we use in those treatments to see if that would have a more potent effect on POMC mRNA.

3.4.5. Stimulation of SLC7A10 (Asc-1) protein levels by D-alanine, in the absence or presence of SLC7A10 vector transfection

The observed increase in the SLC7A10 in AtT20 cells transfected with human SLC7A10 and treated with D-alanine compared to controls (**Figure 3.11**) is of note, as this indicates that the transfection of the human SLC7A10 vector worked. I did not observe a significant difference in transporter levels between cells transfected with SLC7A10 and then treated with D-alanine, and cells treated with just D-alanine. This is possibly due to the fact that SLC7A10 is already expressed in AtT20 cells naturally, and perhaps there was some cross reactivity of the human SLC7A10 western blot antibody that was used, and the mouse SLC7A10 that would have been expressed in the mouse cell line AtT20, and therefore there was not a significant difference in transporter levels in these cells. A potential future study to support this finding could be analysing the band present for SLC7A10 in the AtT20 cells just treated with D-amino acids, perhaps with mass spectrometry, to determine that the protein identified is in fact the mouse form of SLC7A10.

3.4.6. Inhibition of Asc-1 and ASCT2 mRNA in AtT20 cells transfected with SLC7A10 (Asc-1) and treated with D-alanine or D-serine

Decreases in both Asc-1**(Figure 3.12a)** and ASCT2 (**Figure 3.12b**) mRNA in AtT20 cells treated with D-alanine is to be expected but was noteworthy as this is the first time these transporters have been detected in these cells, or in the anterior pituitary. The study by Etoh

et al (2009) that confirmed the presence of D-alanine in ACTH secreting cells of the anterior pituitary hypothesized that D-alanine must be transported there utilizing an amino acid transporter such as Asc-1, and this serves to confirm the presence of such transporters in the anterior pituitary. The decrease in ASCT2 mRNA in AtT20 cells treated with D-alanine in the presence or absence of cells that have been transfected with SLC7A10 is understandable, as the presence of these amino acids may activate these transporters, and activation is often displayed as a decrease in mRNA levels. I have confirmed this by investigating the presence of Asc-1 and ASCT-2 in rodent models and confirming the presence of these transporters in the pituitary gland (**Chapter 5**).

The significant reduction of Asc-1 (**Figure 3.16a**) and ASCT2 (**Figure 3.16b**) in cells overexpressed with SLC7A10, in the presence and absence of D-serine, as well as cells treated with just D-serine when compared to controls shows that the presence of D-serine affects these transporters. D-serine is transported by both these transporters (Suzuki et al., 2019 and Foster et al., 2016), and therefore the suppression of transporter mRNA that have been exposed to D-serine is sensible, as these cells must have the most activated transporter levels.

The activation of both these transporters in cells exposed to high levels of D-serine or D-alanine due to transporter overexpression is thought-provoking, as this could be a potential reason for the observed inhibition of ACTH in AtT20 cells exposed to the same treatment conditions. A potential way of confirming this is to use Asc-1 or ASCT2 knockout mice, and observe the ACTH, POMC and corticosterone levels, to determine if this transporter interacts with the HPA axis in a regulatory way.

3.4.7. Increased GLRA1 protein expression, but decreased GLRA1 mRNA levels in AtT20 cells transfected with SLC7A10 in the presence of D-alanine

The presence of GLRA1, as previously stated in the last chapter, in AtT20 cells, and in general, the anterior pituitary gland, is an exciting confirmation, as Stojilkovic et al., (2010) previously published that this receptor is not found in the anterior pituitary. I further confirm this *in vivo* in **Chapter 5**, where GLRA1 mRNA levels are detected in the pituitary gland of CD1 mice.

The increase in GLRA1 levels in cells transfected with SLC7A10, and then treated with D-alanine **(Figure 3.13**) is unsurprising as D-alanine is an agonist of the strychnine sensitive glycine receptor. Therefore, the higher the presence of D-alanine, the more active binding of this receptor. This relates to the mRNA levels as well (**Figure 3.14**), as I see a decrease of GLRA1 mRNA in both transfected cells, and non-transfected cells treated with D-alanine, compared to controls, with the cells transfected and exposed to D-alanine displaying the greatest suppression. This could be that the time period needed for activation of these cells need to be investigated further, and perhaps most of the regulation is occurring at the gene expression level at basal levels, whereas only the most potent effects - overexpressed transporter treated with D-alanine, have downstream effects reflected in protein levels.

However, due to our previous findings with Taurine in **Chapter 2,** which is the full agonist of the glycine receptor **(Figure 2.16)**, it can be assumed that the suppressive effects of D-alanine on ACTH is not directly via GlyR activation, and the activation observed here is due to the presence of the agonist, D-alanine, being available in great quantities due to overexpression of its transporter, SLC7A10.

3.4.8. Decrease in GLUN3A mRNA levels in AtT20 cells overexpressed with SLC7A10 and treated with D-serine

This further confirms the finding in chapter 2, of the presence of NMDAR glycine subunit GLUN3A in these cells. D-serine has been shown to partially activate the GLUN3A subunit of the NMDA receptor (Smothers and Woodward, 2007), therefore a decrease in mRNA

levels implying activation is expected. D-alanine also binds to the glycine subunit of NMDAR (Barth et al., 2005) but probably to a lesser extent, as D-serine is the endogenous co-agonist of the NMDA receptor. However, since D-serine is the only D-amino acid that has an effect on GLUN3A mRNA levels, it does not explain the D-alanine propagated inhibition of ACTH observed in the previous chapter.

3.4.9. Further studies, Limitations and Concluding remarks

The greatest limitation to this chapter was the COVID-19 pandemic. The ACTH ELISA data for AtT20 cells overexpressed with Asc-1 and treated with D-serine would have been complementary to the data seen in **Figure 3.8,** where overexpression with Asc-1 and exposure to D-alanine reduced ACTH secretion. It would have also been good to have confirmed the transfection in D-serine cells by running these samples for a western blot analysis of human SLC7A10, much like it was done in **Figure 3.14** for D-alanine.

Reproducibility in this cell line was also a challenge, where there was inconsistencies especially with mRNA levels in various genes. This is discussed further in Chapter 6, but AtT20 cells need to be well monitored when growing to ensure that experiments are only performed when cells have reached maturity. Additionally, the potential cross-reactivity of the Human SLC7A10 antibody in the AtT20 mouse cells was a limitation, as changes between transfected and not transfected cells that were activated by D-alanine were hard to differentiate.

Further studies could explore the relationship of HPA axis hormone secretion and Asc-1 transporter *in vivo* by knocking out Asc-1 transporter in the anterior pituitary and observing the effects this would have on ACTH, POMC and corticosterone secretion, to confirm my *in vitro* findings.

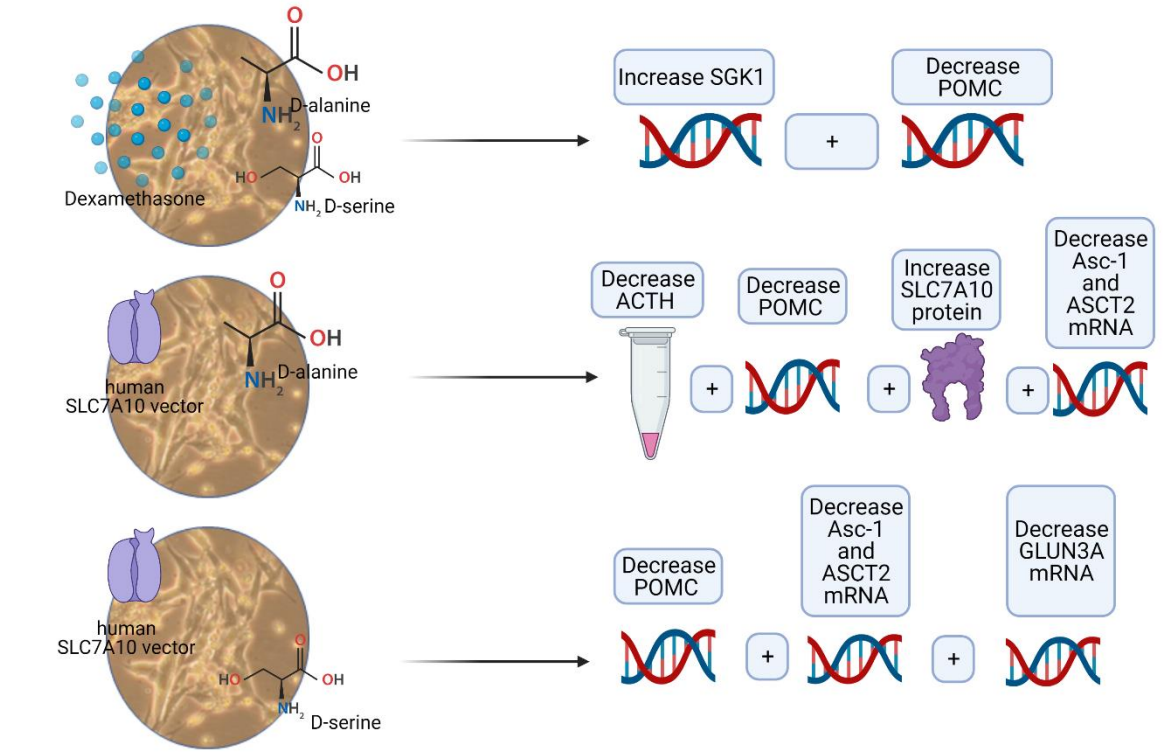


Figure 3.18. A summary of the finding in this chapter. Image made with Biorender.com

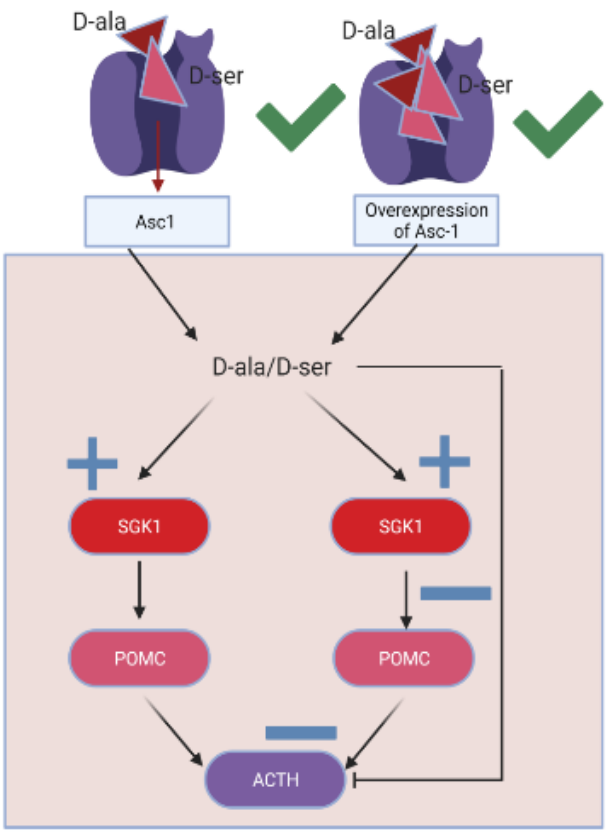


Figure 3.19. An update of the signalling pathways mapped out in the previous chapter. Image made with Biorender.com.

Currently, based on the data it is unlikely that the NMDARs or the GlyRs are involved in inhibition of ACTH by D-alanine or D-serine. Overexpression of Asc-1 served to exacerbate both the increase of SGK1 mRNA and inhibition of ACTH, whilst also inhibiting POMC. therefore the observed inhibition of ACTH is likely through the Asc-1 and SGK1-POMC systems.

CHAPTER FOUR: THE EFFECTS OF D-AMINO ACIDS, D-ALANINE AND D-SERINE ON COLORECECTAL HUMAN CANCER CELLS, CACO-2.

4. 1. Introduction

Following an expansive investigation of D-amino acid effects in the anterior pituitary through AtT20 cells which concluded with the discovery that D-amino acids affect HPA axis hormones, and this was potentially mediated by Asc-1 and ASCT2 transporters and the SGK1-POMC system, I was curious to better understand what effects D-amino acids may have on the site it originally enters – the gut. I delve into the effects D-amino acids have on the gut in this chapter, particularly looking at the human colorectal Caco-2 cell line, especially identifying the presence of transporters and inflammatory effects (by looking at cytokines) so as to ensure that a proposed increase in D-amino acids to manage stress hormones will not have negative effects in the gut.

4.1.1 What is a good in vitro model for investigating amino acid effects in the gut?

Caco-2 cells are an epithelial cell line that have been extensively used in the pharmaceutical industry for drug absorption testing, as well as in laboratory research to investigate pathologies such as IBD, colon cancer, and monitoring of general gut health (Alard et al., 2018, Qi and Ding, 2018 and Jin et al., 2017). These cells are derived from a human colon carcinoma and maintain many features of absorptive intestinal cells during culture; this includes microvillus structure, production of hydrolysis enzymes, and carrier mediated transport systems for a plethora of drugs, amino acids and sugars (Yamashita et al., 2000). Moreover, these cells also attach to each other via tight junctions (TJs) which are formed at the apical side of the monolayer (Tanaka et al., 1995). Because of these kept characteristics this cell line is a good model for absorption, which is why I utilized it in the study to investigate the effects of D-amino acids on the gut.

4.1.2. How are D-amino acids taken up by these cells?

ASCT2 has been cloned from intestinal Caco-2 cells and serves to mediate as a Na^+ exchanger of neutral amino acids (Uchiyama et al., 2005, Kekuda et al., 1997). Features of amino acid transport in Caco-2 cells sufficiently replicate nutrient uptake by intestinal epithelium, and the expression of ASCT2 mRNA in these cells (Uchiyama et al., 2005) validates their use to study enteric uptake of D-serine and D-alanine.

Additional to ASCT2 in the uptake of D-serine and D-alanine is the transporter, Asc-1. It is present in the serosal/basolateral level of the intestine where it transports alanine, serine, cysteine, glycine, threonine, α-aminobutyric acid, β-alanine and D-serine. Asc-1 facilitates the exchange of intracellular glutamate against anionic cystine, along with the transport of alanine, serine and cysteine (Steffansen et al., 2004). In terms of locational presence, Asc-1 has been established in human intestinal tissue, but confirmation of its presence has yet to be determined in the Caco-2 cell line. Fukasawa and co. (2000) determined the presence of Asc-1 in the brain of jcl: ICR mice, whilst Dave et al (2004) reported that in NMRI mice, Asc-1 mRNA was more abundant in the brain than in the intestine.

4.1.3. D-amino acids - an inflammatory trigger in the gut?

With a large component of the immune system residing in the gut it is important to maintain this environment. A considerable aspect of this maintenance involves regulating inflammation, and protecting the environment from inflammatory triggers, so it is prudent to determine the inflammatory effect of D-alanine and D-serine present in the gut.

According to literature, both D-alanine and D-serine exert pro-inflammatory properties, explained further in **Introduction, Chapter 1.** Of interest is that D-serine inflammatory effects has yet to be investigated in the gut, whilst D-alanine has, therefore investigating the effects of D-serine on Caco-2 cells will be of interest.

4.1.4 Do NMDA receptors have a role to play in potential D-amino acid inflammatory effects in the gut?

Now that we see that D-amino acids have exhibited an inflammatory affect in the gut based on literature, this leads us to question what mediates these effects. Perhaps the NMDARs which both D-alanine and D-serine bind could be responsible? Heightened levels of NMDARs, which D-serine and D-alanine bind to, have been noted in microglia following proinflammatory stimuli which implies that they may have a role in the relationship between neuroinflammation and excitotoxicity (Bezzi et al., 2001). D-serine, which is synthesized by serine racemase, appears to contribute to the neurotoxicity exhibited by Schwann cells and amyloid beta (Aβ) or lipopolysaccharide (LPS)-treated microglia (Wu et al., 2005; Wu et al., 2004).

However, NMDARs have been detected in most cells of the ENS and have been shown to be expressed in the parietal, surface and chief cells of the stomach, as well as in neurons in the submucosal and myenteric plexuses (Seo et al., 2011, Liu et al., 1997).

NMDARs have been detected in intestinal cell lines; Qi et al (2016) showed that NMDARs are activated in HT29 human colonic epithelial cells. This activation stimulated an increase in brain derived neurotrophic factor (BDNF). They also found that there was a significant increase of colonic mucosal NMDARs in IBS patients in comparison to control subjects. Ketamine, an antagonist of the NMDA receptor, was found to decrease Ca^{2+} levels and the phosphorylation of CAMK II in human colorectal HT29 and human colorectal adenocarcinoma SW480 cells, strongly implying the existence of NMDARs in these cells (Hu et al., 2019).

4.1.5. Possible non-NMDAR mediated inflammatory effects in the gut

Two proteins with a plethora of roles in the gut, that may also be involved in inflammatory management, are glucagon like peptide 1 (GLP-1) and GLP-2. GLP-1 is predominantly found in the ileum of the small intestine and is released from enteroendocrine L cells following the intake of food. GLP1-R mRNA have been established in the hypothalamus, heart, lung, pancreatic islets, stomach and kidney. GLP-1 receptors (GLP1-R) have been detected in colonic smooth muscle of C57BL6 mice (May et al., 2019). GLP-1 is responsible for the secretion of insulin, and to a lesser effect the control of food intake, gut motility, glucagon secretion, gut growth, immune function and heart rate (Yusta et al., 2019). Subsequently, GLP-1R agonists are used as a treatment for type 2 diabetes (Andersen et al., 2018). Along with the secretion of GLP-1, enteroendocrine L cells secrete GLP-2, the latter which modulates the control of nutrient assimilation, gastro-intestinal (GI) motility attenuation, as well as the stimulation of intestinal crypt cell proliferation. These actions are mediated via the activation of single GLP-2 receptor (GLP-2R) which is expressed in the gut, brain and certain peripheral tissues (Munroe et al., 1999).

Cani et al (2009) reported that alterations in the gut microbiota regulate inflammation in obese mice mediated by GLP-2 stimulated improvement of gut permeability, whilst Müller and co (2019) reported that GLP-1 decreases inflammation, and therefore both these peptides are involved in inflammation regulation and worth investigating in relation to D-amino acids in the gut.

4.1.6. Can D-amino acid effects be reversed by the addition of an NMDA receptor antagonist?

MK-801 is an NMDAR antagonist that is a suggested therapeutic for pathological NMDAR mediated effects. A single dose of MK-801 in Wistar rats which have become tolerant to morphine, reversing the pro-inflammatory effects observed in morphine tolerant rats in the spinal cord, and therefore re-sensitizes neurons to antinociceptive effects of

morphine in rats who have been made tolerant to morphine (Liu et al., 2011). This is interesting as it shows that NMDAR blockage by MK-801 had an anti-inflammatory effect in rodent spinal cord of morphine tolerant mice. Therefore it would be interesting to determine if potential inflammatory effects in the gut in response to D-amino acids are negated by the presence of NMDAR antagonist, MK-801, to determine if these receptors are involved in inflammation in the gut.

4.1.7 Aims of the Chapter

1. The main aim of this chapter is to investigate the effects that D-alanine, and D-serine, which have been established to be present in the brain, may have on their initial location, the gut. In order to achieve this I had to select a viable working model for the gut, so that the D-amino acids are adequately taken up, and the effects they exert may be further elucidated. Upon reviewing the literature, the Caco-2 cells were chosen as a good working model, and these cells were obtained for further experimentation. Prior to elucidating effects, it was imperative that the possibility of D-amino acid uptake is determined in these cells, therefore I sought to identify the existence of amino acid transporters, Asc-1 and ASCT2 by mRNA measurements, or protein expression.

2. A key symptom of many gut diseases is inflammation and is the hypothesis for how some of the more detrimental neurodegenerative effects of dysbiosis is implemented. Therefore, I investigated what, if any, effect D-amino acids have on inflammation in the gut. This was achieved by measuring the mRNA levels of cytokines – TNF-α, IL-6, IL-10 and IL-1β. TNF-α levels were to be measured in culture medium as well, but due to the COVID-19 Pandemic related time/lab space constraints, this was unable to be conducted.

3. After determining if there is an inflammatory response, a mechanistic explanation was sought. Therefore, I investigated receptor involvement in these cells (NMDARs, GLP-1R

and GLP2R), by determining its presence both in mRNA and protein expression, as well as by blocking its activity using an NMDA receptor antagonist, MK-801.

4.2. Methodology

4.2.1. Cell culture

Caco-2 cells (ATCC HTB-37, LGC Standards, United Kingdom) were resuscitated at passage 27, with 10 mls initial plating media of 20% FBS DMEM (Appendix) in a T25 cell culture flask (83.3910.002, Sarstedt, Germany) under the conditions of 5% CO_2 and 37 °C. Cells typically took a week to become confluent and were then transferred to a bigger flask (T75, 83.3911.002, Sarsdtedt, Germany). Cells were left to grow undisturbed in initial plating media until 80% confluence was reached and were then split into another T75 flask where initial plating medium was replaced with growth medium (10% FBS DMEM, Appendix). After splitting these cells once to ensure all DMSO (472301-100ML, Sigma Aldrich, United Kingdom) was removed, Caco-2 cells were plated 50 000 cells per well in a 24 well plate (83.3922.005, Sarstedt, United Kingdom). Cells were left 48 hours to become confluent and then subsequent treatments performed on them.

4.2.2. Protocols for Experimental Treatment of Caco-2 cells

Caco-2 cells were utilized to investigate the effects that D-amino acids have on the gut, and this was achieved by 3 sets of experiments.

In the first set of experiments, the effect D-amino acids have on receptors, transporters, and inflammatory cytokines were investigated by means of protein and mRNA expression (**Figure 4.1a**).

In the next set of experiments, the effect of an overexpression of Asc-1 transporter in the presence of D-amino acids on receptors, transporters and inflammatory profile was also investigated by means of protein and mRNA expression (**Figure 4.1b**).

And lastly, the involvement of NMDA receptors was researched by blocking its activity with the antagonist, MK-801. NMDA receptor activity was measured by both protein and mRNA expression, as well as the effect this antagonism may have on transporters, as well as inflammation in the gut (**Figure 4.1c**).

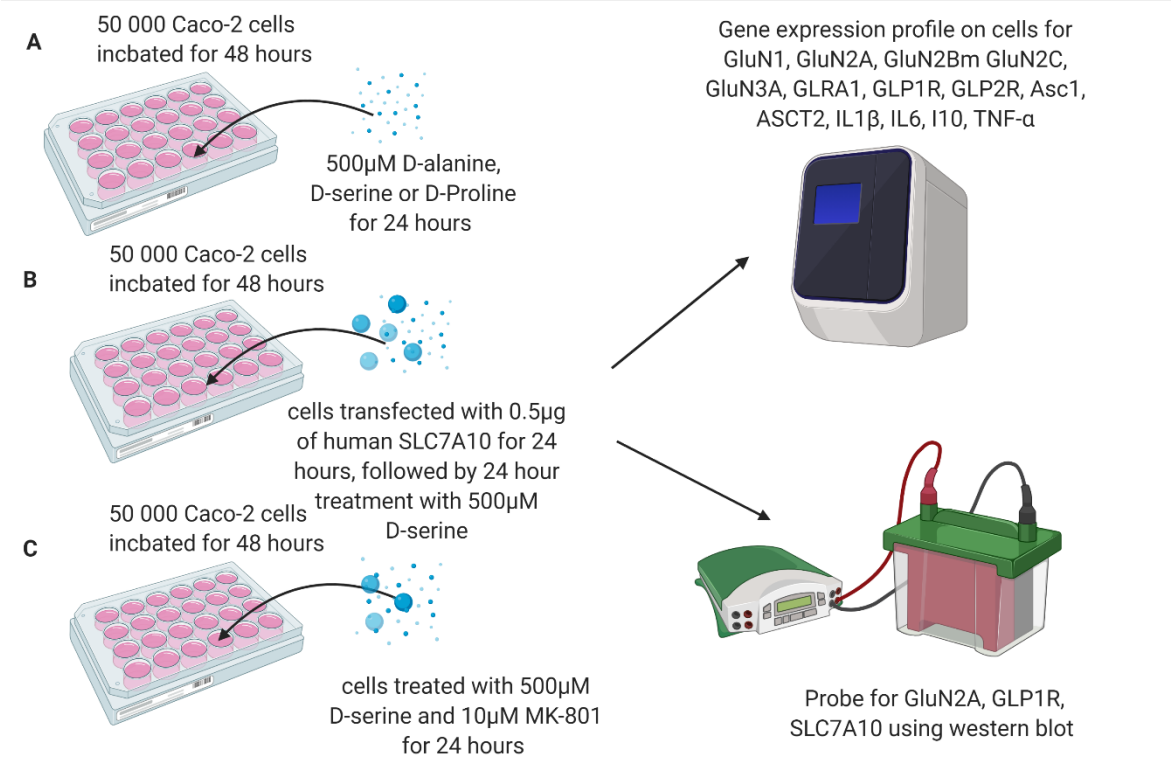


Figure 4.1 - The above figure outlines the experiments conducted on Caco-2 cells in this chapter. Figure made with Biorender.com.

a) 50 000 Caco-2 cells were plated and left to grow to confluency for 48 hours and was then treated with 500 µM of D-alanine, D-serine or D-proline for 24 hours. The second set of experiments **(b)** involved transfecting the cells with human Asc-1 (SLC7A10) (**Figure 4.2)**, and then treating them with D-serine (500µM for 24 hours). The final set of

experiments **(c)** exposed the cells to 10µM MK-801 in the presence or absence of D-serine for 24 hours. For all the experiments, PBS was used as a control, and subsequently gene expression analysis via qPCR (**Figure 4.4)** and protein expression analysis by western blot (**Figure 4.3)** was conducted.

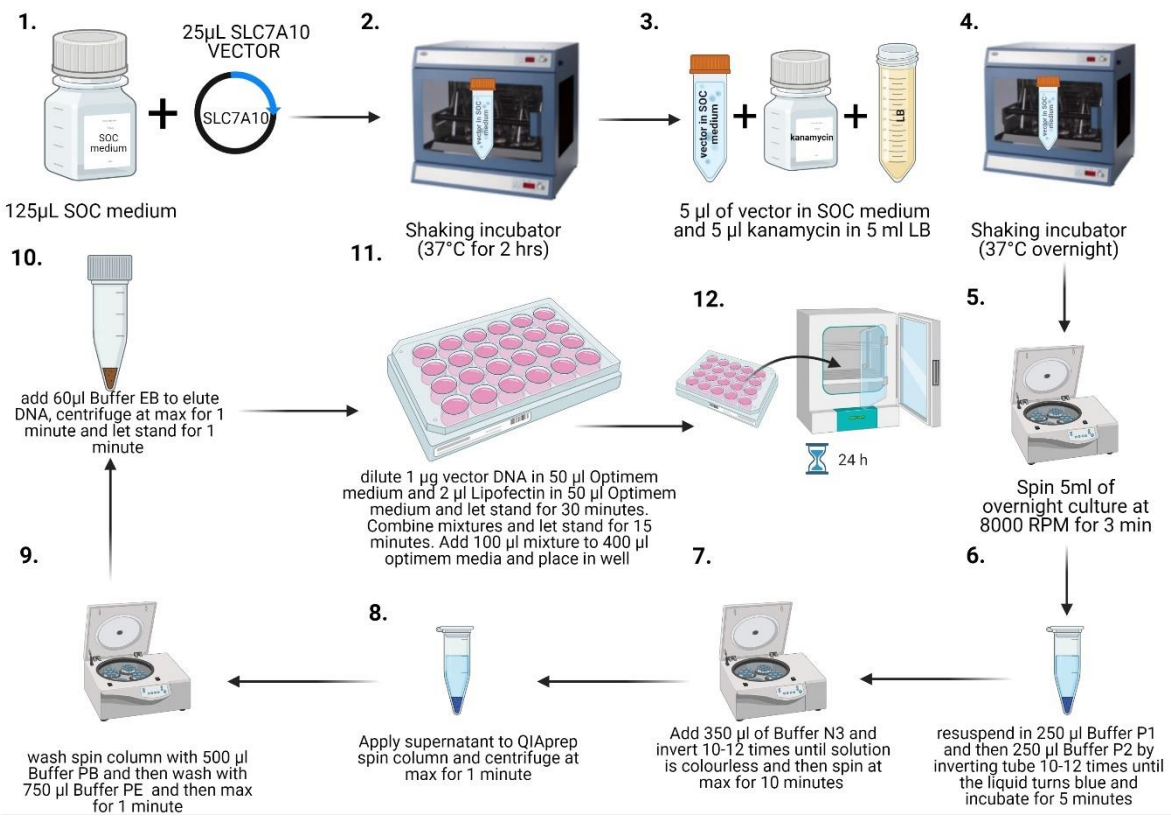


Figure 4.2 - A detailed diagram of the transfection process of SLC7A10 in Caco-2 cells. Figure made with Biorender.com.

As depicted in **Figure 4.2** the first step of transfection involves growth of vector DNA with bacteria – 125 µl SOC medium (#15544034, ThermoFisher Scientific, United Kingdom) and 25 µl SLC7A10 vector (ABIN38169, Antibodies Online, United Kingdom) placed in a benchtop shaking incubator (SI600C, Stuart, United Kingdom) at 37 °C for 2 hrs. 5 µl of this solution and 5 µl of kanamycin (B5264, Sigma Aldrich, United Kingdom) and 5 ml of Lysogeny broth (LB) (L3022, Sigma Aldrich, United Kingdom) overnight. Purification of plasmid DNA was achieved by QIAprep Spin Miniprep Kit High-Yield

(#27104, Qiagen, Germany) according to the Kit protocol described in steps 5 – 10. Vector DNA was diluted in Opti-MEM Medium (#31985062, ThermoFisher Scientific, United Kingdom), whilst Lipofectin (#18292-11, Invitrogen, United Kingdom) was mixed with Opti-MEM and left to stand for 30 minutes. Following this the two mixtures were brought together and left to stand for 15 minutes, then added to Opti-MEM media, and onto Caco-2 cells for overnight transfection with SLC7A10 vector DNA.

4.2.3. Experimental procedures

To discern what effects D-amino acids, along with their transporters and receptors, may have on the gut, protein analysis by Western blot, as well as gene expression utilizing qPCR was conducted. These are briefly described in **Figures 4.3** and **Figures 4.4** below.

4.2.3.1 Western Blot Analysis

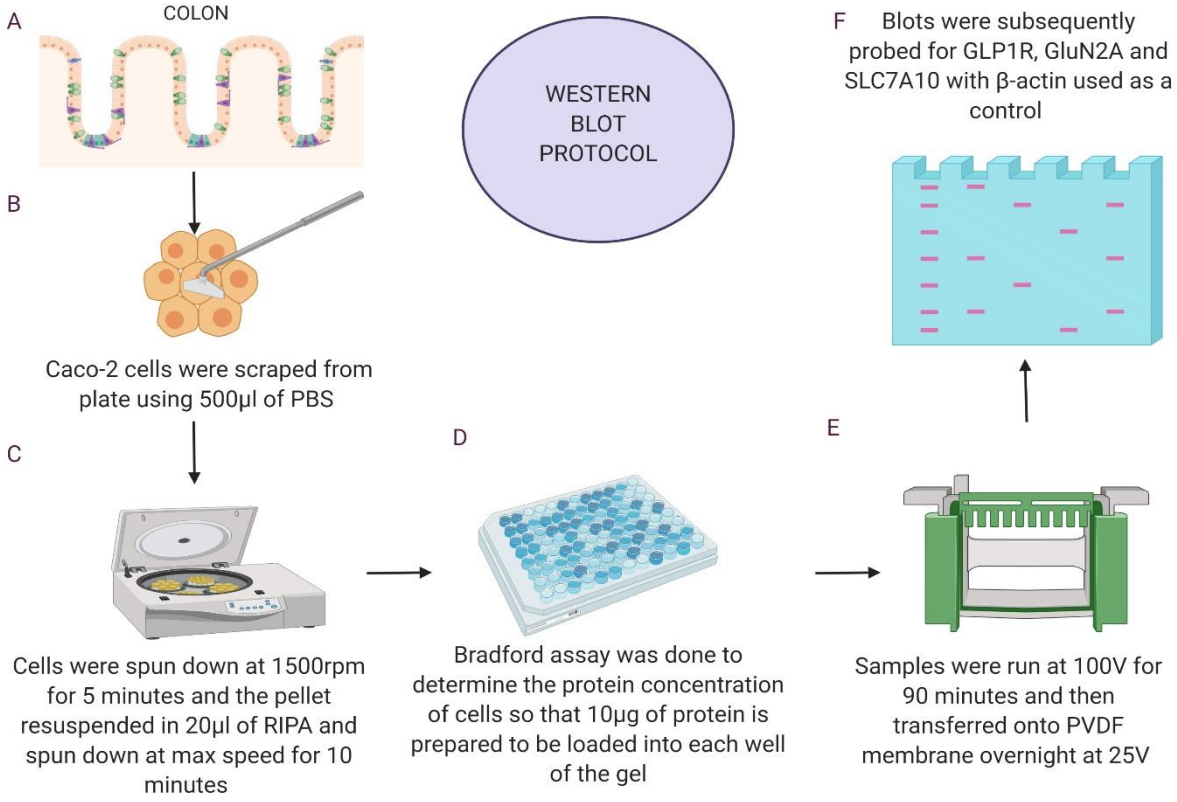


Figure 4.3 - Diagram of Western Blot protocol. Image made with Biorender.com

In **Figure 4.3**, western blot protocol is described: **A**. Caco-2 cells are a colorectal human carcinoma. **B**. 50 000 cells are plated per well in a 24-well plate and exposed to respective treatments. After treatment period, cells were then collected using a cell scraper (83.1830, Sarstedt, United Kingdom) and deposited in 500 µl PBS in 1.5 ml Eppendorf tubes. **C**. Samples were then spun in a centrifuge at 1500 RPM for 5 minutes, resultant pellet was then suspended in 20 µl RIPA buffer (R0278-500ML, Sigma Aldrich, United Kingdom) at max speed for 10 minutes. **D**. Samples were then diluted 1:10 in distilled water. 10 µl of diluted samples was added to each well, with 200 µl Bradford Assay Reagent (#5000205, Bio-Rad, United Kingdom). Samples were then run at 450nm: 590nm on the spectrophotometer (Spectramax 190, DS849B, Beckman Coulter, California USA) and the protein concentration on each sample was determined. Each sample (10 µg) was then calculated in 15 µl of RIPA buffer. **E**. Samples were loaded onto a gel (#4561086, Bio-Rad, United Kingdom) and run at 100 V for 90 minutes. Gels were then transferred to PVDF membrane (IESN07852, Sigma Aldrich, United Kingdom) overnight at 25 V. **F**. The resultant western blot was probed for GluN2A (NMDA receptor subtype 2A) (1:2000, 07-636, Merck Millipore, United Kingdom) with goat anti-mouse (0300-0108P, Bio-Rad, United Kingdom) secondary antibody, GLP1R (1:1000, PA5-89562, Thermo Fisher Scientific, United Kingdom) with goat anti-rabbit secondary antibody (5196-2504, Bio-Rad, United Kingdom), SLC7A10 (3µg/µl, PA5-68097, Thermo Fisher Scientific, United Kingdom) with goat anti-rabbit secondary antibody and the housekeeping protein - conjugated β-actin (1: 1000 000, ab4900, Abcam, United Kingdom). Probing for various antibodies with the same blot was achieved by stripping the blot using stripping buffer (Appendix) between each antibody.

4.2.3.2 Gene Expression

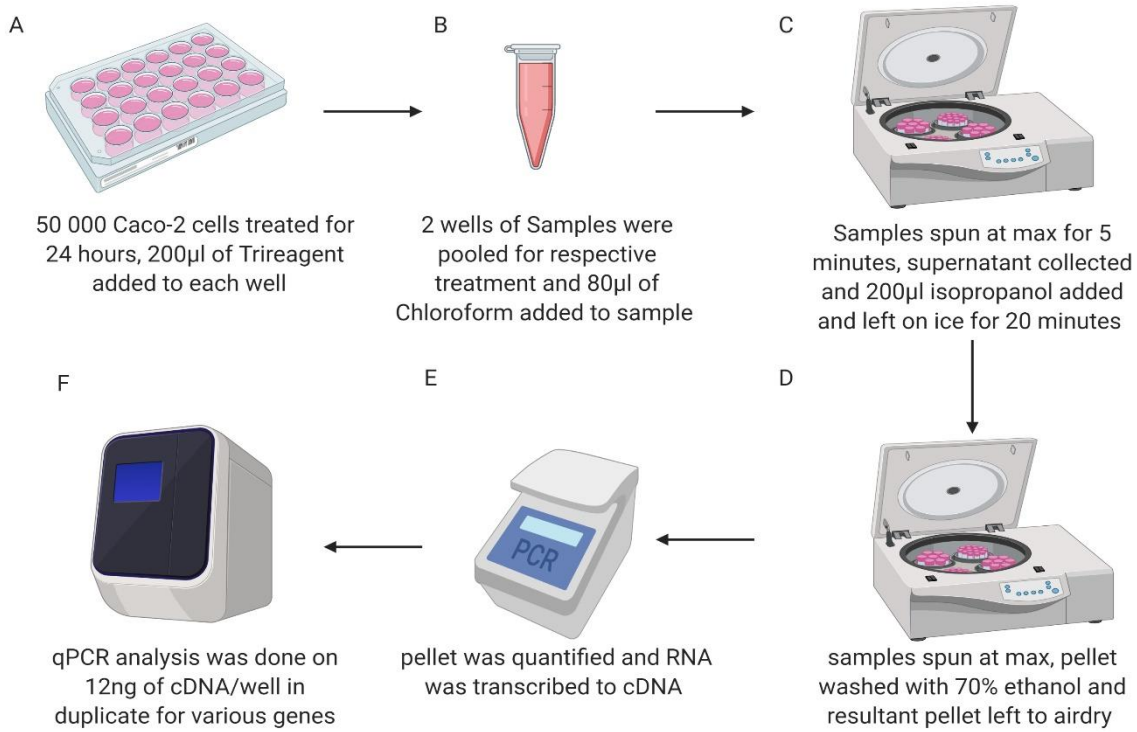


Figure 4.4 – Caco-2 cells undergoing qPCR for gene expression analysis. Image made with Biorender.com.

