Genetic Variation Underlying Anxiety-Like Behaviour in Laboratory Mouse Strains

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PhD 2009
Abstract

**Background and Purpose:** Strain differences have previously been observed in the anxiolytic effect of the Angiotensin Type I receptor (AT₁ R) antagonist losartan in mice. Structural variation in key binding sites, AT₁ receptor binding, AT₁ expression, the effect of a microsatellite in the promoter region of the gene and the influence of angiotensin receptor associated protein (ATRAP) were investigated for association with this observed behavioural variation.

Strain differences in the ability of Angiotensin IV (Ang IV) to improve learning and memory in rats, possibly via an effect on Insulin regulated aminopeptidase has also previously been identified. This study explored potential variation between mouse strains in response to Ang IV and the role of Insulin regulated aminopeptidase (IRAP) and Aminopeptidase N (ApN) in conferring a response. Strain differences in cognitive behaviour due to enzyme activity, inhibitory effect of Ang IV, structural variation in key binding and catalytic domains and expression of the encoding genes for IRAP and ApN were explored.

**Experimental Approach:** Investigations into strain differences in receptor gene sequence and function by gene sequencing and radioligand binding assays respectively and the influence of a microsatellite in the promoter region were carried out. In addition, the role of AT₁ receptor gene expression using a probe based RT-qPCR assay was also investigated. Cognitive effects of Ang IV were assessed using novel object recognition (NOR). IRAP activity and the inhibitory effect of Ang IV were assessed *in vitro* by catalysis of leucine-p-nitroanilide. RT-qPCR and gene sequencing was used for expression and sequence analysis of IRAP and ApN.

**Key Results:** Strain differences in the anxiolytic response to losartan are not attributed to differences in AT₁ receptor sequence variation (AGTR1A: MGI:87964 *n*=6 for BKW *n*=3 for C57/DBA₂). Radioligand binding assays support this result and show no difference in the IC₅₀ of losartan between the strains. The BKW mouse strain displays higher AT₁ expression in
comparison to its counterpart strains, which may account for the increased anxiety and response to losartan. However, this increased expression is not related to microsatellite size in the promoter region \((n=6)\). This study also uncovered a non-synonymous Single Nucleotide Polymorphism (SNP) in DBA\(_2\) and BKW mice which results in a V157A mutation in the Angiotensin II receptor associated protein (ATRAP). Strain differences in the behavioural response to Ang IV were also observed; Two-Way ANOVA revealed that there was a significant effect of Ang IV on NOR \((F=5.15 \ P<0.05)\), where mice of C57 strain showed the best recall and DBA\(_2\) worst \((P<0.05 \text{ Tukey-Kramer})\); while CD mice showed no significant improvement after Ang IV administration in NOR. Non linear regression analysis of aminopeptidase activity showed a significant difference between strains \((F= 22.64 \ P<0.001, \ \text{C57, DBA}_2 \ n=3 \ \text{CD} \ n=6)\). The aminopeptidase \(K_m\) from mice of CD strain differ from C57 and DBA\(_2\) counterparts. Inhibition of aminopeptidase activity by Ang IV was not significantly different across strains \((F=2.10 \ P<0.131)\). There were no differences in the amino acid sequences of IRAP nor ApN \((n=3)\). RT-qPCR analysis showed no difference in IRAP expression across strains but an increased expression of ApN of 40% was observed in CD mice \((P=0.001)\).

**Conclusions and implications:** Strain differences in anxiety may be related to levels of \(\text{AT}_1\) receptor expression. \(\text{AT}_1\) receptor expression is not, however, directly influenced by microsatellite size in the promoter region. The variable \(\text{AT}_1\) receptor expression in BKW and DBA\(_2\) mice may be related to a functional polymorphism in ATRAP although the functional role of this SNP has not been investigated in this study. There are also strain differences in the effect of Ang IV on object recognition in mice. The lack of cognitive response of CD mice to Ang IV cannot be explained through variation within IRAP sequence nor expression. The involvement of ApN in the mechanism of Ang IV remains to be elucidated.
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Acknowledgements

First and foremost, I am eternally grateful to my supervisors Dr Paul Gard and Dr Andrew Overall, without whom my thesis would not exist. They have always been available and willing to devote time and effort to answer my many questions and provide support far beyond the call of duty. They have taught me a great deal about scientific understanding and reasoning, cricket and the importance of a drink in the quest for scientific knowledge. Thank you.

Advice, moral support and enjoyable time in the lab has also been eased thanks to Drs, Jimi, Adu, Marcus Allen, Charlie Chaterjee, Adam Heikal, Jon Mabley Mark Yeoman and Greg Scutt to whom I am most grateful. Thanks extend to Dave Campbell and Roger Clayton for technical support in the lab to allow me to carry out these experiments. In addition technical support and discussions was also sought thanks to Dr Heidi Demaegd and Professor Georges Vauquelin from the Vrije Universiteit Brussels. Thanks also to Graham Brown for the use of the novel object recognition raw data.

Furthermore, thanks to my fellow PhD students for discussions and coffees.

IKC must receive a mention for 2.5 years spent together throughout my thesis life.

Finally, thanks to my parents for everything they have done.

_______

If you’re not part of the solution, you’re part of the precipitate.

_______
Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text (see Chapter 3.7.3), is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

Signed

Dated
Chapter 1

Introduction
1.1 Introduction

The renin angiotensin system (RAS) is a complex endocrine and enzymic cascade that plays a major role in blood pressure regulation, cardiovascular homeostasis, fluid and electrolyte balance and regulation of the sympathetic nervous system (Dinh et al. 2001). Aside from these classical effects, a diverse range of agonists and antagonists of the RAS have been implicated in human and animal behaviours including depression, anxiety and cognition (Gard 2002).

Anxiety represents a complex psychiatric disease that has a prevalence of 10-15% in Western populations and involves an intricate genetic underpinning which accounts for its variability in both humans and animals (Flint 2003). The current pharmacological treatment guidelines for anxiety set by the National Institute for Health and Clinical Excellence (NICE) include the use of Benzodiazepines (BZ), selective serotonin reuptake inhibitors (SSRI) and tricyclic antidepressants; although the latter are not licensed, they are shown to be effective in the treatment of anxiety (www.nice.org.uk 2007). However, BZs are associated with drowsiness, dependence and have a limited use of no longer than 4 weeks, while SSRIs take time before they are effective and are associated with cardiovascular side effects (Gelenberg 2000). Buspirone, a serotonin 5HT1A partial agonist (Jaronczyk et al, 2008) is also used for the treatment of a variety of anxiety-related states in human patients (Groenink et al, 2003) and is specifically effective in the treatment of generalized anxiety disorder (GAD). Buspirone, a azapirone, affects the pre- and post-synaptic serotonin receptors and upon binding to the 5-HT1A presynaptic receptors, reduces synaptic serotonin. Post-synaptically, buspirone is a partial 5-HT1A receptor agonist but also indirectly influences 5-HT2 receptors and interacts with other central nervous system neurotransmitters. It also has an affect on dopaminergic and noradrenergic neurons where it reduces their firing rate. It is however, the decrease in serotonergic activity that is mainly responsible for its anxiolytic effects (Anderer et al, 2000).
Administration of angiotensin type 1 receptor (AT₁R) antagonists has previously been shown to reduce anxiety in rodents and represent a potential novel class of anxiolytic drugs.

As well as demonstrating anxiolytic effects, drugs targeted at other areas of the RAS have shown promising effects on cognition. Cognitive decline is prevalent in many neurological diseases, including Alzheimer’s Disease, Parkinson’s Disease and age-associated dementia, for which currently only a few specific treatments exist (Albiston et al, 2004). Most of the treatments are anticholinesterase inhibitors, the effectiveness of which is limited. The Angiotensin IV receptor (AT₄) and its associated ligands, notably Angiotensin IV and its homologues, provide a potential alternative and novel treatment for cognitive decline in these diseases. This has been demonstrated by their ability to improve cognition and reverse both physically and chemically-induced amnesia in rodents in a variety of cognitive paradigms. The exact mechanism by which these ligands exert their effect is unknown and currently the suggested mechanisms are contradictory. This thesis will explore some of the causes of variation in the RAS underlying murine inter-strain differences in anxiety and cognitive phenotypes.

1.1.1 Renin Angiotensin System

The renin angiotensin system (RAS) is a complex bioenzymic cascade comprised of multiple mediators, multiple-functional enzymes and multiple receptor systems (McKinley et al. 2003). Three distinct RAS systems have been identified: systemic (Dinh et al. 2001), local (Danser 2003) and brain (Baltatu et al. 2003); where nearly all of the RAS components are produced and regulated independently of the systemic circulating system.

The identification of a discrete brain RAS is attributed to the fact that key components of the RAS such as renin, angiotensinogen and angiotensin peptides do not pass from the blood to the interstitium of the brain.
Within the brain the circumventricular organs (CVO) and parts of the hypothalamus have permeable fenestrated capillaries in the blood brain barrier and receptors located here are influenced by peripheral angiotensins (McKinley et al. 2003). Angiotensinogen, the only known source of angiotensins, is also present in the brain and forms the first step in the RAS cascade (Baltatu et al. 2003) (Figure 1.1). It is the brain specific RAS that is responsible for the reported behavioural effects of drugs that perturb the actions of both angiotensin peptides and receptors.

**Figure 1.1. Formation of peptides via the Renin angiotensin system cascade.**

Diagram representing the multiple pathways involved in the metabolism of Angiotensinogen to form the multiple effector peptides and subsequent degradation pathways.

1.1.2 Angiotensinogen

Angiotensinogen, coded for by a single gene Agt, is the only known substrate for the aspartyl protease renin (Morgan et al. 1996) and acts as the precursor of the decapeptide angiotensin I, which forms the rate limiting step in the subsequent production of angiotensin II. Angiotensinogen itself is synthesised in the liver and most regions of the brain, with discrete regions producing more than others (cerebellum and hypothalamus), and forms a constituent part of extracellular fluid and cerebrospinal fluid (CSF).

1.1.3 Renin

Renin, encoded by a single gene Ren1 (Coffman 1998), serves to transform angiotensinogen to the decapeptide angiotensin I by cleaving a leucine-valine bond in the N-terminal region between residues 10 and 11. It is a glycoproteolytic enzyme and is responsible for the first step in the formation of angiotensin II (Griendling et al. 1993) (Figure 1.1). In the human and rodent brain renin, is expressed in two forms: one secreted either as active renin from the secretory/storage granules, or is formed extracellularly from secreted, inactive prorenin; the second is non-secreted, brain-specific intracellular renin (Karamyan et al. 2007). It has previously been shown that while the brain synthesises a novel renin isoform (Bader et al. 20002) whether this synthesising enzyme in the brain is the ‘true’ renin is still under discussion (Bader et al. 2008); while renin immunoreactivity is high in the hypothalamus, renin mRNA levels are exceptionally low (Baltatu et al. 2003). Several other peptidases in addition to renin including cathepsins E and D are also capable of cleaving angiotensinogen (Karamyan et al. 2007) and renin itself can also be converted from kidney derived pro-renin via proteolytic cleavage.
1.1.4 Angiotensin I

Angiotensin I (Ang I) is a decapeptide (Asp¹-Arg²-Val³-Tyr⁴-Ile⁵-His⁶-Pro⁷-Phe⁸-His⁹-Leu¹⁰) liberated by renin cleavage of a leucine-valine bond in the N-terminal region of angiotensinogen. It has no known biological effects but forms the substrate for the angiotensin-converting enzyme (ACE) and is the precursor of angiotensin II.