As seen in **Figure 4.4**: **A**. 50 000 Caco-2 cells incubated for 24 hours with respective treatment. 200 µl of Tri-reagent (T9424-100ML, Sigma Aldrich, United Kingdom) was added to each well. **B**. Wells (2) were pooled together, and 80 µl chloroform (67-66-3, Sigma Aldrich, United Kingdom) added to each sample and mixed well by shaking until the samples are milky pink. **C**. Samples are then spun at max for 5 minutes. Resultant supernatant is collected and 200 µl isopropanol (I9516-1L, Sigma Aldrich, United Kingdom) added to each sample and placed on ice for 20 minutes. **D**. Samples are then spun at max for 10 minutes, and the pellet is then washed with 500 µl 70% ethanol (10041814, Fisher Scientific, United Kingdom). The resultant pellet is left to airdry for 40 minutes and the pellet is resuspended in 20 µl NF water, **E**. The concentration of RNA is calculated using nanodrop (NanoDrop^{TM} 1000 Spectrophotometer, Thermo Fisher

Scientific, United Kingdom), and 2000 ng of sample was reversed transcribed to cDNA using the Thermal Cycler (Veriti Thermal Cycler, Applied Biosystems, Thermo Fisher Scientific, United Kingdom). **F**. Samples were then diluted to 50 ng/µl and 4 ng of each sample is added in duplicate to a 96 well plate and samples were probed for genes of interest on qPCR machine (Quant Studio 6 Flex, ThermoFisher).

4.2.4. Statistical Analysis

For Western Blot, densitometry measures for each band was measured and normalized to housekeeping protein, and then normalization to control to eventually have treatments are presented in relation to control. With regards to qPCR, Ct values were extracted from the qPCR machine and processed on Microsoft excel, and then normalized to housekeeping gene, and then subjected to POWER analysis followed by normalization to control so that treatments are presented in relation to control.

These quantities were then transferred onto SPSS statistics (Version 26) were initial statistics of One-Way ANOVA was run, and if significant ($p > 0.05$), post-hoc analysis via Tukey's Test was done to determine significant differences between treatment groups. Graphs were then drawn using GraphPad Prism (Version 9), with results represented as mean ± SEM.

4.3 Results

4.3.1 The effects of D-amino acids on Caco-2 cells

D-amino acids that have been acquired through nutrition are first absorbed in the gut, therefore I was intrigued to determine the effects such D-amino acids would have in their initial location. As described in **Figure 4.1**, my first lot of experiments investigated the effects D-alanine, D-serine and D-proline have on receptors and inflammatory cytokines in

the Caco-2 cell line. There were no effects on Asc-1 or ASCT2 in Caco-2 cells treated with D-amino acids (**Figure 4.5).** There was an effect on GLP1R in cells treated with D-serine ($F_{3,39}$ = 4.21, p = 0.011, **Figure 4.6b).** There was a significantly higher level of GLP1R mRNA expression in cells treated with D-serine compared to PBS (p =0.016, +3488%) in **Figure 4.6b**.

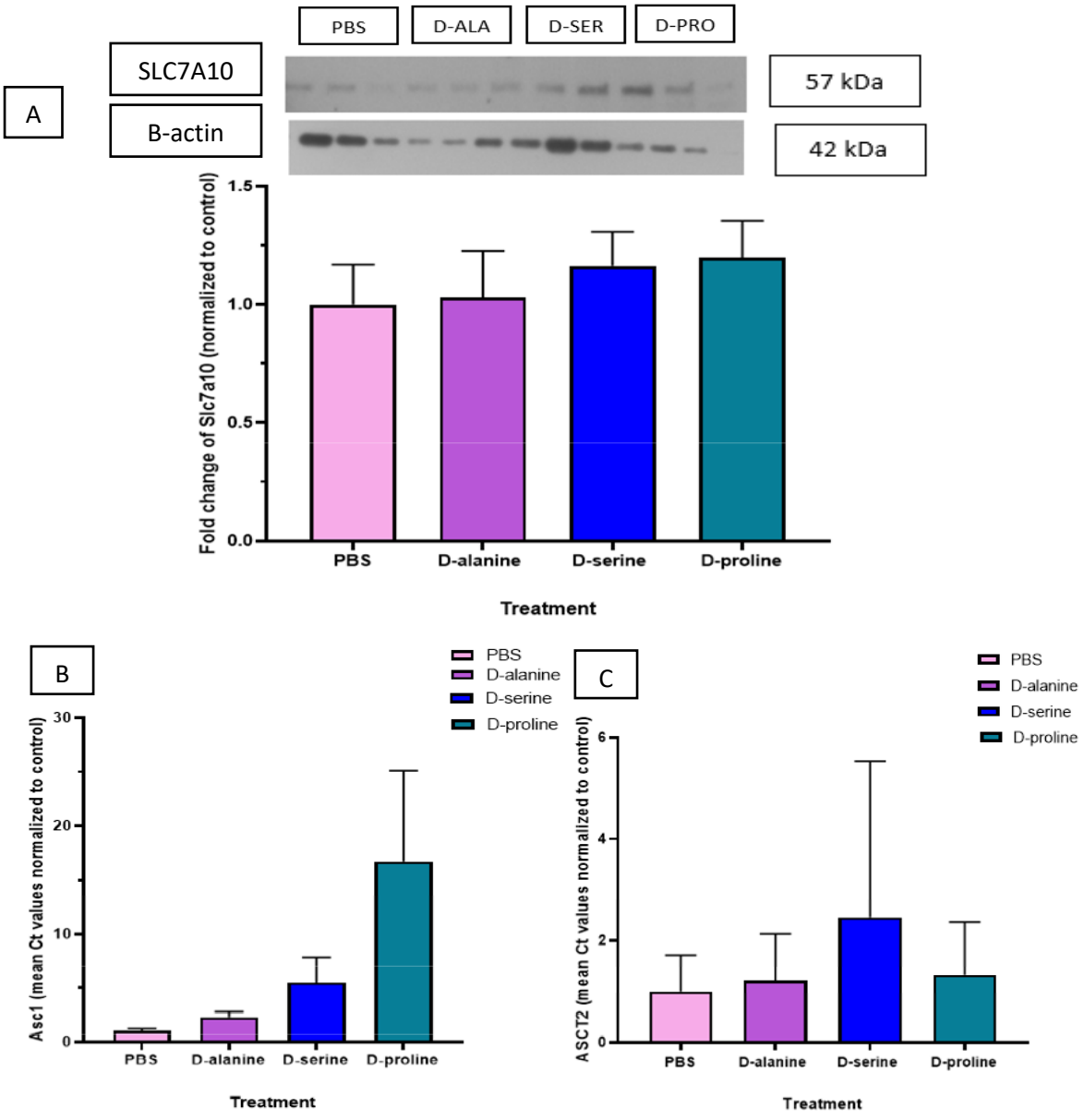


Figure 4.5 - The above graphs display the amino acid transporter expression in 50 000 Caco-2 cells treated with D-alanine, D-serine or D-proline at 500 µM for 24 hours, with PBS as a control (n=6). **A**. SLC7A10 was expressed in Caco-2 cells as shown by western

blot bands (normalized to β-actin) and densitometry readings. Gene expression for Asc-1 **(B)** and ASCT2 **(C)** was investigated in these cells using qPCR. There were no statistical differences between treatment groups.

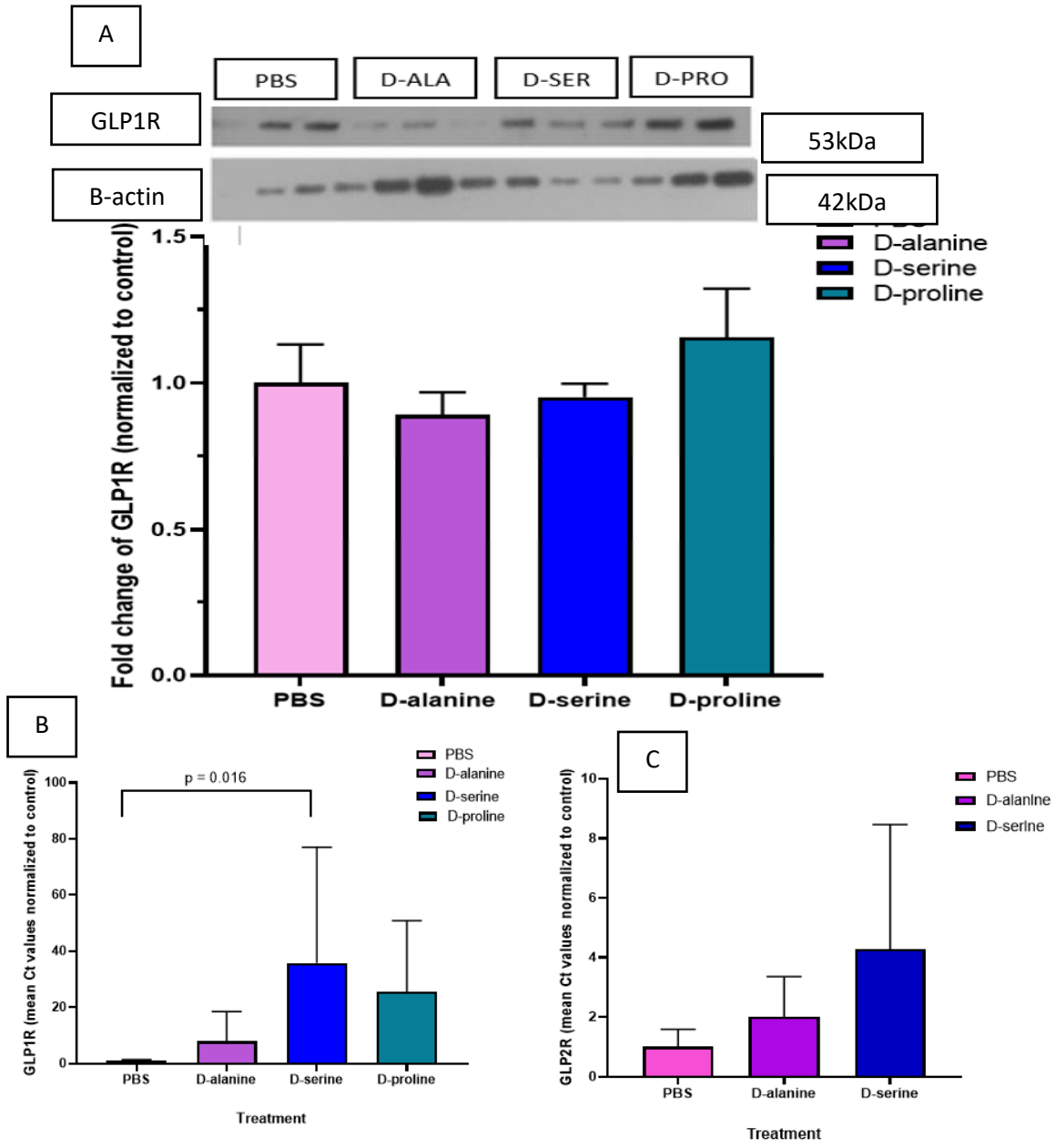


Figure 4.6 - The above graphs display the GLP-1R expression in 50 000 Caco-2 cells treated with D-alanine, D-serine or D-proline at 500 µM for 24 hours, with PBS as a control. **A**. GLP-1R was expressed in Caco-2 cells as shown by western blot bands and normalized to β-actin. Gene expression for GLP-1R **(B)** and GLP-2R **(C)** was investigated

in these cells using qPCR. There was a significantly high level of GLP-1R mRNA (B) in cells treated with D-serine compared to control (p =0.016, +3488%). N = 6

With regards to NMDA receptor gene expression, a D-serine driven effect presented in Caco-2 cells with regards to GluN2A ($F_{2,13}$ = 4.92, p = 0.026, **Figure 4.7b)** and GluN2C ($F_{2,14}$ = 4.60, p = 0.029, **Figure 4.7d)** gene expression. Cells treated with D-serine showed an 82% increase in GluN2A mRNA (p = 0.020), as well as GluN2C mRNA (p = 0.037, +2074%) when compared to PBS control (**Figure 4.7b** and **Figure 4.7d** respectively).

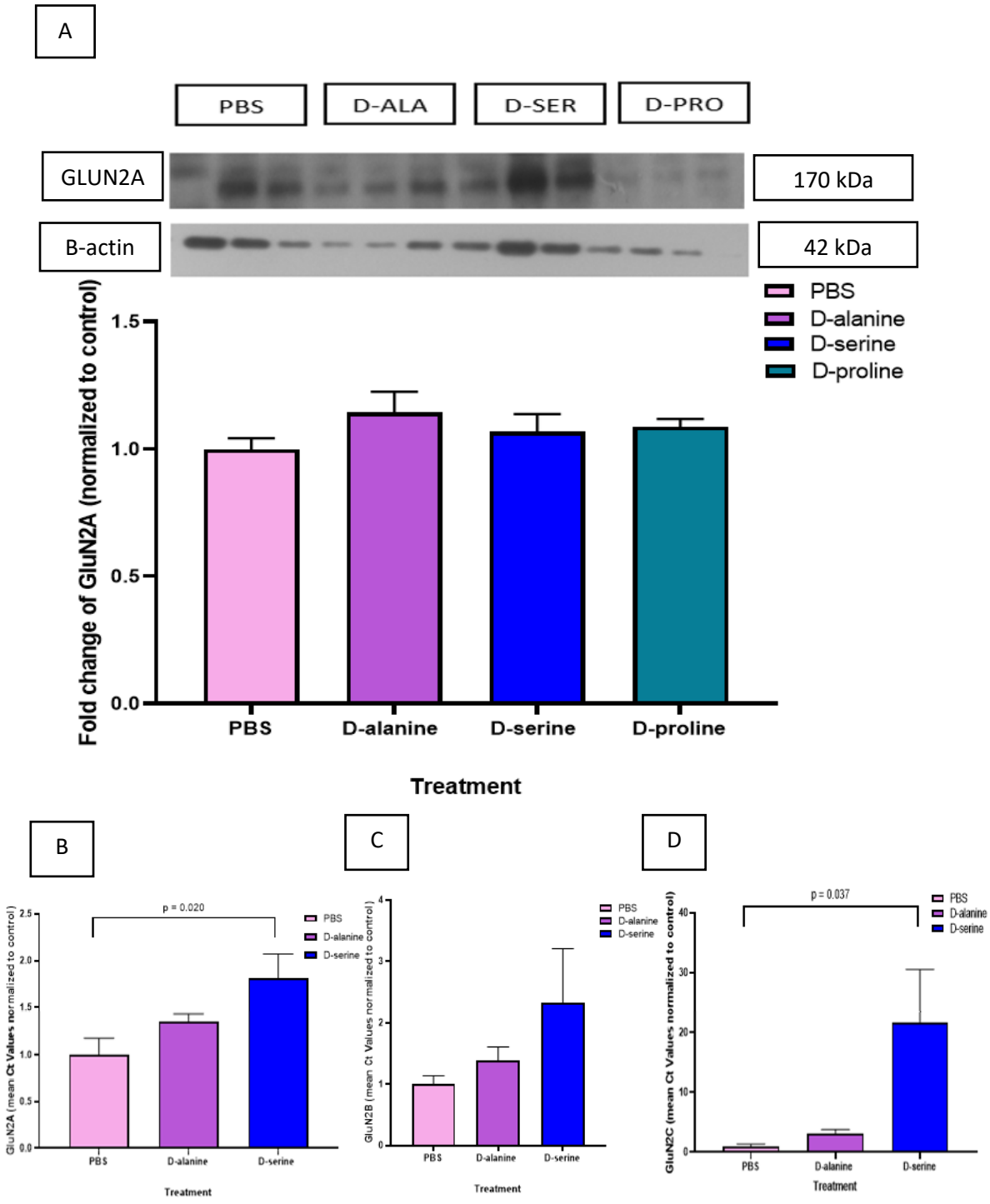


Figure 4.7 - NMDA receptor subtype 2A expression was determined by western blot analysis **(A)** and supported by mRNA expression **(B).** 50 000 Caco-2 cells were treated with D-amino acids at 500 µM for 24 hours. PBS was a control for all. GluN2A gene expression (**B**) and GluN2C gene expression (**D**) was significantly higher in cells treated with D-serine compared to control (p= 0.020, +82% and p = 0.037, +2074% respectively). Data are the mean of 6 replicates.

GluN3A and GluN1 levels were affected by D-serine ($F_{2,14}$ = 7.29, p = 0.007 and $F_{2,13}$ = 13.86, p = 0.01 respectively) were significantly greater in cells exposed to D-serine when compared to both D-alanine (p = 0.038, +359% and p=0.002, +217% respectively) and PBS (p=0.007, +497% and p =0.001, +216% respectively) (**Figure 4.8a** and **Figure 4.8b**).

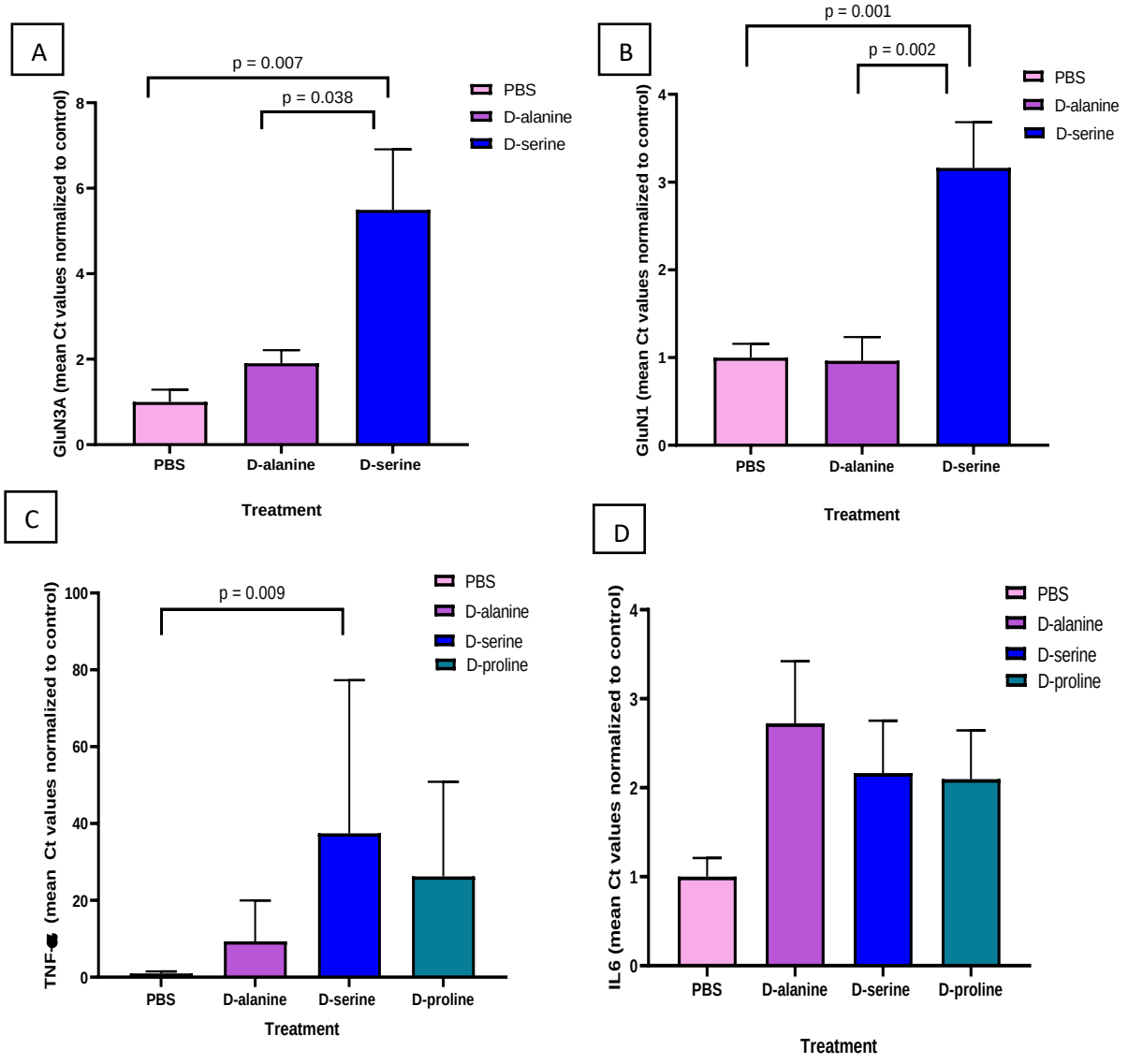


Figure 4.8 - The above graphs display the mRNA levels of GluN3A **(A),** GluN1 (**B**), TNF-α (**C**) and IL6 (**D**) in 50 000 Caco-2 cells treated with D-amino acids for 24 hours. GluN3A and GluN1 levels were significantly greater in cells exposed to D-serine when compared to both D-alanine (p = 0.038, +359% and =0.002, +217% respectively) and PBS (p=0.007, +497% and p =0.001, +216% respectively). TNF-α mRNA (C)levels were significantly higher in cells treated with D-serine compared to control (p =0.009, +3742%). Data are the mean of 6 replicates with PBS as a control.

D-serine displayed a pro-inflammatory effect on Caco-2 cells ($F_{2,13} = 6.71$, p = 0.010), with TNF-α mRNA significantly higher in cells treated with D-serine compared to control (p =0.009, +3742%) as shown in **Figure 4.8c** above.

4.3.2. Overexpression of Asc-1 transporter in the presence or absence of D-serine

Based on the first experiments in Caco-2 cells, D-serine seems to be the D-amino acid with the greatest effect in the gut, when compared to D-alanine and D-proline. D-serine is taken up by Asc-1 (SLC7A10) transporter, therefore Caco-2 cells were transfected with human Asc-1 vector DNA, with the intention that an overexpression of Asc-1 may lead to an increase uptake of D-serine. Curious as to what effect this increased uptake of D-serine exerts in these cells, both the protein and mRNA levels of several receptors and inflammatory cytokines were measured.

Overexpression of Asc-1 did not have an effect on Asc-1 or ASCT, either in protein or mRNA (**Figure 4.9).** In **Figure 4.10b** I found that Caco-2 cells transfected with Asc-1 and treated with D-serine affected GluN2A mRNA levels ($F_{3,19} = 4.33$, p = 0.017). GluN2A mRNA levels were significantly lower in cells transfected with human Asc-1 and treated with D-serine when compared to cells that only received D-serine (p = 0.020, -131%).

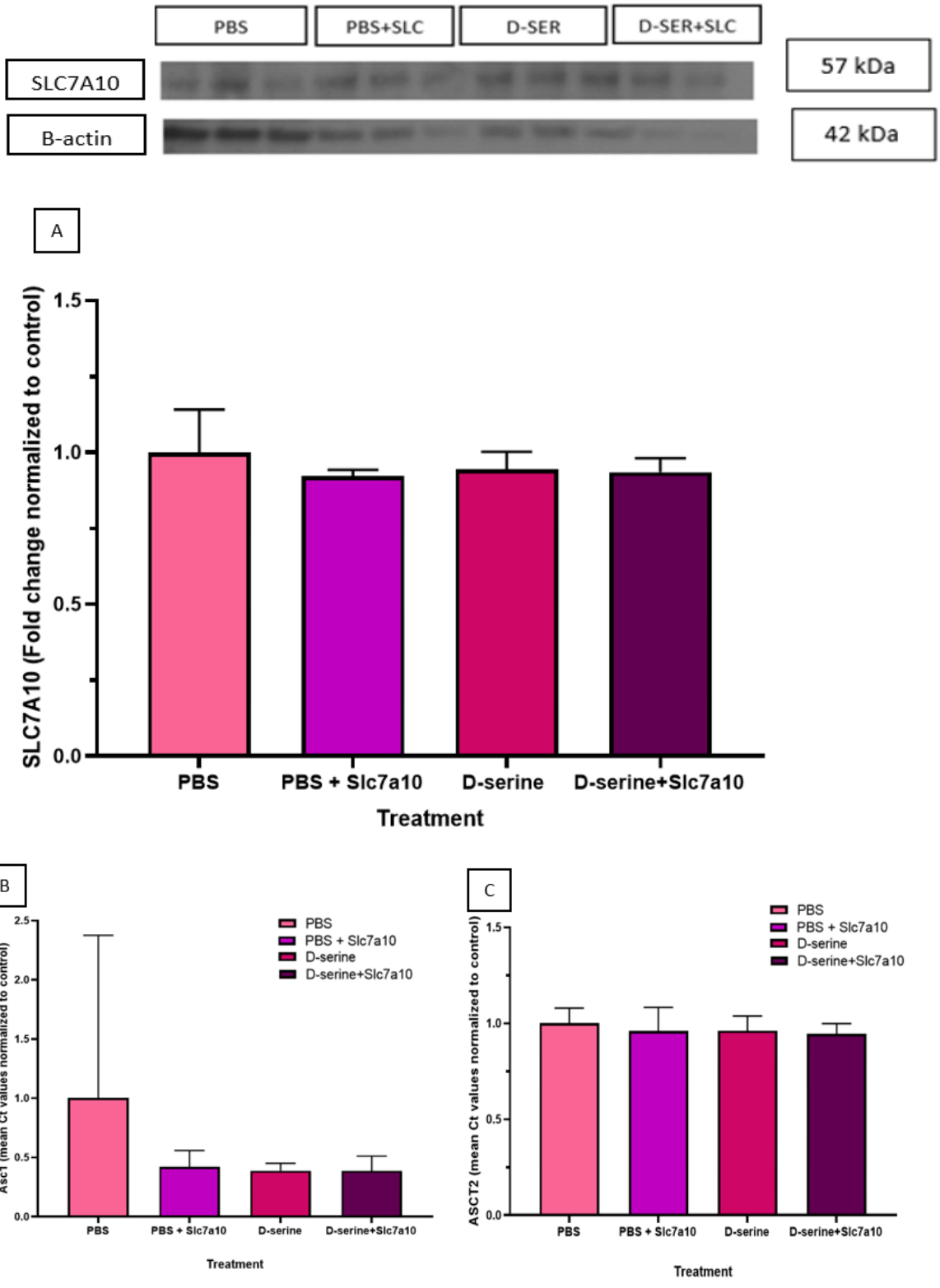


Figure 4.9 - Amino acid transporter expression in 50 000 Caco-2 cells transfected with Human SLC7A10 for 24 hours and then treated with D-serine (500 µM for 24 hours), with

PBS as a control (n=6). **A**. SLC7A10 was expressed in Caco-2 cells as shown by western blot bands and normalized to β-actin (bottom band). Gene expression for Asc-1 **(B)** and ASCT2 (**C)** was investigated in these cells using qPCR. There were no statistical differences between treatment groups.

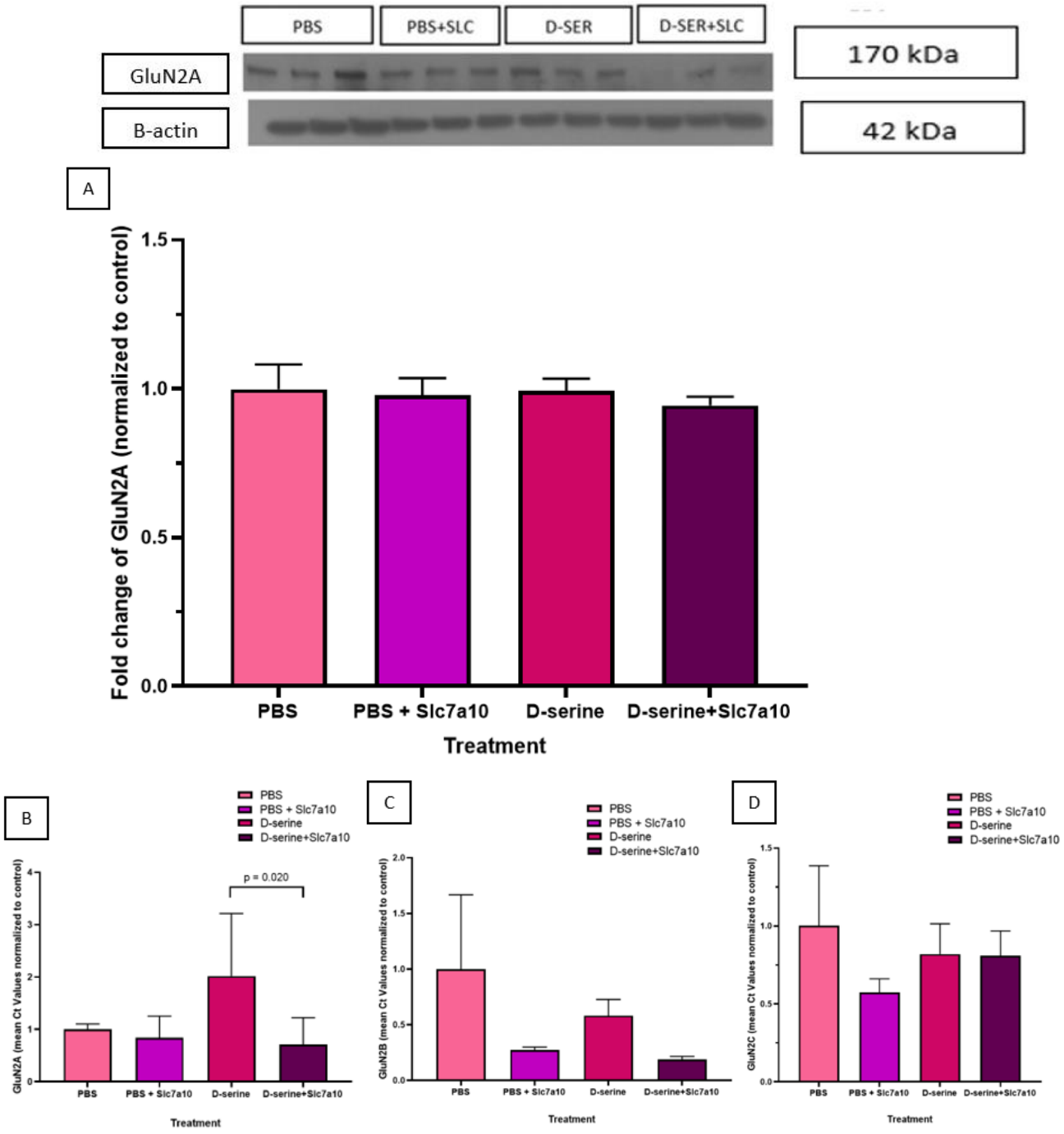


Figure 4.10 - 50 000 Caco-2 cells were transfected with human SLC7A10 for 24 hours, and then treated with D-serine at 500 µM for 24 hours (n=6, PBS used as a control).

GluN2A gene expression levels (**B**) were significantly lower in cells transfected with SLC7A10 and treated with D-serine when compared to cells that only received D-serine (p = 0.020, -131%). There were no statistical significances in protein levels of GluN2A (**A**) or gene expression levels of GluN2B (**C**) and GluN2C (**D**).

In **Figure 4.11a** GLP1R protein levels were affected by D-serine in the presence and absence of Asc-1 vector ($F_{3,20}$ = 7.77, p = 0.001**).** (A) GLP1R protein levels significantly lower in cells treated with D-serine, in the presence and absence of Asc-1 overexpression (p =0.001, -20% and p = 0.015, -15% respectively). GLP1R mRNA levels in **Figure 4.11b (**$F_{3,18}$ = 6.69, p = 0.003) were significantly higher in cells treated with D-serine when compared to PBS (p=0.024, +31%) and PBS transfected with SLC7A10 (p=0.002, +43%).

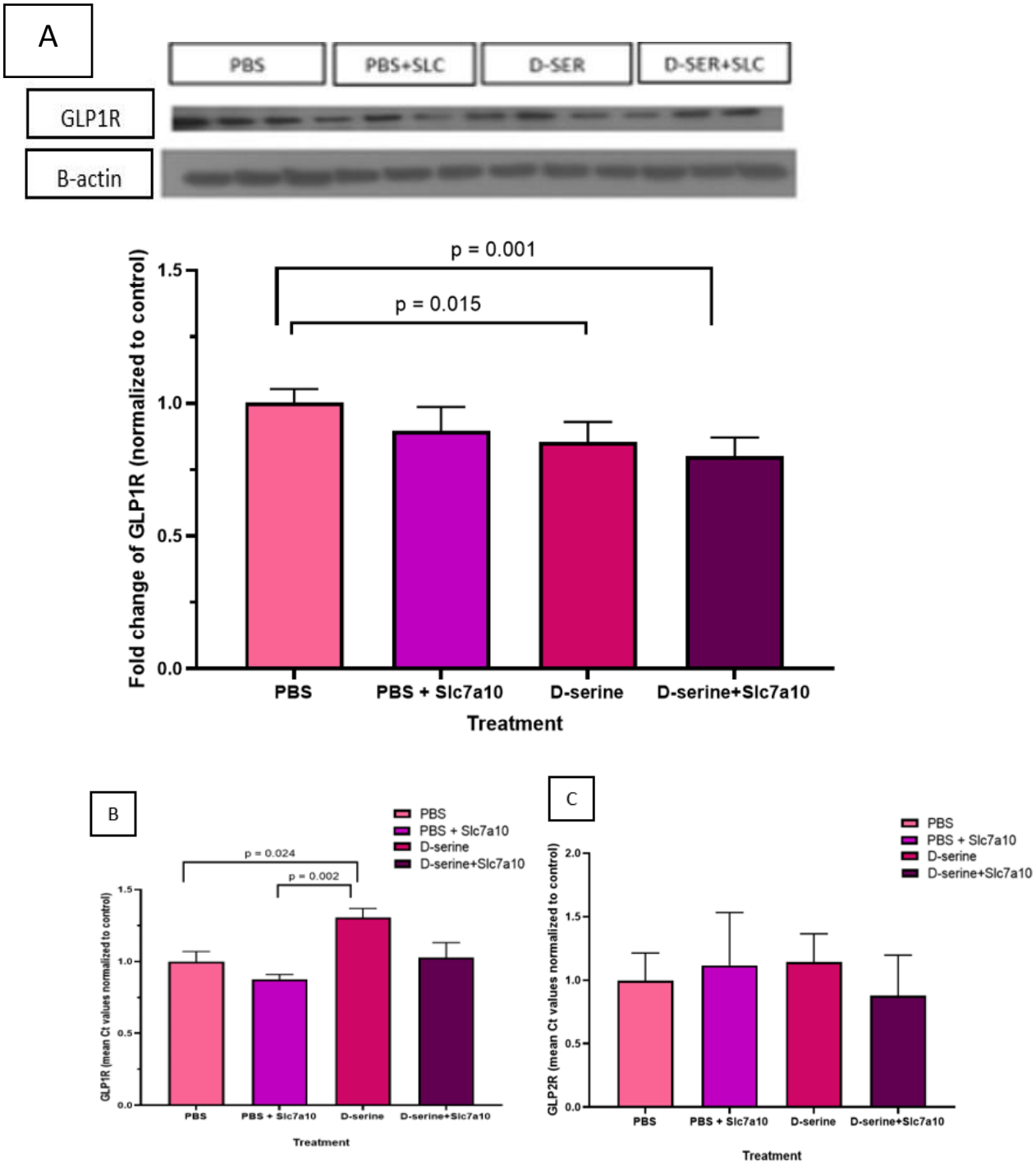


Figure 4.11 - 50 000 Caco-2 cells were transfected with human SLC7A10 for 24 hours, and then treated with D-serine at 500µM for 24 hours (n=6, PBS used as a control). GLP-1R protein levels (A) were significantly lower in cells treated with D-serine, in the presence and absence of vector SLC7A10 (p =0.001, -20% and p = 0.015, -15% respectively). GLP-1R mRNA levels (B) were significantly high in cells treated with D-

serine when compared to PBS (p=0.024, +31%) and PBS transfected with SLC7A10 (p=0.002, +43%).

There were no changes to inflammatory profiles **(Figure 4.12)** and receptors **(Figure 4.13)** in Caco-2 cells transfected with Asc-1 treated with D-serine.

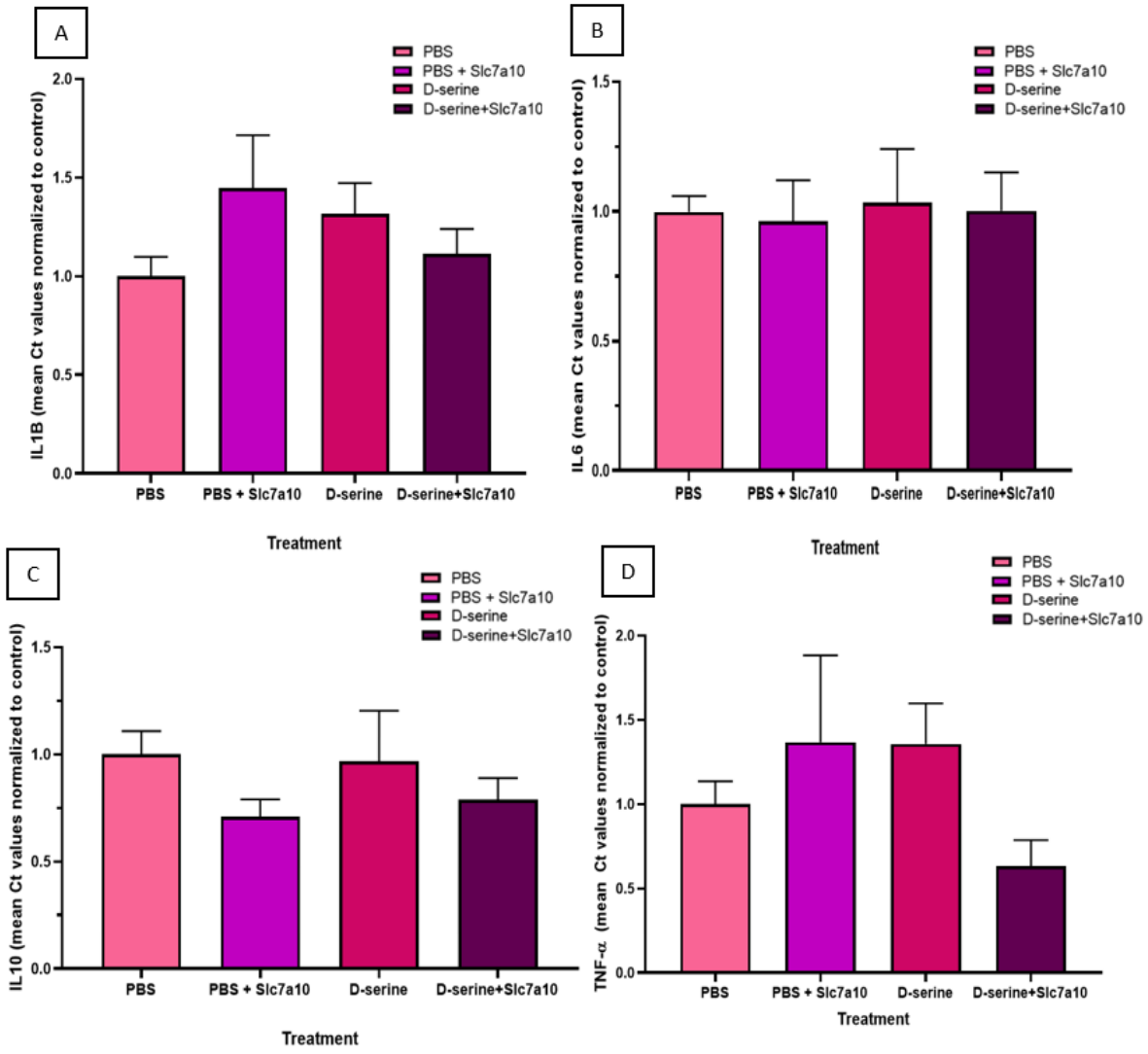


Figure 4.12 - The inflammatory profile of 50 000 Caco-2 cells transfected with human SLC7A10 and treated with 500 µM of D-serine for 24 hours (n=6) was established using qPCR analysis. mRNA levels of IL1β (**A**), IL6 (**B**), IL10 (**C**) and TNF-α (**D**) were analysed and there were no significant changes between the treatment measures. PBS was used as a control.

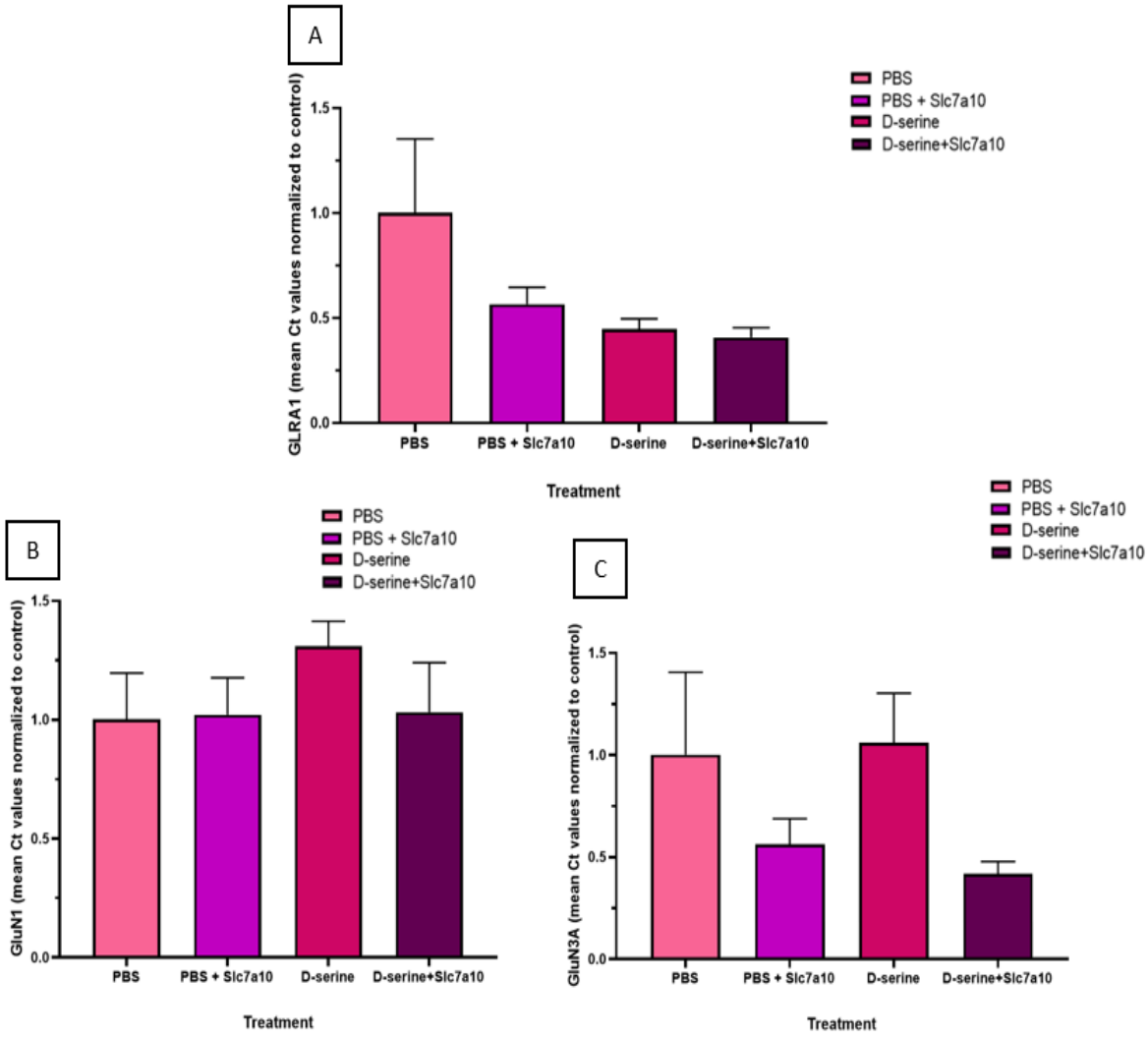


Figure 4.13 - The receptor profile of 50 000 Caco-2 cells transfected with human SLC7A10 and treated with 500 µM of D-serine for 24 hours (n=6) was established using qPCR analysis. mRNA levels of GLRA1 (A), GluN1 (B), and GluN3A (C) were analysed and there were no significant changes between the treatment measures. PBS was used as a control.

4.3.3. MK-801 antagonism of NMDA receptors in Caco-2 cells

Based on the effect D-serine, the endogenous co-agonist of the NMDA receptor, has on the gut, MK-801 was utilized to block the activity of these receptors. Since D-serine binds so

strongly to NMDARs, it may the mediator for observed inflammatory responses by D-serine.

As shown by **Figure 4.14b**, Caco-2 cells treated with MK-801 and D-serine altered Asc-1 mRNA levels ($F_{3,19} = 6.39$, $p = 0.004$). There was a significantly higher level of Asc-1 mRNA expression in cells treated with D-serine and MK-801 compared to PBS ($p = 0.002$, +160%), as well as D-serine when compared to PBS ($p = 0.014$, +140%).

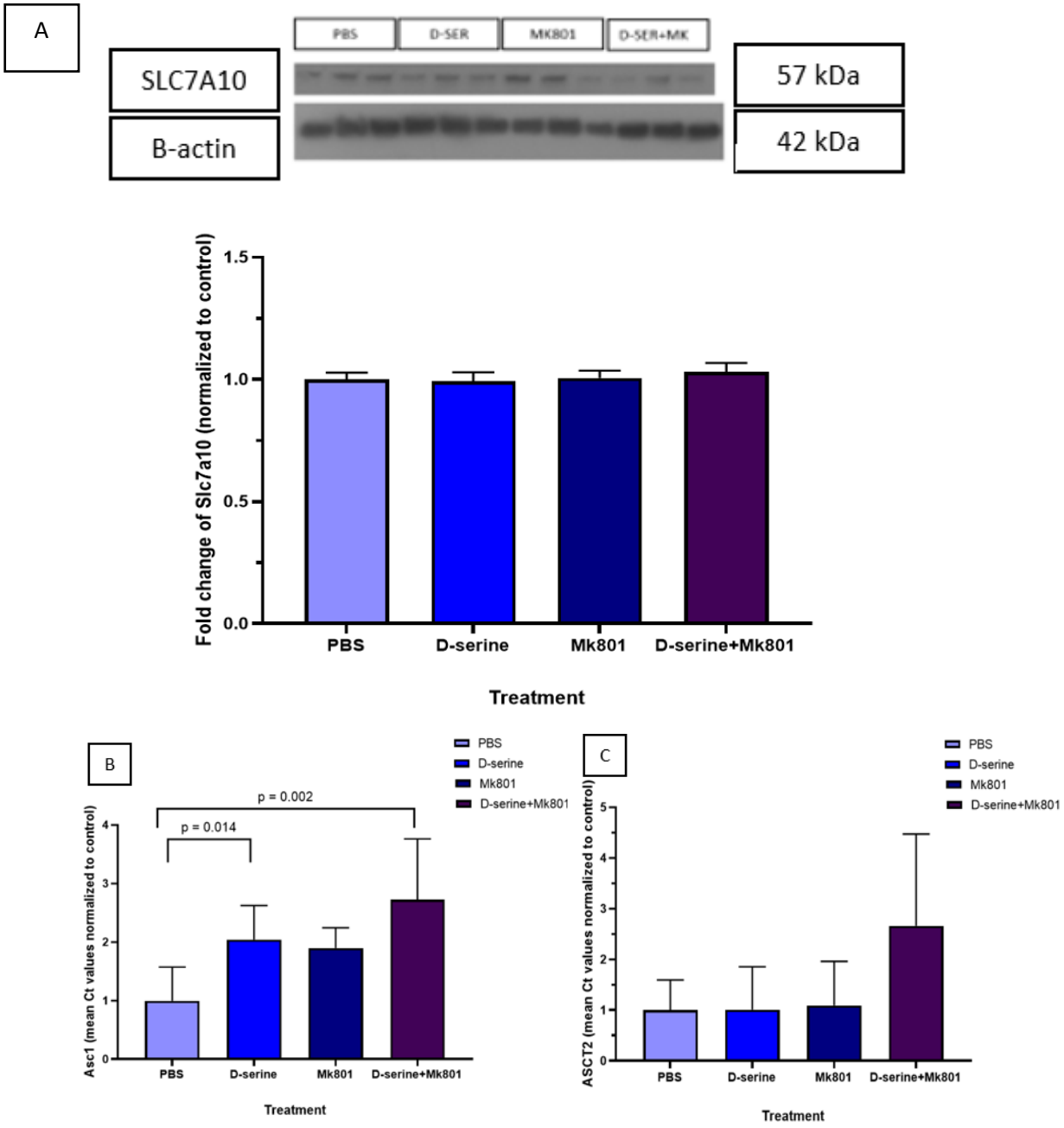
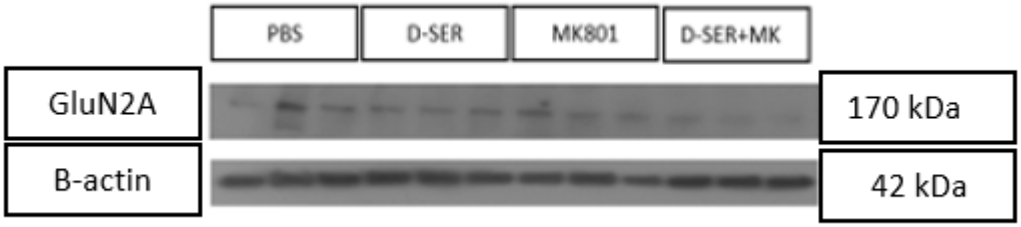
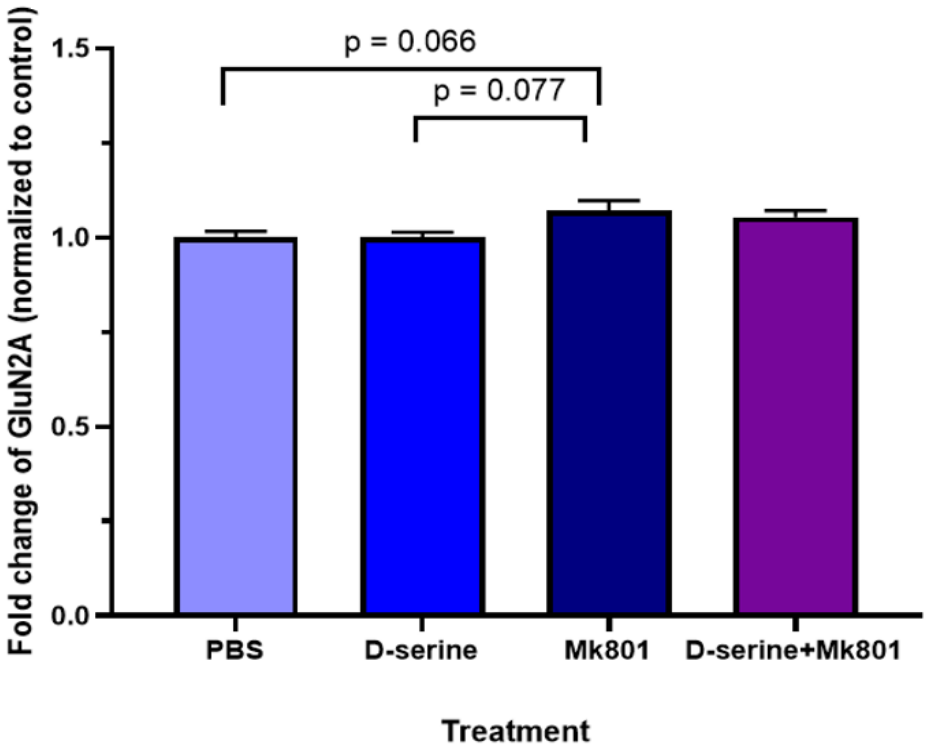


Figure 4.14 - Amino acid transporter expression in 50 000 Caco-2 cells treated with D-serine (500 µM for 24 hours) in the presence of absence of MK-801, NMDA receptor antagonist (10 µM for 24 hours), with PBS as a control. SLC7A10 was expressed in Caco-2 cells as shown by western blot bands and normalized to β-actin (bottom band) (**A**). Gene expression for Asc1 (**B**) and ASCT2 (**C**) was investigated in these cells using qPCR. There was a significantly higher level of Asc1 mRNA in cells treated with D-serine and MK-801 compared to PBS ($p = 0.002$, +160%), as well as D-serine when compared to controls ($p = 0.014$, +140%). Data are mean of 6 replicates.

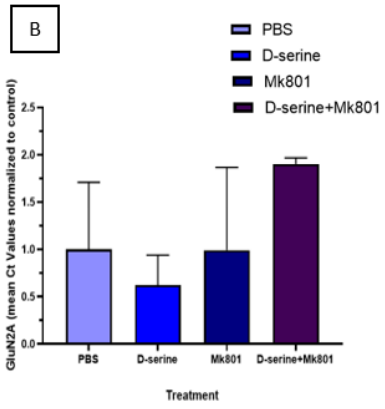
With regards to GluN2A protein expression in **Figure 4.15a**, there was trending increase in cells treated with just MK-801 compared to PBS and D-serine, but this difference was not significant ($p = 0.066$ and $p = 0.077$ respectively).


PBS
D-SER
MK801
D-SER+MK
GluN2A
B-actin
170 kDa
42 kDa

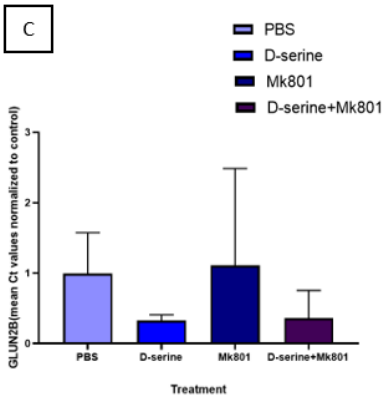
A


p = 0.066
p = 0.077
Fold change of GluN2A (normalized to control)
1.5
1.0
0.5
0.0
PBS
D-serine
Mk801
D-serine+Mk801
Treatment

B


PBS
D-serine
Mk801
D-serine+Mk801
GluN2A (mean Ct Values normalized to control)
Treatment

C


PBS
D-serine
Mk801
D-serine+Mk801
GLUN2B(mean Ct values normalized to control)
Treatment

D

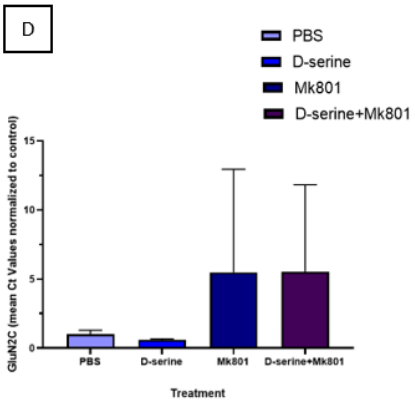

PBS
D-serine
Mk801
D-serine+Mk801
GluN2C (mean Ct Values normalized to control)
Treatment

Figure 4.15 - The above graphs display the GluN2 expression in 50 000 Caco-2 cells (n=6 with PBS as control and treatment lasting 24 hours). Cells were treated with D-serine at 500 µM in the presence or absence of MK-801 NMDA receptor antagonist (10 µM). GluN2A protein expression (**A**) showed a trending increase in cells treated with just MK-801 compared to PBS and D-serine. GluN2A (**B**), GluN2B (**C**) and GluN2C (**D**) mRNA levels had no significant differences between treatment groups.

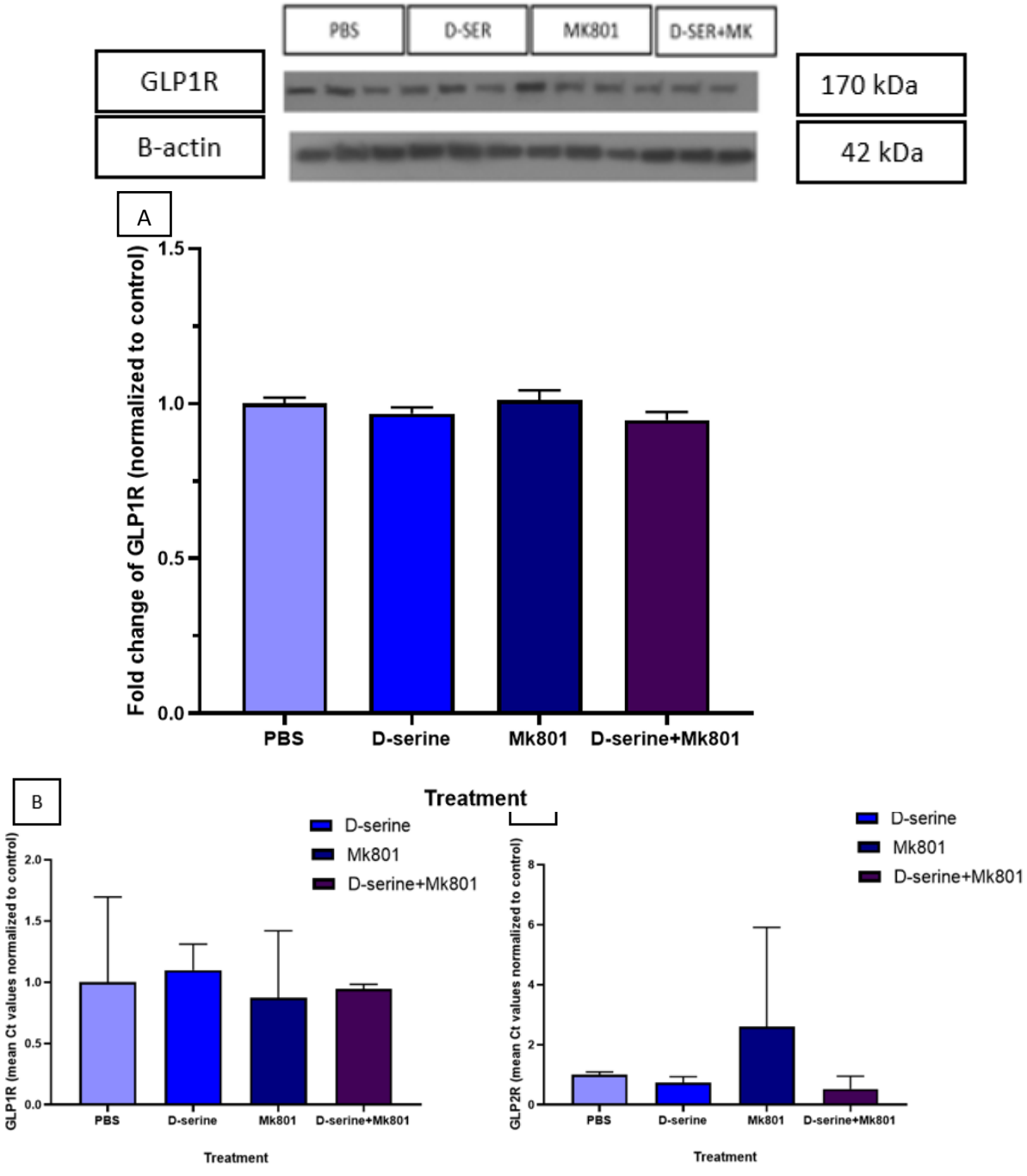


Figure 4.16 - 50 000 Caco-2 cells were treated with 500 µM D-serine in the presence or absence of 10 µM MK-801 (PBS used as a control, treatment duration =24 hours). GLP-1R protein levels (**A**), GLP-1R mRNA levels (**B**) and GLP-2R mRNA (**C**) levels were measured and there were no significant differences between treatment measures. N = 6.

TNF-α mRNA levels were altered in the presence of MK-801 ($F_{3,17} = 3.31$, p = 0.045) In **Figure 4.17d**, there were significantly higher levels of TNF-α mRNA in cells treated with just MK-801 compared to control (p=0.046, +1500%).

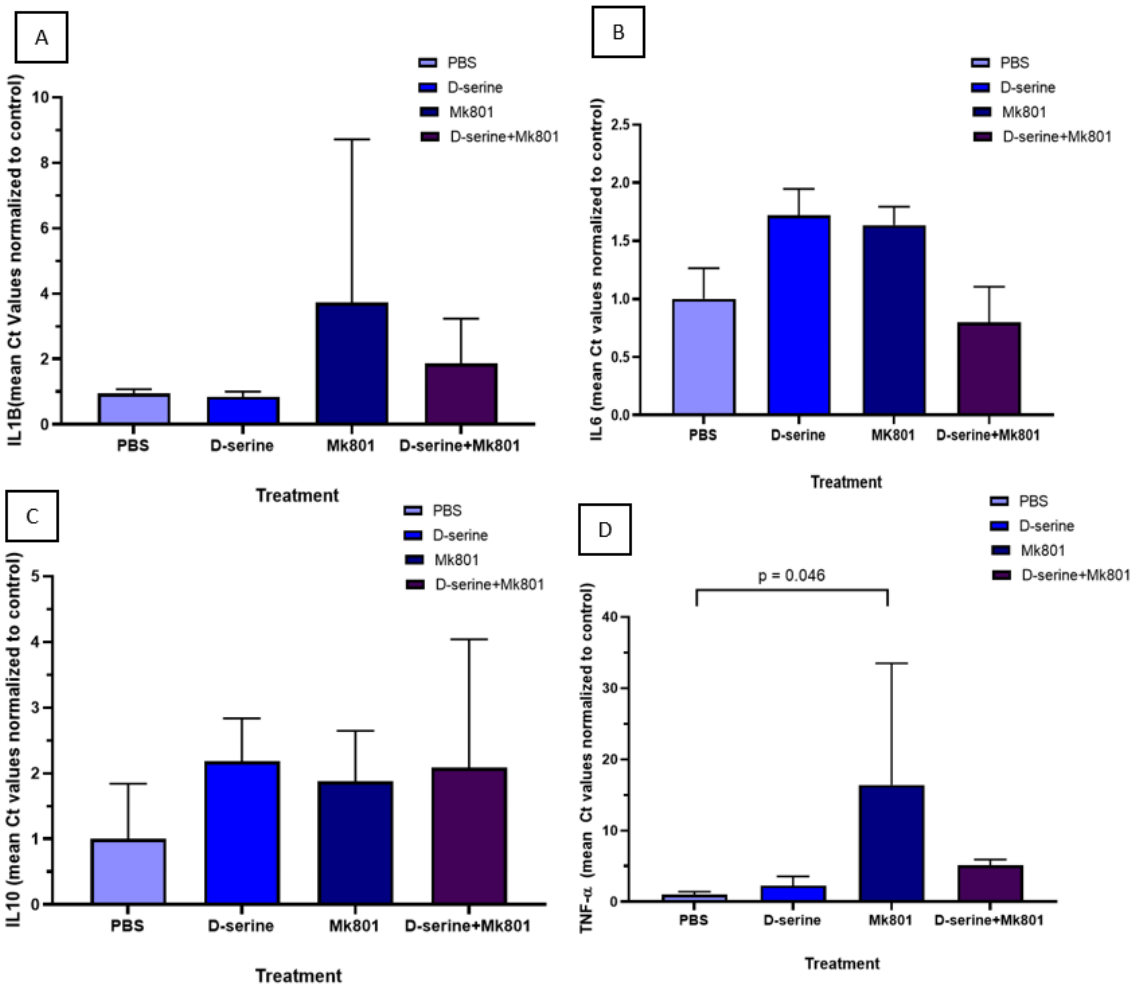


Figure 4.17 - The inflammatory profile of 50 000 Caco-2 cells treated with 500 µM of D-serine in the presence or absence of MK-801 (10 µM) for 24 hours (n=6) was established using qPCR analysis. mRNA levels of IL1β (**A**), IL6 (**B**), IL10 (**C**) and TNF-α (**D**) were

measured. There was significantly higher levels of TNF-α mRNA in cells treated with just MK-801 compared to control (p=0.046, +1500%).

GluN1 mRNA was affected by D-serine and MK-801 ($F_{3,19}$ = 15.07, p = 0.000). GluN1 mRNA levels in cells treated with D-serine and MK-801 were significantly higher than cells treated with just MK-801 (p=0.001, +405%), D-serine (p =0.000, +4194%) and PBS (p =0.000, p =+2859%), as displayed in **Figure 4.18b**.

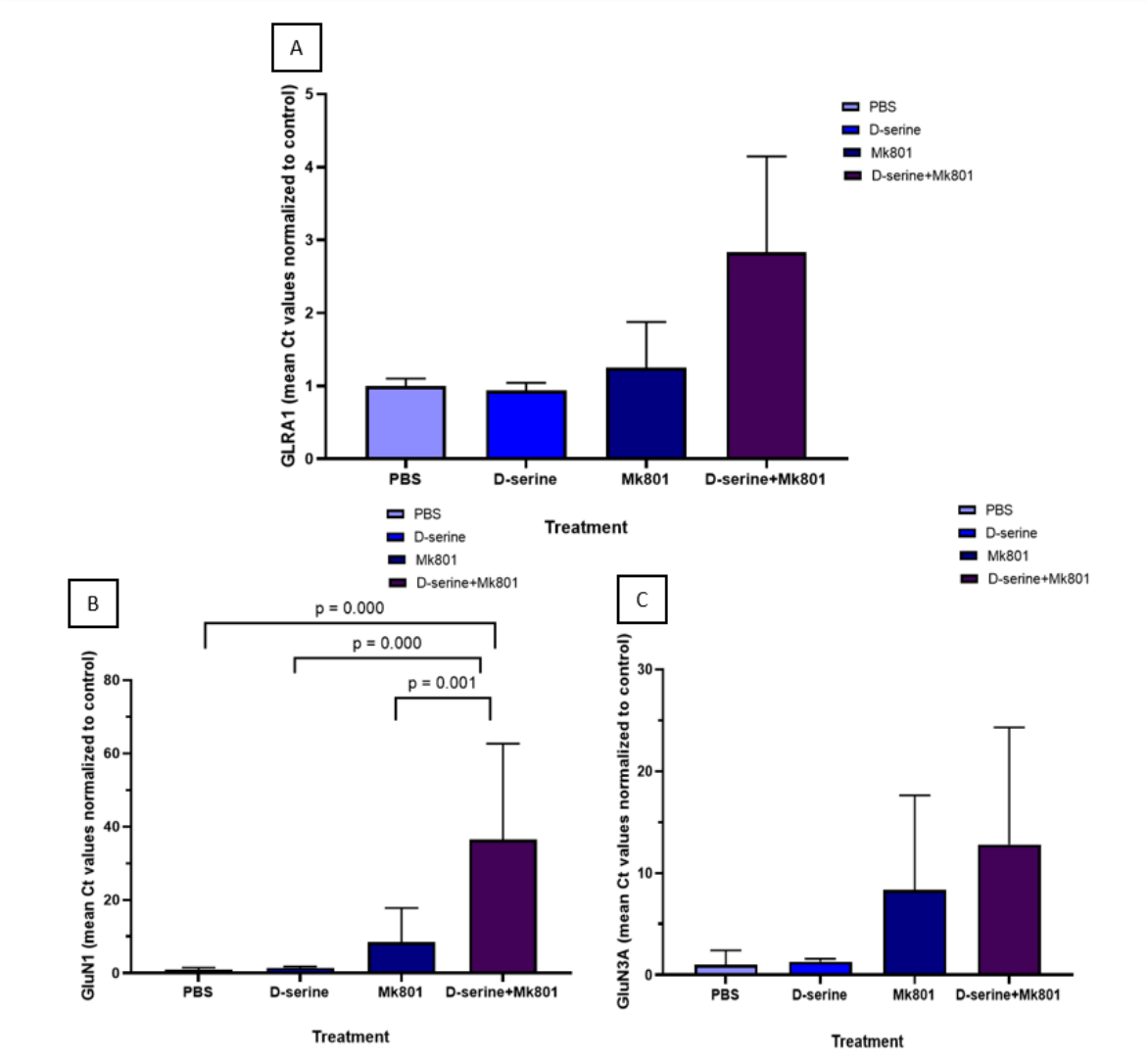


Figure 4.18 - A receptor profile was established using qPCR analysis. The mRNA levels of GLRA1 (**A**), GluN1 (**B**) and GluN3A (**C**) was determined in Caco-2 cells (50 000) treated with D-serine (500 µM) and/or MK-801 (10 µM) for 24 hours. GluN1 mRNA

levels in cells treated with D-serine and MK-801 were significantly higher than cells treated with just MK-801 (p=0.001, +405%), D-serine (p =0.000, +4194%) and PBS (p =0.000, p =+2859%). PBS was used as control and n=6.

4.4 Discussion

4.4.1. Caco-2 cells as a model for D-amino acid uptake

Following the completion of experiments, I was able to answer some of the questions posed at the start of the chapter. The Caco-2 cells serve as a viable model to investigate the effects of D-amino acids in the gut, where we found that D-serine had effects on receptors (NMDA subunits (**Figure 4.7 and Figure 4.8)** and GLP1R (**Figure 4.6**)) and inflammation (**Figure 4.8)** via interaction with TNF-α. This data supports the findings of Uchiyama et al (2005), where they showed ASCT2 mRNA was present in these cells, but also adds to it by detecting Asc-1, in both protein and mRNA. With the presence of both transporters, it can be assumed that D-alanine and D-serine would be robustly taken up in these cells.

4.4.2. D-serine increases TNF-α levels in Caco-2 cells

D-serine was found to have a pro-inflammatory effect in the gut, but not D-alanine. This was shown by a significant increase in TNF-α mRNA expression in cells treated with D-serine (**Figure 4.8)** but failed to repeat this finding in Asc-1 overexpression experiments (**Figure 4.12)** and in the MK-801 experiments (**Figure 4.17)**. With regards to the former, this could be due to difference to treatment protocols, as in **Figure 4.8** Caco-2 cells were treated with D-serine for 24 hrs, whilst in **Figure 4.12** Caco-2 cells were left in growth medium for an extra day whilst other cell wells were undergoing transfection, and then treated with D-serine. Another explanation is the inherent variability of Caco-2 cells, which Briske-Anderson and co (1997) reported in this cell line, though they compared

passage numbers of 22 to passage 72, and I never used cells past passage 40. However, the observation seen in **Figure 4.8** corroborates with the literature, where it was reported that an increase in serine racemase stimulated proinflammatory triggers in microglia and may infer an involvement in neuroinflammation and excitotoxicity. Along these lines, product of serine racemase, D-serine, contributed to the neurotoxicity seen in Schwann cells LPS-treated microglia (Wu et al., 2004).

Further, Davidson et al (2009) conducted a study extensively researching the effects on gene expression which D-serine activates in the forebrain of male F-344 rats. They also used GenMAPP to analyse the pathways D-serine may be interacting with by showing a significant number of differentially expressed genes in activated pathways. They found that D-serine activated the signalling pathways of several proinflammatory cytokines (such as IL-2, IL-3, IL-5, IL-6 and TNF-α).

These reported findings support what was observed in Caco-2 cells, where D-serine has a role to play in the inflammatory response to D-amino acids in the gut, displaying a significantly higher TNF-α mRNA levels in cells exposed to it (**Figure 4.8)**.

4.4.3. Effect of D-serine on GLP-1R in Caco-2 cells

D-serine increased GLP-1R mRNA levels in Caco-2 cells (**Figure 4.6)** and this finding was replicated in **Figure 4.11.** Therefore it would seem that D-serine affects GLP-1 system, but it is not certain whether this is a direct interaction, or through the effect D-serine has on TNF-α, where it increases this pro-inflammatory cytokine resulting in a regulatory decrease of inflammation by upregulation of GLP1-R as preclinical studies found that GLP-1R agonists attenuate atherosclerosis due to its anti-inflammatory activity (Jojima et al., 2017). Patel et al (2018) also showed that co-agonist of GLP-1 and glucagon

caused a decrease in TNF-α and MCP-1 expression in liver, and therefore displayed an anti-inflammatory effect.

GLP-2 was reported by Moran et al., (2012) to have a positive effect on barrier function and tight junction expression in the gut, and therefore could have a protective result against TNF-α effects on the gut. However D-serine doesn't seem to affect this specific subset of the GLP system (**Figure 4.6** and **Figure 4.11).**

Detected GLP-1R in both protein and mRNA was a key finding for these cells, as previous experiments by Wenjin et al., (2018) failed to detect GLP-1R in Caco-2 cells. Future experiments can determine if GLP receptor expression regulates inflammation by over expressing them in Caco-2 cells, and observing the effect it has on TNF-α expression, as the increase we observe in GLP-1R may not be directly due to D-serine, but due to the stimulatory effect D-serine has on TNF-α, which triggers activation of GLP-1R to modulate the inflammation with its anti-inflammatory effects.

4.4.4. D-serine increases mRNA of NMDA subunits, GluN2A, GluN2C, GluN3A and GluN1, in Caco-2 cells

To determine what is mediating this observed D-serine induced inflammatory effect, I investigated NMDAR involvement, as D-serine is a potent co-agonist for this receptor. Whilst the NMDAR has been established in the ENS, it was fairly recent that NMDARs were implied to be present in human colonic epithelial cells (HT29) by noted increases in NMDAR mediated increases in BDNF and the activation of CaMK II (Qi et al., 2016, Chen et al., 2019). The receptor itself has yet to be reported in colonic cells, and therefore my finding of NMDAR subunit mRNA expression in the Caco-2 cells is the first of its kind, thereby supporting the previous literature of downstream effects of NMDA receptors in the gut, but also serving as a potential answer as to *how* D-serine is having its inflammatory effect in these gut cells.

A study by Zhou et al (2006) found that there was increased NMDA receptor expression following colonic inflammation. Looking particularly at GluN1 splice variants in the colon of rats treated with trinitrobenzene sulfonic acid (TNBS) to induce colitis, the GluN1-C1 and GluN1-N1 subunits appeared 14-28 days following TNBS induced colitis, determined by RT-PCR. With regards to protein expression $GluN1_{001}$, $GluN1_{011}$ and $GluN1_{111}$ appeared in TNBS-treated rats with active colitis, while untreated control rats only expressed $GluN1_{001.}$

This supports my findings, where D-serine treated cells showed a significantly greater level of mRNA of NMDAR subtypes: GluN2A (**Figure 4.7)**, GluN2C (**Figure 4.7)**, GluN3A (**Figure 4.8)** and GluN1(**Figure4.8)** mRNA, and this may be a potential explanation for the D-serine induced inflammatory effect observed.

To further investigate if these D-serine effects are NMDAR-mediated, I used the MK-801 NMDAR antagonist, which will be discussed later in this Chapter.

4.4.5 Caco-2 cells transfected with SLC7A10 and treated with D-serine

Following the optimized protocol for transfection utilizing green fluorescent protein (GFP, Appendix), Caco-2 cells were transfected with human SLC7A10 (Asc-1) vector cDNA. These colorectal cells were then treated with D-serine, to observe what a potential overexpression of D-serine due to increased uptake by overexpressed transporters, may do to receptors and inflammatory markers in these cells. Unfortunately, when looking at the protein expression of SLC7A10, there was no difference between cells treated with SLC7A10, and those that were not. This implies that the transfection may not have been successful.

Caco-2 cells have been successfully transfected before, as shown in literature (Cummins et al., 2004). Additionally, Lipofectamine was used to successfully transfect Caco-2 cells (During and Harrison, 2007).

Since GFP was successfully transfected into cells under the same conditions, it is possible that the failure to observe significant alterations in SLC7A10 protein levels may be due to a high presence of naturally occurring SLC7A10 in these cells which are of human origin – transfecting these cells with human SLC7A10 vector may not allow for the contrast seen in in **Chapter 3**, when mouse cells were transfected with human SLC7A10 and then probed with an antibody that reacts to humans. Perhaps to better understand the effects of SLC7A10 in human gut cells knocking out SLC7A10 with siRNA would be better, as it would be easier to determine the differences between cells lacking SLC7A10 (like in the undetectable mouse cells not transfected with human SLC7A10 in **Chapter 3**) and cells that do not have SLC7A10 knocked out i.e. control cells.

4.4.6. D-serine in the presence, or absence, of MK-801 increases Asc-1 mRNA compared to PBS.

The increase of Asc-1 mRNA by D-serine, with or without MK-801 (**Figure 4.14b)** makes sense as MK-801 is meant to act by inhibiting NMDA receptor activation, so cells that receive D-serine displaying higher levels of Asc-1 mRNA is sensible, as this transporter should not be affected by the presence of MK-801, but rather by the presence of D-serine which it transports. However, this is an interesting find as D-serine did not previously have an effect on Asc-1 mRNA (**Figure 4.5b and Figure 4.9b).** This variability in the finding can be confirmed using *in vivo* studies of D-serine treated to mice and determining if there is a difference in Asc-1 gene expression in their gut, as investigation using Caco-2 cells have reported differing outcomes.

4.4.7. MK-801 antagonist effects on Caco-2 cells

The MK-801 experiments were inconclusive as I did not manage to replicate the original increase in GluN2A mRNA or TNF-α mRNA with just D-serine (**Figure 4.7b and 4.8c** respectively). This could be due to passage effects of the cells, or due to too much variability in the samples, as the cells treated with MK-801 had a 15-fold increase in TNF-α mRNA compared to PBS.

MK-801 is shown to be interacting with the NMDA receptor system as effects on GluN1 is observed. There is a trending increase in GluN2A protein levels in cells treated with MK-801, which is understandable as blocking NMDAR by MK-801 may result in feedback to upregulate receptor expression. None of these are able to sufficiently establish an NMDA-mediated link for the D-serine stimulated TNF-α effects, as the inflammatory response with just D-serine was not repeated (**Figure 4.17**). To determine the mechanism for TNF-α increase in cells treated with D-serine, it may be better to do functional study of NMDARs using calcium signalling, to determine if these receptors are more active in these cells, as MK-801 failed to paint a clearer picture of NMDAR involvement.

4.4.8. Concluding remarks, Limitations and Future studies

In conclusion, D-serine causes an increase in GluN2A (**Figure 4.7)**, GLP-1R (**Figure 4.6)** and TNF-α (**Figure 4.8)**. The increase in TNF-α may be NMDA receptor mediated, but the data as it stands from MK-801 study is inconclusive. GLP-1R seems to have a role to play when D-serine is involved, and this receptor involvement should be further investigated, as described above by overexpressing this receptor and observing the inflammatory response, or by using a GLP-1R antagonist such as Exendin-3 (Jones et al., 2018), to determine if this antagonism may have an effect on D-serine mediated TNF-α levels, and if its increase is to regulate inflammation in these cells.