1.1.5 Angiotensin Converting Enzyme

Angiotensin converting enzyme (ACE) is a dipeptidyl carboxypeptidase that cleaves the C terminal of Ang I to form the potent vasoconstrictor angiotensin II (Ang II) (Griendling et al. 1993). ACE is found throughout the central nervous system (CNS) and in the brain is found extensively in the circumventricular organs where Ang I from the periphery can be locally converted to angiotensin II to affect receptors located here (Mckinley 2003). ACE is also capable of cleaving peptides without a free C-terminal carboxy group including substance P and neurotensin (Skidgel et al. 2004) and also inactivates the vasoactive peptide bradykinin (BK) via its conversion to BK-(1-7) and further to BK-(1-5) (Kuoppala et al. 2000).

A homologue of ACE, ACE-2, has also been identified that shows 42% homology with ACE and has a different biochemical profile. It is also highly specific for Ang I cleavage and also allows the formation of Angiotensin 1-7 (Asp¹-Arg²-Val³-Tyr⁴-Ile⁵-His⁶-Pro⁷) (Rice et al. 2004).

1.1.6 Angiotensin II

The octapeptide angiotensin II (Asp¹-Arg²-Val³-Tyr⁴-Ile⁵-His⁶-Pro⁷-Phe⁸) is the major biological metabolite involved in RAS signalling (Dinh et al. 2001) formed by the removal of two terminal (His⁹-Leu¹⁰) residues from angiotensin I by ACE. It is the major effector peptide for AT₁ receptors through which it mediates its actions. Circulating Ang II regulates blood pressure and electrolyte balance by mediating vascular tone, aldosterone...
secretion, renal sodium handling, thirst, water intake, and sympathetic activity and vasopressin release. Further to these classical effects, angiotensin II is also implemented in cardiovascular remodelling (via AT₂ receptors), renal function and mediating lutenising hormone release (Phillips et al. 1995). Through the AT₁ receptor, angiotensin II can also stimulate multiple signalling pathways, activate several growth factor receptors and promote the formation of reactive oxygen species (ROS) and other pro-inflammatory responses (Riberiro-Oliverira et al. 2008). It is also suggested to play a role in behavioural effects of anxiety, depression and cognition (Gard 2002).

1.2 Angiotensin Receptors - Receptor binding

The actions of Angiotensin II are mediated by the Angiotensin receptors that are specific seven-transmembrane helices that form G protein coupled receptors (Hauser et al. 1998). These receptors are divided into two pharmacological classes: AT₁ and AT₂ with AT₁ receptors mediating nearly all of the physiological effects of Ang II (Timmermans et al. 1995). Neuronal AT₁ receptors are primarily localised in specific nuclei of the hypothalamus and brainstem including the sub fornical organ (SFO), supra optic nucleus, paraventricular nucleus (PVN), supra optic nuclei (SON), the rostral ventral lateral medulla (RVLM), the nucleus tractus solitari (NTS) (Summers et al, 2002). Receptors are also located in the spinal cord, dorsal root ganglia and the sciatic nerve (Pavel et al, 2008); and on both noradrenergic and adrenergic neurons (Yang et al, 1997).

The functional role of the AT₂ receptors is not fully understood, but they are generally reported to mediate opposing or counterbalancing effects of the AT₁ receptors including vasodilatation, nitric oxide release and inhibition of cell growth (Fyhrquist et al. 2008). Although they exhibit a similar binding affinity for Ang II, AT₁ and AT₂ share only 32-34% identity at the amino acid level and pharmacologically AT₁ receptors exhibit a low affinity to tetrahydroimidazolepyridines and a high affinity for a class of compounds called “sartans”. Conversely, AT₂ receptors show a very low
affinity for biphenylimidazoles and a high affinity for tetrahydroimidazole-pyridines. A further aspect that differentiates the two is the localisation of AT$_2$ receptors. AT$_2$ receptors are localized in numerous embryonic and neonatal tissues but their expression declines rapidly after birth and is then restricted to certain organs and brain areas. In the adult rat, AT$_2$ receptors are localized in high quantities in peripheral organs such as the kidney, uterus, and adrenal medulla (Gallinat et al. 2000).

Mice have two isoforms of the AT$_1$ receptors which share 94% genetic similarity: AT$_{1a}$ localised in the brain and AT$_{1b}$ predominantly found in glandular tissue, although both are thought to regulate the same biological function (Baltatu et al. 2003). These two isoforms exhibit high sequence identity in the coding region, with most of the amino acid differences being restricted to the intracellular COOH–terminal domain. The binding signatures of the two isoforms are essentially indistinguishable from each other.

Figure 1.2. Receptors of the Renin Angiotensin System.
The receptors of the RAS, effector peptides, associated signalling cascades and the resulting events. These include Angiotensin II, III and IV and the AT$_{1r}$, AT$_2$ and AT$_4$ receptor. Adapted from Vauquelin et al. 2002.
other despite being the products of different genes, namely: Agrt1a and Agrt1b, which are differentially expressed and regulated (Davisson et al. 2000). In the brain AT$_{1A}$ expression exceeds that of AT$_{1B}$ except in the pituitary gland. This differential localization and unique neural circuitry for AT$_{1A}$ and AT$_{1B}$ is coupled with differences in downstream ligand-receptor interaction and signalling that may explain the different functions of these isoforms (Burson et al. 1994). The different isoforms also have unique functions with AT$_{1A}$ mainly regulating blood pressure effects and AT$_{1B}$ being involved in the drinking response. However the AT$_1$ isoforms present in rodents are absent in humans, where a single AT$_1$ receptor is present.

The human AT$_1$ gene is structurally similar to the mouse AT$_1$ gene being composed of five exons and four introns, which have been found to be highly polymorphic (Baudin 2004). Recently, identification of three variants of the AT$_1$ receptor gene that induce a loss of function phenotype has been found. The three variants show decreased affinity and functional potencies for Ang II and the anti-hypertensive agents irbesartan and telmisartan. The substitution of the amino acid Glycine (G) for Arginine (R) at position 45 (G45R) results in a variant that shows reduced but detectable cellular expression, failure to bind Ang II and a complete lack of signalling. F204S showed reduced cell surface expression, binding affinities and potency and efficacy in functional assays; while the C289W variant located in the seventh transmembrane domain showed reduced surface expression and decreased binding affinities (Hansen et al. 2004).

1.2.2 AT$_1$ Receptor binding

The binding of peptide and non-peptide ligands to the AT$_1$ receptor are defined by specific binding residues within the extracellular (EC) and transmembrane domains (TM) that form the receptor itself (Figure 1.3). Peptide ligands bind to transmembrane helices and extracellular loops, while non-peptide antagonists bind to a common pocket within the transmembrane region of the receptor; providing the basis for the
antagonism by ‘Sartan’ compounds, such as losartan (Zoumpoulakis et al. 2003).

Multiple residues within the pharmacophore contribute to the integrity of the binding pocket and are required for both optimal agonism and antagonism. Specificity of both Ang II binding and losartan appears to produce a partial overlap of the binding site between non-peptide and peptide ligands, currently described as an overlapping but non-identical binding site model (Noda et al. 1995).

Ang II binding in contrast to losartan binding involves different epitopes located in the N-terminal extension of the receptor, adjacent to the top of TMI and in the third extracellular loop close to the top of TMVII. Substitution of residues in these domains results in a 5000-20,000 fold decrease in affinity for the peptide agonist Ang II, yet does not affect the binding of non-peptide antagonists (Hjorth et al. 1994).

Figure 1.3. Diagram of Rat AT$_{1A}$ receptor.

Schematic diagram of rat AT$_{1A}$ receptor, solid circles represent amino acid residues conserved across species (Human, Bovine, rat, mouse, turkey and Xenopus as well as between species subtypes) Image source: Hjorth et al, 1994.
The binding of peptide ligands to the receptor also involves interactions with the extracellular regions, while most of the non-peptide ligands appear to interact with residues in the transmembrane helices that form the binding pocket. The binding of non-peptide antagonists is specifically confined to the region between the transmembrane regions, with essentially no contribution from the extracellular domains. It is accepted that the residues required for non-peptide binding are not critical for the binding of peptide antagonists (Hunyady et al. 1996). It is important to note therefore, that while the non-peptide binding domain is distinct from the receptor domain involved in Ang II binding (Kwang-Lae et al. 2002), a degree of overlap in binding domains does occur allowing the competitive binding nature of the antagonists.

The amphibian Ang II receptors also bind peptide ligands with similar affinity to mammals, but have an affinity for losartan several orders of magnitude lower, suggesting the non-peptide binding domain is largely distinct from the receptor domain involved in Ang II binding. This difference may arise as Ang II is a larger compound than the AT$_1$ antagonists and therefore is likely to involve the extracellular loops of the receptor. Receptor mutagenesis studies exemplify several important common features of peptide receptors wherein there is overlap, but non-identity of binding peptide and non-peptide ligands. It is apparent that peptide ligands are bound by transmembrane helices and extracellular loops, while non-peptide antagonists bind to a common site within the transmembrane region of the receptor (Flower. 1999).

Binding of the tetrazole anion (CN$_4$H$_2$ side group) of losartan involves multiple contacts in the receptor including Lys$^{199}$ and His$^{256}$ (Karnick et al, 1996). Studies investigating the replacement of Asn$^{111}$ and Ser$^{115}$ within TM-III show this does not alter the binding affinity for peptidic analogues but, interestingly, modifies the ability of the receptor to interact with losartan (Bihoreau et al. 1993). Mutational studies have highlighted the importance of specific residues in transmembrane domains III, IV, V, VI and VII that are involved specifically in losartan binding. This is shown by the marked attenuation in losartan binding in mutant receptors when
substituting the amino acids at specific positions: Val$^{108}$, Ala$^{163}$, Thr$^{198}$, Ser$^{252}$, Leu$^{300}$ and Phe$^{301}$. This defines the binding site of losartan as the epitopes located within the membrane spanning regions of the receptor, which are distinct from the site at which peptide ligands bind (Ji et al. 1994). Further studies have shown that an additional six residues in receptor TMs II (Ala-73), III (Ser-109, Ala-114, Ser-115), VI (Phe-248), and VII (Asn-295) are also important in losartan binding (Ji et al. 1995). Other investigations have extended the range of amino acids to implicate Lys$^{199}$ and His$^{256}$.

The mechanism of binding, while known to involve specific residues in the pharmacophore, is not fully characterised. It is probable that losartan binds to the receptor after insertion and diffusion through membrane bilayers. This involves incorporation and interaction with membrane bilayers and then diffusion and docking to the receptor. The process of losartan binding is summarised thus: interaction of Lys$^{199}$ NH$_3$ group with the tetrazole, interaction of Lys$^{102}$ NH$_3$ group with the hydroxymethyl group of losartan and the interaction of Val$^{108}$ with the biphenyl system. It is concluded that losartan is incorporated into the bilayer interface that is situated within the membrane and laterally diffuses to reach the active site of the AT1 receptor (Zoumpoulakis et al. 2003).

### 1.2.3 Localisation

Distribution and localisation of the AT$_1$ receptors within the brain has been studied extensively. In humans, receptors are primarily found in forebrain regions including the paraventricular nucleus (Barnes et al. 1993) and elsewhere in the body including the adrenals, heart, vasculature and kidney; with distribution in the mouse as with other mammalian species following a similar pattern (Hauser et al. 1998). Binding studies have shown that the affinity of mouse AT$_1$ receptors, for Ang II, is similar to that in rat and human and in the mouse brain, as in other mammalian species, there is discrete localization of both AT$_1$ and AT$_2$ receptors (Table 1.1).
1.2.4 AT₁ signalling

There are five classical signal transduction mechanisms for the AT₁ receptor: activation of phospholipase A₂, phospholipase C, phospholipase D, L type Ca²⁺ channels and inhibition of adenylate cyclase (Dinh et al. 2001). The signalling effects of AT₁ receptors result from the interaction with heterotrimeric G proteins that induce second messengers such as inositol trisphosphate, diacylglycerol and ROS (reactive oxygen species). Various intracellular protein kinases, such as receptor and non-receptor tyrosine kinases and serine/threonine kinases and various other protein kinases are also activated (Higuchi et al. 2007).

Table 1.1. AT₁ and AT₂ Receptor localisation in mammalian brain.

Table showing the localisation of AT₁ and AT₂ receptors in specific nuclei of the mammalian brain. Image source (Gard 2002).
1.3 Angiotensin receptor associated protein (ATRAP)

The angiotensin receptor associated protein (ATRAP/AGTRAP) acts as a negative regulator of AT$_1$ receptor signalling. It is a small membrane protein, 161 amino acids in length and contains three hydrophobic domains at the amino terminal and a hydrophilic cytoplasmic carboxyl tail.

Overexpression of ATRAP results in a marked decrease of agonist induced AT$_{1A}$ mediated activation of phospholipase C and acts as a negative regulator of AT$_1$ receptor signalling (Daviet et al. 1999). Overexpression also reduces the number of AT$_1$ receptors at the cell surface, suggesting a mechanism that promotes downregulation of the receptor (Tanaka et al. 2005) and negatively regulates promoter transcription and protein synthesis. This suggests that ATRAP acts as a negative regulator of AT$_1$ receptor signalling and expression. Similar to other G-protein receptors, AT$_1$ receptors undergo desensitisation and internalisation after binding of agonist (Cui et al. 2000). In vascular smooth muscle cells (VSMC) overexpression of ATRAP does not significantly affect the density of cell surface AT$_1$ receptors; however, Ang II-induced decrease of AT$_1$ receptors is exaggerated, suggesting that ATRAP enhances AT$_1$ receptor internalisation. After ligand binding to the receptor and subsequent activation of heterotrimeric G proteins, additional steps determine the responsiveness of the receptor. These include phosphorylation of intracellular loops and the cytoplasmic tail, which is mediated by G protein receptor kinases (GRKs). This is followed by binding of the receptor to arrestin, internalization of the receptor, and the subsequent dephosphorylation and recycling of receptors to the cell surface. In the case of AT$_1$ receptors, these are internalized via clathrin-coated vesicles (Hunyday et al. 2000). Functionally, ATRAP is able to induce a decrease in the generation of inositol 1,4,5-triphosphate (IP3) in an agonist-dependent manner and enhances AT$_1$ internalisation. Studies suggest a modest but consistent effect of ATRAP on the inhibition of phospholipase C in response to Ang II and a corresponding ability to decrease guanosine triphosphate binding to membrane preparations. These mechanisms
suggest a novel interacting partner of the AT₁ receptor with the potential ability to modulate some of the biological actions of Ang II (Lopez-IIlasaca et al. 2003).

1.4.1 Aminopeptidase A (ApA)

Aminopeptidase A is a homodimeric membrane spanning zinc aminopeptidase that specifically cleaves acidic amino acid residues from the N-terminal aspartyl residue. It forms a critical step in the formation of the heptapeptide Ang III from Ang II and is also implicated in blood pressure regulation and pain perception and is shown to be inhibited by Angiotensin IV (Reaux et al. 2000; Goto et al. 2006).

1.4.2 Angiotensin III (2-8)

Angiotensin III (Ang III) Arg₂-Val³-Tyr⁴-Ile⁵-His⁶-Pro⁷-Phe⁸ normally binds to AT₁ receptors with more affinity than AT₂ receptors (Padia et al. 2007) and elicits pressor effects and pituitary hormone release. However, it is still not established whether these effects of Ang III are due to direct action of this peptide at angiotensin receptors or if they are mediated by smaller peptides formed as a consequence of its degradation.

1.4.3 Aminopeptidase N (ApN)

Aminopeptidase N (ApN/CD13/Anpep EC 3.4.11.2) is a type II zinc dependent integral membrane metallopeptidase and forms a critical step in the formation of Ang IV. It has a ubiquitous distribution throughout the body and is found extensively in the brain and spinal cord. Its physiological role is hydrolysing the unsubstituted N terminal neutral or basic side chains and generally acting on Ang III to form Ang IV; it is also capable of cleaving N terminal residues from Ang IV itself (Ardaillou et al. 1998). In this way it is capable of both Ang IV catabolism and metabolism.
Acting as an ectoenzyme, and forming a dimer via a covalent bond, it anchors its N-terminus to the cell membrane with the catalytic domain outside the cell. The zinc activity is conferred by the binding of a zinc ion via the \[(\text{HEXXH}(X)_{18}E)\] amino acid motif (Kotlo et al. 2007; Luan et al. 2007), which it utilises for peptide cleavage.

1.4.4 Angiotensin IV (Ang 3-8)

Angiotensin IV (Ang IV) is a hexapeptide (Val\(^3\)-Tyr\(^4\)-Ile\(^5\)-His\(^6\)-Pro\(^7\)-Phe\(^8\)) formed by the actions of ApN on Ang III and is a biologically active peptide that binds to the angiotensin IV receptor (AT\(_4\)) identified as the Insulin regulated aminopeptidase (IRAP) (see later section 1.5.1) (Albiston et al. 2001; McKinley et al. 2003). Ang IV has a raft of identified functions including: renal vasodilation, activation of NF-kB and important regulatory functions in cognition, renal metabolism and cardiovascular damage (Fyhrquist et al. 2008). Other effects of Ang IV administration include: increased renal cortical blood flow and mean arterial pressure (Bundel et al. 2009), increased cerebral blood flow via nitric oxide (NO) release (Naveri et al. 1994; Krammar et al. 1997; Krammar et al. 1998) and effects on cell proliferation.

1.5 AT\(_4\) receptor

The C-terminal 3–8 hexapeptide fragment of angiotensin II (Ang IV) was initially found to bind saturably, reversibly, specifically and with high affinity to membrane-binding sites in a variety of tissues from many species (Swanson et al. 1992). Distinct binding sites were later defined on vascular smooth muscle cells (Kaiser et al. 1992), heart membranes and guinea pig brain. These binding sites were found to be pharmacologically distinct from the classic angiotensin receptors (AT\(_1\) or AT\(_2\)) and show different ligand specificities; high affinity (1-10 nM) for Ang IV and much lower affinity for Ang II and related antagonists (> 10\(^{-6}\) nM) as well as distinct tissue distribution (Harding et al. 1994). The characterisation and isolation of the specific AT\(_4\) receptor was achieved in bovine adrenal
Glands by covalent photoactive labelling of an Ang IV analogue (Zhang et al. 1998).

Distribution of the receptor has been identified in rat, guinea pig and primate brain sections, with Ang IV-specific binding along the lateral olfactory tract, cerebral cortex, dentate gyrus, medial habenula, thalamic nuclei, including anteriomedial, posteromedial and ventromedial nuclei, superior and inferior colliculi, cerebellum and periaqueductal grey and less intense labelling in arcuate nucleus of the hypothalamus and locus coeruleus in the mesencephalon and pons (Roberts et al. 1995). Similar distributions were found in the monkey, in areas involved in motor, sensory and cholinergic systems, which implies a major and integral role in the function of in the central nervous system (Moeller et al. 1996). Immunohistochemistry confirmed the earlier findings by Roberts et al 1995 in rats (see Table 1.2)(Fernando et al. 2008).
<table>
<thead>
<tr>
<th>Brain region</th>
<th>Relative labelling</th>
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<td>Olfactory region</td>
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<td>Basal lateral nucleus</td>
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<td>Main olfactory bulb</td>
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<td>Anterior olfactory nucleus</td>
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<td>Islands of Calleja</td>
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<td>Olfactory tubercle</td>
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<td>Basal ganglia</td>
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<td>Nucleus accumbens shell</td>
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<td>Caudate-putamen</td>
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<td>Globus pallidus</td>
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<td>Subthalamic nucleus</td>
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<td>Substantia nigra (pars compacta)</td>
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<td>Amygdala</td>
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<td>Central nucleus</td>
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<td>Lateral nucleus</td>
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Table 1.2. Localisation of the AT₄ receptor

Table showing the localisation of AT₄ receptors and the relative labelling in the rat. Image adapted from (Fernando et al. 2008).
The work of Albiston et al (Albiston et al. 2001) utilising specific peptide sequencing showed that the AT₄ receptor was in fact insulin regulated aminopeptidase (IRAP) and demonstrated that IRAP transfected cells gained AT₄ receptors. IRAP distribution in the brain also parallels that of the AT₄ receptor and AT₄ receptor ligands inhibit IRAP activity. IRAP distribution is not however limited to the brain and is also found in the heart, adrenal cortex and vascular smooth muscle (Moelller et al. 1998).

1.5.1 Insulin regulated aminopeptidase -IRAP

IRAP is a type II membrane-bound leucine aminopeptidase classified as a member of the M1 family of zinc metallopeptidases (Turner et al. 2002). Nine mammalian aminopeptidases belong to this family with high degrees of sequence homology around two conserved motifs. Human genes for these enzymes are located contiguously around chromosome 5q15 suggesting a gene duplication and divergence over evolutionary time. The characterized motifs consist of a catalytic domain; the exopeptidase motif formed of five amino acids (GAMEN) and a second zinc binding motif (HEXXH(X)₁₈E). The zinc binding motif comprises two histidine residues and a second glutamate residue, which together form the two zinc coordinating residues (Goto et al. 2006). The extracellular catalytic site formed of the GAMEN motif stabilises peptides in the cleft and the zinc domain allows specific leucine cleavage via hydrolysis.