Study limitations were a failure to establish if Caco-2 cells were successfully transfected, or if MK-801 effectively blocked NMDARs. Overcoming these limitations will involve using different experimental procedures to determine the effect of Asc-1 (using gene knockout) and NMDARs (using calcium imaging) in Caco-2 cells, which are much more effective than western blots and qPCR for evaluating such mechanisms.

TNF-α mRNA levels were meant to be supported by TNF-α protein levels by ELISA, but due to COVID-19 Pandemic related time constraints, these experiments were not done.

To further consolidate the D-serine mediated GLP effects, GLP-2R protein expression could have been measured, along with GLP-1R, to confirm if D-serine is only affecting the GLP-1R system, as is reflected in the mRNA data (**Figure 4.6)**.

There is an interesting contrast between D-amino acids in the anterior pituitary and D-serine in the gut, where the former seems to have an inhibitory effect on hormonal secretion, while the latter has a stimulatory effect on inflammatory response. The lack of effect of D-alanine on the gut maybe due to the fact that it is predominantly stored in the anterior pituitary gland and the pancreas in the body, where it may affect hormonal signalling for ACTH and insulin, and therefore the Caco-2 cells are less receptive to its presence than the likes of D-serine. It would be interesting to confirm the pro-inflammatory effects of D-serine in the gut *in vivo* using ELISA analysis of plasma. If this pro-inflammatory finding of D-serine is true, this will support increasing D-alanine uptake over D-serine uptake in the body to regulate stress axis hormones (as both these D-amino acids have been shown to regulate HPA axis hormones in **Chapter 2)**, as D-alanine does not have pro-inflammatory effects on the gut like D-serine may potentially have (**Figure 4.8)**.

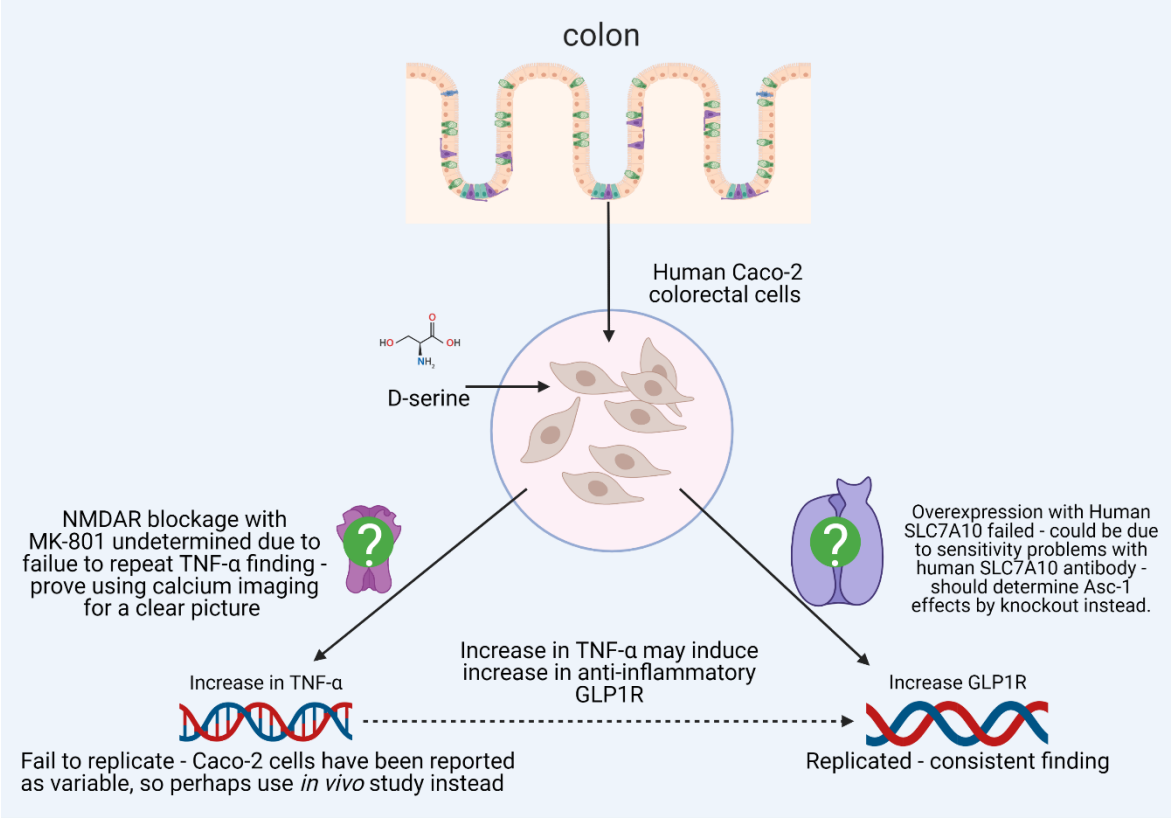


Figure 4.19 - A summary of the findings of studies involving D-serine and Caco-2 cells.

CHAPTER FIVE: AN *IN VIVO* STUDY ON THE EFFECTS OF D-AMINO ACIDS: THEIR PRESENCE IN CD1 MICE AND THEIR ABSENCE IN GERM-FREE MICE

5.1. Introduction

In this chapter I will investigate how D-amino acids effect an *in vivo* system – that of CD1 mice and Germ Free (GF) mice. Particularly, the regional differences of receptors (GlyRs and NMDARs) and transporters (Asc-1 and ASCT2) observed in the brain, and most importantly the effect of D-amino acids on corticosterone levels in the plasma of CD1 mice. I also observe the SGK1-POMC system, as it is related to ACTH secretion, which would affect corticosterone production.

5.1.1. What molecular factors should be monitored when studying the D-amino acid effects on the HPA axis in GF and CD1 mice?

Firstly, Asc-1 and ASCT2 transporter levels (and potential changes) should be confirmed in brain regions of interest, as any D-amino acid effects in these areas would be facilitated by uptake by these transporters.

As stated earlier in this thesis, the microbiome influences the activity of the HPA axis. Therefore it is important to investigate HPA axis hormone secretion *in vivo,* as well as known regulators of ACTH such as POMC, which have been well studied in stress response experiments thus far. Studies have demonstrated the excess release of ACTH and corticosterone in GF mice following restraint stress (Sudo et al, 2004). However, Vagnerová et al (2019) did not observe higher expression of POMC in the pituitary of nine-week old GF mice when compared to SPF male BALB/c mice, following acute restraint stress, which was in contrast with their previous findings of an upregulation of pituitary POMC following chronic psychosocial stress (Vodička et al., 2018), so it would be interesting to investigate POMC in the pituitary of GF mice to add to this story. Reiter et al (2011) discovered that SGK1 stimulated POMC transcription and ACTH release in

murine anterior pituitary AtT20 cells, which is contradictory to the usual relationship between GCs and POMC. Therefore SGK1 levels should be measured accordingly in relation to POMC.

D-amino acids fail to effect NMDARs in AtT20 cells, so it would be interesting to establish their effects in other brain regions, especially those sensitive to D-serine, such as the frontal cortex. Sudo et al (2004) reported that GluN1 (in the cortex) and GluN2A (in the hippocampus and cortex), decreased in GF mice when compared to SPF mice. So it would be interesting to see if my study confirms this, and what effect D-amino acids would have on these receptors in CD1 mice. GluN2B levels decrease in the amygdala of GF mice, so this brain region was also analysed to determine the effects a lack of (GF mice) or an increase of (D-amino acid treated CD1 mice) D-amino acids will have on NMDARs in the amygdala (Neufeld et al., 2010).

This chapter, therefore, investigated the changes in ACTH, POMC, Sgk1, NMDARs, Asc1 and ASCT2 in GF mice, and in CD1 mice fed D-amino acids. The GLRA1 gene was also investigated as D-alanine is a known agonist.

5.1.2. What brain regions should be investigated?

This is important to determine as unlike *in vitro* models, there is a limit to sample accessibility *in vivo* and one must think about the best ways to divide the brain, particularly based on regions of interest to be studied. My primary region of interest was of course the anterior pituitary gland, as I wanted to determine translatability of anterior pituitary *in vitro* findings. Additionally Etoh et al (2009) established that D-alanine was stored in corticotrophic cells of the rodent anterior pituitary, therefore it would be profoundly important to investigate this region both in CD1 mice and GF mice. Unfortunately, CD1 mice anterior pituitaries were beyond my dissection expertise, so

whole pituitaries were used instead, and I was unable to attain the anterior pituitaries of GF mice.

The levels of D-serine in the cerebellum and brain stem increase from birth until post-natal day 14, and the decreases to the adult concentration which is low owing to the abundant expression of the catabolic enzyme, DAO (Schell et al., 1997). There is strong evidence that NMDA receptor subunits are higher in areas that contain D-serine, since it is an endogenous agonist for this receptor, such as the cortex and hippocampus (Nagata et al., 1993). However, I was also curious to determine the effect D-serine treatment or lack of gut microbiota (GF mice) may have on NMDARs in brain regions not accustomed to high D-serine content, and perhaps less sensitive to this amino acid, such as the cerebellum and brain stem.

D-serine has been established to be present in high concentrations in the medial frontal cortex (Fujihira et al., 2007), and the serine levels in the frontal cortex was of special interest with regards to schizophrenia, as serine racemase levels in the post-mortem frontal cortex were shown to be 39% lower in patients with schizophrenia than the control group, whilst hippocampal serine racemase was 21% lower in people with schizophrenia compared to controls.

Noradrenergic neurons that originate chiefly from the nucleus of the solitary tract in the brainstem play a crucial role in activating the HPA axis (Herman et al., 2016). The fast-synaptic inhibition that occurs in the brainstem and the spinal cord are due to the presence of the inhibitory chloride channel glycine receptor, which D-alanine binds to (Hurdiss et al., 2015).

Therefore, I investigated the D-amino acid effects in the frontal cortex, hippocampus, cerebellum and the pituitary of CD1 mice, and the cortex and brainstem of GF mice.

5.1.3. What can GF mice tell us about D-amino acids

D-alanine in the mammal has been established to have a microbial origin (Konno et al., 1990). Therefore, the quantity of D-alanine present in the body is dependent on the body's microbiota. Microbiota consist of viral, bacterial, fungal and archaeal components that occupy various niches in the human body, predominantly the gastrointestinal tract, where over 100 trillion commensal bacteria inhabit. The microbiota has been proven to be involved in inflammatory regulation, infectious and metabolic diseases, and seems to play a crucial role in potentially causing, propagating or preventing human illnesses (Bhattarai and Kashyap, 2016; Lai et al., 2014; Norman et al., 2014).

GF mice models came into existence to test if microbiota have a causative or correlative factor in disease states. They are bred in isolators which fully inhibit exposure to microorganisms, the intention being they remain free of detectable bacteria, viruses and eukaryotic microorganisms (Kennedy et al., 2018). Since these animals are void of bacteria, they should be lacking in D-alanine, and therefore serve as a 'D-alanine knock out model'. They were first established in the 1940s, following the conceptualization by Louis Pasteur in 1885 (Yi and Li, 2012). GF mice allow for the study of absolute absence of microbes, or alternatively for the generation of gnotobiotic animals exclusively colonized by distinguished microbes.

Martínez-Augustin et al (2009) found that GF mice display a heightened response to forskolin/cAMP response, which implies that commensal gut microbes may influence colonic ion transport, via cAMP-mediated responses. Which means they may serve as a good model to investigate cell signalling as well, which is imperative for studying D-amino acid effects on hormone secretion.

SPF mice are raised with normal functioning microbiota but not with specific pathogens (Sudo et al., 2004). They were introduced in the 1960s to reduce disease and infection as a variable in biomedical research. Modern laboratory mice are usually SPF, which entails a defined list of mouse pathogens that are excluded from the animal colonies, but these lists of pathogens are vary between institutes (Letson et al., 2019). SPF mice are a good control model for GF mice - whilst being free of certain pathogens, they still maintain a microbiota, which GF mice lack.

5.1.4. The aims of the chapter

1. To test if the previous findings in AtT20 cells would translate *in vivo*, the main aim of the chapter was to determine whether D-amino acids affected hormone secretion and gene expression in the pituitary of CD1 mice. The abundance of mRNAs encoding SGK1, POMC, the amino acid transporters Asc-1 and ASCT2 and the receptors, GluN1, GluN3A and GLRA1 was determined with qPCR. Corticosterone concentrations were measured in the plasma of these mice by ELISA.

2. To determine if there are regional differences with regards to effects of D-amino acids in the brain. Therefore the hippocampus, cerebellum and frontal cortex of CD1 mice were also probed to determine the mRNA levels of the above-mentioned genes.

3. To have a better understanding on the role of D-amino acids in mammals, animals that should have no D-alanine, GF mice, were studied. The receptor changes were determined by both protein and mRNA levels in the cortex and brainstem and compared to SPF controls. The mRNA levels were also investigated for amino acid transporters, ASCT2 and Asc-1, as well as ACTH regulators SGK1 and POMC.

5.2. Methodology

5.2.1. Treatment parameters for CD1 mice treated with D-amino acids

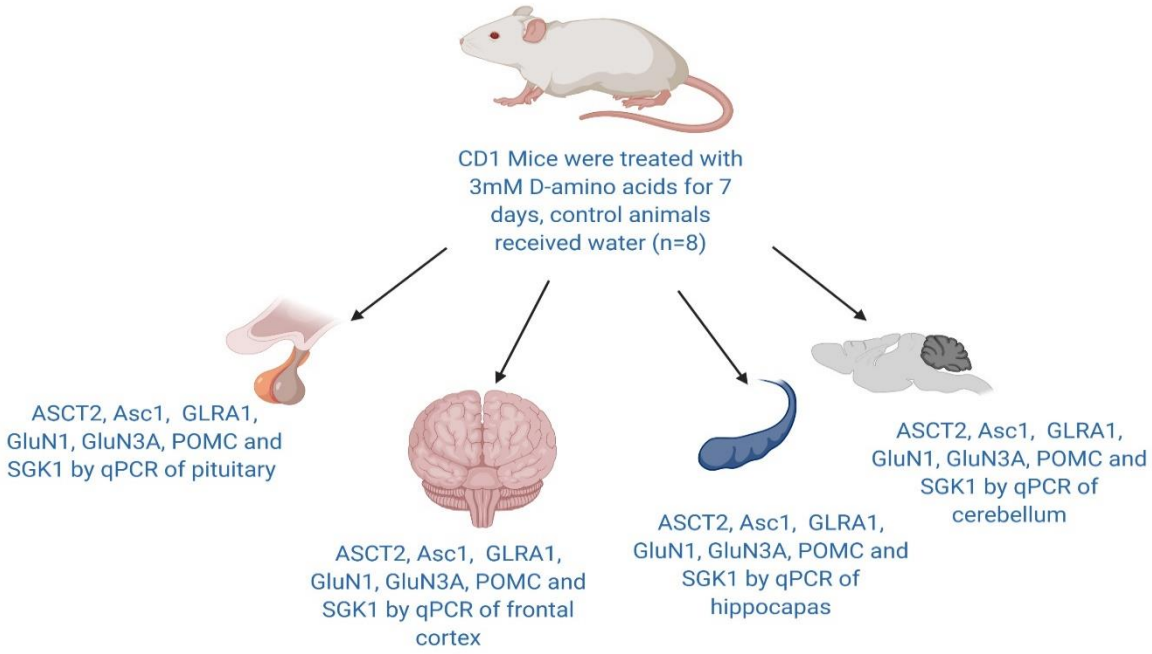


Figure 5.1 - CD1 mice D-amino acid study depicting brain regions and genes studies. Image made with Biorender.com.

As seen in **Figure 5.1** above male, adult (25-30g) CD1 mice (Envigo RMS Ltd, UK) were provided with 3mM D-amino acids in their drinking water for 7 days, with control rodents receiving water as usual. Each cage contained two animals and water bottles were weighed every day and concentrations adjusted to ensure each mouse received 50mg/kg D-amino acids. Brains and pituitaries were collected, and the frontal cortex, hippocampus, and cerebellum were dissected out. These samples were then processed using the method described in **Figure 5.2** below and analysed for specific gene expression levels by qPCR. Rodent plasma was also collected, where corticosterone levels were measured, as illustrated in the **Figure 5.3** below. Should be noted that I did not conduct the animal handling portion of these experiments due to licensing restrictions but received samples of brain tissue to be processed.

5.2.2. Dissection of brain regions and sample preparation

Brain regions were dissected as per training under supervisor, Prof Phil Burnet. This was done on dry ice using a surgical blade. Once dissected, brain regions were placed in appropriately labelled Eppendorfs and weighed. This is important as sample weight determined how much trireagent would be needed for the RNA extraction step later on, or how much RIPA buffer would be used for the protein extraction step. Samples were processed as described in **Figure 5.2** below.

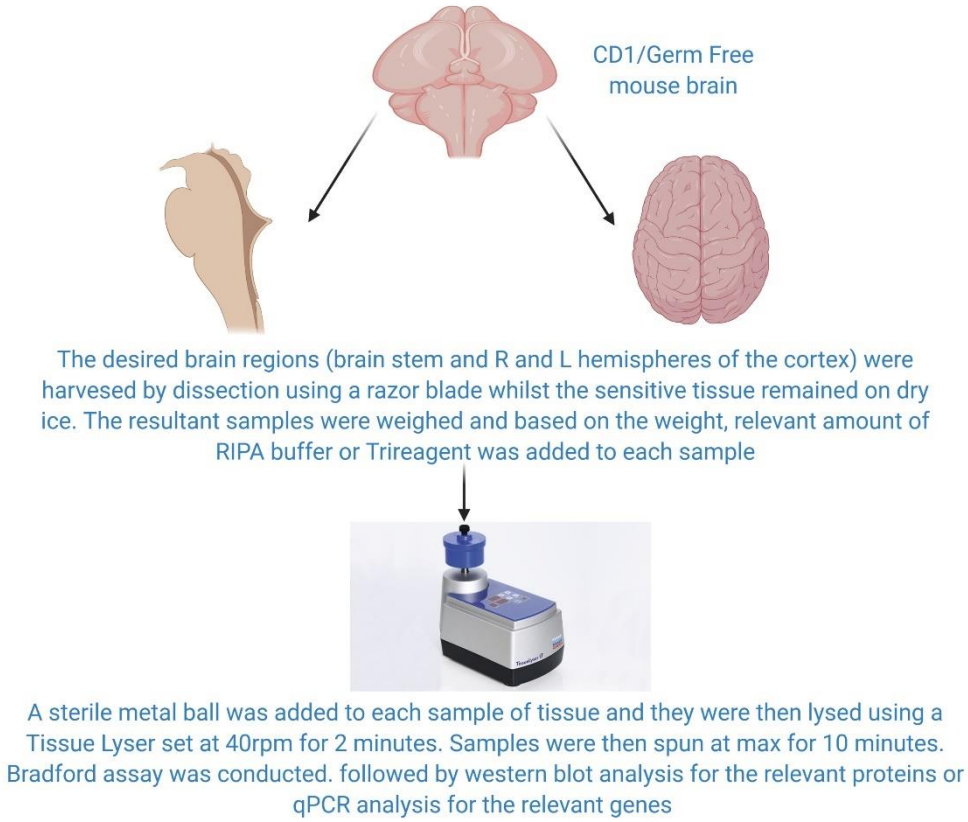


Figure 5.2 - Brain tissue processing. Following weighing of brain regions, RIPA buffer for protein analysis (R0278-500ML, Sigma Aldrich, United Kingdom) or Tri-Reagent for mRNA analysis (T9429, Life Technologies Ltd, United Kingdom) is added according to the size of the sample, and then lysed on the Tissue Lyser (Tissue Lyser LT, Qiagen) at 40RPM for 2 minutes. Samples were then analysed for protein presence via western blot

analysis, or gene expression levels by qPCR analysis described in detail in **Methods section of Chapter 2**. Image made with Biorender.com.

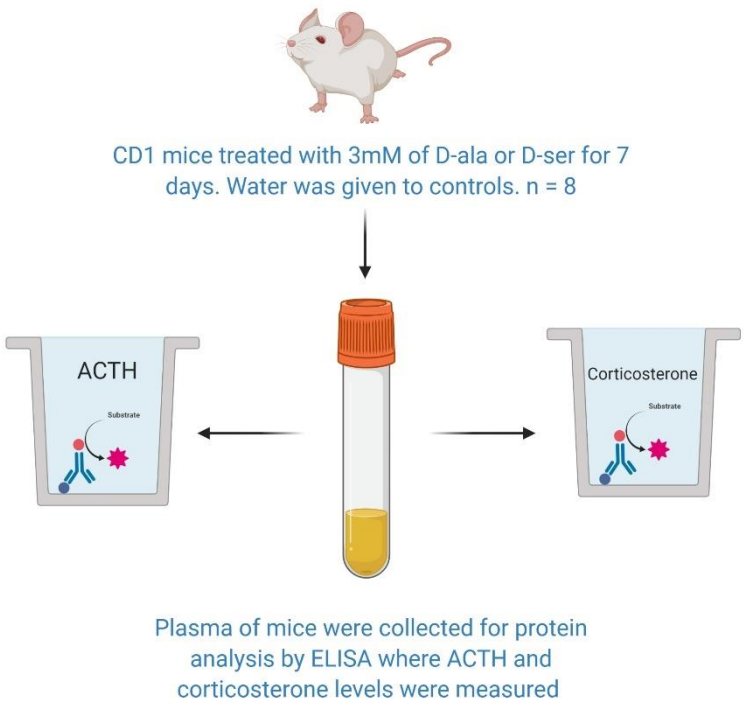


Figure 5.3 - Plasma of mice were collected and analysed for ACTH (EA100849, Origene, United Kingdom) or corticosterone (ADI-900-097, Enzo Life Sciences, United Kingdom) by ELISA. Image made with Biorender.com.

5.2.3. Corticosterone Assay

Corticosterone measurements were achieved using ELISA assay mentioned in **Figure 5.3.** Plasma was collected prior to euthanization of rodents following their 7-day treatment with D-amino acids at 3mM. This plasma was then analysed for corticosterone levels using the following steps, based on the protocol of the kit (ADI-900-097, Enzo Life Sciences, United Kingdom):

1. Reagents were left for 20-30 minutes on the benchtop so as to acclimatize to room temperature

2. 40 µl of each sample was added to a microfuge tube
3. 2ml of 1:100 steroid displacement reagent was made just before it was required in deionized water
4. 40 µl 1: 100 steroid displacement reagents was added to each tube
5. Samples were vortexed and left to stand for 5 minutes
6. 320 µl of EIA buffer was added to each plasma tube.
7. Final sample concentration at 1:10, each sample and kit provided standard was added to a 96-well plate at 10 µl in triplicate
8. Samples were read on spectrophotometer (Spectramax 190, Beckman Coulter, DS849B, California, USA) at 450 nm
9. Standard curve generated using Microsoft Excel, corticosterone levels were measured in pg/ml and averages calculated according to treatment, which was then analysed on SPSS statistics to generate the graph below in **Figure 5.5**.

5.2.4. GF mice procurement

Brains from male GF and SPF control mice brains (n =5) were a generous gift from Ms Claire Pearson, Oxford Centre for Microbiome Studies. Unfortunately, the samples were not provided with pituitaries and so the analysis was limited to the cortex and brainstem only. **Figure 5.4** explains briefly how these mice were reared.

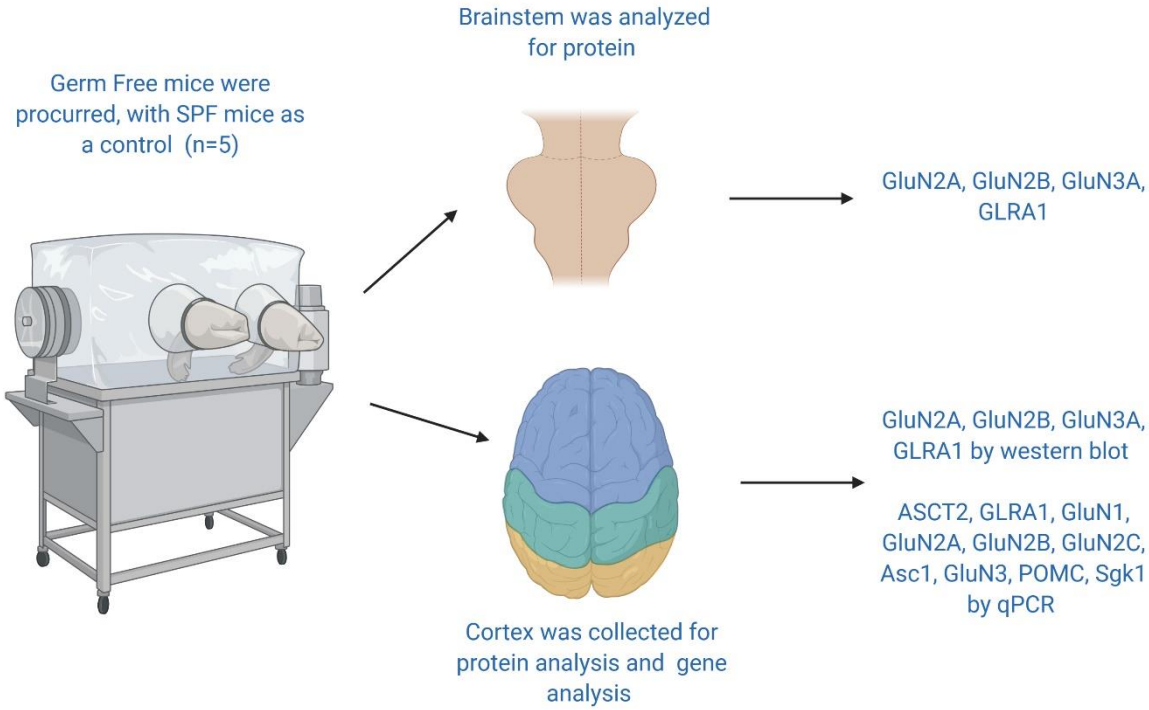


Figure 5.4 - Experiments involving GF mice. Adult male GF and SPF mice reared at the same time was used as a control. There were 5 replicates of each treatment group, and the brainstem, and right and left cortical hemispheres were harvested for analysis.

5.2.5. Statistical analysis

Data were extracted from Microsoft Excel and analysed on SPSS statistics (version 13). For CD1 mice with 3 experimental groups (Saline, D-alanine and D-serine) One-way ANOVA was used initially so significance was ascertained ($p < 0.05$). Tukey's test was then used post-hoc to find the significance between treatment groups. With the GF study, there were only 2 groups (GF and SPF mice) so Independents Sample's T-test was used to determine significance instead. Graphs were then drawn using GraphPad Prism (Version 9). Results are represented as mean ± SEM.

5. 3 Results

5.3.1. D-amino acid effects on plasma corticosterone

There was a significant effect of treatment on corticosterone in the plasma of CD1 mice administered D-amino acids ($F_{2,9}$ = 7.62, p = 0.012). As seen in **Figure 5.5.** D-serine (p = 0.021, -84%), as well as D-alanine (p = 0.019, - 86%) levels decreased compared to controls.

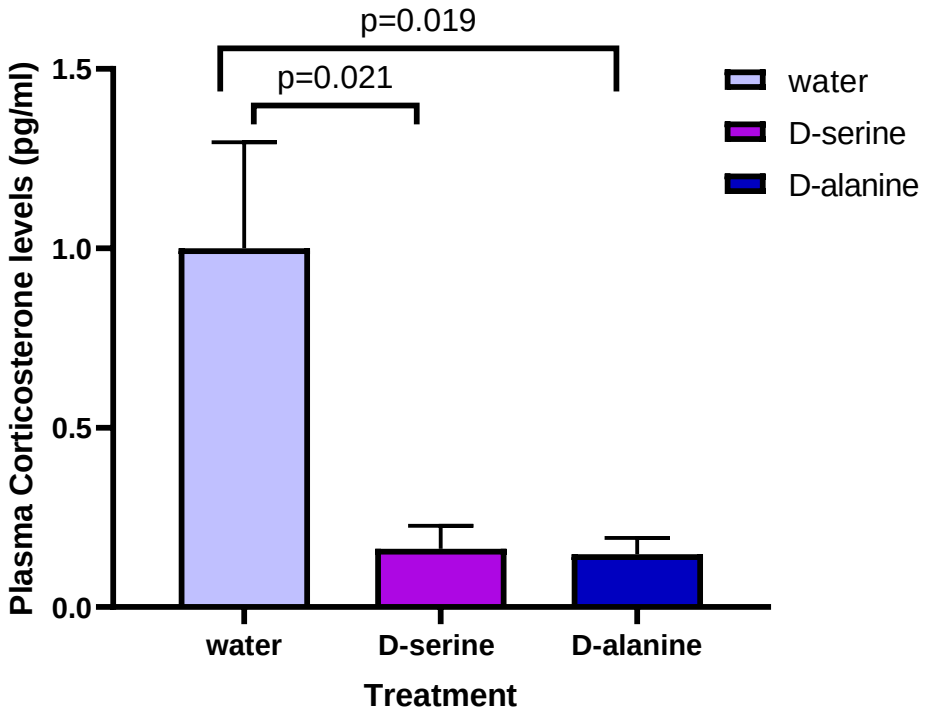


Figure 5.5 - Corticosterone levels were measured in plasma of CD1 mice treated with D-alanine or D-serine (50mg/kg) for 7 days. CD1 mice receiving water was used as a control, where n=8. There was a significantly lower level of corticosterone in the plasma of mice treated with D-serine (p = 0.021, -84%), as well as D-alanine (p = 0.019, - 86%).

5.3.2. D-amino acid effects on receptor gene expression in brains of CD1 mice

There were no changes in POMC or SGK1 (**Figure 5.6)** and amino acid transporters (**Figure 5.7)** in CD1 mice treated with D-amino acids. From the genes quantified by qPCR (POMC, SGK1, Asc-1, ASCT2, GluN1, GluN3A and GLRA1), D-amino acids effected the mRNA levels of the GLRA1 receptor subtype (**Figure 5.8c**, $F_{2,15} = 4.33$, $p = 0.033$) in the pituitary. There was a significantly lower abundance of GLRA1 mRNA in mice treated with D-alanine compared to the control ($p = 0.031$, -72%). However, there was also a decreased trend in pituitary GLRA1 mRNA levels of mice treated with D-serine compared to control ($p = 0.077$, -59%).

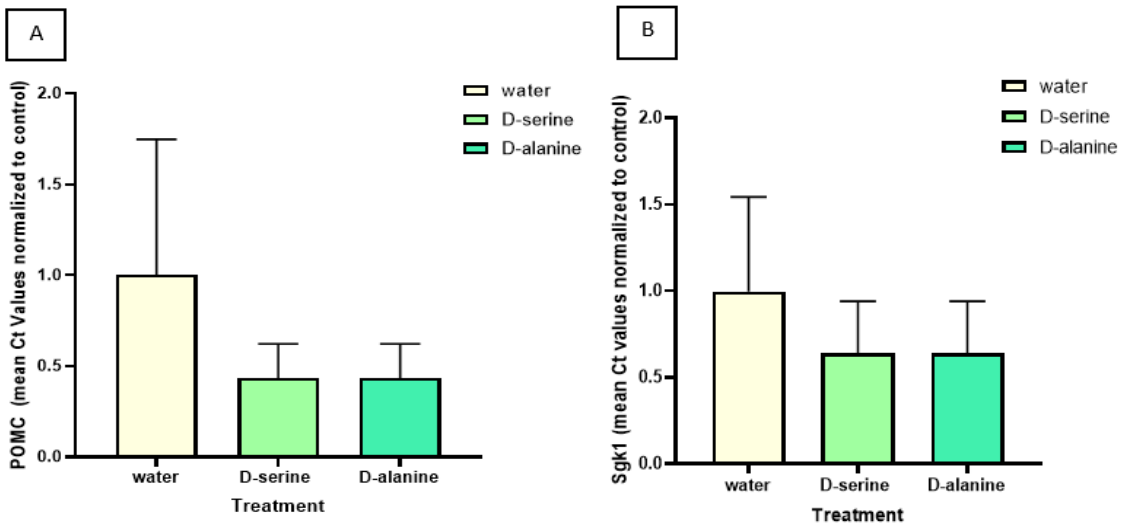


Figure 5.6 - POMC (A) and SGK1 (B) mRNA abundance in the pituitary of CD1 mice. Mice were administered 50mg/kg D-alanine or 3mM D-serine via the drinking water for 7 days, with mice receiving water as a control. Samples are the mean of 8 replicates. There is no statistical difference between the treatment groups in both POMC and SGK1 expression.

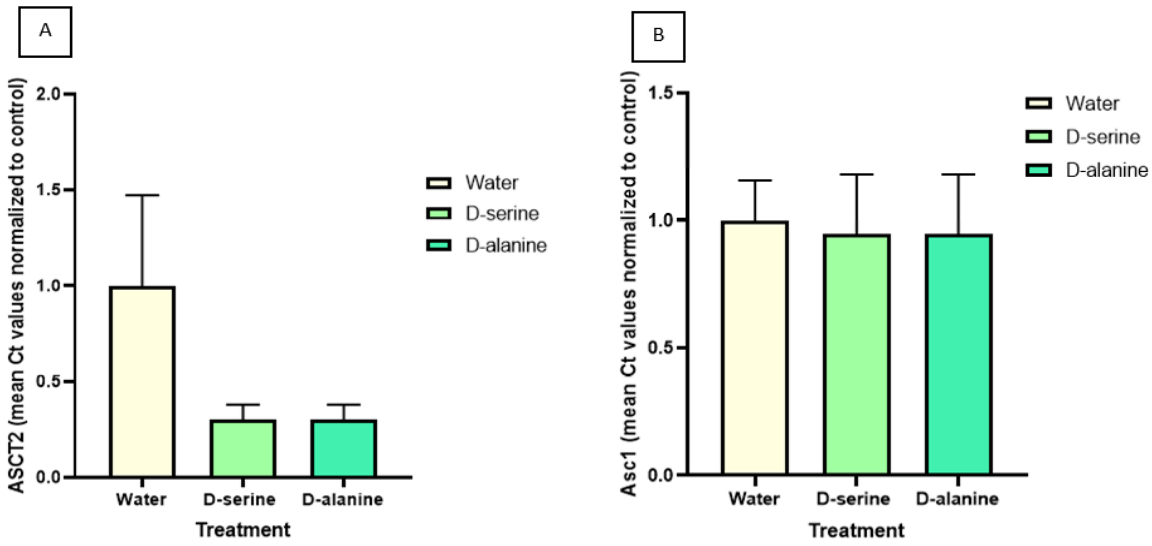


Figure 5.7 - ASCT2 (A) and Asc-1 (B) gene expression were measured using qPCR analysis, in the pituitary of CD1 mice. CD1 mice were treated with D-alanine or D-serine (50mg/kg) for 7 days, with water used as a control (n = 8). There were no significant differences between treatment groups.

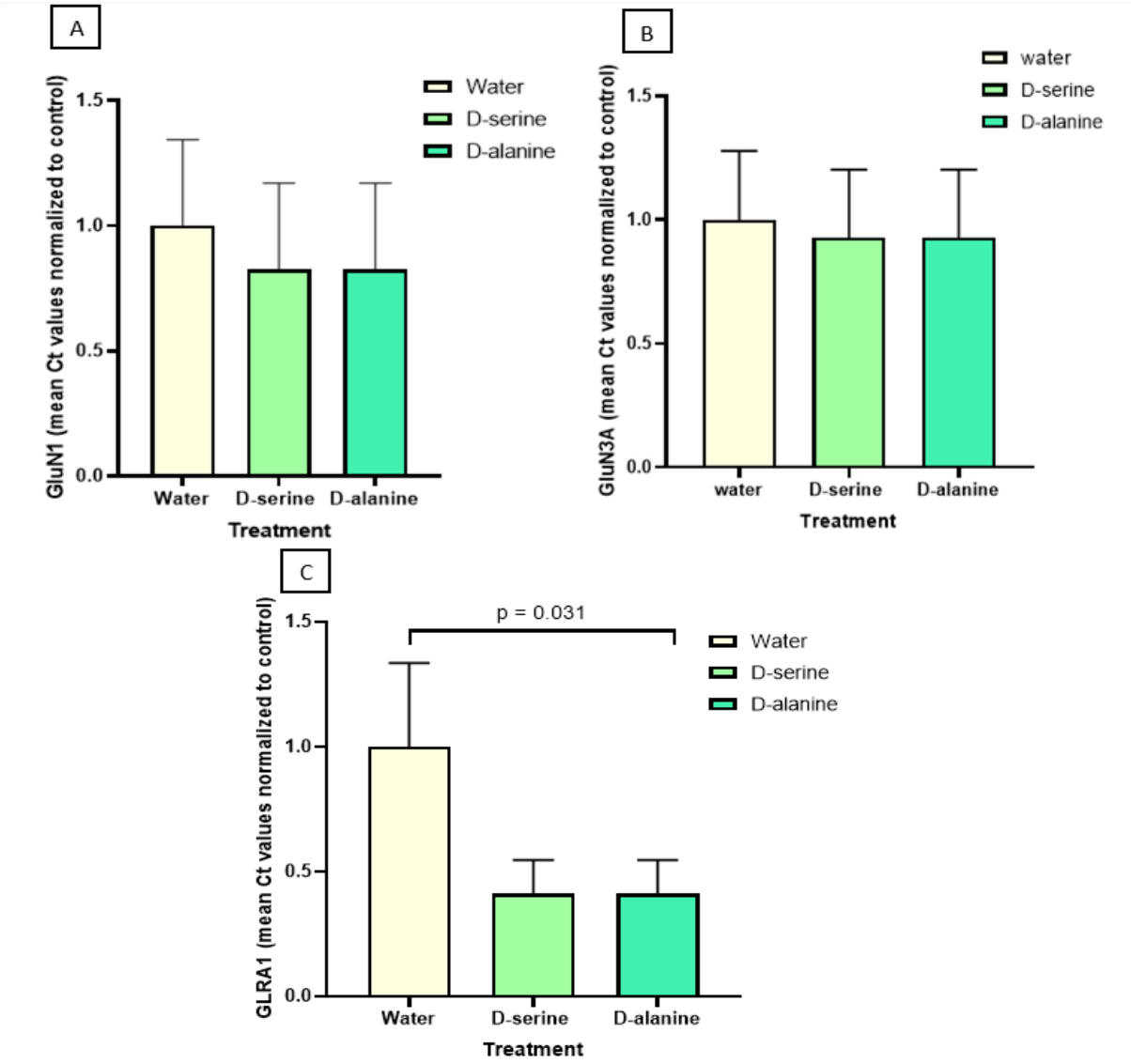


Figure 5.8 - GluN1 (A), GluN3A (B) and GLRA1 (C) mRNA abundance in the pituitary of CD1 mice. Mice were treated with D-alanine or D-serine (50mg/kg) for 7 days, or water as a control for the same duration of time. It was determined that there were no significant differences between the groups in GluN1 and GluN3A levels. There was a significantly lower level of GLRA1 mRNA in mice treated with D-alanine compared to the control ($p = 0.031$, -72%) and a trending decrease in GLRA1 mRNA levels of mice treated with D-serine compared to control ($p = 0.077$, -59%). Samples are the mean of 8 replicates.

There were no significant effects of D-amino acids on gene expression in the frontal cortex (**Figure 5.9 – Figure 5.11)**, or the cerebellum (**Figures 5.12 -Figure 5.14)**.

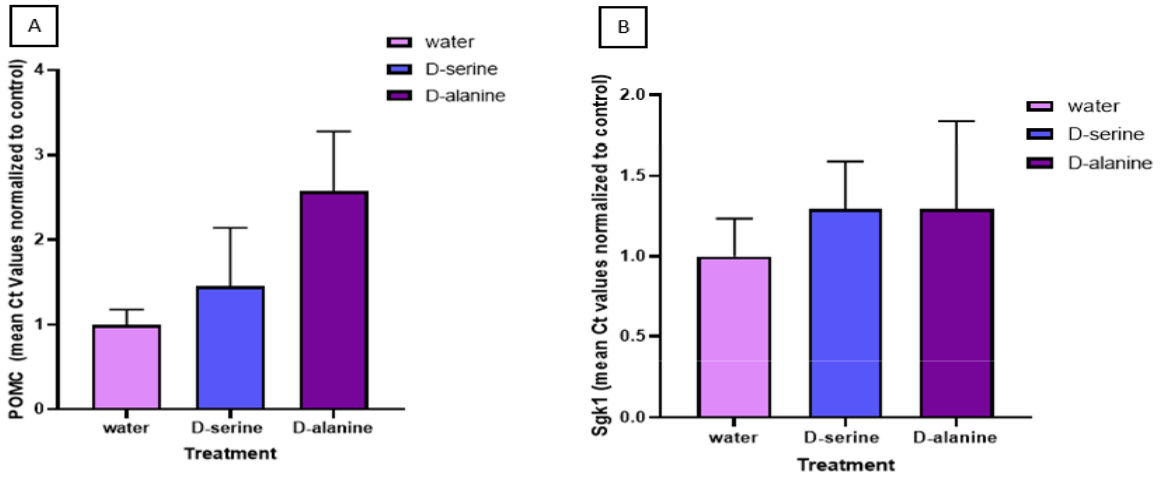


Figure 5.9 - POMC (A) and SGK1 (B) mRNA abundance were measured using qPCR analysis in the frontal cortex of CD1 mice. CD1 mice were treated with D-alanine or D-serine (50mg/kg) for 7 days, with mice receiving water as a control. Samples are the mean of 8 replicates. There is no statistical difference between the treatment groups in both POMC and SGK1 expression.

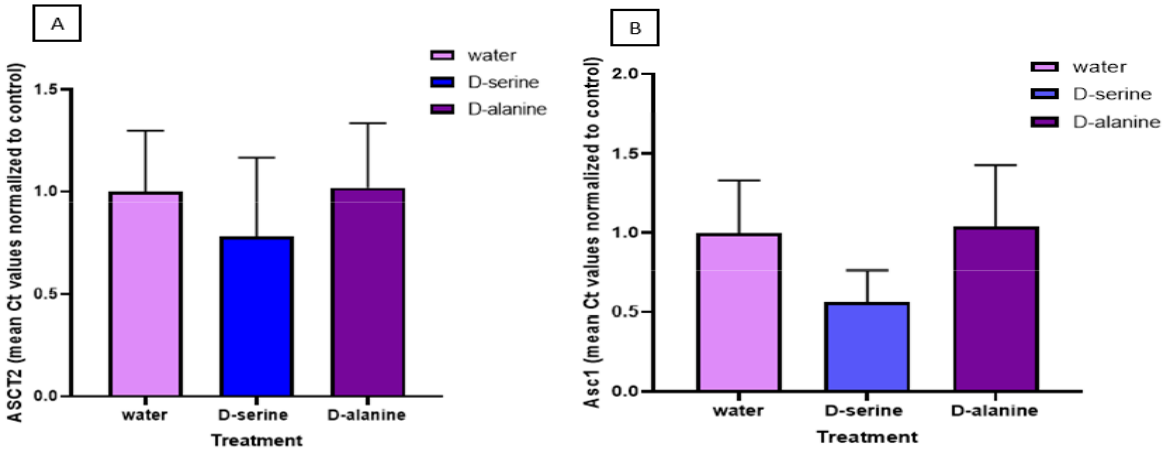


Figure 5.10 - The abundance of ASCT2 (A) and Asc-1 (B) mRNAs in the frontal cortex of CD1 mice of the amino acid transporters. CD1 mice were treated with D-alanine or D-serine (50mg/kg) for 7 days, with water used as a control (n = 8). There were no significant differences between the treatment groups.

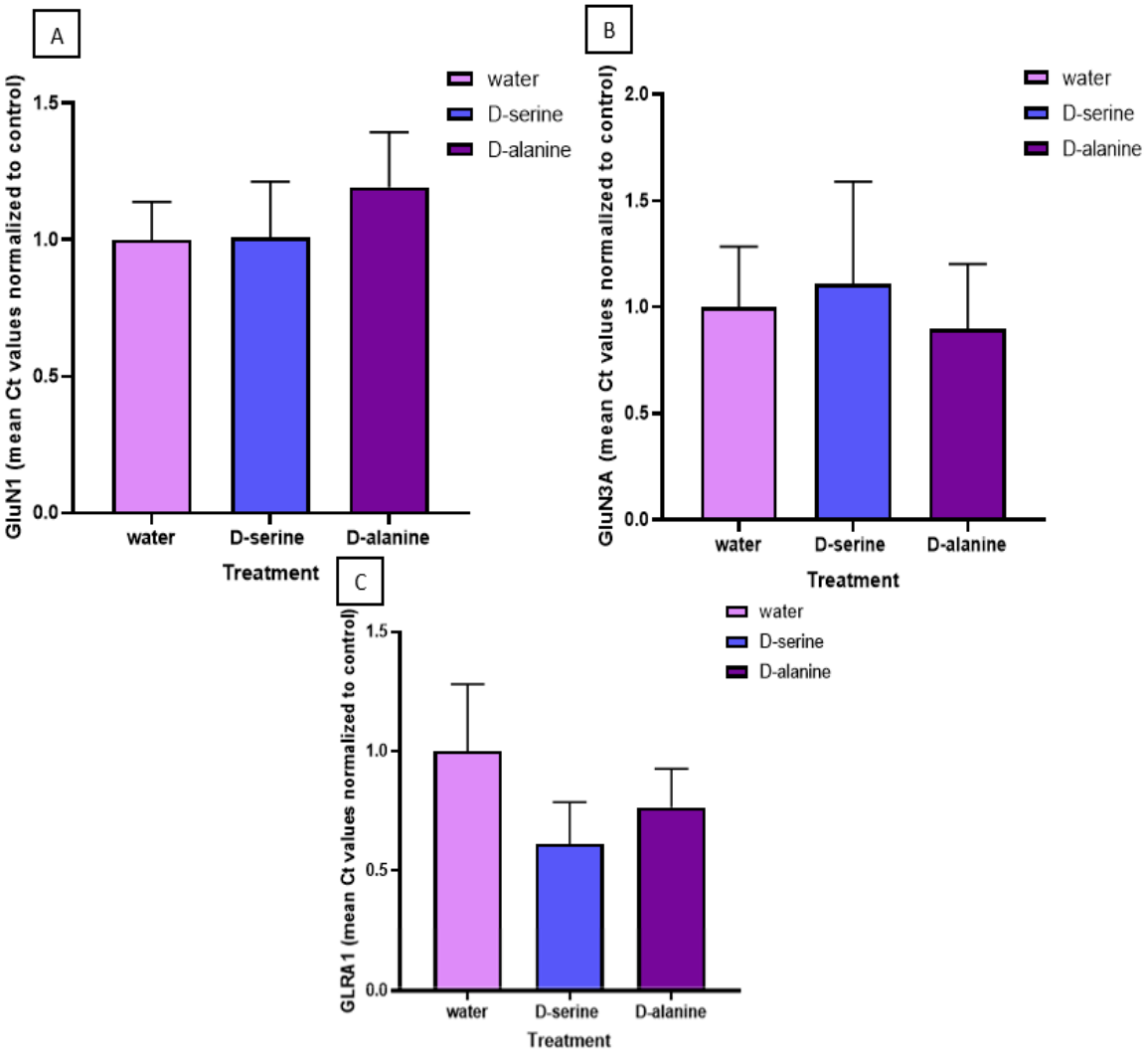


Figure 5.11 - GluN1 (A), GluN3A (B) and GLRA1 (C) mRNA abundance in the frontal cortex of CD1 mice. Mice were treated with D-alanine or D-serine (50mg/kg) for 7 days, or water as a control for the same duration of time. There were no significant differences between any of the treatment groups for any of the genes. Samples are the mean of 8 replicates.

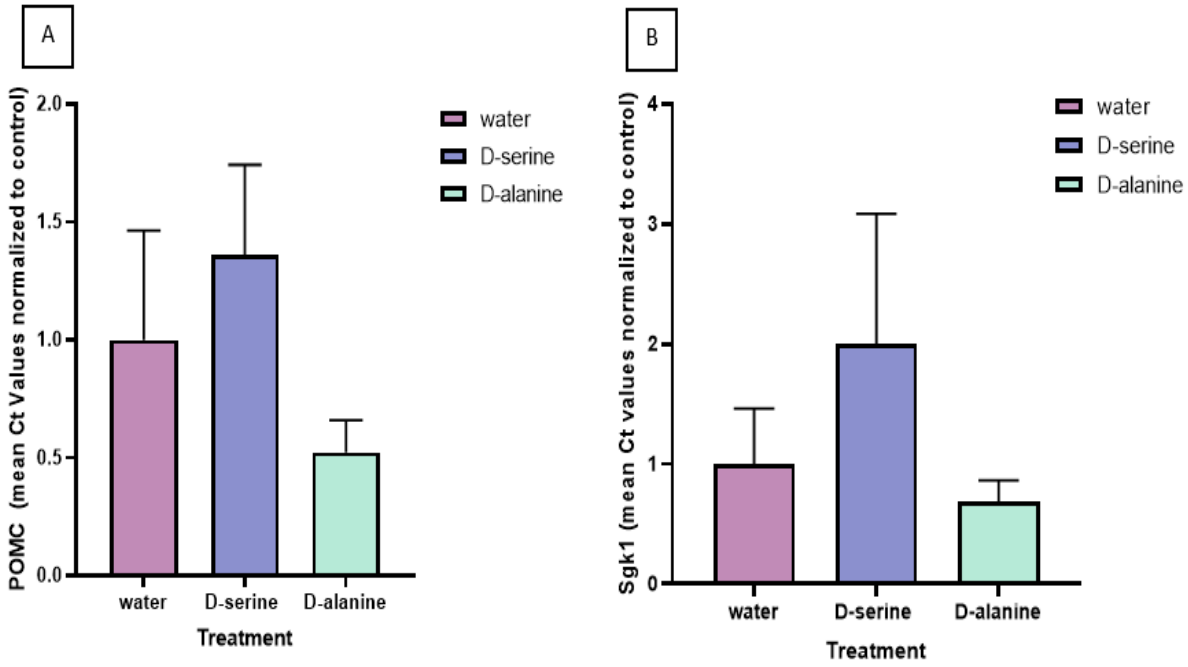


Figure 5.12 - POMC (A) and SGK1 (B) gene expression in the cerebellum of mice administered with D-amino acids (50mg/kg). The data are a mean of 8 replicates. There were no changes in either POMC or SGK1.

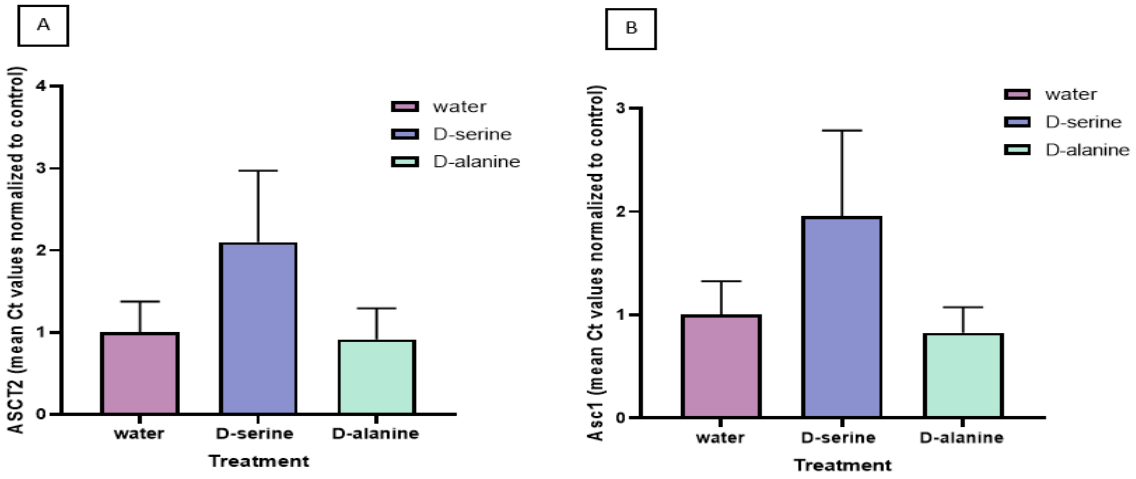


Figure 5.13 - ASCT2 mRNA (A) and Asc-1 mRNA (B) were detected in the cerebellum of these mice. CD1 mice were treated with D-alanine or D-serine (50mg/kg) for 7 days, with CD1 mice receiving water for the period time as a control. Samples are the mean of 8

replicates. There were no significant differences between the treatment groups in either amino acid transporter.

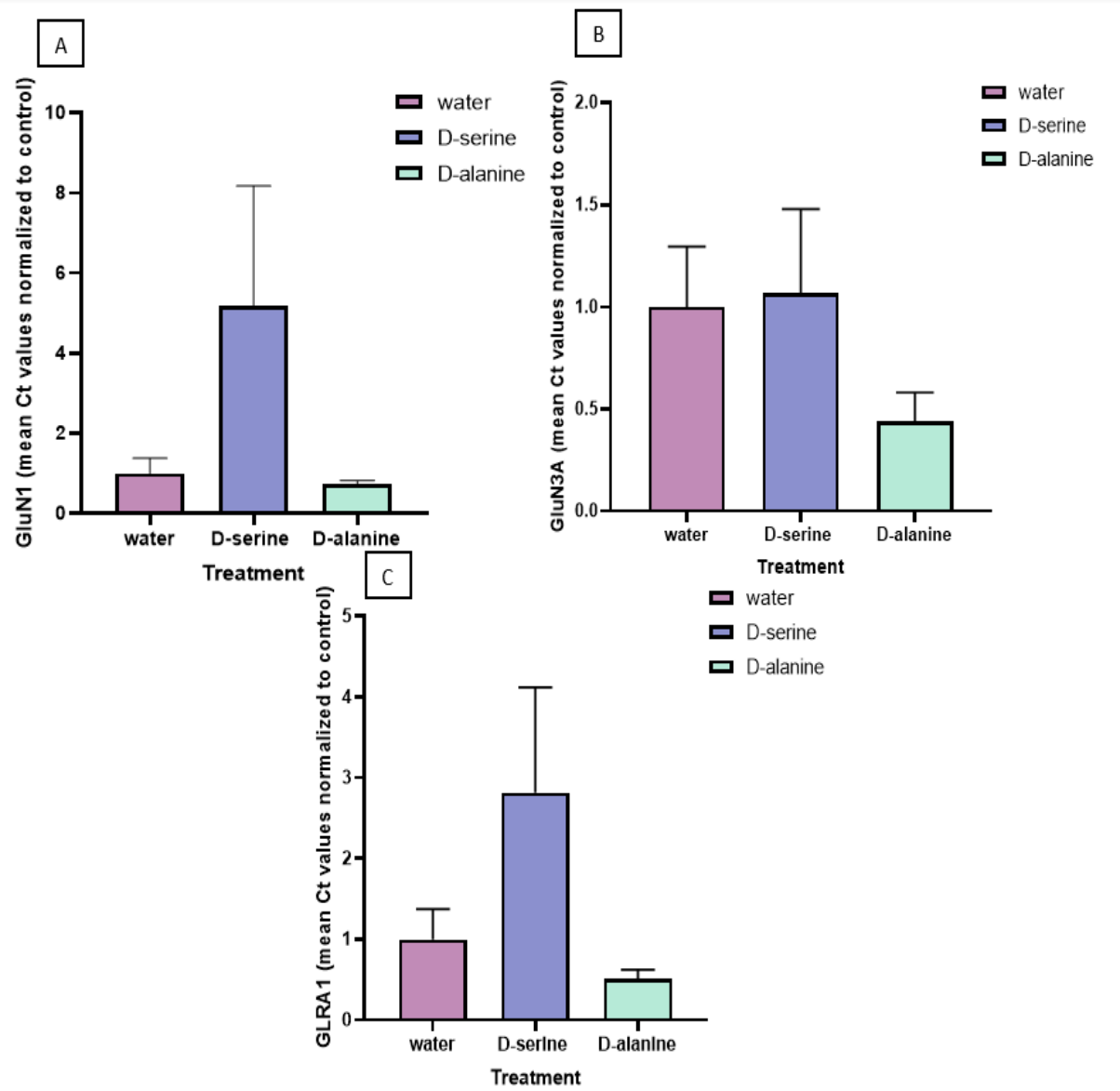


Figure 5.14 - GluN1 (A), GluN3A (B) and GLRA1 (C) mRNA abundance in the cerebellum of mice administered with D-amino acids (50mg/kg). Water was used as a control and samples were the mean of 8 replicates. There were no statistically significant changes in GluN1, GluN3A or GLRA1 levels in the cerebellum between any of the treatments.

The mRNA levels (POMC and SGK1 in **Figure 5.15** and amino acid transporters in **Figure 5.16)** in the hippocampus of rodents treated with D-amino acids were not changed. There were D-serine effects on GlyRs observed in the hippocampus (**Figure 5.17c,** $F_{2,20}$ = 5.55, p = 0.024). GLRA1 mRNA in mice treated with D-serine were higher in the hippocampus than that of control mice (p = 0.030, +365%). There was also a trending increase of GluN1 expression in mice administered D-serine compared to controls (**Figure 5.17a,** p = 0.062, +164%).

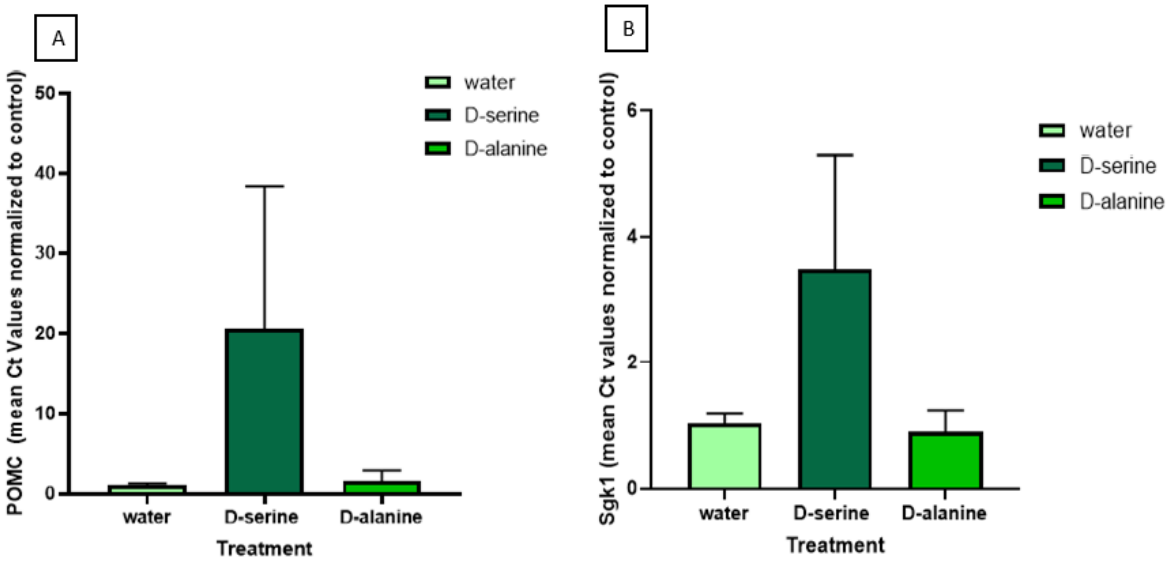


Figure 5.15 - mRNA levels of POMC (A) and SGK1 (B) were measured in the hippocampus using qPCR. CD1 mice were treated with D-alanine or D-serine (50mg/kg) for 7 days, with water as a control. The data are a mean of 8 replicates. There were no significant differences between treatment measures.

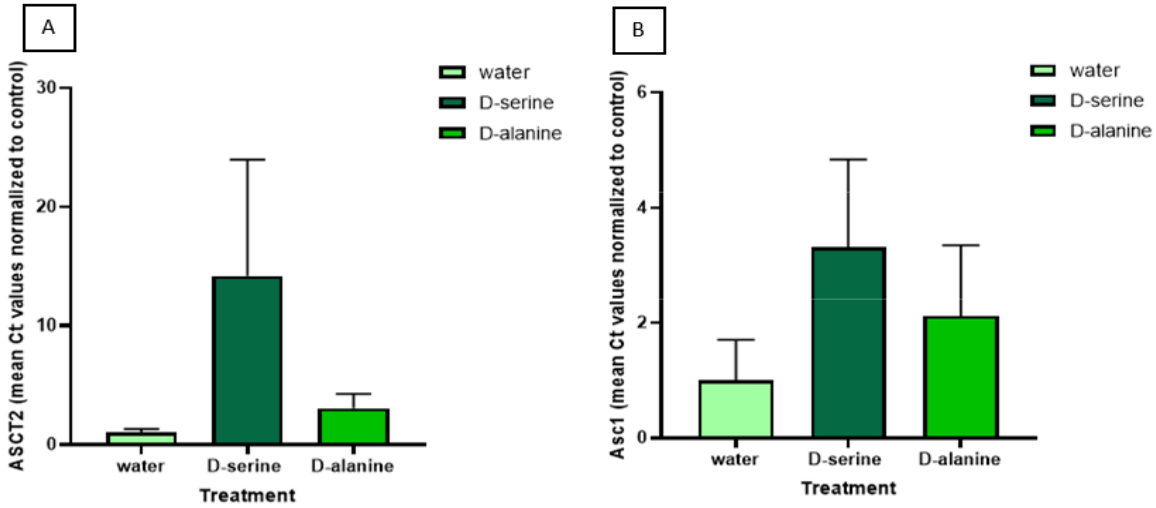


Figure 5.16 - mRNA levels of ASCT2 (A) and Asc-1 (B) were measured in the hippocampus using qPCR. CD1 mice were treated with D-alanine or D-serine (50mg/kg) for 7 days, with water as a control. The data are a mean of 8 replicates. There were significant differences between treatment measures of either amino acid transporter.

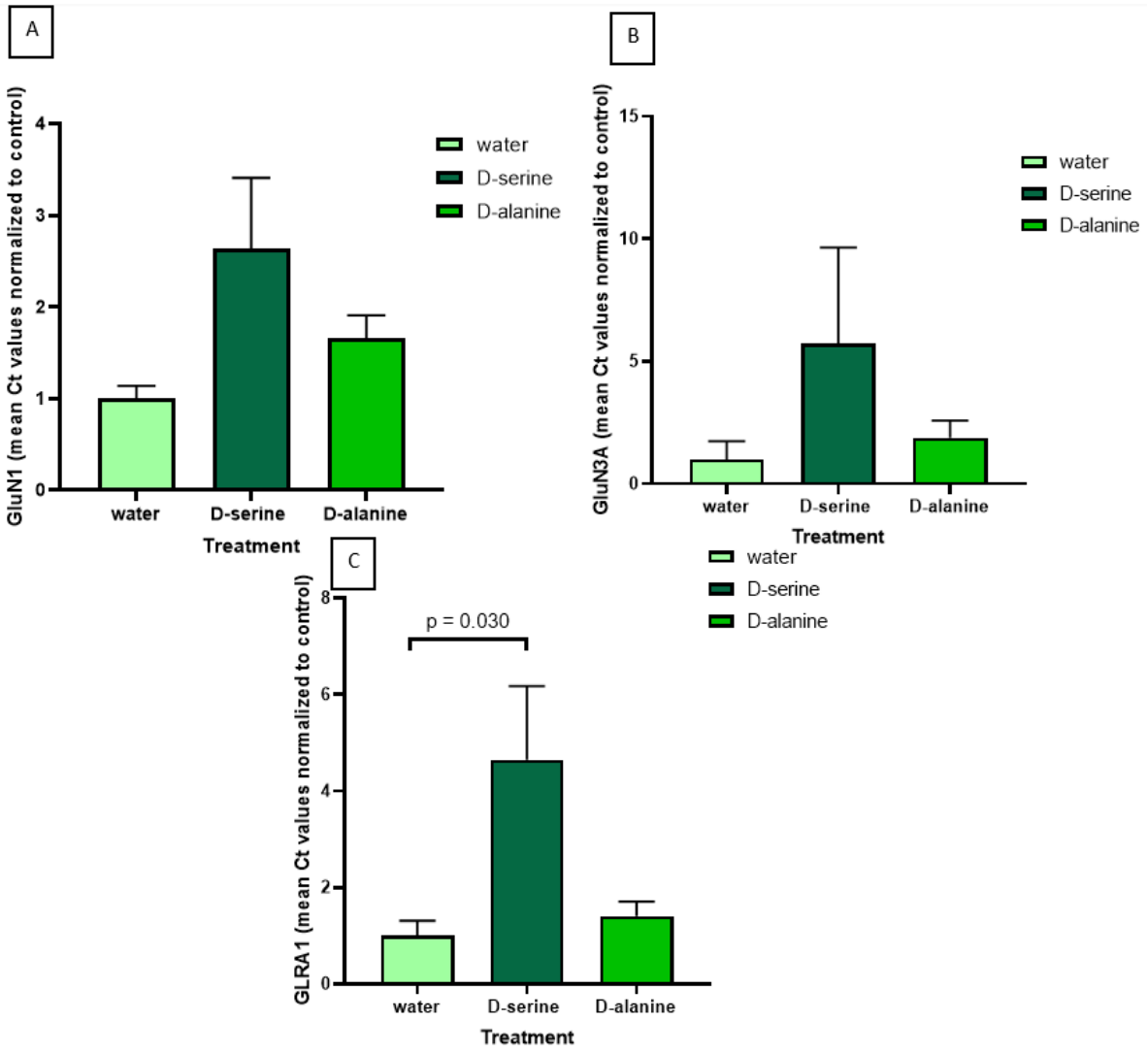
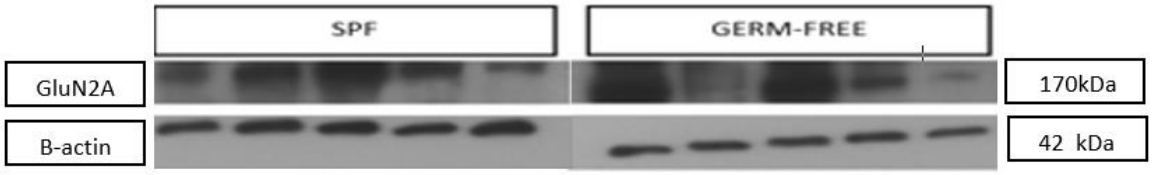


Figure 5.17 - Gene expression of receptors in the hippocampus of CD1 mice treated with D-alanine or D-serine (50mg/kg), with water as a control (n=8, mice treated for 7 days). Receptors of interest measured are GluN1 (A), GluN3A (B) and GLRA1 (C) in the hippocampus. There were no significant changes in mRNA levels of NMDAR receptor subunits, but there was a trending increase of GluN1 in mice treated with D-serine compared to control ($p = 0.062$, +164%). There was a significant increase in GLRA1 mRNA in mice treated with D-serine compared to control ($p = 0.030$, +365%).

5.3.3. GF study

As explained earlier in the chapter, GF mice have no microbiota, so are predicted to have no D-alanine in their systems, and potentially serves as a D-alanine knockout model, to determine if a lack of D-alanine has an effect on receptor, transporter, POMC and SGK1 levels in the mammalian cortex and brainstem.

Whilst there were no significant differences between the mice subtypes in terms of protein expression in the cortex (**Figure 5.18 – Figure 5.26)**, the presence of NMDAR subtype GluN3A (**Figure 5.25)** in cortical GF mice has yet to be published.



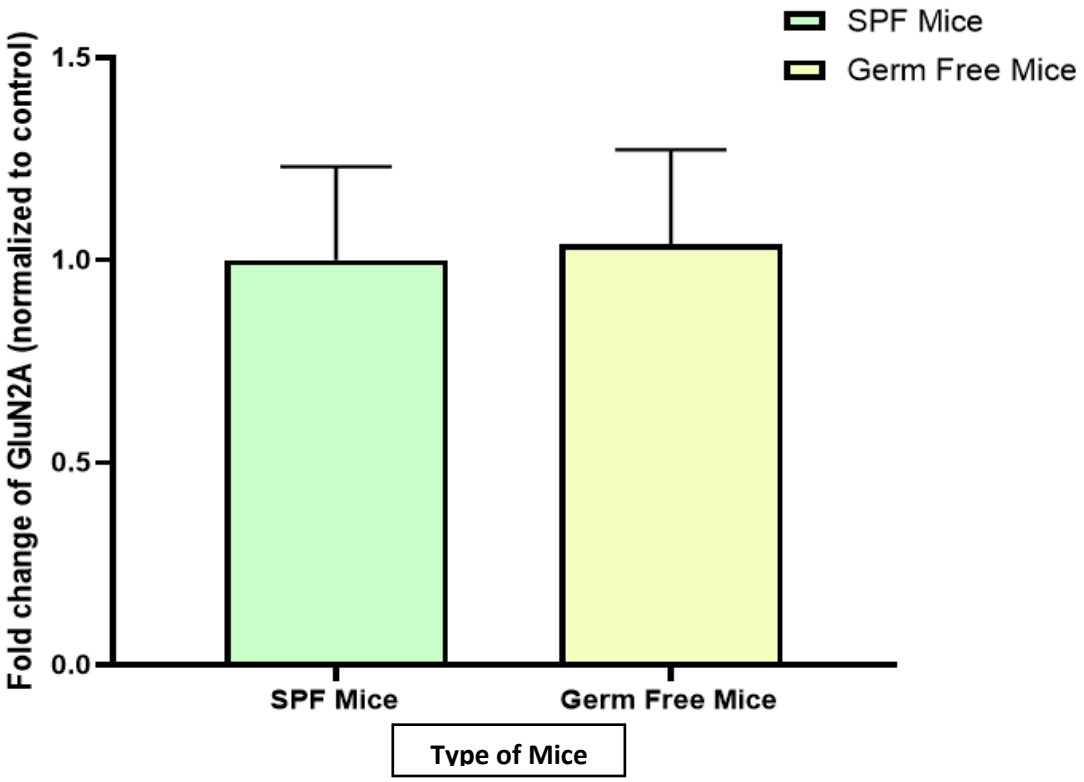


Figure 5.18. - Western blot of GluN2A levels present in the brainstem of GF mice. SPF mice were used as a control (n=5), with β-actin as the housekeeping protein for the NMDA receptor subtype GluN2A. There were no significant differences between receptor levels between the different mice types.

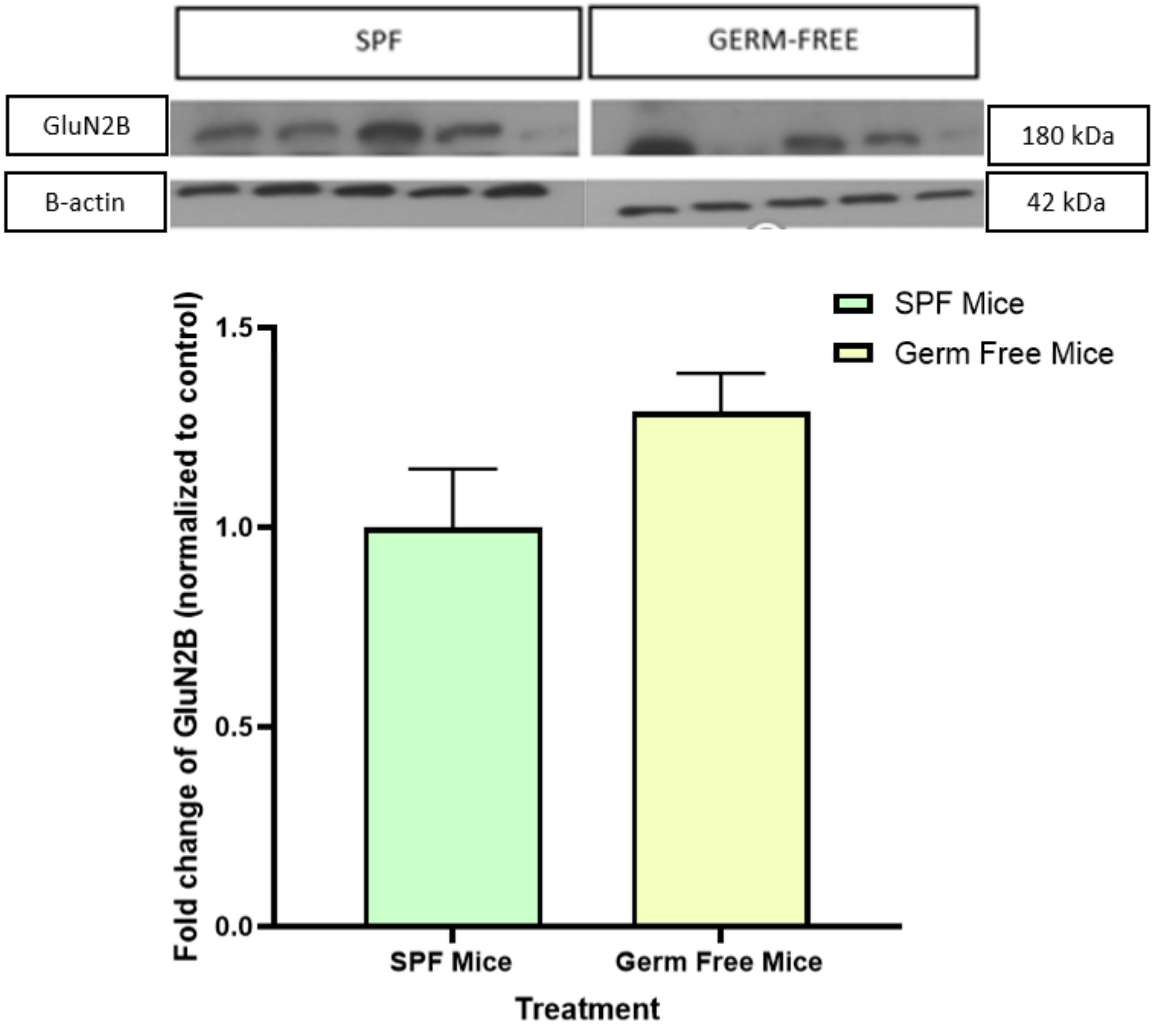


Figure 5.19 - The NMDA receptor subunit GluN2B protein levels were measured in the brainstem of mice (GF or SPF mice (control)). This was achieved by western blot analysis, with β-actin as a control, and n=5. There was no statistical difference between the SPF and GF mice GluN2B presence, and this was determined using an Independent's Samples T Test.

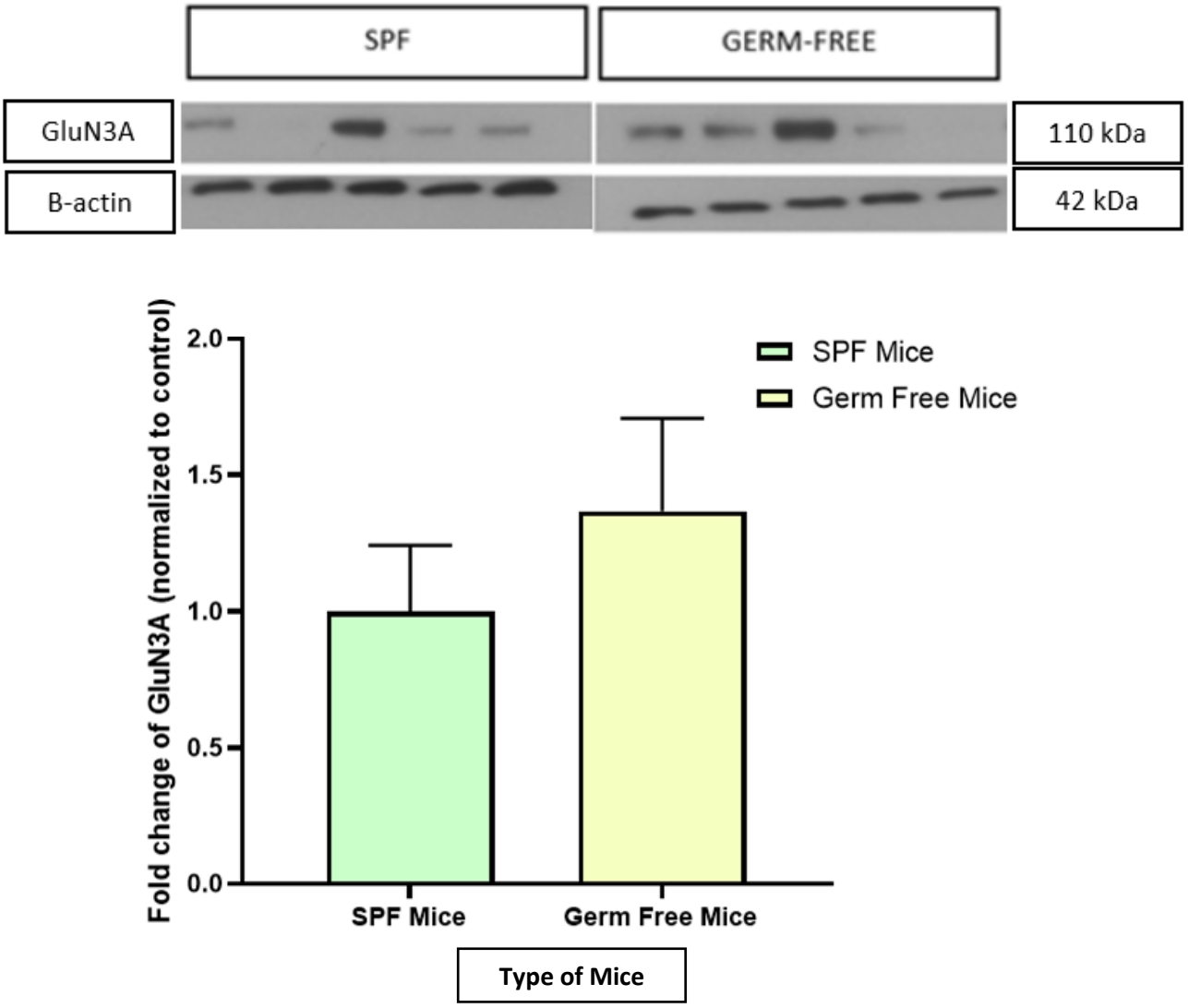


Figure 5.20 - GluN3A protein levels in SPF mice (control) and GF mice (n=5). As can be seen by the bands, the protein levels were measured by western blot analysis. The mice were x days old at culling. There were no significant differences between SPF mice and GF mice GluN3A levels in the brainstem.