Mutational analysis at both of these sites has shown an effect on peptide binding and enzyme activity (Laustsen et al. 2001; Axen et al. 2007). A similar effect is seen in the reduced catalytic activity of other structurally similar aminopeptidases on mutation of the zinc binding domains (Wang et al. 1993). Site directed mutation of the structural GAMEN domain also results in marked affects on enzyme activity. The Met, Glu and Asn residues are essential for enzyme activity while Gly and Ala are important for the binding of substrate and inhibitors (Katrien et al. 2007). Investigations using the zinc ion chelator 1,10-phenanthroline highlight the importance of this motif as zinc ion chelation reduces catalytic activity (Laeremans et al. 2005). Peptide inhibitors, while not
substrates for the enzyme, bind competitively to the GAMEN catalytic site and inhibit its catalytic activity. Interestingly, and somewhat paradoxically, Ang IV only binds with high affinity to the Zn$^{2+}$-deleted apo enzyme, while enzymic activity requires Zn$^{2+}$ to allow substrate hydrolysis (Demaegdt et al. 2004a).

The localisation of IRAP is often associated with the glucose uptake transporters 4 (GLUT4) and immunohistochemical techniques have revealed that the expression of IRAP is restricted to specific cell types in different tissues. A specific characterisation of IRAP is its distinct intracellular localisation and the ability to redistribute to the cell surface in response to a variety of stimuli (Keller 2003). Specifically, Demaegdt has demonstrated the ability of insulin to produce a time and concentration dependent increase in the exposure of IRAP at the surface of recombinant HEK293 cells (Demaegdt et al. 2008).

1.5.2 IRAP trafficking

In the absence of insulin, IRAP is localised to secretory vesicles and retained in the vesicle membrane. In this basal state, cargo proteins slowly recycle to and from the cell surface membrane. However, insulin stimulation increases the rate of exocytosis, resulting in a marked redistribution of these proteins to the cell surface (Liu et al. 1995). In fat and muscle cells insulin causes a marked translocation of GLUT4 from an intracellular location to the plasma membrane. Due to the colocalisation of IRAP and GLUT4, IRAP also markedly translocates in response to insulin. This effect is reportedly due to the cytoplasmic carboxy terminal domain of GLUT4, which contains a motif that directs its intracellular retention in unstimulated cells; this same motif also occurs in the cytoplasmic tail of IRAP (Ross et al. 1997). After de novo synthesis both IRAP and GLUT4 are sorted into GLUT4 storage vesicles that continuously move to and from the plasma membrane. In response to insulin these vesicles undergo robust exocytosis to the plasma membrane to deliver both GLUT4 and IRAP to proteolyse specific circulating hormones (Tsung-Yin et al. 2007). This insulin regulated translocation results in a 6-8 fold increase in IRAP and a 10-20 fold increase in GLUT4 at the plasma membrane (Wallis et al. 2007).
As well as the trafficking effect of insulin, vasopressin and oxytocin can induce the translocation of IRAP to the plasma membrane. Vasopressin and oxytocin, acting via their respective receptors (V₂R and OTR), are able to induce a two to three fold increase of IRAP at the cell surface for vasopressin and a three to four fold increase for oxytocin (Nakamura et al. 2000; Masuda et al. 2003).

1.5.3 IRAP substrates

IRAP has a range of in vivo substrates, although the endogenous ligand for IRAP is not fully known; Lys-bradykinin, Ang III, met-enkephalin, dynorphin A, neurokinin A and neuromedin B are however hydrolysed by IRAP in vitro (Matsumoto et al. 2001). The use of IRAP knockout mice (-/-) has shown that the NH₂ terminal cleavage of vasopressin is performed by IRAP and that this regulates circulating levels of vasopressin; insulin therefore increases the clearance of vasopressin (Keller 2004). Interestingly, the processing of oxytocin and Ang IV is not fully ablated in IRAP -/-, and the processing of Ang IV is due to the ubiquitously expressed aminopeptidase N.

1.5.4 IRAP signaling mechanisms

The signaling mechanisms of IRAP via Ang IV are poorly defined. It is therefore difficult to elucidate whether the signaling response seen is directly attributable to IRAP or results from IRAP inhibition preventing the degradation of other peptides, thereby enhancing efficacy at their cognate receptors. This is particularly prevalent as in some instances, concentrations of Ang IV in the nanomolar range can also activate AT₁ receptors and induce signalling effects that are blocked by losartan (Li et al. 2005).

1.5.5 IRAP and ApN

IRAP and ApN both belong to the same class of type II zinc aminopeptidases characterised by their catalytic and zinc binding domains. They can be
discriminated from each other based on their $K_m$ ratios for the synthetic substrates, L-Leu-$\beta$NA and L-Ala-$\beta$NA, while IRAP showed a clear preference for L-Leu-$\beta$NA; ApN is non-selective (Demaegdt et al. 2006). Specific differentiation of IRAP from ApN can be facilitated using $[^{125}\text{I}]-\text{Ang IV}$, but while useful, only binds with high affinity to the Zn$^{2+}$-depleted ‘apo-enzyme’ that the divalent cation chelators EDTA and 1,10-PHE act in a synergistic fashion to produce (Demaegdt et al. 2004a); $[^{125}\text{I}]-\text{Ang IV}$ binding to IRAP does not therefore occur under physiological condition.

1.5.6 Hemorphins

Hemorphins represent naturally occurring peptides with a N-terminal valine or leucine and a C-terminal phenylalanine that are potent competitors of both AT$_4$ receptors and the catalytic site of ApN. Thus, Ang IV and hemorphins share the same receptor and the same degradation pathway and hemorphins can act as endogenous analogues of Ang IV. Indeed, LVV-hemorphin 7 and Ang IV possess similar structures and both can serve as substrates for ApN, decreasing the rate of hydrolysis of synthetic substrates. Any hemorphin possessing a valine or a leucine at their N-terminal end is therefore a substrate for ApN and inhibits competitively the activity of the enzyme (Garreau et al. 1998). Another feature of ApN is its ability to induce a transmembrane signal transduction pathway that results in intracellular calcium release, an effect inhibited by tyrosine kinase inhibitors. The in vivo ligand that induces the cascade is yet to be defined, however (Santos et al. 2000).

Aminopeptidase B, an aminopeptidase in the same family as ApN, is also able to synthesise Ang IV from Ang III but is only present in the cytosol and thus is not likely to play any role in the degradation of extracellular peptides (Ardaillou et al. 1998)
1.6.1 Anxiety Behaviour

Anxiety is a normal reaction to threatening situations and serves as a physiological protective function; pathological anxiety, however, is an extension of this normal physiological response but with a bias to interpret ambiguous situations as threatening, either by avoiding situations that are perceived to be harmful or by exaggerated reactions to threat (Wood et al. 2001). This manifests as an emotional state that is subjectively experienced as unpleasant or threatening and encompasses changes in mood and cognition that are generally accompanied by physiological and behavioural changes including palpitations, sweating and hypervigilance. Typically this response occurs to various stressors (either physiological or environmental) and although it is normally a common emotional phenomenon in the human population it can often reach a pathological state (Clement 1998). At this point anxiety becomes irrational, disruptive and interferes with the ability to function normally (Pratt 1992).

There is support for a genetic underpinning in anxiety disorders seen by familial aggregation that is to some extent heritable. This view is reinforced by twin studies which implicate a genetic source of this aggregation (Hettema et al. 2001). The underlying causes of anxiety are diverse: dopamine, serotonin, corticotrophin releasing hormone (CRH), genetic factors, gamma-aminobutyric acid (GABA) and various neuropeptides are all implicated as causative mechanisms of the disease; but none are yet implicated as the major causative factor.

1.6.2 Animal models of anxiety

A few classical models to test anxiety in rodents are routinely used: the elevated plus maze (EPM), light/dark choice test (LD) and the open field test (OF) all exploit the natural aversion of rodents to exposed fields (Carola et al. 2002).

The EPM is based on the conflict displayed by rodents between the drive to explore a new environment and the fear of open elevated areas (Hinojosa et al. 2006). It is one of the best documented and pharmacologically validated animal
models of anxiety; in which both rats and mice perform similarly and strain distribution analyses indicate that there is a genetic contribution to basal levels of anxiety (Finn et al. 2003).

Rodents in the EPM express anxiogenic behaviour via open arm avoidance, which can be pharmacologically mediated via anxiolytics and anxiogenics. It is important to highlight that pharmacological agents that reduce anxiogenic behaviour are not sedative, i.e. the number of crossings between closed arms and the centre platform remains similar for pre and post treatment. The mouse EPM highlights anxiety through comparisons in percentage of open arm entries and time spent in open arms before and after treatment. Anxiolytics aim to reduce open arm avoidance selectively without inducing sedation (Rodgers 1997).

The light/dark aversion test is an alternative to the EPM, based on the natural aversion of mice to brightly illuminated areas and on their spontaneous exploratory behaviour in novel environments. The number of entries into the bright chamber and the duration of time spent there are used as indices of bright-space anxiety in mice. The light/dark transition test and elevated plus maze tests are reported, however, to assess different aspects of anxiety-like behaviour, such as bright-space anxiety and open-space anxiety-like behaviour (Takao et al. 2006).

1.6.3 AT₁ Antagonists and Anxiety

The selective AT₁ antagonists have displayed anti-anxiety properties after administration to rodents in a variety of paradigms. Pre-treatment of mice in the LD aversion test with losartan 45 minutes before testing induced anxiolytic behaviour that was devoid of any sedative side-effects (Barnes et al. 1990). A similar effect is also reported by Gard et al (2001) using losartan in the LD and EPM.

In rats, losartan administered alone, and followed five minutes later with Ang II, has shown a significant decrease in anxiety, but only in tests using aversive
stimulation (passive and active avoidance) (Kulakowska et al. 1996). This effect is demonstrated by earlier work where an anxiolytic response to losartan was seen in rats which importantly did not influence blood pressure, produce sedation or muscle relaxation and was suggested to involve the AT$_1$ receptor subtype (Kaiser et al. 1992). An anxiolytic response is also found with administration of candesartan, an alternative AT$_1$ antagonist, to rats which also significantly enhanced the time spent in open arms of the EPM, showing a clear anxiolytic effect. This led to the hypothesis that antagonism of brain AT$_1$ receptors could lead to a new treatment of anxiety (Saavedra et al. 2005; Saavedra et al. 2006). Indeed, AT$_1$ receptor blockade of hypertensive rats has previously been shown to produce a beneficial effect in controlling anxiety and may be attributed to better central effect and pre-synaptic regulation of neurotransmitter release. In this case, specific blockade of AT1 receptors by losartan was more effective and produced a far more pronounced effect than blocking the precursor enzyme by ACE inhibitors (Srinivasan et al. 2003).

1.6.4 Angiotensin II and anxiety

While AT$_1$ antagonists show an anxiolytic effect, the reverse is true for Ang II and the AT$_2$ receptor, which demonstrate anxiogenic effects. Ang II administration microinjected into the right CA1 hippocampal area of rats, at dose of 0.5µg, in the absence of an AT$_1$ antagonist produced pronounced anxiogenic effects in the EPM (Belcheva et al. 1998). AT$_2$ knockout mice also demonstrate increased anxiety-like behaviour via a mechanism thought to involve neuromodulation of $\alpha_1$ adrenoreceptors via AT$_1$ receptors (Okuyama et al. 1999). This increased anxiety-like behaviour was also suppressed by the ACE inhibitor captopril.

The role of angiotensin II in the context of anxiety can be seen further in experimentally induced hypertensive rats. Induced hypertension, by renal vein occlusion, significantly decreased the number of entries and time spent in open arms, but did not alter the closed arm activity when compared to normotensive animals. Losartan at doses of 5mg/Kg and 10mg/Kg produced significant anxiolytic activity in these normotensive animals (Srinivasan et al. 2003).
Interestingly, losartan produced anxiolytic activity in normotensives only at higher doses, whereas in the hypertensives the anxiolytic effect was seen in a dose dependent manner. Importantly, losartan, enalapril and diazepam gave a better anxiolytic response in hypertensive animals, indicating a higher tone of angiotensin II and decreased BZ/GABA system in the hypertensive state.

Overexpression of AT$_1$ receptors in mice lacking AT$_2$ receptors also affects behaviour and results in increased levels of anxiety, suggesting that AT$_1$ receptor stimulation specifically enhances anxiety and specifically that these receptors regulate the autonomic and hormonal as well as the behavioural response (Okuyama et al. 1999).

In support of this hypothesis, an increase in both plasma and brain angiotensins (Ang I and II) can be seen in the transgenic rat TGR(mREN-2)27, in which the entire ren-2 gene from the DBA mouse is inserted into their genetic profile. This results in elevated renin levels in the adrenals, increased angiotensin I and II levels in both the medulla and the hypothalamus and an altered ratio of brain angiotensins. Behaviourally the (mREN-2)27 rat is of a more anxiogenic nature and shows a different behavioural profile compared to its outbred controls on the EPM. Again administration of captopril to the transgenic (mREN-2)27 rat, reversed their anxiety-like behaviour (Wilson et al. 1996).

1.6.5 Angiotensin Converting Enzyme (ACE) and Anxiety

In line with the involvement of RAS systems in anxiety, treatment with the angiotensin converting enzyme (ACE) inhibitor, enalapril, shows a significant decrease in hyperactivity and anxiogenicity in animals tested in the EPM. However, the direct blocking of the AT1 receptor may have a better effect in attenuating anxiogenicity in hypertensives rather than inhibiting enzyme action (Srinivasan et al. 2003). The role of the RAS in anxiety is not limited to rodents as efficacious effects are also seen in humans. Treatment of individuals with high arterial blood pressure, that significantly associates with anxiety, with the ACE inhibitors enalapril and captopril were seen to reverse anxiogenic effects (Braszko et al. 2004).
1.6.6 Suggested involvement of AT₁ receptor in anxiety

The hypothalamus-pituitary-axis (HPA) is an interactive system that regulates hormone release in response to stress. The release of corticotrophin releasing factor (CRF) by the hypothalamus causes the pituitary to release adrenocorticotrophin hormone (ACTH) and facilitates glucocorticoid release (Matthes et al. 2008). Glucocorticoid receptors are specifically implicated in the regulation of anxiety and despair-related behaviours but different signalling pathways or circuits modulate the responses (Boyle et al. 2006). The HPA axis itself is also directly implicated in anxiety as abnormalities show increased CRF levels in some disorders.

AT₁ receptor antagonists are reported to exert their anti-stress and anti-anxiety properties by modulating, in part, the HPA axis and three interacting cortical systems, CRF, GABA Type A receptors (GABA₆), and noradrenaline (Saavedra et al. 2006). Stimulation of AT₁ receptors in the paraventricular nucleus (PVN) by Ang II increases CRF production and is important for the induction of anxiety; indeed, CRF₁ receptor antagonists decrease stress-induced anxiety (Holsboer et al. 2008).

The inhibition by AT₁ receptors is sufficient to block stress-induced changes in CRF₁ receptors and restores the inhibitory effect of the cortical GABA₆ system. In the cortex, CRF negatively modulates the activity of the GABA₆ complex that forms the main central inhibitory system. The effects of CRF antagonists are similar to the effect of benzodiazepines, which stimulate central BZ sites as part of the GABA₆ receptor complex and are shown to be effective in anxiety treatment. Stimulation of central BZ receptors increases the affinity of GABA for its binding site through positive allosteric effects, potentiating GABAergic transmission. Conversely, a decrease in BZ binding also decreases GABAergic transmission and leads to stress and anxiety (Nutt et al. 2001).

The CRF and GABA systems are tightly interconnected and in the PVN GABA₆ receptors co-localise with CRF neurons. The PVN is also a brain region that shows discrete localisation and high numbers of AT₁ receptors and importantly is where losartan has been shown to cross the blood brain barrier and exert its
effects (Li et al. 1993; Polidori et al. 1996). AT₁ receptors are also concentrated in all key hypothalamic areas of the HPA axis (Tsutsumi et al. 1991) and are all of the AT₁A subtype (Johren et al. 1995; 1996). Also, numerous AT₁ receptors are present in the SFO, a region outside the blood brain barrier and capable of sending projections to the PVN in the hypothalamus (Bains et al. 1995). The AT₁ receptors in the CVO are sensitive to peripheral circulating Ang II and AT₁ receptors in the PVN as with other areas inside the blood brain barrier, respond to stimulation by brain derived Ang II. These peripherally and centrally responding brain AT₁ receptors are also inter-connected through the receptor band running through the lamina terminalis (Saavedra et al. 2005). AT₁ receptor antagonists are anxiolytic, attributed to their ability to influence the HPA axis via AT₁ receptors in these specific brain regions. Ang II can also potentiate neurotransmission; hence, blockade of the Ang II system with specific antagonists may hyperpolarise the membrane potential of sympathetic neurons, resulting in normalisation of their activity. Further, AT₁ receptor facilitates catecholamine release throughout the AT₁ pre-synaptic receptor mechanism and blockade of this via losartan may have a beneficial effect (Srinivasan et al. 2003).

1.6.7 Genetics of Strain Differences

Studies involving comparisons between mouse strains have shown strain differences in anxiety related behaviours (van Gaalen et al. 2000; Carola et al. 2002) and important inter-individual variations in the responses to tests of anxiety. This variability is partially caused by genotypic variations, since different strains react differently when submitted to different tests (Hinojosa et al. 2006). Selection studies of these behavioural traits strongly suggest a genetic influence where many genes contribute to variations in behaviour and differences in baseline anxiety (Lesch 2001; Hinojosa et al. 2006).

Within-strain and inter-strain variation in the responses of rodents to various paradigms of anxiety is evident; while some strains of mice, such as the C57BL/6, have been described as non-emotive, the contrary has been described for the BALB/C and A/J mouse strains (Belzung et al. 2001). While it has been established that differences in strain ranking are sometimes observed in studies
from different laboratories using the same apparatus, an example being the EPM, which strongly depends on lighting conditions (Clement et al. 2002), it is widely accepted that strain variation in behavioural anxiety in animals is prevalent. Many studies do in fact show important within-strain variations in the responses of rodents to tests of anxiety and depression. It is hypothesised, therefore, that inbred strains may harbour alleles that influence many traits, and the phenotype of an individual may be influenced by genetic background in addition to the targeted allele (Homanics et al. 1999).

These comparisons between different inbred strains of mice expose remarkable differences in measures of anxiety-related behaviours; such differences between strains can be attributed to a number of factors, notably environmental influences. Selective breeding of mice for many generations produces differences between high and low anxiety lines that steadily increase within each generation. Selection studies of behavioural traits strongly suggest a genetic influence and the idea that many genes contribute to variations in behaviour (Lesch 2001).

Owing to the fact that anxiety disorders are heritable traits, inter-strain differences in anxiety-related behaviour should not be unexpected. Genetically different strains of mice and rats behave differently in a variety of tests of anxiety. Interstrain comparisons between mice highlight an ‘order’ of anxiety, whereby some mice strains show higher levels of anxiety compared with others. The order of mouse strain, in terms of levels of anxiety-like behaviours, are task dependent and different tests measure different aspects of anxiety (Gordon et al. 2004); this is seen experimentally where strain differences between C57BL/6 and DBA/2J inbred mice. When tested in two different assays of anxiety: open field and the light/dark box, it was established that DBA2 mice exhibit higher basal levels of anxiety-like behaviour (DuBois et al. 2006); a finding that supports the work of Gard et al (2001).

Observations of C57BL/6J and BALB/C also show that mice from the C57BL/6 strain are non-anxious, while mice from the 129 strains are much less active and generally more anxious. Mice from the 129 strain are therefore characterised as more anxious relative to their C57 BL/6 counterparts when tested in LD and
EPM; in addition all three inbred strains differ from outbred Swiss-webster strain (Rodgers et al. 2002). These findings suggest that performance in EPM and LD is markedly influenced by genetic variation between strains.

Analysis of an anxiety test battery involving four mouse strains revealed that strains differed in measures of anxiety-related behaviour, exploratory behaviour and locomotor activity. SW/J mice were the most active, while the A/J mouse strain exhibited a high degree of anxiety-related behaviour and the C57BL/6J an intermediate level. Although a clear strain dependent effect was observed, the order was more variable and task dependent, suggesting that different tasks may measure different aspects of anxiety-related behaviour (van Gaalen et al. 2000). A comparative analysis of anxiety-related behaviour in four inbred mice strains also revealed a genetic spectrum in terms of anxiety levels in which the inbred FVB/N strain was shown to exhibit an extremely low level of anxiety (Kim et al. 2002)

1.6.8 Strain Differences to Losartan

While the involvement of AT$_1$ receptors and the effects of AT$_1$ antagonists have been demonstrated in anxiety, Sheperd et al (1996) could find no support for any functional relationship between selective antagonism of either AT$_1$ or AT$_2$ receptors and an anxiolytic effect. This was in contrast to the anxiolytic response of losartan reported by Barnes et al (1990) and Gard et al (2001). A differing response to losartan has been observed when multiple strains are compared. BKW, C57 and DBA2 mice were observed in the EPM and L/D tests where it was found that BKW mice showed the greatest reduction in anxiety after losartan administration. Isolated descending colon from BKW mice also showed an attenuated contractile response to Ang II leading to the hypothesis that BKW mice have an endogenous functional deficit in Ang II activity, which may render them more susceptible to the effects of Ang II antagonism (Gard et al. 2001). This presents an interesting caveat in that despite the BKW strain displaying the least AT$_1$ receptor function, it was the most responsive to the anxiolytic effects of losartan.
1.6.9 Cytochrome P450

Cytochrome P450 (CYP) are a super family of haemoproteins that function as metabolising enzymes (Miners et al. 1998) and are implicated in the metabolism of losartan. They are arranged into families and subfamilies on the basis of percentage amino acid sequence identity (Nebert et al. 2002). The nomenclature for CYP2C9, one of the cytochrome enzymes responsible for losartan metabolism, adopts the following format: the CYP prefix indicates cytochrome P450; the 2 indicates the genetic family; the C indicates the sub family and the 9 the specific gene.