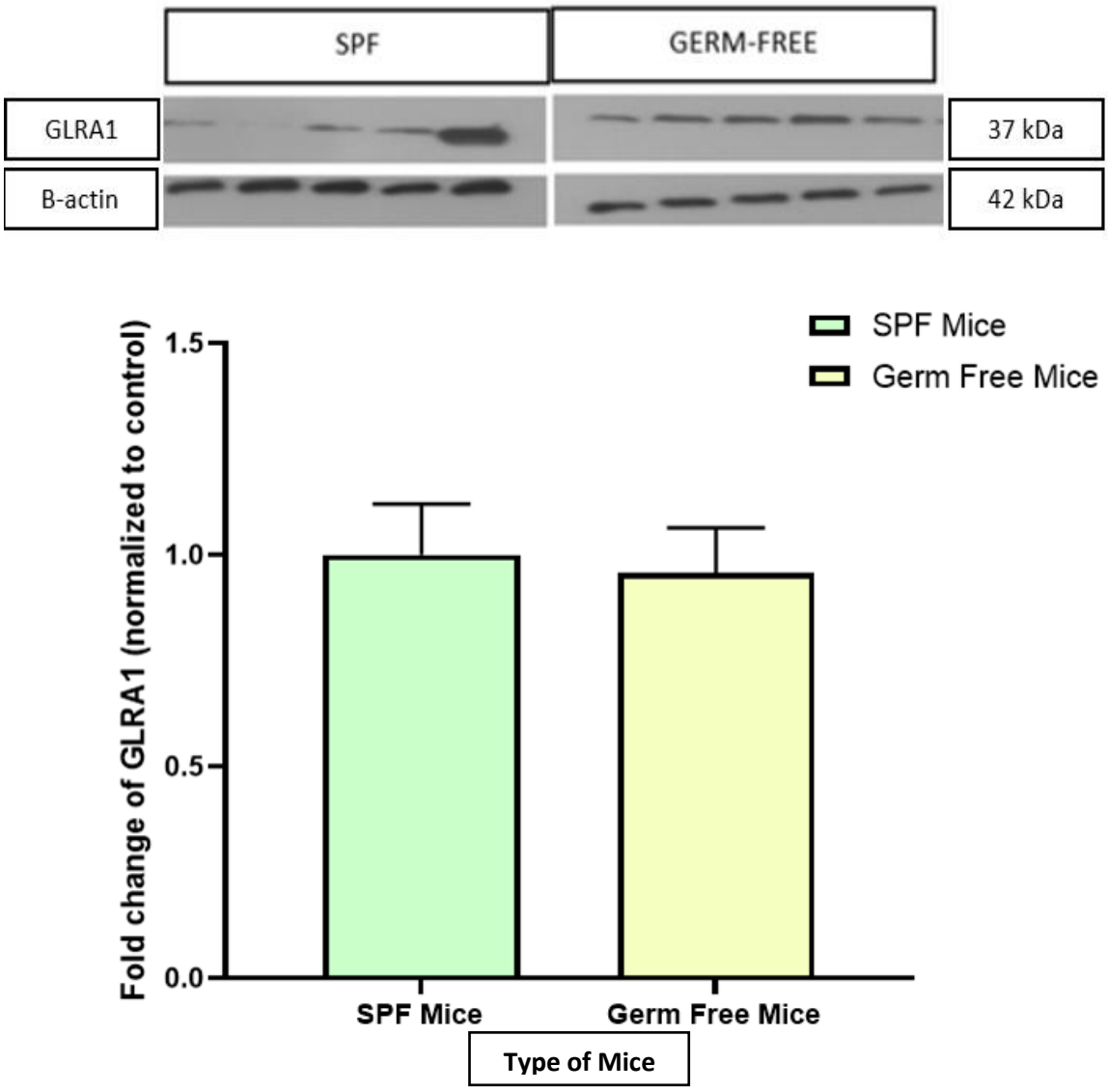


Figure 5.21 - The glycine receptor GLRA1 was measured in the brainstem of SPF mice (control) and GF mice using western blot analysis. There were 5 replicates of each type of mice. There were no significant alterations in GLRA1 detected by protein analysis.

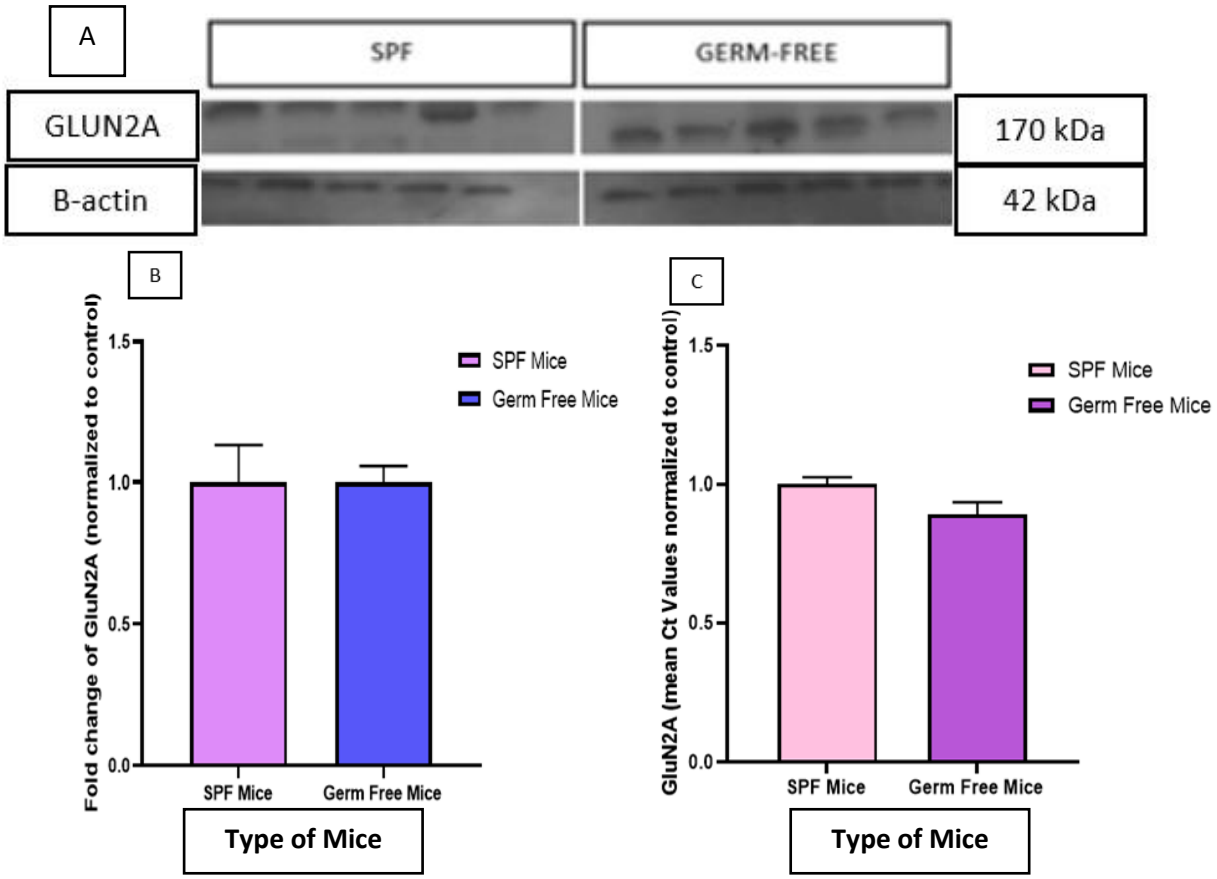


Figure 5.22 - The NMDA subtype (GluN2A) was measured in the cortex of GF mice and SPF mice (fed just water and used as a control) by both western blot analysis (A & B) and qPCR analysis for gene expression (C). There were 5 replicates of each type of mice.

The mRNA levels of GluN3A (**Figure 5.25)**, POMC (**Figure 5.28a)** and SGK1 (**Figure 5.28b)** were altered in the cortex. There was a significant difference in GluN3A gene expression, where there was a significantly higher level of GluN3A in GF mice compared to SPF mice ($p = 0.016$, + 43%).

There was a statistical significance in both POMC and SGK1 in the cortex (**Figure 5.28)**. There was a statistically higher level of cortical POMC in GF mice compared to SPF mice control ($p = 0.030$, +120%). There was also a significantly higher level of cortical SGK1 gene expression in GF mice compared to SPF control ($p = 0.004$, +57%).

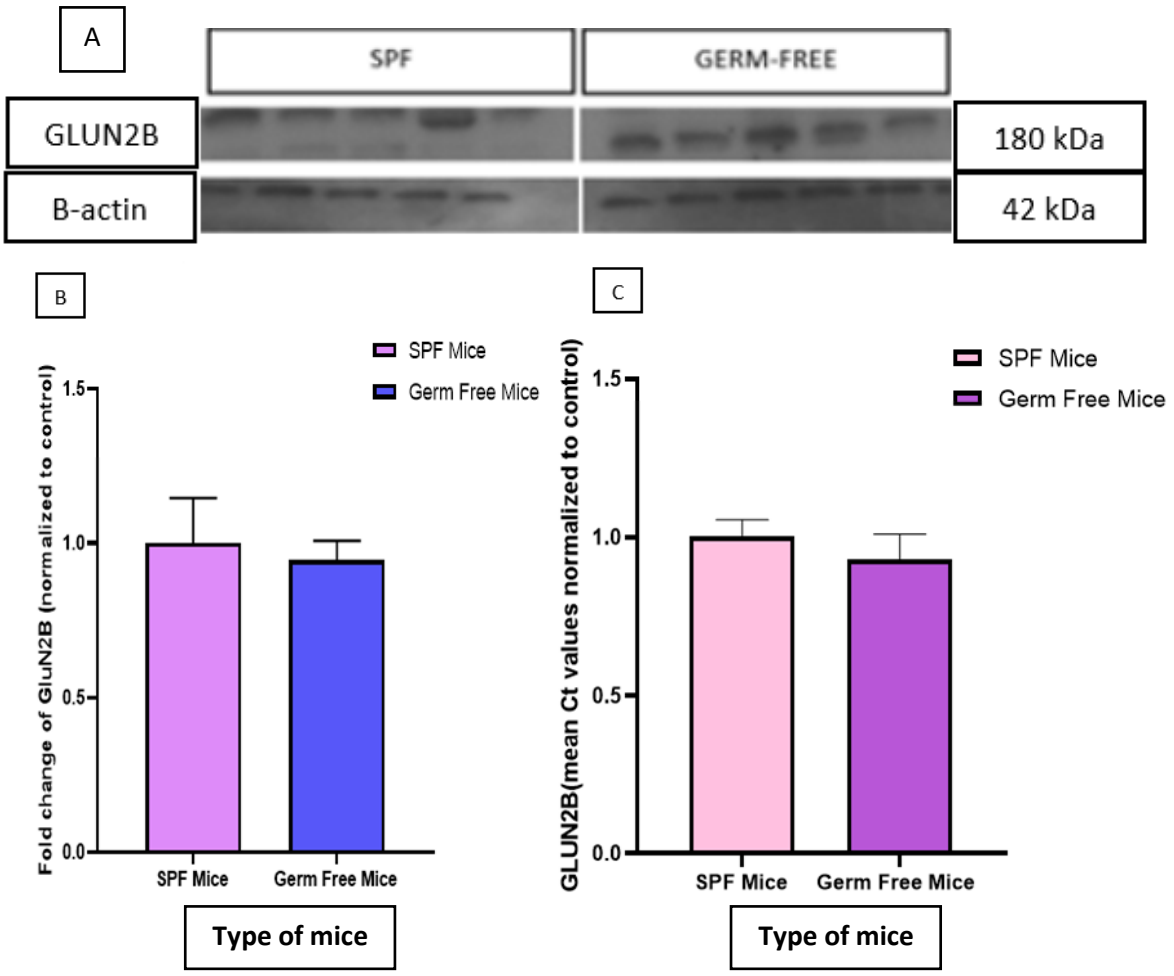


Figure 5.23 - Western blot analysis (A & B), as well as gene expression analysis (C) for the subtype of the NMDA receptor GluN2B. There were no significant differences between the two types of mice.

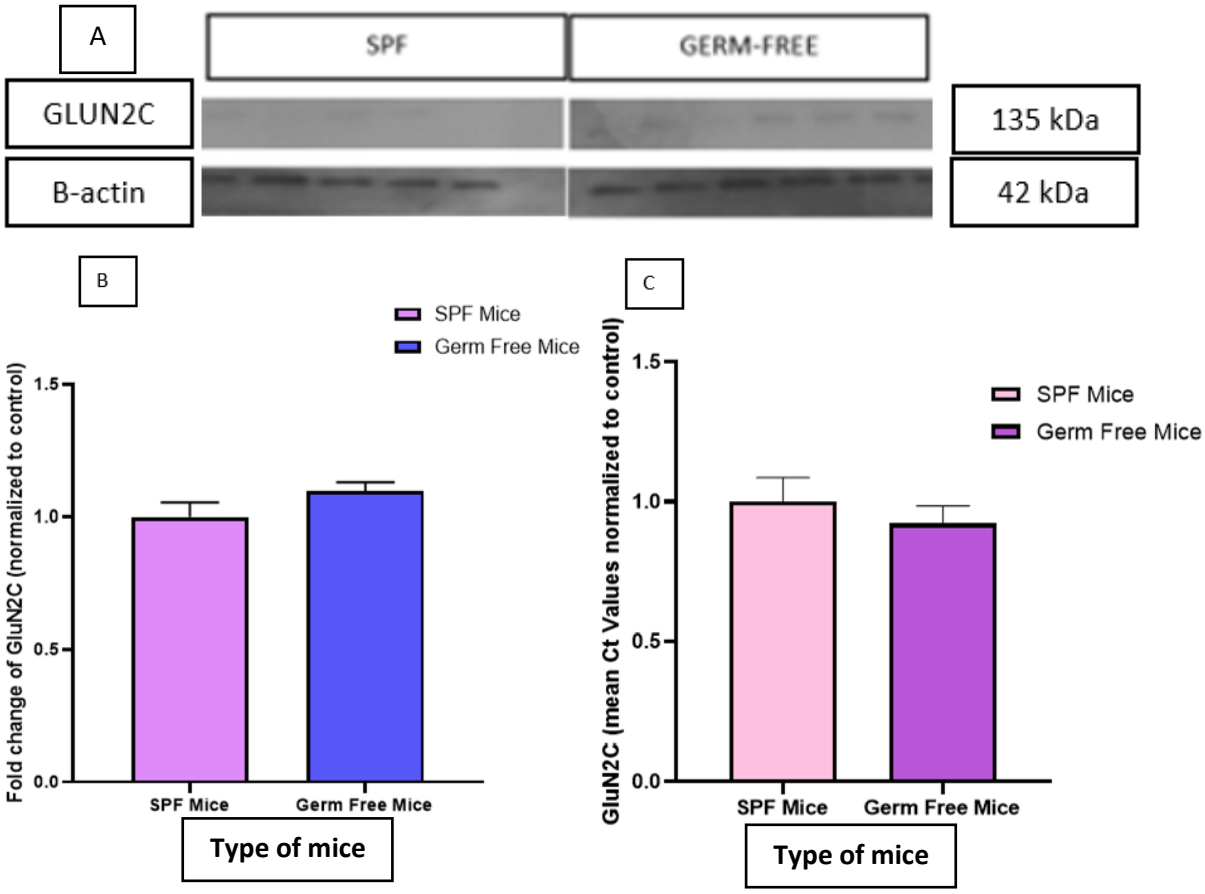


Figure 5.24 - The above graph depicts GluN2C levels in the cortex of mice (Germ-free and SPF). SPF was used as the control, and data are the mean of 5 replicates. GluN2C levels were measured for protein (A & B) and gene expression (C). There were no significant differences between the two mice types.

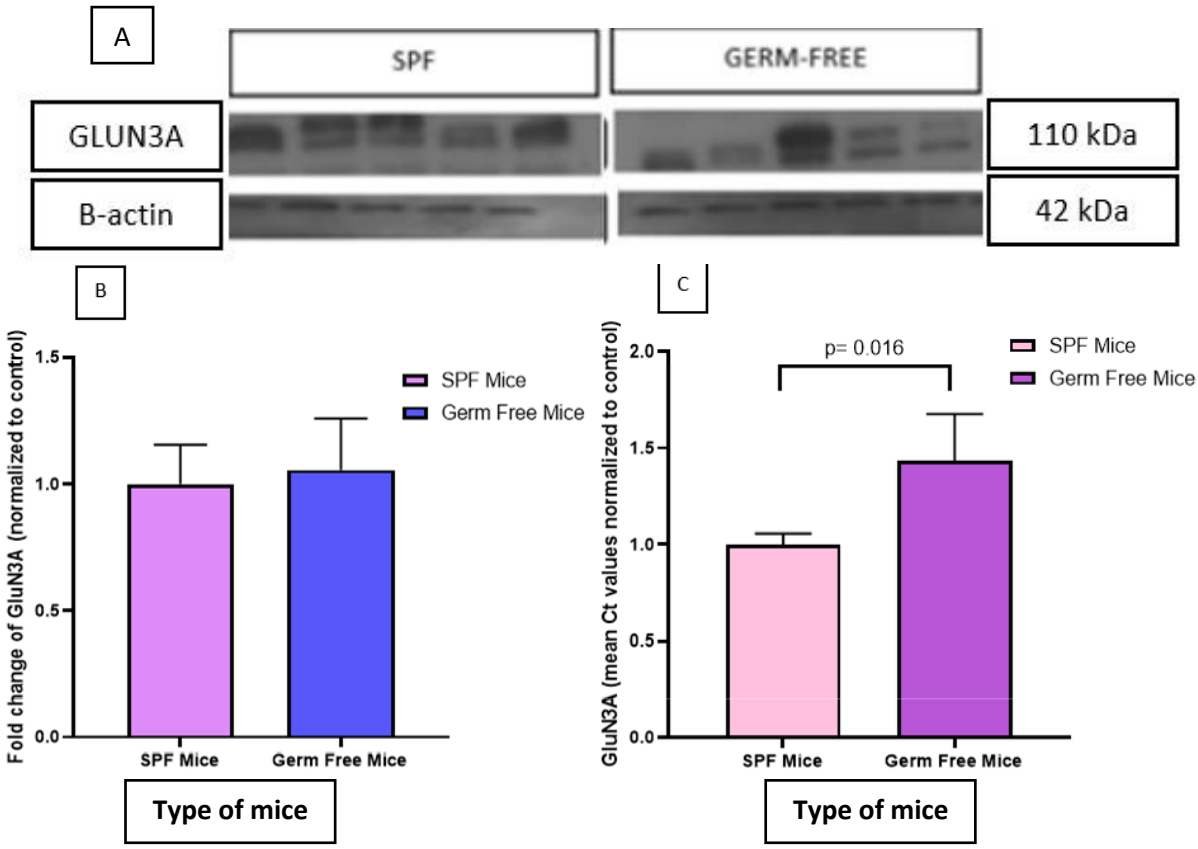


Figure 5.25 - Western blot analysis (A & B), and mRNA analysis (C) for the subtype of the NMDA receptor GluN3A. (n=5). There were no significant differences between the two types, with SPF mice used as a control, in the protein expression levels, but there was a significant difference in GluN3A gene expression, where there was a significantly higher level of GluN3A in GF mice compared to SPF mice ($p = 0.016$, + 43%).

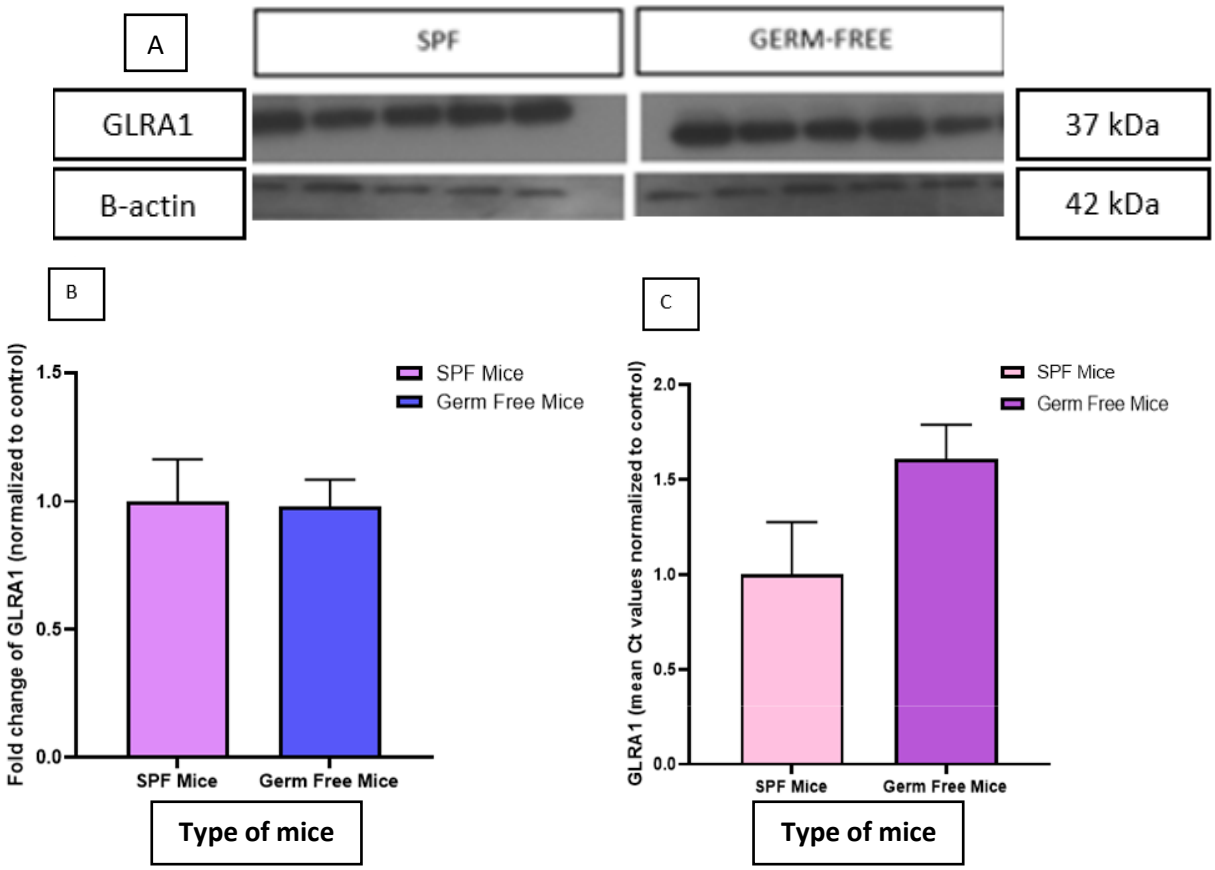


Figure 5.26 - GLRA1 levels in the cortex of mice (GF and SPF). SPF was used as the control, and data are the mean of 5 replicates. GLRA1 levels were measured for protein (A & B) and gene expression (C). There were no significant differences between the two mice types.

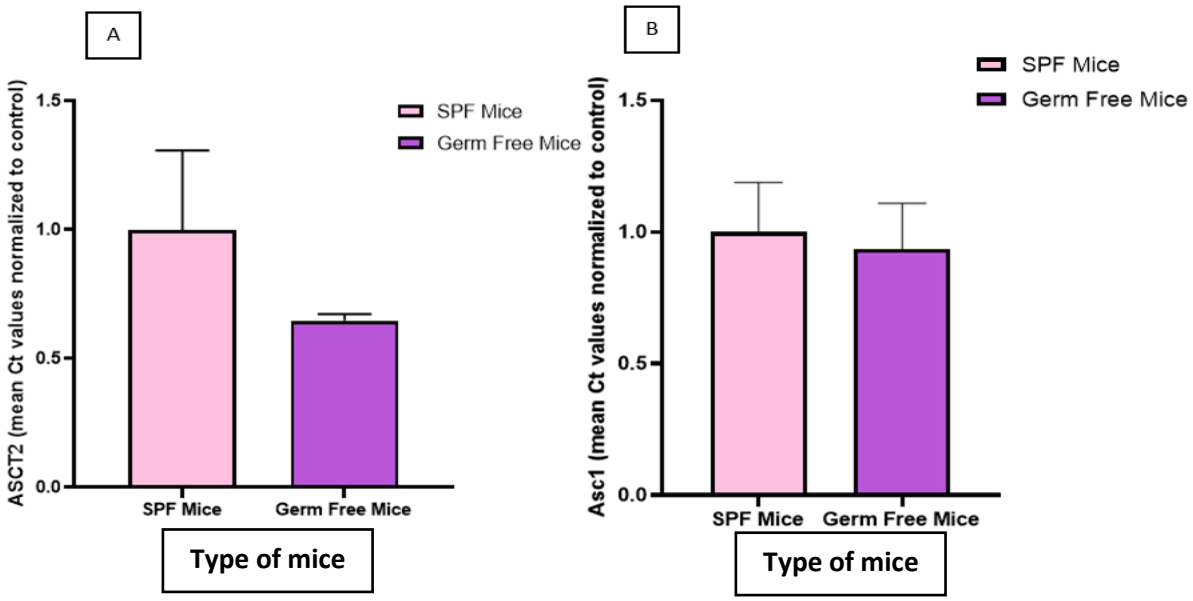


Figure 5.27 - The mRNA levels of D-amino acids transporters (ASCT2 and Asc-1) in the cortex of GF mice and SPF mice (control). Samples are a mean of 5 replicates. There were no statistical significances between the two types.

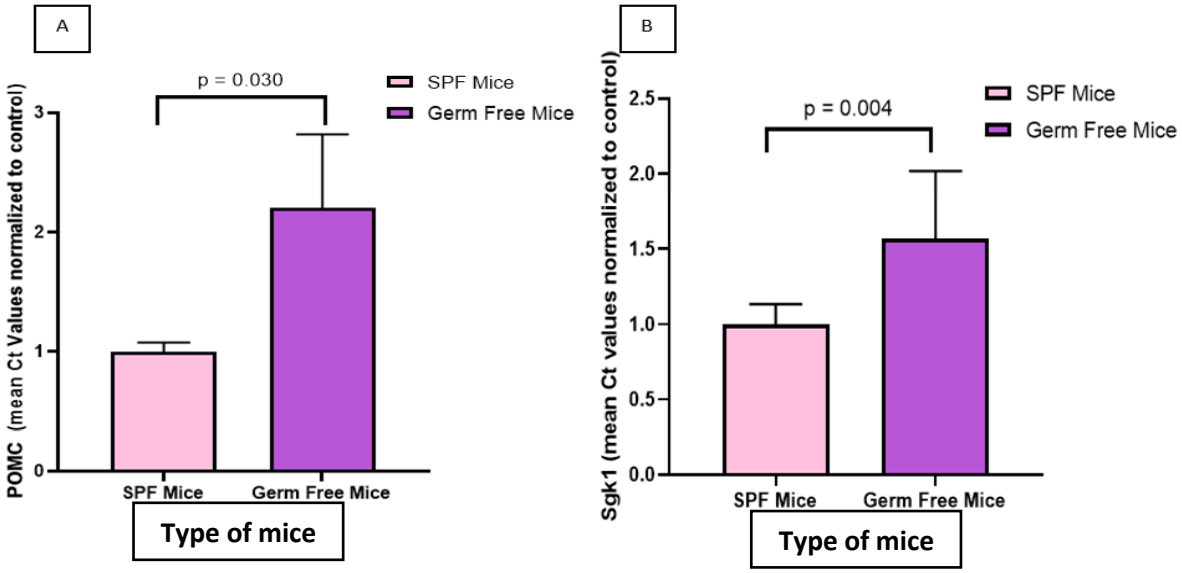


Figure 5.28 - POMC (A) and SGK1 (B) mRNA levels in the cortex of mice (GF and SPF). SPF was used as the control, and data are the mean of 5 replicates. There was a statistical significance in both POMC and SGK1 in this brain region. There was a statistically higher level of cortical POMC in GF mice compared to SPF mice control (p = 0.030, +120%).

There was also a significantly higher level of cortical SGK1 gene expression in GF mice compared to SPF control ($p = 0.004$, +57%).

5.4. Discussion

5.4.1 Decreased corticosterone levels in CD1 mice treated with D-serine and D-alanine

The inhibitory effect of D-amino acids on corticosterone supports the data in Chapter 2, where D-amino acids had an inhibitory effect on another stress hormone, ACTH, in AtT20 cells. The inhibitory effect of D-alanine was likely due to the increased storage of the D-amino acid in the anterior pituitary that resulted in a more robust inhibition of ACTH (as we observed in AtT20 cells in Chapter 2), which then had downstream effects on corticosterone levels. This is interesting, as this is contradictory to what is expected from feedback inhibition. A decrease in ACTH should signal the HPA axis to increase ACTH secretion, which would eventually lead to an increase in corticosterone levels. However, because of the possible persistent presence of inhibitory D-amino acids, a sustained inhibition is achieved in ACTH, which is being maintained through the HPA axis hormones. It would be intriguing to detect D-alanine in AtT20 cells 24hrs following treatment, and determine if the increased presence of this D-amino acid is still there 24 hrs later, and supports the theory that amino acid transporters Asc-1 and ASCT2 are involved, as D-alanine uptake is their responsibility.

Unfortunately, the plasma concentrations of ACTH in CD1 mice treated with D-amino acids could not be accurately determined using the ACTH kit procured, where plasma readings were too varied (following dilution) to be considered reliable. Sudo et al (2004) showed an increase in plasma ACTH and corticosterone in GF mice following restraint stress, however this mimics the activated HPA axis system, whilst my study involved GF mice at basal levels. It would be of interest to measure the plasma corticosterone levels of

GF mice, as they lack D-alanine, to confirm the finding of D-alanine driven inhibition of basal ACTH levels in AtT20 cells (**Figure 2.8, Chapter 2)**. GF mice are therefore predicted to present with higher plasma corticosterone levels in response to lack of D-alanine, even at basal levels.

The reduction of corticosterone by both D-serine and D-alanine, just like the reduction in ACTH by both these D-amino acids in AtT20 cells, implies that a system that influences both these D-amino acids are implicated in their regulation of hormones, which is why the receptors they bind to and transporters that take them up were investigated further. The SGK1 and POMC system was also investigated, as POMC is a precursor for ACTH, and ACTH influences the release of corticosterone, so regulation of these hormones may occur at the POMC level.

The effects that D-alanine exert on ACTH are not as surprising as it is co-localized to the same cells, but the D-serine effects are harder to explain. D-serine is found in the rodent pituitary gland, but at lower concentrations than D-alanine (24.7 ± 3.3 nmol/mL vs 69.9 ± 11.3 nmol/mL) (Karakawa et al., 2015). Capitão et al (2020) found that following 4 hr treatment with D-serine in a clinical study, individuals showed a trending increase in salivary cortisol when compared to D-alanine, but not the control ($p = 0.052$). This shows that D-serine interacts with the HPA axis, even if it may not be as well localized as D-alanine to do so. Additionally, in Chapter 2, D-serine, like D-alanine, stimulated SGK1 in AtT20 cells, which regulates POMC. Like D-alanine, D-serine is also transported by Asc-1, which I've shown to have an inhibitory effect on POMC and ACTH secretion in Chapter 3**.** Therefore it is likely via Asc-1 and SGK1-POMC system, that both D-alanine and D-serine interact with, that results in reduced plasma corticosterone levels.

5.4.2 Suppression of GLRA1 by D-alanine in the pituitary of CD1 mice

The observed reduction of GLRA1 mRNA by D-alanine, is consistent with the down regulation of a receptor following over-exposure to its agonists i.e. a homeostatic response. Pituicytes in the neural lobe of the pituitary are rich in the amino acid taurine, which is also a full agonist of the glycine receptor (Song and Hatton, 2003). The earlier finding that taurine reduced GLRA1 expression in the anterior pituitary AtT20 (**Figure 2.15b)** cells is supported by the similar effects D-alanine had on this receptor *in vivo*, however it is in contrast to the 'stimulatory' effect D-alanine had on glycine receptor in AtT20 cells (**Figure 2.8b)**. This may be due to D-alanine having to compete with Taurine to bind with GlyRs in the whole pituitary gland, rather the isolated *in vitro* anterior pituitary system, so that the stimulatory effect observed *in vitro* is not translatable due to the potential presence of Taurine in the pituitary exerting it's inhibitory effect on GlyRs.

5.4.3. Effect of D-serine on glycine receptor in the hippocampus of CD1 mice

D-serine is abundant in the hippocampus (Schell et al., 1995) and is proven to be the main co-agonist of hippocampal NMDA receptors (Duffy et al., 2008). D-serine is not known to bind to the strychnine sensitive GLRA1, but the presence of D-serine increases GLRA1 in the murine hippocampus (**Figure 5.17c)** when compared to saline control. This increase of GLRA1 is unlikely due to direct action on the receptor, as it's known agonist D-alanine had no effect on GLRA1 in the hippocampus, so this increase of GLRA1 may be compensatory to an increase of excitatory GluN2 subunits of NMDARs that D-serine binds to. The increase in excitatory receptors stimulates a compensatory feedback of increase in inhibitory GLRA1 in the hippocampus, and this could be shown by studying the functionality of receptors in the hippocampus, and utilizing NMDAR antagonists like MK801 to see if the D-serine induced increase of GLRA1 persists in the hippocampus when NMDARs are inhibited.

5.4.4 GF mice as a 'D-alanine knockout' model

Karakawa et al (2013) investigated the presence of D-alanine in germ free mice and found its presence to be in very low, negligible quantities, indicating that it may serve as a good model of 'D-alanine knock-out'. In my study, the lack of pituitary gland and plasma make it difficult to prove the effectiveness of D-alanine knock-out, as the effects in the cortex and brainstem cannot be compared to the effects in anterior pituitary AtT20 cells, but there is no reason to doubt the robust findings by Karakawa et al (2013) at present.

Whilst GF mice are an excellent model to investigate microbe-microbe and microbe-host interaction in health and disease, there are a few limitations. Studies have shown that GF mice have biochemical and physiological abnormalities which result in an altered immune system (Mazmanian et al., 2005), mild chronic diarrhoea, disrupted metabolism and reduced reproductive abilities (Martínez-Augustin et al., 2009). These may evoke concern, but these alterations are likely a response that is necessary for these mice to physiologically survive the GF state. These physiological parameters are altered when GF mice are introduced to microbial communities, to the point they then resemble conventionally raised mice. This serves to support that they are a good model system to investigate the effects of microbes on host development and function. Human babies start in a GF state and obtain a microbiota just before, or at birth, from their environment. This resembles what happens when GF mice are introduced to microbial communities, and therefore, whilst they are concerns, like they are with any animal model, gnotobiotic mice are a crucial tool in understanding host-microbe interactions in health and disease, as well as a preclinical model to test microbiota directed therapies (Martínez-Augustin et al., 2009).

5.4.5. Lack of D-alanine did not change NMDA and glycine receptor expression in the brainstem

GLRA1 and GluN3A are strongly expressed in the brainstem (Wong et al, 2002; Piechotta et al., 2001; Schmid et al., 1991). GF mice have been reported to present with altered NMDAR expression, particularly decreased GluN2B levels in the amygdala compared to SPF mice (Neufeld et al., 2010). Sudo et al (2004) showed a reduction in GluN2A and GluN1 in the cortex, and reduction of GluN2A in the hippocampus of GF mice exposed to restraint stress when compared to SPF mice. Kawase et al., (2017) reported a higher level of D-serine in the plasma of GF mice compared to SPF mice, implying that the gut microbiota may have an inhibitory effect on endogenous D-serine, whilst they also found that the brainstem of GF mice had higher L-serine levels compared to brainstem of SPF mice. Changes in NMDARs in the brainstem of GF mice have yet to be reported, and perhaps the increase presence of L-serine in the brainstem, which is a source of D-serine, combats the lack of D-alanine derived from the gut microbiota, and these balance each other out so that NMDARs expression is maintained in the brainstem of GF mice at basal levels.

5.4.6 Increased expression of GluN3A, POMC and Sgk1 in the cortex of GF mice

The GluN3A subunit is highly expressed in the fifth lamina of the cerebral cortex (Wong et al., 2002), however its expression levels in the cortex of GF mice has not been reported. D-serine levels are higher in the cortex of GF mice than SPF mice (Kawase et al., 2017). When expressed along with GluN1, D-serine inhibits GluN3A (Chatterton et al., 2012). Perhaps the combination of a lack of D-alanine agonist, and the increased presence of D-serine which can inhibit this NMDAR subtype, results in a compensatory upregulation of GluN3A.

Whilst GluN3A mRNA levels increase in the cortex of GF mice compared to SPF controls (**Figure 5.25c)**, there were no detected protein changes (**Figure 5.25b).** Perhaps this is due to the D-serine inhibition of GluN3A receptors, the cortex is compensating by upregulating transcription of GluN3A, but D-serine is acting on the receptor by interfering with the translation process so there is no change in the protein levels of GluN3A.

The higher levels of cortical POMC mRNA in GF animals works with the proposed hypothesis that D-alanine inhibits ACTH, possibly via SGK1 and POMC regulation. Additionally, as mentioned above, there are high levels of D-serine in the cortex of GF mice, and D-serine is shown to stimulate SGK1 **(Figure 2.11a).** Reiter at al (2011) has reported that SGK1 may have stimulatory effects on POMC, and perhaps this is also what is contributing to the overall increase in POMC and SGK1 mRNA expression.

5.4.7 Comparisons between relevant brain regions of CD1 mice treated with D-amino acids and GF mice

The cortex was investigated in both CD1 mice treated with D-amino acids, as well as GF mice, and it was found that there were only notable gene expression changes in GF rodents, however based on the findings by Kawase et al (2017) GF animals may have higher levels of D-serine, therefore it is difficult to deduce whether observed changes are due to increase in D-serine despite a lack of D-alanine, as I have found both these D-amino acids to be important to the HPA axis.

5.4.8 Further studies and Limitations

The greatest limitation for this chapter was the lack of pituitaries and plasma from GF mice as this would have been ideal to validate D-amino acid effects observed in AtT20 cells in Chapter 2 and Chapter 3. The plasma of GF mice would have complemented the plasma of D-amino acid fed CD1 mice plasma with an increase in corticosterone levels

(due to the lack of D-alanine), seeing as D-amino acids caused an increase in corticosterone levels in mice.