The non-peptide AT₁ antagonist losartan is metabolised by two specific cytochrome P450 enzymes: CYP2C9 and CYP3A4 (Stearns et al. 1995; Yun et al. 1995). Three pathways have been identified for the metabolism of losartan: 1) oxidation of the alcohol to the carboxylic acid, 2) monohydroxylation of the butyl side chain and 3) glucuronidation of the tetrazole moiety (Stearns et al. 1992). The oxidative biotransformation of losartan generates a metabolite, the carboxylic acid E3174 that is 10-fold more potent than its parent compound—hence the potential relevance of these enzymes in mediating the anxiolytic response to losartan.

Significant species differences exist in the metabolism of losartan. In humans, metabolism is not via a single metabolic pathway, as with the rat and monkey, but via the production of an equal distribution of oxidized and glucuronidated metabolites via different metabolism routes (Stearns et al. 1992). In humans, the gene coding for CYP2C9 carries numerous polymorphisms that have significant functional effects and appreciable frequencies (Kirchheiner et al. 2005). Three examples include Arg144/Ile359, Cys144/Ile459 and Arg144/Leu359. These variants are commonly referred to as CYP2C9*1, CYP2C9*2 and CYP2C9*3.
(Miners et al. 1998). Individuals homozygous for Leu359 (CYP2C9*3) are likely to have impaired clearances of losartan. This effect can be identified by a reduced area under curve clearance. The rate of losartan metabolism and importantly the rate of formation of the more potent intermediate and subsequent clearance is therefore directly affected by polymorphism in these CYP genes. The variability of these enzymes is an important facet to consider when investigating strain dependent effects of drugs. It is important to consider the role of cytochrome enzymes in any strain dependent effects due to the direct effect in influencing drug availability.

1.7.1 Renin angiotensin system and cognition.

Previous reports show that there are strain differences in the effects of Ang IV on cognition in the mouse, as demonstrated by the novel object recognition task (Braszko et al. 2004). Potential bases for the observed strain differences were manifold and include the variable experiences between strains purchased from different suppliers; differences in pharmacokinetics; drug metabolism and genetic differences in the drug targets. The aim of this present study was to examine the effect of Ang IV on novel object recognition in a range of different mouse strains reared under identical conditions, to explore possible pharmacokinetic variability using a dose-response relationship and to investigate possible variation in the molecular structure and function of the AT₄ receptor and the associated enzyme aminopeptidase N (ApN).

The positive effect of Ang IV on behaviour was first proposed in 1986 when Harding et al. suggested that the previously reported effects of Ang II on cognition may be due to the generation of metabolites, as inhibition of aminopeptidase A, which converts Ang II to Ang III, attenuated the response. Braszko et al. (1988) first demonstrated that Ang IV considerably improved learning of conditioned avoidance responses and facilitated recall of passive avoidance behaviour in rats. Memory facilitation by i.c.v. Ang IV administration in passive avoidance conditioning was supported by later work of Wright et al. (1996) and the observation that Ang IV caused a dose dependent improvement in passive avoidance in rats (Tchekalarova et al. 2001). It has now been shown in
rats that the cognitive effects of Ang II occur after its conversion to Ang IV (Braszko 2006). LVV-hemorphin 7, an Ang IV analogue, has also been shown to promote robust enhancing effects on spatial learning and facilitates memory retention and retrieval (Lee et al. 2004) and chronic i.c.v infusion of the Ang IV agonist, Norleucine\(^1\)-Ang IV, also facilitated the rate of acquisition in the water maze, an effect which was blocked by divalinal (Wright et al. 1999). Des-Phe\(^6\)-Ang IV (i.c.v.) shows similar effects to Ang IV on passive and conditioned avoidance, and both improve novel object recognition (Braszko 2004). Further evidence in support of the cognitive effects of Ang IV and its analogues is the reversal of scopolamine- (Pederson et al. 1998; Pederson et al. 2001; Albiston et al. 2004), ischemia- (Wright et al. 1996) and knife lesion-induced deficits in the water maze (Wright et al. 1999).

Ang IV and its analogues also significantly enhance long term potentiation (LTP) in vitro in a dose and time dependent manner which can be prevented by pre-treatment with divalinal (Wayner et al. 2001). Activation of AT\(_4\) receptors by Nle\(^1\)-AngIV has also been demonstrated to enhance synaptic transmission and LTP (Kramar et al. 2001).

The definitive mechanisms underlying the effects of Ang IV on cognition are unclear, and a matter of some ongoing debate but Albiston et al (2001; 2003) speculated that the cognitive effect of Ang IV results from inhibition of the enzyme IRAP, which protected bioactive peptides from cleavage. This preserves such in vivo substrates as oxytocin and vasopressin, both of which have effects on learning and memory in their own right (De Weid et al. 1987; Lew et al. 2003). IRAP is synonymous with oxytocinase (OTase), leucyl/cystinyl aminopeptidase (Lnpep) and placental leucine aminopeptidase (P-LAP) and specific binding and inhibition of the catalytic activity of IRAP by Ang IV has been demonstrated (Lew et al. 2003; Demaegdt et al. 2004b; Demaegdt et al. 2006; Axen et al. 2007) and this is thought to be a viable mechanism for the cognitive improvement (McKinley et al. 2003).

Vauquelin (2008), and indeed Wright et al (2008b), however, argue that the concentrations of Ang IV and the onset time of cognitive response are incongruent. In rats, Ang IV displays some degree of inhibition of IRAP,
typically at μM concentrations (Stragier et al. 2004), while the physiological and indeed behavioural effects are seen at nM concentrations (Braszko et al. 2004). The onset of physiological effects would also be expected to be slow to allow the accumulation of ligands to proceed; yet Ang IV-mediated effects are rapid; occurring in less than a minute (Wright et al. 2008b).

An alternative mechanism suggested by Vauquelin et al (2008) considers IRAP as a “classical” receptor producing intracellular signalling events in response to ligand activation. Importantly, enzymes other than IRAP demonstrate the ability to form dimerised aminopeptidases to act as receptors; for example, the structurally-related ApN is able to mediate trans-membrane intracellular signalling events. Meanwhile, Wright et al (2008b) suggest a different Ang IV receptor altogether. Following a homology search with Ang IV analogues they identified a similarity to hepatocyte growth factor (HGF) that acts via the tyrosine kinase receptor c-Met. In support of this the authors demonstrated that Norleual, an AT$_4$ receptor antagonist, is capable of inhibiting c-Met in the picomolar range (Wright et al. 2008b).

Other possibilities for the mechanism of action of Ang IV are modulation of neurotransmission of glutaminergic, dopaminergic and cholinergic synapses; regulation of cellular proliferation and gene expression and finally cellular uptake of glucose.

Activation of AT$_4$ receptors produce changes in glutamine release, postsynaptic receptors, or receptor-intracellular signal coupling (Wright et al. 1999) whilst striatal dopamine release is also observed after administration of Ang IV, Divalinal and LVV-hemorphin-7 (Stragier et al. 2004). The importance of functional D$_1$ and D$_2$ dopamine receptors in cognitive effects of Ang IV has also been highlighted (Braszko 2004; 2006). Enhanced depolarisation-evoked release of acetyl choline, which is also implicated in cognition, also occurs following Ang IV or LVV-hemorphin-7 stimulation of AT$_4$ receptors (Lee et al. 2001).

Ang IV and IRAP may also be involved in the regulation of the cellular proliferation and/or gene expression processes that play a key role in consolidation of memory. AT$_4$ ligands can induce DNA synthesis in neuronal...
cell lines and in VSMC Ang IV has also demonstrated the ability to upregulate several genes including ICAM-1, IL-6, TNF-α, MCP-1, and PAI-1 via the NF-κB pathway (Esteban et al. 2005; Ruiz-Ortega et al. 2007) raising the possibility of gene expression changes associated with cognition. Importantly, both NF-κB and TNF-α are specifically implicated in the processes of learning and memory and affect LTP in the hippocampus (Albensi et al. 2000; Bracchi-Ricard et al. 2008).

Finally there is the finding that AT₄/IRAP ligands cause an increase in glucose uptake in pyramidal neurons (Fernando et al. 2008), which supports such a mechanism for cognitive improvement. A recently synthesised selective IRAP inhibitor, HF-419, invokes uptake of glucose into hippocampal neurons and significantly improves novel object recognition in rats (Albiston et al. 2008). Increased availability of glucose within neurons as a means to improve learning and memory is an established concept (Leclerc et al. 2006).

The ability of Ang IV to cross the blood brain barrier has not been investigated but Nle¹-Ang IV, an Ang IV analogue, does not cross the blood brain barrier due to its large size and hydrophilic nature (Wright et al. 2008a). Passive movement across the blood brain barrier is dependent upon size, general hydrophicity and the number of hydrogen bonds. Brain regions susceptible to peripheral administration of Ang IV situated within the CVO were therefore targeted.
1.8 Aims.

Strain differences to the anxiolytic response to losartan were investigated in mice focusing on areas of genetic variation that may influence strain specific responses to losartan. This includes: AT$_1$ receptor-ligand interactions, receptor sequence variation, receptor signalling proteins, cytochrome P450 enzymes and receptor expression differences between strains.

Previously reported strain differences in cognition in response to Ang IV and potential mechanism of cognitive improvement were also investigated. This included differences in the structure of IRAP and ApN at key binding sites, hypothalamic IRAP and ApN gene expression and potential differences in IRAP enzyme activity and inhibition across strains.
Chapter 2

Methods
2.1 General Methods

To investigate strain variation in the RAS associated with anxiety-like behaviour and learning and memory a variety of pharmacological and molecular methods were used. These include radioligand binding, enzyme biochemistry, gene sequencing and gene expression techniques as outlined below.

2.2 Animal Husbandry

Male mice from the following strains: DBA₂, C57, BKW (supplier B&K Universal www.bku.com) and CD-1 mice (supplier Charles river laboratories www.criver.com) were used for all experiments. Mice were housed under identical conditions in North Kent M1/M2 cages on flake sawdust bedding in an air-conditioned room (approx 19°C +/- 1°C; humidity 50% +/-10%) under a 14/10-h light/dark cycle, commencing 07:00 to 21:00. The subjects had free access to food and water.

2.3 Inbred and Outbred mouse strains

Inbred mice are classified as such when they have been mated brother×sister for twenty or more consecutive generations (F20). At 20 generations, on average, at least 98.6% of the loci in each mouse are homozygous (Beck et al. 2000). In contrast, outbred mice are defined as a closed population for at least four generations of genetically variable animals that are bred to maintain minimum inbreeding, while maintaining genetic background of the strain (Chia et al. 2005).

In any genetic study, information on the genetic background of the mouse strain is important in order to rule out involvement of genes known to influence the phenotype of interest. The DBA₂ and C57 mice strains are both inbred; the DBA strain originated in 1909 from a stock segregating for coat colour, while the specific DBA₂ substrain was established in 1929-30 when crosses were made between other DBA substrains; although differences between these other
substrains are probably too large to be accounted for by mutation, they are probably due to substantial residual heterozygosity following the crosses between substrains. The C57 strain was established by Little in 1921 from the mating of female mice from strain 57 with male mice from strain 52 that originate from Miss Abbie Lathrop’s stock. BKW, in contrast to C57 and DBA₂, is an outbred strain. Unfortunately, little information is currently available on its origins or background: originally bred at B&K prior to 1977 and developed from outbred albino mice obtained initially from BDH, ICI and Schofield that has been maintained as a closed colony since (B&K Universal product catalogue, 2005). CD-1 mice were sourced from Charles River where the original group of Swiss mice that served as progenitors of this stock consisted of two male and seven female albino mice derived from a non-inbred stock in the laboratory of Dr. de Coulon, Centre Anticancereux Romand, Lausanne, Switzerland.

http://www.criver.com/.

2.4 Humane treatment of animals

All behavioural experiments were licensed under the UK Scientific Procedures (Animals) Act, 1986.

2.5 Drugs, Chemicals and consumables

Acetic acid (A9967 Sigma UK)
Ammonium persulfate (Sigma UK)
Amplitaq Gold (Applied biosystems)
[tyrosyl-3,5-³H]-Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) 250 µCi (TRK733 Amersham UK)
Angiotensin IV (Val-Tyr-Ile-His-Pro-Phe-OH) (batch number; S: 22-24/25, 0566586) Bachem, (Germany)
Bradford reagent (B6916 Sigma UK)
Chloroform (C7559 Sigma UK)
100bp DNA Ladder (Fisher UK)
dNTP 100mM mix set (Fisher BPE2564 UK)
Ethanol (51976 Sigma UK)
Isopropanol (19516 Sigma UK)
L-Leucine-para-nitroanaline (185310 Sigma UK)
Metaphore agarose (Cambrex UK)
2-Methoxyethanol. (284467 Sigma UK)
Methanol (322415 Sigma UK)
Para-nitroanalide (61910 Sigma UK)
Platinum Taq polymerase (10966-018 Invitrogen UK)
Primers/Probes - MWG biotech (Eurofins) ·
Gel loading dye (Eppendorf)
Sodium Acetate (S2889 Sigma UK)
Starscint Scintillation fluid (Perkin-Elmer)
Turbo DNase I – (Invitrogen UK)
Trizol reagent (15596-026 Invitrogen UK)
IScript Reverse Transcriptase- (Bio-Rad UK)
IQ Supermix for Probes (Bio-Rad UK)
IQ SYBR Green supermix -(Bio-Rad UK)

2.5.1 PCR Consumables

PCR tubes and tips -(Eppendorf UK)
UVettes –(952010077)(Eppendorf UK)

2.5.2 RT-qPCR consumables:

Barrier tips (MBP –UK)
DEPC treated water (Ambion UK)
96-well PCR plates – (BIORAD UK)
Optical sealing tape – (BIORAD UK)

2.5.3 Other

20G Disposable syringe (Fisher Scientific UK)
96 well microplates – (Fisher Scientific UK)
1.5ml microcentrifuge tube (Eppendorf UK)
15ml microcentrifuge tube (Fisher Scientific UK)
50ml Ultracentrifuge tube (Beckman Coulter UK)

2.5.4 Kits

Qiagen DNeasy blood and tissue DNA extraction kit (Qiagen UK)
Qiagen QIAquick PCR cleanup kit (Qiagen UK)
Ready to pour Sequagel 4.5 (National Diagnostics)

2.5.5 Buffers

All salts for buffers - Fisher Scientific UK.

TBE 5X
0.5 M ethylenediaminetetraacetic acid (EDTA), 444.7 mM Boric acid, 445.7 mM Tris base pH 7.5.

Radioligand Binding buffer
150mM Sodium Chloride
50mM Sodium dihydrogen phosphate
10mM Magnesium chloride
5mM EGTA
0.4% (w/v) BSA
IRAP extraction buffer
50mM Tris, 0.25M Sucrose, 140mM NaCl pH 7.5

IRAP enzyme buffer
50mM Tris, 140mM NaCl pH 7.5

2.6 Data analysis and statistical procedures

Statistical comparisons were performed using ANOVA followed by post-hoc Tukey-Kramer and Dunnetts analysis using Minitab 15 (Minitab Solutions). Non linear regression and one site competitive binding analysis was performed using GraphPad prism (GraphPad software U.S.A). Bayesian analysis and RT-QPCR calculations were analysed using Microsoft Excel (Microsoft). $P<0.05$ was considered statistically significant.

2.7 Pharmacological Methods

2.7.1 AT$_1$ Radioligand binding

Radioligand binding uses a radioisotope labelled ligand that associates with a receptor. One site competitive ligand binding assesses a single concentration of labelled ligand in the presence of various concentrations of unlabeled ligand (displacer), in this method [$^3$H]-Angiotensin II is used as the label with varying concentrations of losartan acting as the displacer. This method allows receptor ligand interactions to be investigated and provides information on receptors numbers ($B_{max}$) and ligand affinity (IC$_{50}$). One site competitive binding analysis provides a means to identify any differences in receptor ligand and receptor antagonist interactions.

Mice were sacrificed and the liver dissected, weighed and placed into ice cold buffer (150mM sodium chloride, 50mM sodium dihydrogen phosphate, 10mM magnesium chloride, 5mM EGTA and 0.4%w/v BSA buffered to pH 7.4 with 10M NaOH). Tissue was homogenised in a Ystral GmbH homogeniser at full speed and then centrifuged at 40,000g for 10 minutes at 4°C. Supernatant was
removed and the pellet was resuspended with buffer and centrifuged again at 40,000g for 10 minutes at 4°C. The supernatant was removed and the pellet was subsequently resuspended to 50mg wet weight tissue per ml of buffer. Tissue was then transferred to LP3 tubes at a volume of 0.25ml and was incubated with 0.1ml [³H] radiolabelled angiotensin II at a concentration of 6 µM with buffer and Losartan at concentrations varying from 1 pM to 1 µM. All concentrations were performed in triplicate. Tissue preparations were then incubated at room temperature on a shaking water bath for 1 hour. Samples were then harvested with ice cold buffer using a Brandel harvester through polyethylenimine (PEI) soaked filters to reduce non-specific binding, transferred to scintillation vials containing 2mls of Starcsint scintillation fluid (Perkin-Elmer) and left for 24 hours. Samples were counted in a liquid scintillation counter (Beckman-Coulter) that detects ionizing radiation from the beta emitting tritium. The tritium radionuclide dissipates its energy by decay in the liquid scintillation cocktail containing solvent and fluor. The aromatic solvent absorbs most of the energy of the beta particle, and the energy of excitation of the solvent is transferred to the scintillator (fluor) molecules. On de-excitation, emitted photons of visible light are detected by the Photomultiplier tubes. The intensity of the light flashes is directly proportional to the original nuclear energy dissipated in the flour cocktail and the number of nuclear decays/time or the sample radioactivity is reported as disintegrations per minute (DPM). Usual counting efficiency for tritium is between 30-50% (L’Annunziata, et al 2000).

Initial problems with the binding assays included low binding and erratic and limited displacement. This was sourced to potential discrepancies in freezing of tissues post mortem by the supplier. The duration before freezing could have allowed for significant denaturing of receptors coupled with subsequent thawing of tissue. Repeating the binding experiments with fresh tissue produced more favourable results yet the binding remains low compared to the rat with a high degree of non-specific binding. The Hillslope quantifies the steepness of a competitive binding curve derived using the equation \( Y = \text{Bottom} + \frac{\text{Top}-\text{Bottom}}{1+10^{(X-\text{LogEC50})}} \) where a standard competitive binding curve that follows the law of mass action has a slope of -1.0.
These differences may occur due to differences in receptor density between mice and rats, clearly equal wet-weight of liver comparisons between rat and mice show much greater values and displacement in the rat (Figure 2.1.). The rat binding data reinforce the fact that omission of guanosine triphosphate (GTP) from the binding buffers is acceptable. The addition of GTP or its analogues can steepen G-protein receptor curves, but the rat data suggest acceptable binding is still produced with its omission. The lack of protease inhibitors and the reliance upon low temperatures and an excess of BSA to preserve receptors may also affect binding characteristics if the protease activity is high; however, omitting protease inhibitors from the rat assay still produced high specific binding values.

**Figure 2.1.** One site competitive ligand binding in rat.

One site competitive radioligand binding in rat liver tissue using fixed concentration of $[^3]H$-Angiotensin II and increasing concentrations of losartan. $n=2$ Error bars ± S.E.Mean.
2.7.2 Leucine-aminopeptidase Enzyme Kinetics assay

To determine aminopeptidase activity and the inhibitory effects of Ang IV, a method based on the cleavage of L-leucine-p-nitroanilide into L-leucine and p-nitroaniline was employed (Stragier et al. 2007). The latter P-nitroaniline compound shows a characteristic absorption at 405nm upon cleavage by aminopeptidases. Whole brains were removed immediately post mortem and placed in ice cold extraction buffer (50mM Tris, 0.25M Sucrose, 140mM NaCl pH 7.5). This was followed by homogenization at full speed for one minute with a Ystral homogeniser. Homogenate was centrifuged at 4000g for 8mins at 4°C. The supernatant was removed and centrifuged at 100,000g for 40 minutes at 4°C. The pellet was then washed and re-suspended in assay buffer (50mM Tris, 140mM NaCl pH 7.4). Protein concentration was assayed using Bradford reagent (Sigma-Aldrich, UK). 25μg of membrane homogenate was incubated with varying concentrations of leucine-p-nitroanilide (Sigma-Aldrich UK). Ang IV
inhibition assays were carried out using 1.2mM substrate aliquoted into 96 well plates at 37°C with a range of Ang IV concentrations. The absorbance was read every 10mins using an ASYS plate reader at 405nm. $K_m$ and $IC_{50}$ values were estimated using iterative non-linear regression using Graphpad prism (V4.0 GraphPad Software, San Diego California USA, www.graphpad.com). Mice numbers were as follows: CD: $n=6$ C57/DBA$_2$: $n=3$, in triplicate for $K_m$ and $IC_{50}$.

2.8 Molecular Biology Methods

2.8.1 RT-qPCR

The use of Real Time PCR (RT-qPCR), which uses template DNA derived from messenger RNA (mRNA), allows the quantification of specific gene expression. The variation of mRNA due to changes in gene transcription level can therefore be quantified to identify changes in the regulation of specific target genes. The use of primers specific for a target gene allows a multitude of targets to be identified, providing primers can be designed. All primers were designed to ensure target specificity and to ensure optimal primer conditions.

2.8.2 Primer Design

Primer design for PCR is an important step in optimising the success of amplification of specific template DNA. Important factors to consider during the design of any primer pair is specificity to the flanking regions of the sequence of interest, primer dimer formation, hairpin loop formation, annealing temperature ($T_m$) and the minimization of $T_m$ differences between primer pairs. All of these parameters can be optimised using software-based algorithms and the use of basic logical alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis to ensure primers do not anneal to unwanted and non-specific sites. Primer dimer formation, where primers anneal to one another, must also be avoided as these can cause non-
specific amplification and extension from the primers and not the target sequence. Primer dimer formation can be checked using Gibbs free energy analysis (-ΔG) where a more negative ΔG indicates easier formation of primer dimers. Hairpin loop formation must also be avoided to allow successful priming, in particular large hairpin loop formation where more than three primer bases cross anneal with their complementary bases. For RT-qPCR analysis, amplicon size is usually restricted to 50-175 base pairs to ensure efficient amplification.