Similarly, due to the COVID-19 pandemic gut tissue was not analysed, and it would have been an interesting counterpart to the *in vitro* data in Chapter 4. It would be interesting to confirm that D-serine exerts the pro-inflammatory effects on the gut *in vivo* and whether D-alanine remains to have no effect on inflammation in the gut.

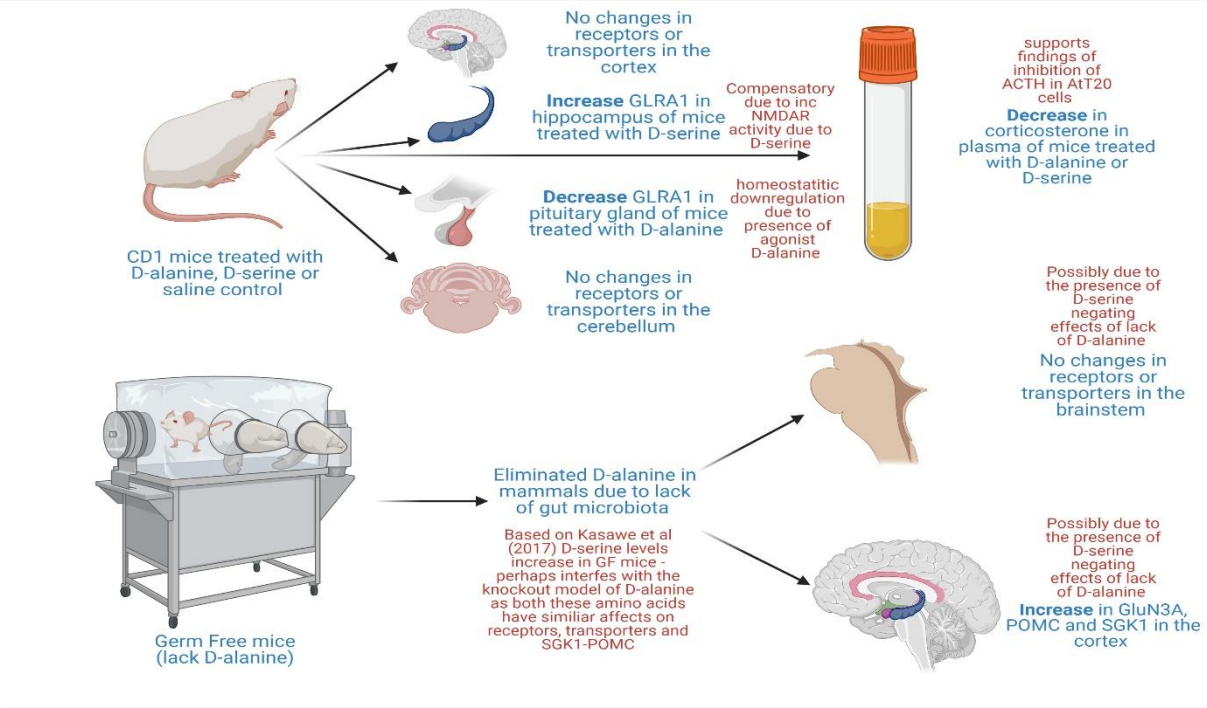


Figure 5.29 - Summary of *in vivo* findings reported in this chapter. Made with Biorender.com

Perhaps a study inhibiting D-serine in the GF mice by inhibiting serine racemase may assist in elucidating the effect a lack of D-alanine and D-serine may have on the mammalian brain, as it seems that these two D-amino acids act on similar systems and allow for compensatory increases in one when there is a decrease in the other.

CHAPTER SIX: DISCUSSION

6.1. D-alanine – an inhibitor of ACTH secretion?

A core finding of this thesis was the inhibition of unbound ACTH by both D-alanine and D-serine, as shown in **Figure 2.7**. This finding occurred after extensive optimization of treatment protocols on AtT20 cells, where I found that the levels of ACTH secretion were dependent on the passage number of cells, with younger passages (earlier than passage 7) secreting high levels of ACTH that were out of the sensitivity range of the ACTH kit **(**Appendix**)**. Following this clarification, in order to attain consistency between experiments, AtT20 cells were only used for treatment protocols when they were passage 7 and older, but not older than passage 15 as cells looked morphologically different under the microscope at this later stage. Various *in vitro* studies show the importance of passage number (Briske – Anderson et al., 1997, Chang – Liu and Woloschak, 1997, Yu et al., 1997). A paper by O'Driscoll et al (2006) observed differences in MIN6 cells between low passage (18) and high passage (40) cells, where they found 1000 genes to be differently expressed depending on whether they were at a low passage state, or at high passage state, which is greatly affected many aspects of the cell's physiology, resulting in alteration in secretory products. I hypothesized that this passage dependent effect observed in AtT20 cells may be due to immature receptor maturation, which are responsible for regulating many processes in the cells, especially ACTH secretion, which is notably high in these early passages, and lower in later passages, implying regulatory mechanisms are developed later on. This could be determined by measuring the electrical activity of various receptors involved in ACTH secretion (MC2R, NMDARs etc.) between younger passage AtT20 cells and older passage AtT20 cells and determining whether receptors are not as active in the younger passage cells.

The experiments that provided the data for **Figure 2.7**, where D-amino acids were administered to AtT20 cells for 24 hours, had a sample size of 12, which was twice as many as the rest of the experiments. This was because I ran replicates of the experiment to confirm the findings which became the basis of follow-up experiments. However, the treatment criteria of 50 000 AtT20 cells treated with D-alanine at 500 µM was repeated 3 times in the following experiments:

1. Later in Chapter 2 when I investigated the effects of CRH on these cells in **Figure 2.12**.
2. When I investigated the effects of the glycine receptor (GlyR) antagonist strychnine in **Figure 2.18**.
3. Lastly in Chapter 3 when investigating the effects of overexpressing the Asc-1 transporter in AtT20 cells in **Figure 3.8**.

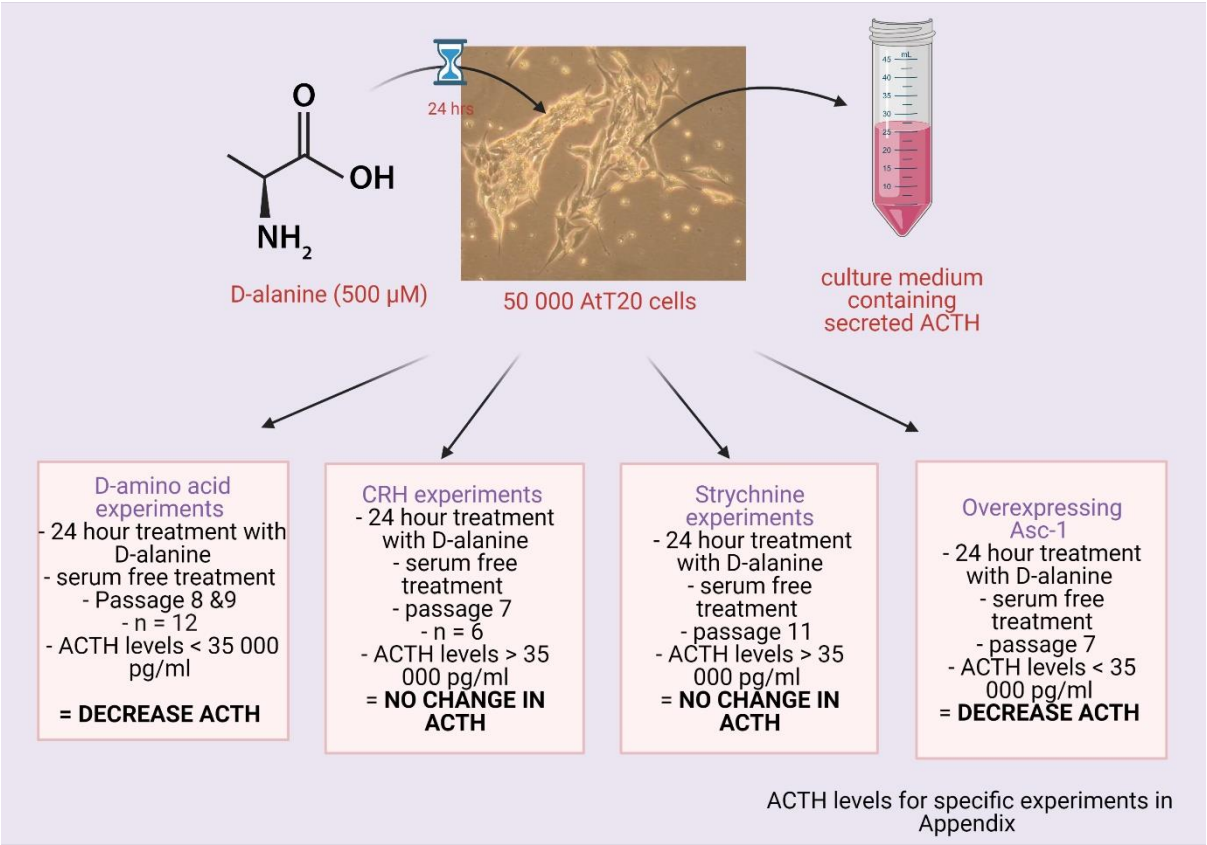


Figure 6.1 - Summary of experiments that involved D-alanine treated to ACTH cells at the same treatment measures. Diagram made with app.Biorender.com

Was the finding of the inhibitory effect on ACTH by D-alanine consistent throughout these experiments? As seen in the summary in **Figure 6.1** above, it was not. I found a significant reduction of unbound ACTH in cells treated with D-alanine in D-amino acid experiments **(Figure 2.7**) and overexpression with Asc-1 experiments **(Figure 3.8**), but there were no changes in unbound ACTH levels in the CRH experiments (**Figures 2.12**) and the strychnine experiments (**Figure 2.18**). This was interesting as the initial finding in **Figure 2.7** of a decrease in ACTH when cells were treated with 500 µM was robust with a sample size of 12. Additionally, in **Figure 3.8**, when AtT20 cells were transfected with transporter Asc-1, there was further reduction in ACTH in cells overexpressing Asc-1 and treated with D-

alanine compared to both control and control cells overexpressed with Asc-1, implying an increase in D-alanine uptake may be responsible for this ACTH inhibition. However the failure to replicate findings in the CRH and Strychnine experiments cannot be ignored.

It cannot be assumed that this is due to low passage numbers as following the initial optimization protocol showing that early passage numbers may not be mature enough for experiments, going forward passages of 7 and over were used. This begged the question, were the difference in ACTH levels observed in AtT20 culture media purely due to passage number? Woods et al. (1992) conducted a study evaluating the different strains of AtT20 cells – D1 (suspension cells), D16v (derived from D1 cells but grown as a monolayer, and the cell line strain utilized for my experiments) and D16:16 (subcloned from D16v on the basis of high β–endorphin content). Woods and colleagues (1992) were interested in the differences between the strain's ability to hormonally respond to secretagogues such as CRH. They found that the ACTH response in D16v to CRH was more variable than that of D16:16, and some experiments could not show an increase of ACTH to CRH. Phillips and Tashjian's (1982) study failed to show a reduction of ACTH following treatment with synthetic glucocorticoid, Dexamethasone (which is a pinnacle of the relationship between glucocorticoids and ACTH), in AtT20 D16 cells. They hypothesized the reason for this is the variability in the serum of culture medium, stating that 'difference in medium has a substantial effect on the morphology and hormone release'. Therefore Woods et al (1992) repeated the experiments with medium starved of serum but these experiments were not successful, and they deemed D16v cells to be too inconsistent to use in their hormonal studies, and used D16:16 instead, which had not displayed any discrepancies following replication.

Matsuuchi and Kelly (1991) found that 60% of basal hormone release is derived from regulated pathways, but Woods et al (1992) suggests that this portion of basal secretion

derived from regulatory ACTH pathways may vary from experiment to experiment, which would explain the hormonal response variability detected in these cells in my experiments. It was determined that differences in ACTH levels were only detected using the ELISA assay when levels remained lower than 35 000 pg/ml, which we originally assumed to be an effect of passage number, as earlier passages showed levels that ranged between 30 000 – 200 000 pg/ml in our optimization treatments, and our later passages had more consistency and averaged less than 30 000 pg/ml. As seen in **Figure 6.2** below, these passages were all grown from one parent stock, and perhaps cells exhibit regulatory control of ACTH secretion post passage 7 in this specific parent stock, and maturation times are not translatable between different parent stocks, so that each time a different parent stock of these cells are resuscitated, experiments should measure ACTH levels and define which are the viable passage numbers in these stocks, as it might be much later than passage 7.

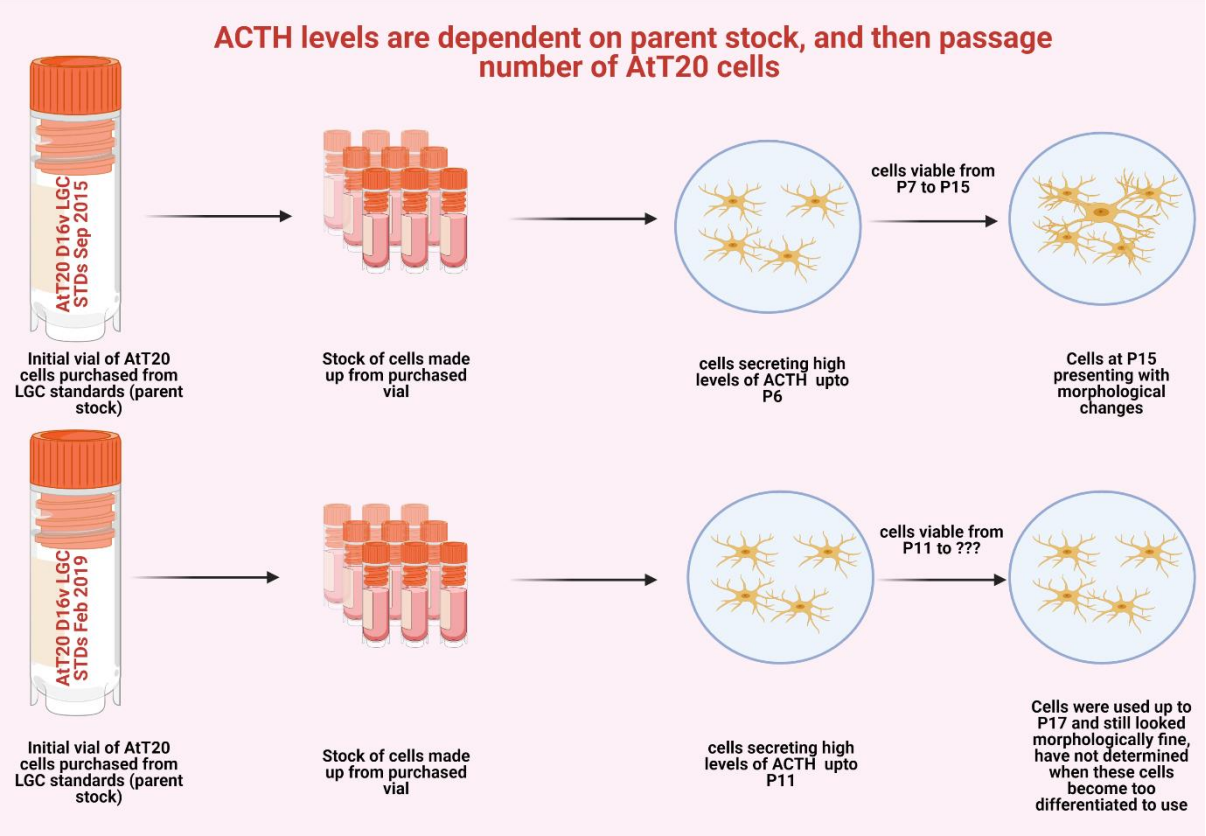


Figure 6.2 - Diagram explaining the ACTH variability depending on parent stock and passage number displaying some of the limitations of these cells as an in vitro model of the anterior pituitary. Diagram made using app.Biorender.com

How can we overcome the variation in basal secretion of ACTH? When the basal ranges are too high they fall out of range for the ELISA kit to be viable. This could be dealt with by further diluting the medium, but this would mean there would be inconsistent protocols between replicated experiments, as some cell's medium would be diluted further than the medium of cells from a different experiment, which defeats the purpose of replication. Additionally, ideally, we want cells to be as similar to each other to maintain consistency between experiments, so using cells that may not have matured ACTH regulatory mechanism would not be a good model for the anterior pituitary. Therefore further dilution may not be the best solution, but instead, monitoring basal ACTH secretion within each parent passage, to know when the ACTH regulation occurs in these cells (it may be passage 7 for one parent stock that was stored down, but passage 11 for another).

Knowing that these cells have a variability to hormones, can my findings be trusted? I think they can, as the inhibition seen in **Figure 2.7** has a robust sample size (n=12), and in **Figure 3.8** the reduction in ACTH is more pronounced with cells overexpressed with Asc-1 transporter and treated with D-alanine, showing more D-alanine caused a greater inhibition of ACTH. Furthermore, the *in vivo* findings of D-alanine inhibiting corticosterone release (**Figure 5.5**) in CD1 mice support what was observed *in vitro*. Therefore the lack of reproducibility of a reduction in ACTH stimulated by D-alanine in the CRH and the strychnine experiments is likely due to high basal ACTH secretion in these cells, which were out of the sensitivity range for the ELISA kit, and it is possible that these specific cells mature later than the cells in the other experiments, and retrospectively were not viable to be used for experimentation at the times that they were used.

6.2. Lack of POMC genomic changes in response to D-alanine treatment

Looking at POMC mRNA levels in the same experiments mentioned above (D-amino acid experiments (**Figure 2.11d**), CRH experiments (**Figure 2.14c**), strychnine experiments (**Figure 2.20d**) and overexpression of Asc-1 experiments (**Figure 3.10**)) there was a consistent finding of no change in mRNA levels when comparing cells treated with D-alanine to the control. However, in **Figure 3.10**, when AtT20 cells were overexpressed with Asc-1 and *then* treated with D-alanine there was a reduction in POMC mRNA compared to control. Additionally, in Figure **3.15**, POMC mRNA levels decrease in AtT20 cells overexpressed with Asc-1 and treated with PBS *or* D-serine, but not in cells treated with just D-serine, so perhaps the transporter Asc-1 may have a role to play in POMC regulation. POMC does not need a transporter for movement, but instead their vesicles are anterogradely transported along microtubules by motor proteins (Cawley et al., 2016), so its relationship with Asc-1 transporter may not be that of a direct vehicle-passenger system. Whilst not as concentrated as D-alanine, D-serine is found in the anterior pituitary (Miyoshi et al., 2011), and perhaps D-serine can be endogenously produced in these cells, unlike D-alanine which is dependent on microbiota of the gut to enter the body, and the presence of excess Asc-1 transporter increases D-serine uptake in AtT20 cells, which we've shown to also affect ACTH release (**Figure 2.7**). This can be proved by detected D-serine in these cells and observing if there is an increase in D-serine levels following Asc-1 transfection.

The failure of D-alanine to effect POMC in D-amino acid experiments (**Figure 2.7**) and Overexpression of Asc-1 experiments (**Figure 3.10** and **Figure 3.15**) is perplexing, as they both show a potent reduction in ACTH levels. ACTH is a product of POMC, and it is assumed that changes in ACTH are linked to changes to its precursor, POMC. This is exhibited by the most potent stimulator of ACTH, CRH, which alters POMC gene expression levels via a signalling cascade that eventually effects orphan transcription factors

Nur77 and Nurr1. These factors act on the POMC gene, which effects POMC translation into protein, and therefore ACTH production (Bonfiglio et al., 2011). Thus a reduction in ACTH secretion may likely also exhibit a parallel reduction in POMC mRNA. Steven and White (2009) showed that the relationship between CRH, POMC and ACTH has its complexities. They observed POMC and ACTH levels in AtT20 cells and found in the basal environment there is a greater amount of intracellular POMC compared to ACTH. Following 2 hr treatment with CRH there is a 1.3-fold increase in intracellular ACTH levels and 2-fold increase in secreted ACTH levels with no change in POMC levels, but following a longer treatment with CRH (72 hrs) there is 1.2-fold and 1.3-fold increase in secretion of POMC and ACTH respectively (but no change in intracellular levels). This shows that CRH stimulates ACTH release in a nongenomic manner, as it managed to increase secretion of ACTH irrelevant of POMC changes, and perhaps D-alanine is doing a similar thing when it inhibits ACTH production.

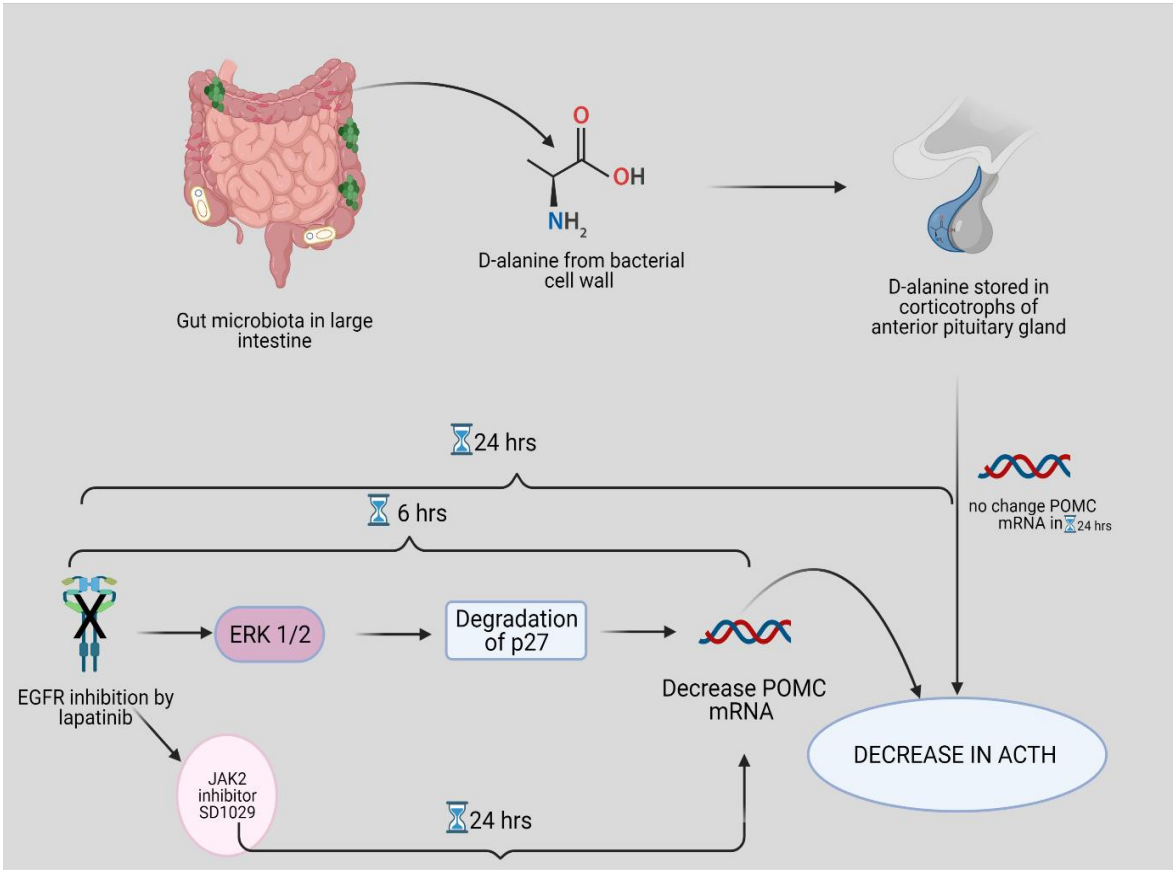


Figure 6.3 - Signalling mechanism that regulate POMC mRNA. EGFR inhibition (Asari et al., 2019) leads to POMC decrease in 6 hrs, whilst downstream inhibition (JAK2) (Asari et al., 2017) leads to POMC mRNA decrease in 24 hours. My experiments show no change in POMC mRNA following 24 hr treatment with D-alanine, but reduction in ACTH release, so perhaps it is following signalling mechanisms similar to EGFR inhibition or further upstream, where POMC mRNA levels are changing earlier (like 6 hrs). Information from Ma et al., 2015. Made with app.Biorender.com.

As seen in **Figure 6.3** above Asari et al (2019) investigated the effects of Lapatinib, a tyrosine kinase inhibitor of epidermal growth factor (EGFR) on POMC and ACTH levels in AtT20 cells. EGFR has been implicated in Cushing's Syndrome, which is identified by increased levels of ACTH production, and inhibiting it may be an effective anti-tumour agent in the pituitary. Asari and colleagues (2019) found that Lapatinib successfully decreased both POMC and ACTH levels in these cells, but what was interesting was that POMC mRNA levels decreased following 6 hr treatment with Lapatinib and this reduction disappeared at the 24 hr mark, whilst ACTH reduction occurred at the 24 hr mark. Asari et al (2017) also looked at ACTH and POMC levels in AtT20 cells by observing the effects a selective Jak2 inhibitor may have on this hormone. This time, POMC mRNA levels were significantly decreased after 24 hr treatment with the Jak2 inhibitor, SD1029, and ACTH release also significantly decreased after 24 hrs. The Jak–STAT pathway is located downstream to EGFR (Asari et al., 2017), therefore Jak2 inhibition acts lower down than the Lapatinib inhibition, and perhaps this affects the time taken for POMC mRNA levels to change. If the inhibition happens further downstream, it takes longer for POMC mRNA to be altered and we see inhibition at the 24 hr mark, but further upstream the changes to POMC happen earlier (6 hr mark) and within 24 hrs there is sufficient time for regulatory pathways

to try and bring POMC mRNA levels to homeostasis. This is perhaps what occurs with our data, where changes in POMC mRNA are not detected because 24 hrs is enough time for the cells to regulate their POMC mRNA levels to normal, and possibly a shorter duration may show POMC mRNA alterations, but unfortunately shorter durations (Appendix) are not sufficient for an effect on ACTH release, as is the case in Asari et al's (2017, 2019) studies as well. However, the fact that POMC mRNA levels consistently do not change at the 24 hr mark may indicate that the inhibition by D-amino acids on ACTH levels observed may be due to an inhibition of a factor higher up in the signalling cascade (like EGFR) rather than lower down (like Jak2). This could be ascertained by shorter treatments of D-alanine just to observe POMC mRNA levels, and perhaps blocking a signalling factor higher up in the ACTH release cascade, such as MEK1/2, by using an inhibitor like selumetinib (Sale and Cook, 2013).

6.3. What is responsible for the inhibition of ACTH by D-amino acids, D-alanine and D-serine?

The inhibition of ACTH by D-alanine and D-serine in AtT20 is an exciting discovery, as it supports the idea that D-amino acids, whilst less common than their L-configured counterparts, have a role to play in the physiological management of the body.

6.3.1. NMDARs?

My initial theory behind the mechanism of the core finding of ACTH inhibition was that of a mutual receptor – the NMDARs, which both these amino acids bind to (Lin et al., 2019). This would be a sensible deduction, as D-alanine and D-serine are affecting both ACTH (**Figure 2.7**) and corticosterone (**Figure 5.5**) in an inhibitory way, and we would assume it was through the same pathway. NMDARs are best known for their excitatory effects, but they do have inhibitory subunits (GluN3) and have exerted their inhibitory capability on synapses (Adesnik et al., 2008) and platelet aggregation (Zhu et al., 2015). However, whilst

GluN1 and GluN3A were detected in AtT20 cells, there were no changes in protein (**Figure 2.10b** and **Figure 2.9b** respectively) or mRNA (**Figure 2.10c** and **Figure 2.9c** respectively) when AtT20 cells were treated with D-alanine or D-serine. However, the finding of GluN3A in this cell line was novel, as previously, the only NMDAR subunit to be detected in AtT20s was GluN1 (Zhou and Lapingo, 2014), and further supported in the *in vivo* study, where CD1 mice expressed GluN1 and GluN3A in their pituitary (**Figure 5.8**).

6.3.2. GlyRs?

Based on the inhibitory interaction between D-amino acids and hormone secretion, the inhibitory GlyR involvement was then investigated, as D-alanine is an agonist of GlyRs. D-alanine is localized to the β- cells of the Islets of Langerhans of the pancreas at a concentration of 1500 – 3000 nmol/g (Yan-Do et al., 2016). For perspective, that is higher than D-serine presence in the cerebrum (300 nmol/g), where D-serine plays an endogenous role in NMDAR modulation (Morikawa et al., 2007). The GlyR subunit GLRA1 is expressed in the these same β- cells (Yan-Do et al., 2016) and Taneera et al (2015) found that GLRA1 is downregulated in donors with Type 2 Diabetes, as well as in human pancreatic islets which receive a high glucose dose. They also found a positive correlation of GRLA1 expression and insulin secretion. Inhibition of the glycine receptor resulted in disrupted glucose-stimulated insulin secretion and treating islets with glycine resulted in an increase in glucose-stimulated insulin secretion (Yan-Do et al., 2016). Both these studies show the importance of GLRA1 in the pancreas, in the same cells where D-alanine is stored, with regards to insulin secretion, and perhaps a similar mechanistic regulation occurs in the anterior pituitary with regards to ACTH release. Maybe GlyRs regulate hormone secretion in the anterior pituitary, similarly to how they regulate it in the pancreas.

However, Stojilkovic and colleagues (2010) stated that glycine receptor was not found in the anterior pituitary, which shook the foundation of the above hypothesis. I wished to confirm this and investigated the anterior pituitary GLRA1 expression. I found it to be expressed in both AtT20 cells (**Figure 2.8**, **Figure 2.13**, **Figure 2.15** and **Figure 2.19**) and the pituitary of CD1 mice (**Figure 5.8**). This was an exciting finding and strengthened the hypothesis of a GlyR mediated regulation of ACTH via its agonist, D-alanine.

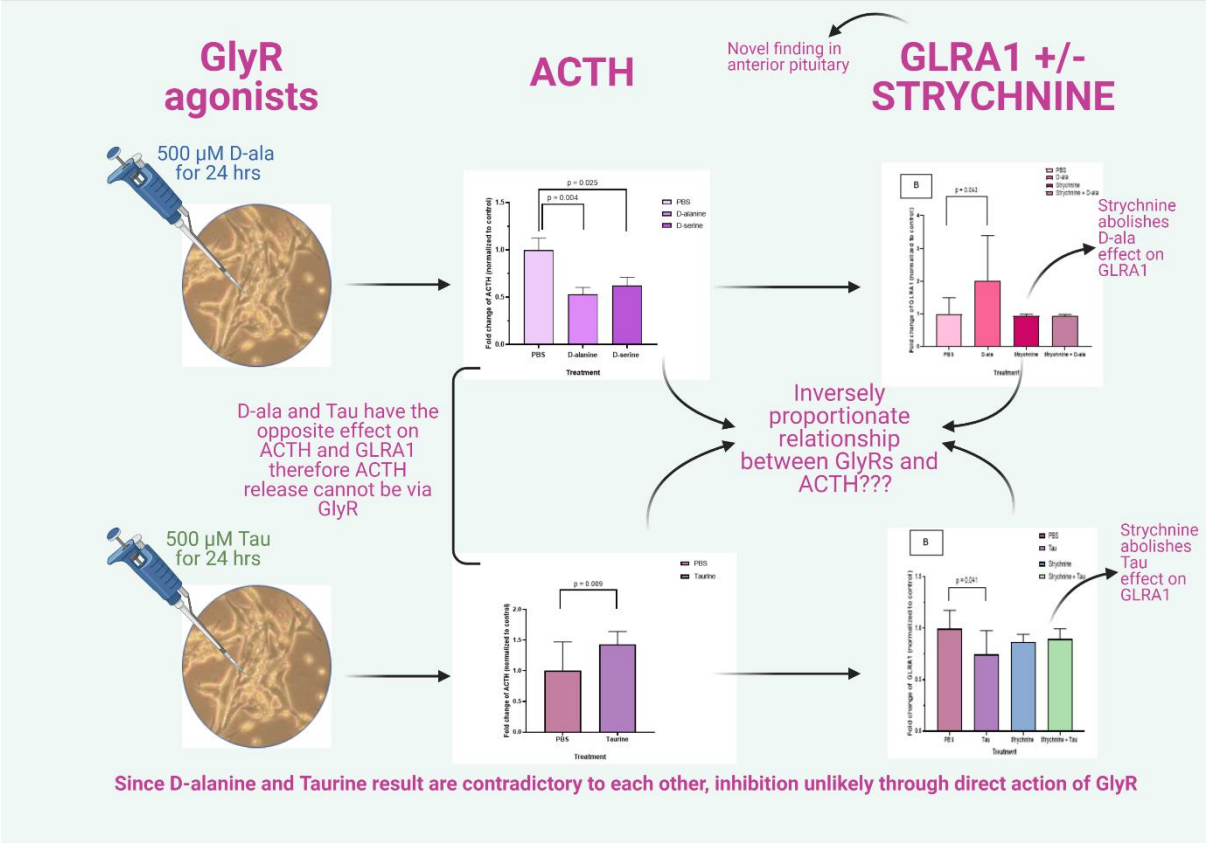


Figure 6.4 - How GlyR agonists interact with AtT20 cells to effect ACTH and GLRA1. Diagram made with app.Biorender.com

As shown in **Figure 6.4**, to ascertain GlyR involvement, another GlyR agonist, taurine (Han et al., 2004) a GlyR antagonist, strychnine (Ito et al., 2020) were used to investigate the relationship between GLRA1 expression and ACTH release. Taurine stimulated ACTH release in AtT20 cells (**Figure 2.16**) whilst inhibiting GLRA1 (**Figure 2.15**). In perfect

contrast, D-alanine inhibited ACTH release (**Figure 2.7**) and stimulated GLRA1 (**Figure 2.8** and **Figure 2.19**) in AtT20 cells. In the pituitary of CD1 mice GLRA1 mRNA decreased in the presence of D-alanine (**Figure 5.8c**) but this was a less localized investigation of GLRA1 expression as it involved the whole pituitary gland and not just the anterior lobe. There appears to be an inversely proportionate relationship between GLRA1 mRNA expression in the anterior pituitary gland and ACTH release, but this effect is currently causal through amino acid treatment and should be more directly investigated to be proven. However, because the agonists of GlyRs are behaving differently to ACTH, I postulate that it is unlikely that GLRA1 that is directly responsible for D-amino acid driven inhibition of ACTH. Unfortunately, the ACTH data (**Figure 2.18**) we have for the strychnine experiment cannot be used as the ACTH levels were out of the sensitivity range for the experiment (explained above). However the inhibition of GLRA1 by strychnine was successful as usual stimulation of GLRA1 with D-alanine was inhibited in the presence of strychnine (**Figure 2.19)**, and the inhibition of GLRA1 due to taurine was negated in the presence of strychnine (**Figure 2.15**). There is no ACTH data for taurine + strychnine experiments as I was not able to conduct these experiments due to the COVID-19 pandemic. Therefore we don't have data on the effect that blocking GlyRs has on ACTH but the opposing ways in which D-alanine and taurine interact with ACTH indicate the inhibition of ACTH is not directly mediated through GlyRs. However, there does seem to be some inversely proportionate relationship between ACTH and GlyRs, which can further be explored by looking at how the GlyR antagonist, strychnine, affects ACTH release using known positive (CRH) and negative (dexamethasone) regulators of ACTH secretion.

6.3.3. SGK1 and POMC?

Moving away from the inhibition of ACTH facilitated by direct action by GlyRs, I then investigated the more upstream mechanisms of ACTH regulation, that of POMC regulation.

Despite being stimulated by the presence of GCs, SGK1 is found to have a contradictory effect of the usual GC mediated negative regulation of POMC, where SGK1 increases POMC activity (Reiter et al., 2011). The findings by Reiter et al (2011) were interesting, as they discovered that SGK1 is found in corticotroph cells of the anterior pituitary (much like D-alanine and ACTH) and regulated basal POMC levels, but not basal ACTH release. However in an overexpression model of SGK1, ACTH release is then increased, which shows that SGK1 may have a compensatory role to play in protecting the body in states of high GC presence. This is not the first time SGK1 behaved dissimilarly to a GC; it displayed anti-apoptotic effects on HEK293 cells (Brunet et al., 2001), whilst GCs are known for their proapoptotic effects (Drouin et al., 1993). Therefore I investigated the potential role of SGK1 in D-amino acid driven ACTH release from AtT20 cells, in both the basal system and the activated system (in the presence of Dexamethasone) as it was shown to affect ACTH release differently depending on HPA axis activity (Reiter et al., 2011).

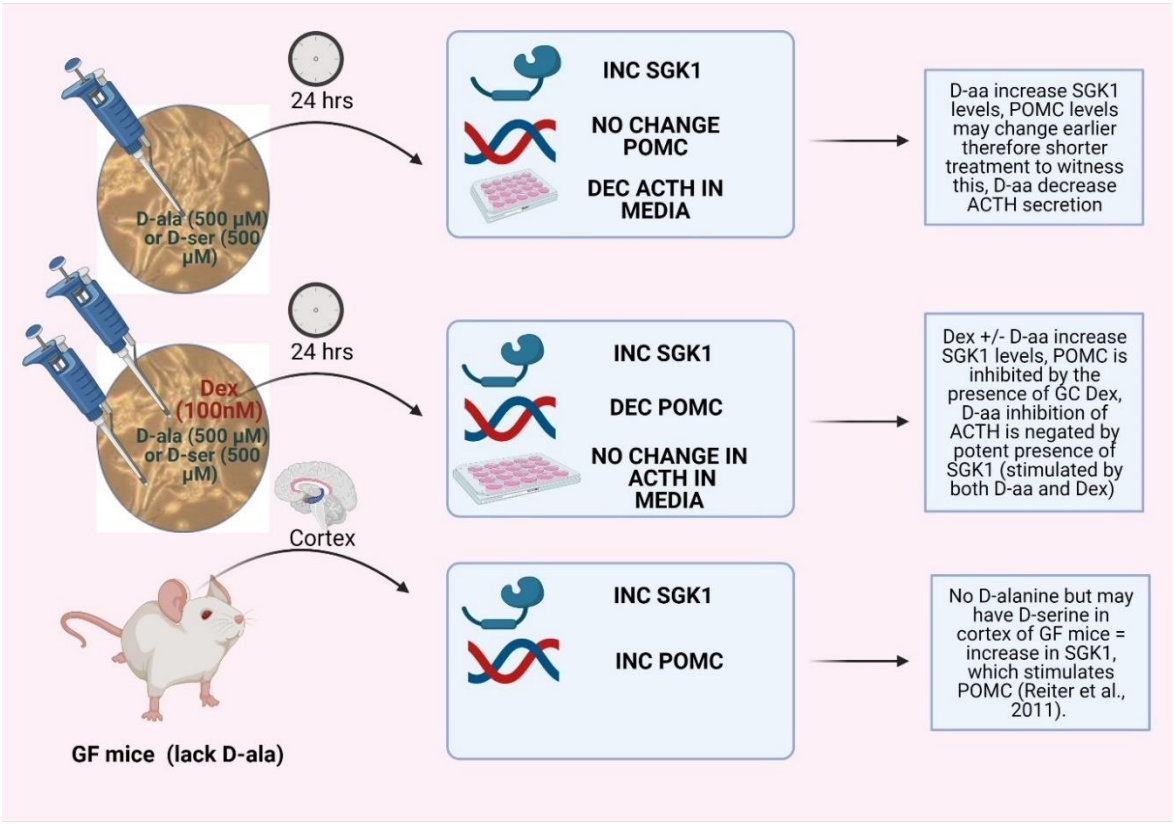


Figure 6.5. - Summary of SGK1 and POMC expression in relation to D-alanine. Made with app.Biorender.com

I found that both D-alanine and D-serine increased SGK1 mRNA levels (**Figure 2.11**) but D-alanine failed to have an effect on SGK1 in the CRH experiments (**Figure 2.14d**) and strychnine experiments (**Figure 2.20e**), but the variability observed in the CRH and strychnine experiments were addressed above, and these cells are thought to be, in retrospect, not mature enough to be utilized for experimentation, hence the failure to reproduce findings. In the activated system, the presence of Dexamethasone negated the ACTH inhibition by D-amino acids in AtT20 cells (**Figure 3.5**) and increased SGK1 both in the presence, and the absence, of D-alanine or D-serine (**Figure 3.6a**). Interestingly, POMC is greatly inhibited in the same cells where D-alanine and D-serine failed to affect POMC previously due to a hypothesised time dependent effect, where it was proposed that 24 hrs is too long a duration for POMC gene regulation to be observed. In the activated system POMC levels remain significantly low at the 24hr mark, but ACTH release is no longer affected. This is intriguing as Reiter et al (2011) found that SGK1 stimulates POMC expression and ACTH release. The causal effect of an increase in SGK1 via the stimulation of GC Dexamethasone, which also negated the inhibition of ACTH, supports what Reiter et al (2011) found: that SGK1 increases ACTH release in an activated system and therefore negates the D-amino acid inhibition (**Figure 3.5)**, but did not support the stimulation of POMC mRNA, it in fact achieved the opposite (**Figure 3.6b)**. Since the effect observed on POMC mRNA isn't directly linked to overexpressing or knocking out SGK1, but rather D-amino acid effects in an active system, it would be presumptuous to deduce SGK1–POMC interactions in this system. The inhibition of POMC is likely due to the high presence of dexamethasone which is a known negative regulator of this gene, and a stimulator of SGK1. However, dexamethasone presence should exacerbate the decrease of D-amino driven

ACTH release, which is not happening, and this could be due to the dexamethasone-induced SGK1 presence, where SGK1 may be affecting ACTH release. This could be proven by knocking out SGK1 and observing the dexamethasone effects on ACTH release, but for now it hasn't cancelled out the possible regulatory involvement of SGK1 in the D-amino acid driven ACTH inhibition.

The *in vivo* study of D-amino acids – where D-alanine and D-serine were given to CD1 mice - did not show any effects on SGK1 or POMC signalling in the pituitary gland (**Figure 5.6**), frontal cortex (**Figure 5.9**), hippocampus (**Figure 5.15**) or cerebellum (**Figure 5.12**). SGK1 cortical (Licznerski et al., 2015), hippocampal (Wu et al., 2021), cerebellar (Wu et al., 2021) and pituitary (Reiter et al., 2011) expression has been proven in literature, whilst POMC expression has also been published in the hippocampus (Jiaxu and Weiyi, 2000), cortex (Jiaxu and Weiyi, 2000), pituitary (Reiter et al., 2011) and the cerebellum (Mountjoy, 2015). The brain regions (aside from the pituitary) were observed as I was curious to see how D-alanine and D-serine may affect the SGK1-POMC system in areas where there was no ACTH secretion, so as to see if these D-amino acid driven effects on SGK1 and POMC observed *in vitro* are direct, and not just a causal effect via ACTH hormone regulation that occurs in the anterior pituitary. Perhaps the concentrations of D-alanine and D-serine (3mM in drinking water for 7 days = 50mg/kg) in CD1 mice were not the optimum dosage levels to effect SGK1 or POMC gene expression in the above mentioned brain regions, as they exert most of their effects in brain regions rich, and potentially sensitive, to these D-amino acids (specifically the cortex and anterior pituitary).

Additionally, I also observed the SGK1-POMC system in a 'D-alanine knockout model' of GF mice, which lack a gut microbiota and therefore should lack D-alanine. I compared GF mice to SPF mice and found that both SGK1 *and* POMC mRNA levels increased in the cortex of GF mice when compared to SPF mice (**Figure 5.28**). The effect that is observed

in the cortex of GF mice is interesting, as our studies in AtT20 cells show the stimulation of SGK1 in the presence of D-amino acids (**Figure 2.11**), whilst the absence of D-alanine in the cortex of GF mice is *also* stimulating SGK1 (**Figure 5.28**). This could be due to the fact that D-serine levels are highest in the cerebrum (Suzuki et al., 2017), which the cortex belongs to, and D-serine can be synthesized in the body and therefore is not dependent on microbial content, like D-alanine is. Therefore GF mice may have D-serine derived from diet. D-serine has had a stimulatory effect on SGK1 in AtT20 cells (**Figure 2.11),** so perhaps the strong presence of D-serine in the cortex is sufficient to stimulate SGK1 levels there. Interestingly, POMC mRNA also increases (**Figure 5.28**) in mice lacking D-alanine (GF mice), which follows what Reiter et al (2011) stated; that SGK1 has a stimulatory, rather than inhibitory effect on POMC.

In summary (**Figure 6.5** above), in the basal AtT20 studies, D-amino acids had no effect on POMC (**Figure 2.11b**), but in the activated AtT20 studies, POMC mRNA levels decreased (**Figure 3.6b**), and in the *in vivo* studies, GF mice showed an increase in POMC mRNA in the cortex (**Figure 5.28**), whilst SGK1 was consistently stimulated in each of these experiments (**Figure 2.11a**, **Figure 3.6b** and **Figure 5.28**). Therefore, it does seem that D-amino acids do exert an effect on the SGK1-POMC system in areas which are high in (and therefore sensitive to) D-amino acid content (D-alanine in the anterior pituitary and D-serine in the cortex), and with regards to POMC, this effect is dependent on whether the HPA axis is activated or not, but I cannot know for sure until a more direct interaction is determined, perhaps by knocking out SGK1 and exposing these *in vitro* or *in vivo* models to D-amino acids and observing effects on POMC and ACTH.

6.3.4. Amino acid transporters?

The D-amino acid effects with regards to POMC were inconsistent as they seem to depend the on activity state of the HPA axis, therefore the inhibition of ACTH by D-amino acids were further explored via their transporters – Asc-1 and ASCT2, since this was a system which interacted with both D-alanine and D-serine, and the assumption is that the ACTH inhibition occurs through a mutual pathway. This posed a challenge as I wished to investigate Asc-1 activity in particular but could only do so by mRNA levels in AtT20 cells as they were no murine antibody for protein analysis, however this was overcome by transfecting murine AtT20 cells with the human vector of Asc-1, and there was an Asc-1 western blot antibody that reacted with human Asc-1.

There was an inhibition of Asc-1 mRNA in AtT20 cells treated with D-alanine compared to control (**Figure 2.20c and Figure 3.12a**) and ASCT2 mRNA also decreased in cells treated with D-alanine, as well as cells overexpressed with Asc-1 and treated with D-alanine, when compared to control (**Figure 3.12b**). This could be due to the presence of D-alanine, as there is now an increased demand for Asc-1 and ASCT2 transporter, and the resultant lower mRNA levels mean that mRNA is being translated into its relevant proteins, which we see reflected in the increase in Asc-1 protein levels in **Figure 3.11**, where Asc-1 protein increased in AtT20 cells treated with D-alanine in the presence or absence of transfection with Asc-1 human vector. D-serine also expressed inhibitory effects on Asc-1 and ASCT2 mRNA levels (**Figure 3.16)** where it inhibited transporter levels in cells with or without transporter overexpression. Interestingly, control cells transfected with Asc-1 also decreased transporter levels (**Figure 4.3.3.2**), but this could be due to the increased presence of Asc-1 transporter in these cells affecting the mechanics of the transporter expression, where the cells may register the increase of Asc-1 transporter and act to downregulate it genetically.

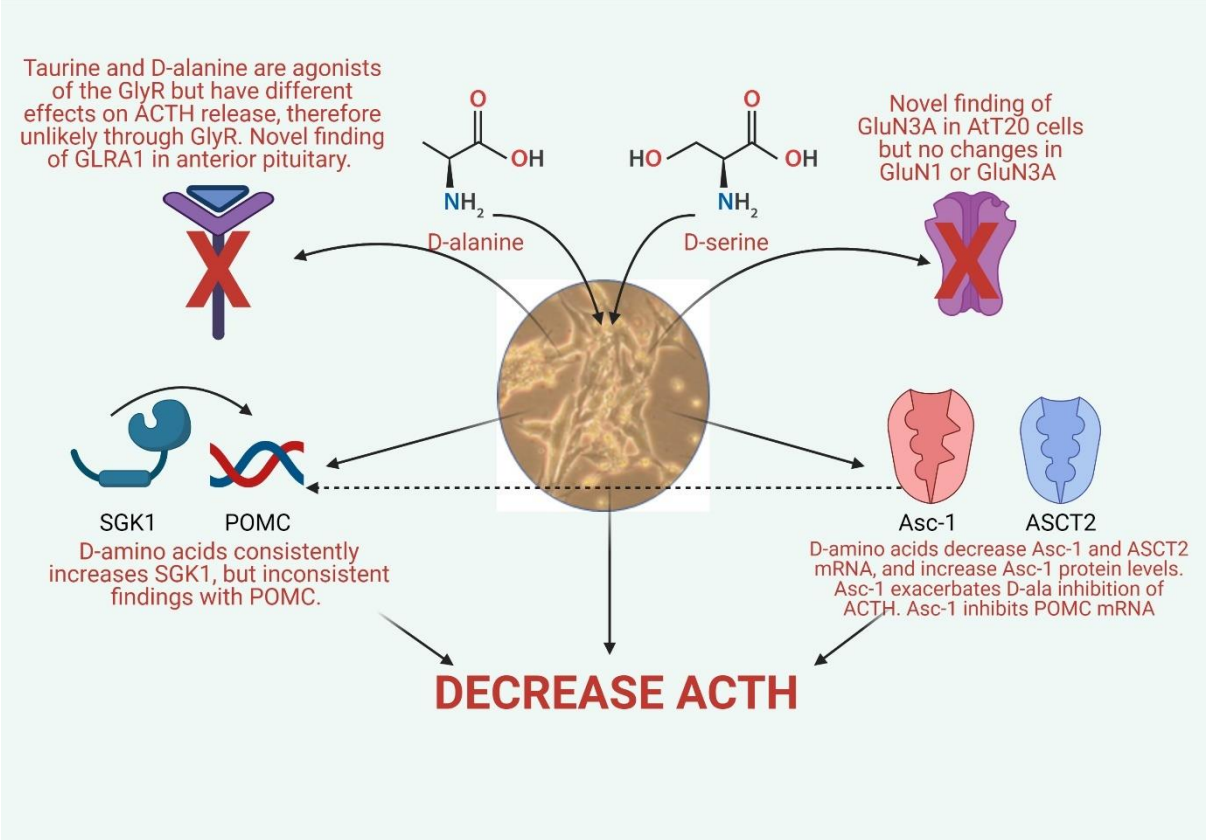


Figure 6.6. - Summary of key players involved in inhibition of ACTH driven by D-amino acids in the anterior pituitary. Diagram made with app.Biorender.com

It is unsurprising that D-alanine and D-serine are affecting Asc-1 and ASCT2 as they are known to interact with this transporter, but what is exciting is the results displayed in **Figure 3.8**, where AtT20 cells transfected with Asc-1 and treated with D-alanine show a further reduction in ACTH levels when compared to both PBS control and transfected AtT20 cells treated with PBS. Additionally, as mentioned above, POMC mRNA seems to be linked to Asc-1, as cells overexpressed with Asc-1 have noted inhibitory effects on POMC (**Figure 3.10** and **Figure 3.15**). Therefore it would seem that Asc-1 and ASCT2 are interacting with POMC-ACTH pathways, whether directly or through their agonists is still a mystery. Adding to the mystery is whether SGK1 is also involved, as it interacts with POMC and ACTH, and now it is found to interact with D-amino acids as well. This can be solved by venturing further into *in vivo* studies by overexpressing Asc-1 transporter and observing if

an increase in transporter alters HPA axis hormones, as it appears to interact with POMC mRNA independently of D-amino acid presence **(Figure 3.15)**, and also observing SGK1 effects in this system.

6.4. How does D-amino acid uptake effect the gut?

Now that a better understanding of the effects of gut microbial by-products had been established, I investigated the effects D-amino acids have at their primary entry site – the gut, using Caco-2 human colorectal cells. This is of importance, as any suggestion of using D-amino acids as a potential therapeutic to manipulate the HPA axis would involve understanding any safety limitations that excess presence of these D-amino acids may present in the gut. Firstly, the presence of Asc-1 (**Figure 4.5a** and **Figure 4.5bb**) and ASCT2 (**Figure 4.5c)** were established in these cell lines, which supported the finding by Uchiyama et al (2005) with regards to ASCT2, whilst our finding of Asc-1 transporters in Caco-2 cells is novel.

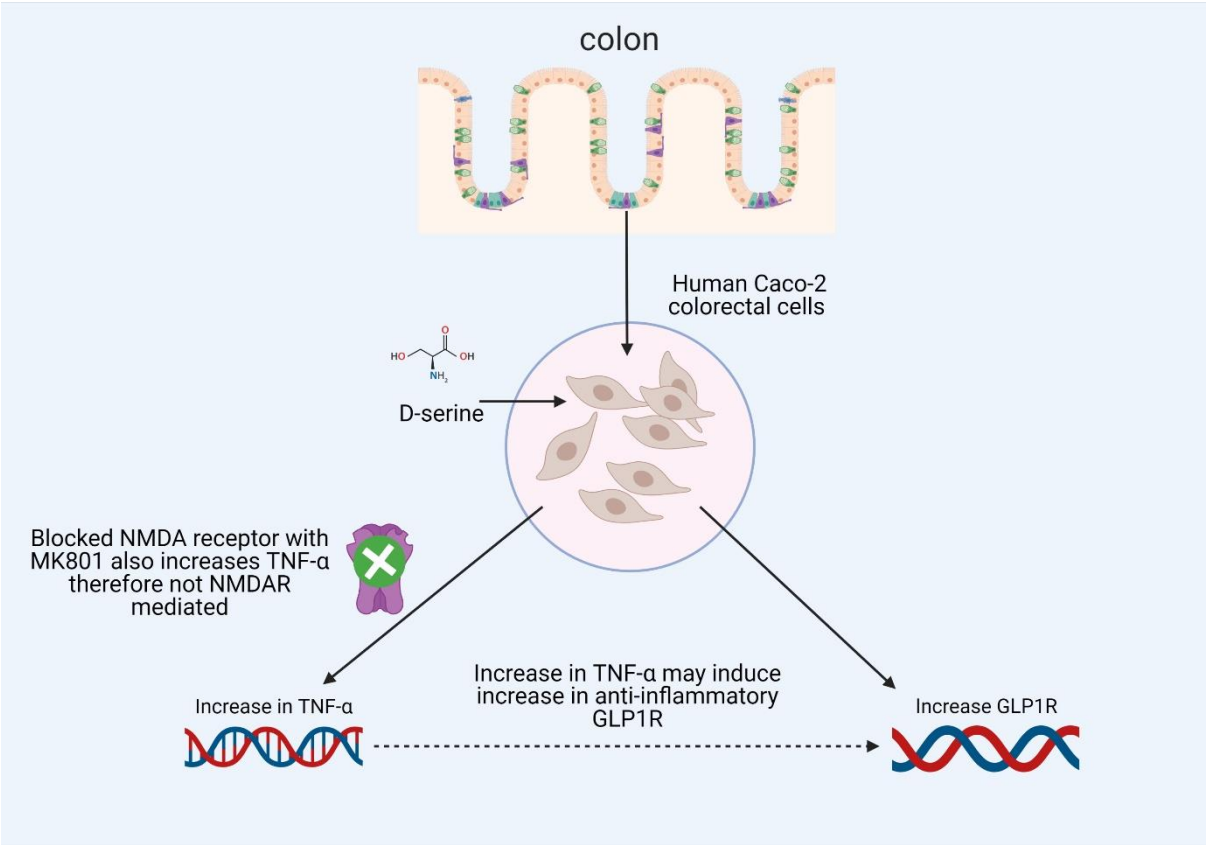


Figure 6.7 - Inflammatory effects of D-serine in Caco-2 cells. Diagram made with app.Biorender.com

D-alanine did not affect Caco-2 cells, probably due to a lack of sensitivity to this D-amino acid as it is only present in high concentrations in the anterior pituitary and the pancreas, and seems to spend little time in the gut, whist D-serine affected Caco-2 cells in a plethora of ways, but of particular interest was the pro-inflammatory effect it had on TNF-α mRNA (**Figure 4.8c**), which can be further proven by ELISA (which was not done due to COVID-19 time limitations). This is of importance, as if D-serine is suggested as a therapeutic intervention based on its ability to regulate the HPA axis hormones, it could cause the gut to be inflamed over time, so an anti-inflammatory component should be paired to any such therapeutic intervention, however the cells seems to attempt to regulate this for themselves, by increased GLP1R (**Figure 4.6b** and **4.11b**).

GLP1R has been extensively studied in relation to IBD, where it was found that it is expressed in nitrergic neurons of myenteric plexus in human colon smooth muscle where it inhibits large intestine motility when activated (Amato et al., 2014). It's agonists were also found to regulate enteric immune response which may influence the gut microbiome and intestinal inflammation (Yusta et al., 2015) and exert anti-inflammatory effects (Jojima et al., 2017) – however it is uncertain if the increase in GLP1R observed in this study is directly achieved by D-serine, or is due to D-serine induced inflammation via excess TNF-α.

Jander et al (2000), looked at TNF-α and IL-1β and for the first time showed that NMDAR signalling induces inflammatory cytokine gene expression in the brain. I found that D-serine significantly increased GluN2A and GluN2C (**Figure 4.7)**, which is an expected finding as D-serine is an NMDAR agonist, however detecting NMDARs in colonic cells was a novel finding. On the basis that D-serine was exerting inflammatory effects in Caco-2 cells

possibly via NMDARs, MK-801 NMDAR antagonist was used, which interacted with GluN2A (**Figure 4.15)** and GluN1 (**Figure 4.18)** but seemed to stimulate TNF-α (**Figure 4.17d).** If the NMDAR antagonist is also causing an increase in TNF-α, inflammatory effects are unlikely to be NMDAR mediated. However, the D-serine driven increase in TNF-α (**Figure 4.8)** failed to be repeated in **Figure 4.12** (in overexpression of Asc-1 experiments) or **Figure 4.17** (in MK-801 experiments). This finding will be best confirmed using TNF-α ELISA kit instead of mRNA, as this is a better way of measuring cytokine expression.

In conclusion (**Figure 6.7** above**)**, D-serine stimulates GLP1R expression (**Figure 4.6 and Figure 4.11b**) but the finding of TNF-α stimulation was not replicated, and this should be confirmed using ELISA. Asc-1 and ASCT2 are expressed in Caco-2 cells, and this should be further confirmed *in vivo.* If D-serine does in fact have an inflammatory effect in the gut via TNF-α, this is unlikely through NMDARs as its antagonism by MK-801 did not abolish this effect, in contrast, it also increased TNF-α. D-serine interaction with GLP1R shows that it may exert anti-inflammatory effects in the gut and affect the gut microbiome in this way, which can be confirmed *in vivo,* or the GLP1R effects maybe a response to TNF-α increase. To better understand the dynamics between D-serine, TNF-α and GLP1R, a GLP1R antagonist can be used and TNF-α measures recorded in response.

6.5. Further studies, limitations and Concluding Remarks

As discussed in this chapter, AtT20 D16v cells are not the best strain to measure corticotroph secretion, and AtT20 D16:16 cells should have been used instead as they had more consistency in ACTH secretion (Woods et al., 1992). However, AtT20 D16v cells are still adequate to study D-amino acids as they successfully secrete ACTH and express Asc-1 and ASCT2 transporters, but care needs to be taken to ensure experiments are done using the same parent stock.

The COVID-19 pandemic was a great limiting factor for this project as it infringed on time allocated to further investigate various aspects, especially in Chapter 4, with regards to the effects of D-serine on the gut.

The inhibitory effect of ACTH by D-amino acids mediated by Asc-1 transporter via POMC-SGK1 should be further investigated, as the link between Asc-1 and POMC can be validated by knocking out Asc-1 in AtT20 cells and measuring POMC and SGK1 levels at the 6 hr mark (where I hypothesize changes to POMC mRNA may occur) and the 24 hr mark (which is the amount of time necessary for SGK1 changes). The Asc-1 mediated inhibition of ACTH can be confirmed by measuring ACTH levels in cells overexpressed with Asc-1 and treated with D-serine, as well as investigated *in vivo* by knocking out Asc-1 and observing ACTH and POMC effects in the anterior pituitary, to determine if changes in ACTH are due to Asc-1 transporter.

In conclusion, most aims of this Thesis was addressed. An inhibitory effect of ACTH and corticosterone by D-amino acids were established. This inhibition is unlikely through NMDAR or GlyRs (based on taurine and strychnine experiments) but has a strong case for SGK1-POMC and Asc-1 transporter involvement. Therefore D-amino acids may influence the HPA axis via combination of these 2 systems, which should further be explored. The safety of D-amino acids as a therapeutic was investigated, and it was found that D-serine, and not D-alanine, had pro-inflammatory effects in Caco-2 colorectal cells, however the mechanism for this inflammation was not elucidated. Therefore D-alanine is proposed over D-serine as a potential therapeutic to regulate the HPA axis.

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APPENDIX

METHODOLOGY

1. **Initial plating medium for AtT20 cells**:

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. **Initial plating medium for Caco-2 cells:**

20% FBS (FBS, Sigma Aldrich, F9665, Missouri USA) and 4mM L-glutamine (ThermoFisher, 25030081, Massachusetts, USA) in DMEM (high glucose, Sigma Aldrich, D5546, Missouri USA).

3. **Growth medium for AtT20 and Caco-2 cells**:

10% FBS and 4mM L-glutamine in DMEM

4. **Treatment medium**:

0.02% FBS and 4mM L-glutamine in DMEM

5. **Preparing D-alanine and D-serine 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 500µM D-alanine:

C1V1 = C2V2

(100mM) V1 = (0.5mM)(2000µl)

V1 = (0.1)(2000)/(100)

= 10µl

Therefore, for 500µM in 2ml media, add 10µl 1mM 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 500µl of above solution in 1ml for 0.1M stock solution

For 500µM D-serine:

C1V1 = C2V2

(100mM) V1 = (0.5mM)(2000µl)

V1 = (0.5)(2000)/(100)

= 10µl

Therefore, for 500µM in 2ml media, add 2µl 1mM stock solution

6. **Preparing 1 X Running Buffer for Western Blot**:

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).

7. **Preparing Transfer Buffer for Western Blot**:

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

8. **5% Blocking buffer for Western Blots:**

5g of dry non-fat milk in 100ml of PBS-T

9. **2% Blocking buffer for Wash Buffer for Western Blots**:

2g of dry non-fat milk in 100ml PBS-T

10. **PBS-T for Wash buffer for Western Blots and ELISA:**

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

11. **PBS**:

10 tablets of PBS mixed into 1 litre of distilled water

12. **ECL substrate for Western Blot**:

2mls of Substrate A mixed with 2mls of Substrate B

13. **Stripping Buffer for Western Blot**:

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

14. **Stock solution of CRH**:

Molecular weight is 4757.45. Therefore, for 1M mix 4.757g 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 CRH:

C1V1 = C2V2

(0.1mM) V1 = (0.0001mM)(2000µl)

V1 = (0.0001)(2000)/(10)

= 2µl

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

15. **5X loading buffer for Western Blot gel**:

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.

16. **Stock solution of dexamethasone**:

Molecular weight is 392.46. Therefore 1M mix 0.392g in 1 mL of PBS. Dissolve 10 µl of above solution in 1 mL for 0.01M stock solution.

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

For 100nM DEX:

C1V1 = C2V2

(0.1mM) V1 = (0.0001mM)(2000µl)

V1 = (0.0001)(2000)/(10)

= 2µl

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

17. **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:

C1V1 = C2V2

(10mM) V1 = (0.01mM) (2000µl)

V1 = (0.01) (2000)/ (10)

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

18. **Developing Western Blots in Dark Room**

Make up Fixer (1:10 in water) and Developer (1:5). Pour Fixer and Maker into a shallow dish to develop film in a bit later. Make sure developer is not exposed to direct light using a cover. Place film in western blot cassette for 1 minute. Let film sit in the fixer for 1 minute, wash with water, then Developer for 1 minute and then wash and leave to dry. If too exposed, place film in cassette for less than 1 minute, or if too weak, longer than 1 minute, and adjust accordingly.

19. **Reaction Mix for RT PCR protocol:**

	Positive	Negative
RT Buffer	48 µl	10 µl
RT Random Primer	48 µl	10 µl
dNTP	19.2 µl	4.0 µl
RNAse-in	24.0 µl	5.0 µl
NF water	52.8 µl	21.0 µl
RT	48.0 µl	00.0 µl

Figure 1. Reaction mix for RT PCR, samples without RT will be used as negative controls.

20. Primer sequences for genes for qPCR (5’ to 3’)

GLUN2A reverse: AAC TTA GCC AAA GGG AAA GCT CCC CAT

GLUN2A forward: GCT TTC CTT GAA CCC TTC AG

GLUN2B reverse: ACC TTC TGC CTT CTT AGA GCC

GLUN2B forward: TTG GTG AGG TGG TCA TGA AG

GLUN2C reverse: AAT GGT TGT GAT CGC TCT CA

GLUN2C forward: GAG GCT TTC TAC AGG CAT CTG

GLRA1 reverse: GTC TCA GCG ATG GAA CCG AA

GLRA1 forward: CAT TGT ATT CTT CAG CCT TGC TGC

GLP2R reverse: GAG GAG ATT CTG GAA GAG GAG AGT GAG

GLP2R forward:	GCT GGG ATC GAG CAG GG
GLUN1 reverse:	AGC AGA GCC GTC ACA TTC TT
GLUN1 forward:	ATC ATC CTG CTG GTC AGC GA
IL10 reverse:	CGA TCA TTT CCG ATA AGG CT
IL10 forward:	TGG ACA ACA TAC TGC TAA CC
IL6 reverse:	GAA GTA GGG AAG GCC GTG G
IL6 forward:	CTG CAA GAG ACT TCC ATC CAG TT
TNF-α reverse:	TTT GCT ACG ACG TGG GCT A
TNF-α forward:	GCC TCC CTC TCA TCA GTT CTA T
IL-1β reverse:	GAT CCA CAC TCT CCA GCT GCA
IL-1β forward:	CAA CCA ACA AGT GAT ATT CTC CAT G
B2M reverse:	AAT GTG AGG CGG GTG GAA CTG
B2M forward:	CAT GGC TCG CTC GGT GAC C
ASCT2 reverse:	GCC AGT CCA CGG CCA AGA TC
ASCT2 forward:	GCT TGG TCG TGT TCG CTA TA
Asc-1 reverse:	TCA TTG TGG CTT CGA GGG CTT
Asc-1 forward:	ATG GCC GGC CAC ACG CAG CAG
SGK1 reverse:	GCT CCC TCT GGA GAT GGT AGA ACA
SGK1 forward:	CAA GGC ACA AGG CAG AAG AAG CAT
POMC reverse:	AAG TGA CCC ATG ACG TAC TTC
POMC forward:	GAG ATT CTG CTA CAG TCG CTC
GLUN3 reverse:	CTC TGT CCT GCT TAC AG
GLUN3 forward:	ACA TAG TGC ACA GAC TGC TG
GLUN1 reverse:	AGC AGA GCC GTC ACA TTC TT
GLUN1 forward:	ATC ATC CTG CTG GTC AGC GA

20. **JM 109 competent cells used to express GFP**

- Thawed 1 tube of JM109 E. *coli* on ice

- split each tube in half to give 2n X 25 µl E. *coli* in 1.5 ml screw cap tubes

- Added 5 µl ligation mix to each tube (1-4)

- Incubated on ice for 30 minutes

- Heat shocked in water bath for 20s at 42 °C

- Put on ice for 2 minutes

- Added 500 µl room temperature SOC medium

- incubated at 37 °C with shaking at 200 rpm for 2 hrs

-Add this to 5 mL of LB and 5 µl of ampicillin and leave shaking in incubator overnight

- Purify DNA as described in Chapter 4 using the QIAGEN Miniprep Kit

RESULTS

1. ACTH optimization based on AtT20 passages showing that later passage expressed lower levels of ACTH than earlier passages.

		OD 540nm	pg/ml diluted	pg/ml			OD540nm	pg/ml dilu	pg/ml
P3	sal1	1.5666	361.15	3611.48	**P7**	sal1	0.7354	144.88	1448.83
	sal2	1.7645	403.64	4036.36		sal2	0.6289	121.91	1219.09
	sal3	1.6375	376.37	3763.70		sal3	0.5331	101.24	1012.43
	sal4	1.6416	377.25	3772.50		sal4			
	sal5	1.5438	356.25	3562.53		sal5	0.7383	145.51	1455.09
	sal6	1.55	357.58	3575.85		sal6	0.2673	43.90	439.05
	Dala1	1.9915	452.37	4523.70		Dala1	0.5903	113.58	1135.83
	Dala2	1.8423	420.34	4203.38		Dala2	0.767	151.70	1517.00
	Dala3	1.761	402.88	4028.84		Dala3	0.5972	115.07	1150.71
	Dala4	1.707	391.29	3912.91		Dala4	0.3723	66.56	665.56
	Dala5	1.7397	398.31	3983.11		Dala5	0.1834	25.81	258.06
	Dala6	1.894	431.44	4314.38		Dala6	0.279	46.43	464.29
	Dser1	1.7283	395.86	3958.64		Dser1	0.4077	74.19	741.92
	Dser2	1.5375	354.90	3549.01		Dser2	0.389	70.16	701.58
	Dser3	1.592	366.60	3666.01		Dser3	0.5683	108.84	1088.37
	Dser4	1.6844	386.44	3864.39		Dser4	0.4453	82.30	823.03
	Dser5	1.9088	434.62	4346.15		Dser5	0.8583	171.40	1713.95
	Dser6	1.745	399.45	3994.49		Dser6	0.8089	160.74	1607.39
	Dasp1	1.7296	396.14	3961.43		Dasp1	0.416	75.98	759.83
	Dasp2	1.7111	392.17	3921.71		Dasp2	0.5316	100.92	1009.20
	Dasp3	1.5486	357.28	3572.84		Dasp3	0.8234	163.87	1638.67
	Dasp4	1.6282	374.37	3743.73		Dasp4	0.7982	158.43	1584.31
	Dasp5	1.617	371.97	3719.69		Dasp5	0.4373	80.58	805.77
	Dasp6	1.4313	332.10	3321.01		Dasp6			

Figure 2. Comparison of ACTH ELISA between P3 and P7

		OD 540nm	pg/ml diluted	pg/ml			OD540nm	pg/ml diluted	pg/ml
P5	sal1	1.3846	322.07	3220.75	**P13**	sal1	1.1297	229.94	2299.42
	sal2	1.4736	341.18	3411.82		sal2	1.3372	274.70	2747.04
	sal3	1.3858	322.33	3223.32		sal3	0.9781	197.24	1972.39
	sal4	1.4115	327.85	3278.50		sal4	0.9731	196.16	1961.60
	sal5	1.5638	360.55	3605.47		sal5	0.753	148.68	1486.80
	sal6	1.55	357.58	3575.85		sal6	0.8114	161.28	1612.78
	Dala1	1.4929	345.33	3453.26		Dala1	0.9032	181.08	1810.81
	Dala2	1.2988	303.65	3036.54		Dala2	1.1336	230.78	2307.83
	Dala3	1.4599	338.24	3382.41		Dala3	0.755	149.11	1491.12
	Dala4	1.6159	371.73	3717.33		Dala4	1.387	285.45	2854.47
	Dala5	1.7301	396.25	3962.50		Dala5	0.7057	138.48	1384.77
	Dala6	1.6385	376.58	3765.85		Dala6	0.4591	85.28	852.80
	Dser1	1.5848	365.06	3650.56		Dser1	0.3691	65.87	658.65
	Dser2	1.6275	374.22	3742.23		Dser2	0.6397	124.24	1242.39
	Dser3	1.4928	345.30	3453.04		Dser3	0.8955	179.42	1794.20
	Dser4	1.3875	322.70	3226.97		Dser4	1.1617	236.84	2368.45
	Dser5	1.1722	276.47	2764.75		Dser5	1.3581	279.21	2792.12
	Dser6	1.4128	328.13	3281.29		Dser6	0.7529	148.66	1486.59
	Dasp1	1.5303	353.36	3533.55		Dasp1	0.8078	160.50	1605.02
	Dasp2	1.1672	275.40	2754.01		Dasp2	0.4645	86.44	864.45
	Dasp3	1.3046	304.90	3049.00		Dasp3	0.458	85.04	850.43
	Dasp4	1.1438	270.38	2703.77		Dasp4	0.1869	26.56	265.61
	Dasp5	1.3594	316.66	3166.65		Dasp5	0.9354	188.03	1880.27
	Dasp6	1.2962	303.10	3030.96		Dasp6	0.4245	77.82	778.16

Figure 3. Comparison of ACTH ELISA between P5 and P13

		OD 540nm	pg/ml diluted	pg/ml			OD540nm	pg/ml diluted	pg/ml
P6	sal1	1.9787	449.62	4496.22	**P9**	sal1	0.9614	193.64	1936.36
	sal2	2.1543	487.32	4873.22		sal2	0.4531	83.99	839.86
	sal3	2.0807	471.52	4715.20		sal3	0.7612	150.45	1504.49
	sal4	1.9888	451.79	4517.90		sal4	0.7349	144.78	1447.76
	sal5	2.3011	518.84	5188.38		sal5	0.5828	111.96	1119.65
	sal6	2.2373	505.14	5051.41		sal6	0.951	191.39	1913.93
	Dala1	2.3492	529.16	5291.65		Dala1	0.5112	96.52	965.19
	Dala2	2.2564	509.24	5092.42		Dala2	1.3111	269.07	2690.73
	Dala3	2.1487	486.12	4861.19		Dala3	0.9102	182.59	1825.91
	Dala4	2.0402	462.83	4628.26		Dala4	0.8706	174.05	1740.49
	Dala5	2.1614	488.85	4888.46		Dala5	0.8568	171.07	1710.72
	Dala6	2.5551	573.37	5733.69		Dala6	0.3095	53.01	530.08
	Dser1	2.0178	458.02	4580.16		Dser1	0.3277	56.93	569.34
	Dser2	1.911	435.09	4350.88		Dser2	0.7594	150.06	1500.61
	Dser3	1.9288	438.91	4389.09		Dser3	0.8571	171.14	1711.37
	Dser4	2.5191	565.64	5656.41		Dser4	0.6922	135.56	1355.64
	Dser5	1.8078	412.93	4129.32		Dser5	1.0913	221.66	2216.58
	Dser6	2.1965	496.38	4963.82		Dser6	0.3563	63.10	631.04
	Dasp1	2.2386	505.42	5054.20		Dasp1	0.5092	96.09	960.88
	Dasp2	2.183	493.48	4934.83		Dasp2	0.792	157.09	1570.93
	Dasp3	2.3	518.60	5186.02		Dasp3	0.9353	188.01	1880.06
	Dasp4	2.1788	492.58	4925.82		Dasp4	0.891	178.45	1784.50
	Dasp5	2.5215	566.16	5661.56		Dasp5	0.8291	165.10	1650.96
	Dasp6	2.1893	494.84	4948.36		Dasp6	1.0118	204.51	2045.08

Figure 4. Comparison of ACTH ELISA between P6 and P9

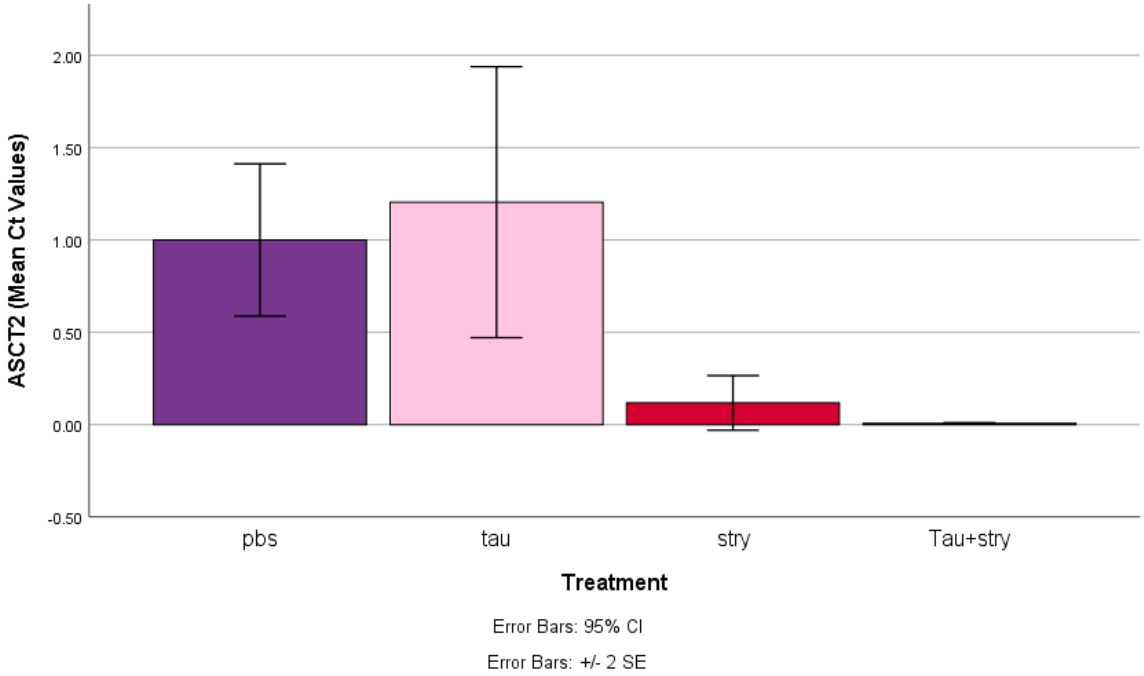


Figure 5. qPCR for mRNA abundance was done on AtT20 cells treated with 500 µM Taurine in the presence or absence of 100 µM Strychnine for 24 hours. ASCT2 levels were not detected in cells treated with Taurine and Strychnine, and were very low for strychnine, but were detectable in Taurine and PBS. Data are mean of 6 replicates.