Real-time, quantitative or reverse transcription Polymerase Chain Reaction (PCR) is the most powerful and sensitive method for amplifying and detecting trace amounts of mRNA. One of the most important aspects of a RT-qPCR assay is that it is specific for the detection of coding DNA (cDNA) which unlike genomic DNA (gDNA) has the introns spliced out leaving only exons. It is imperative that cDNA is used as the template and not genomic DNA as cDNA is derived from the variable mRNA levels in a particular tissue or cell. The specific detection of cDNA over genomic DNA can be achieved through the use of the enzyme DNase I that removes any contamination by digestion of gDNA. However digestion is never 100% efficient and use of this method alone can produce non-specific amplification of gDNA. For this reason it is important to optimize primer design to ensure primer amplification is cDNA specific. This can be achieved by designing primers on alternate exons such that amplification of gDNA would incorporate an intron than if a cDNA target is amplified. If design allows, the size of the intron should be large enough to not allow amplification, or secondly produce a product that is larger than that generated from cDNA. If, however, introns are too small the design of primers that span exon-exon boundaries with a base pair overlap can be used to ensure that any amplification is cDNA specific. Genomic DNA will not be amplified through non-specific priming. A combination of DNAse I treatment and careful primer design ensures that any PCR amplicon relates to cDNA even if gDNA contamination occurs.

The use of intercalating chemistries in RT-PCR, such as SYBR green, brings with it its own problem, which is the non-descriptive nature of detection. SYBR green, a commonly used intercalating dye, will bind to any double stranded product
including primer dimers. This can lead to non-specific detection of product and must be avoided through careful primer design. The use of melt curve analysis, which shows specific product specificity graphically, should also be used.

Reliable RT-qPCR data requires that primer efficiency is calculated for each primer pair and lies between 90-100%. Optimisation of primers within this range allows for the use of the $2^{\Delta \Delta C_t}$ method and primer pairs must be optimised to produce efficiencies within this range. Primers were designed taking into account the above considerations using PerlPrimer, Primer3 (http://frodo.wi.mit.edu/) and in-silico PCR software (http://genome.ucsc.edu/cgi-bin/hgPcrcommand=start) using sequence data from mouse genome informatics (http://www.mgi.com). List of primer pairs used in this study are listed in tables 2.2-2.8.

2.8.3 Primer pairs.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>MGI Ref</th>
<th>Location</th>
<th>Polypeptide size (amino acids)</th>
<th>Exons</th>
<th>Size (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin II Type 1A Receptor (AT$_{1A}$)</td>
<td>87964</td>
<td>13 30428310-30474736</td>
<td>359</td>
<td>3</td>
<td>46427</td>
</tr>
<tr>
<td>AT$_{1A}$ Promoter Region</td>
<td>87964</td>
<td>13 1000bp downstream from 30428310</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Glyceraldehyde Dehydrogenase (GAPDH)</td>
<td>95640</td>
<td>6 125111869-125116485</td>
<td>333</td>
<td>7</td>
<td>4617</td>
</tr>
<tr>
<td>Insulin Regulated Aminopeptidase (IRAP /Lnpep)</td>
<td>2387123</td>
<td>17 17664687-17761453</td>
<td>1025</td>
<td>18</td>
<td>96767</td>
</tr>
<tr>
<td>Aminopeptidase N (ApN /Anpep)</td>
<td>96749</td>
<td>7 86966689-86993096</td>
<td>966</td>
<td>20</td>
<td>26408</td>
</tr>
<tr>
<td>Angiotensin Receptor associated Protein (ATRAP)</td>
<td>339977</td>
<td>4 147451170-147462140</td>
<td>161</td>
<td>5</td>
<td>10971</td>
</tr>
<tr>
<td>Cytochrome P450 (CYP2C55)</td>
<td>1919332</td>
<td>19 39081509-39117183</td>
<td>490</td>
<td>9</td>
<td>35675</td>
</tr>
</tbody>
</table>

Table 2.1. Gene regions and locations.

Table showing the genes studied, the MGI references, chromosome location, base pair, polypeptide size, exon and gene size. Sourced from C57BL/6J strains.
### Table 2.2 Primer Sequences: AT1 Sequencing

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5'→3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1 Main F</td>
<td>CCC TGG CTG ACT TAT GCT TT</td>
</tr>
<tr>
<td>AT1 Main R</td>
<td>TCC CCA GAA AGC CGT AAA A</td>
</tr>
<tr>
<td>AT1 Main Fb</td>
<td>CTT TGT TTT CTC CAG GTG CAT</td>
</tr>
<tr>
<td>AT1 Extracellular F</td>
<td>CCA ACT CAA CCC AGA AAA GC</td>
</tr>
<tr>
<td>AT1 Extracellular R</td>
<td>GAG ACA CGT GAG CAG GAA CA</td>
</tr>
<tr>
<td>AT1 Tail F</td>
<td>GGC GTC ATC CAT GAC TGT AA</td>
</tr>
<tr>
<td>AT1 Tail R</td>
<td>ATT GCC AGC AGG CTT TGA AC</td>
</tr>
</tbody>
</table>

Table 2.2. AT1 sequencing primers

### Table 2.3 AT1 Microsatellite

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5'→3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1 Microsat F</td>
<td>TCT GGC CTC GTG ATA CAC TG</td>
</tr>
<tr>
<td>AT1 Microsat R</td>
<td>TTT ACA CGT GGC TCA AGC TG</td>
</tr>
<tr>
<td>AT1 Microsat F4</td>
<td>GGT GAC AGG CAG ATG ATG TTT ATG</td>
</tr>
<tr>
<td>AT1 Microsat R4</td>
<td>TCA GAT GTG GTG ACA AGG AAC CTA C</td>
</tr>
</tbody>
</table>

Table 2.3. AT1 Microsatellite primers
### Table 2.4 Cytochrome P450 Sequencing

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp 55 Ex 7 F</td>
<td>GGG AGG GGA GTT TGT GTG TT</td>
</tr>
<tr>
<td>Cyp 55 Ex 7 R</td>
<td>TGT TGG TGG AAG AGT AGA ATC A</td>
</tr>
<tr>
<td>Cyp 55 Ex 8 F</td>
<td>CTC CAT TTC CTG TCC CTG AA</td>
</tr>
<tr>
<td>Cyp 55 Ex 8R</td>
<td>AAC AGT GCC CCC AAA AAG TT</td>
</tr>
</tbody>
</table>

Table 2.4. Cytochrome P450 Sequencing primers.

### Table 2.5 IRAP/ApN Sequencing

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRAP Ex 2 F</td>
<td>TGA AGA AGA GCC AGA TGT GGT A</td>
</tr>
<tr>
<td>IRAP Ex 2 R</td>
<td>GCT GGA TGA GTT CTG CTG AC</td>
</tr>
<tr>
<td>IRAP Ex 6 F</td>
<td>ATT GCT GAT CGG GTC TTC CT</td>
</tr>
<tr>
<td>IRAP Ex 6 R</td>
<td>GGG CGT TTA TGA GAT GAG ACA</td>
</tr>
<tr>
<td>IRAP Ex 7 F</td>
<td>TCT TCA ATT AGC CTG ATA GTC CA</td>
</tr>
<tr>
<td>IRAP Ex 7 R</td>
<td>ATG TGG CTA TTC CCT CCT CA</td>
</tr>
<tr>
<td>APN 6 F</td>
<td>CAT GGA GGT AGT GCC AGG AT</td>
</tr>
<tr>
<td>APN 6 R</td>
<td>GTC ACA GAC ACT CCC GCT CT</td>
</tr>
<tr>
<td>ApN Ex 7 F</td>
<td>GGT TGG ATG TGA TTA GTG ATG G</td>
</tr>
<tr>
<td>ApN Ex 7 R</td>
<td>AGC TCA GAA GGG GTG TGT CA</td>
</tr>
</tbody>
</table>

Table 2.5. IRAP/ApN Sequencing primers.
### Table 2.6 ApN Transcript Sequencing

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1 F</td>
<td>AGC TAT TCC GAG CTC CCT GT</td>
</tr>
<tr>
<td>S1R</td>
<td>CTG ATG CTC GGA AAT CCT TT</td>
</tr>
<tr>
<td>S2F</td>
<td>CGT CAG TAC GAG ATG GAC AG</td>
</tr>
<tr>
<td>S2R</td>
<td>AAC TGG GGT CTG GTG ACC TA</td>
</tr>
<tr>
<td>S3F</td>
<td>GGG TGA TTA CGC ACT GAA CG</td>
</tr>
<tr>
<td>S3R</td>
<td>CTA TCA TGG ACC GCT GGA TT</td>
</tr>
<tr>
<td>S4F</td>
<td>TAC TCG AAC ACC GTT TAT CTG G</td>
</tr>
<tr>
<td>S4R</td>
<td>CCA TGA AGA GGT ATC TGA AGA AGC</td>
</tr>
<tr>
<td>S5F</td>
<td>GGT CAA AGA GGC GGA GTA CA</td>
</tr>
<tr>
<td>S5R</td>
<td>CTG TTT GAG AAT TAC GGT GG</td>
</tr>
<tr>
<td>S6F</td>
<td>CTC CAC CAT CAT CAG CAT TG</td>
</tr>
<tr>
<td>S6R</td>
<td>GGA TGA AGT CTC CAG CCT GT</td>
</tr>
</tbody>
</table>

Table 2.6. ApN sequencing primers.

### Table 2.7 ATRAP Sequencing

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATRAP F</td>
<td>ATGCACATGTCTGTCTCCTC</td>
</tr>
<tr>
<td>ATRAP R</td>
<td>TATGGGGTAGGAGGGAAAAA</td>
</tr>
</tbody>
</table>

Table 2.7. ATRAP primers
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1 RT F</td>
<td>ATT GCC AGC AAG CTT TGA AC</td>
</tr>
<tr>
<td>AT1 RTR</td>
<td>CCA TCA CCA GAT CAA GTG C</td>
</tr>
<tr>
<td>AT1 RTF2</td>
<td>AGA GTT AAG GGC CAT TTT GCT TT</td>
</tr>
<tr>
<td>AT1 Probe FAM –BHQ1</td>
<td>TGA GAC CAA CTC AAC CCA GAA AAG CA</td>
</tr>
<tr>
<td>GAPDH RT F</td>
<td>ATG TAA CTG TGC CTG CCA G</td>
</tr>
<tr>
<td>GAPDH RT R</td>
<td>GAG AAA CCT GCC AAG TAT GAT GAC</td>
</tr>
<tr>
<td>GAPDH Probe HEX –BHQ1</td>
<td>GAA GGT GGT GAA GCA GGC ATC T</td>
</tr>
<tr>
<td>ApN RT F</td>
<td>ACA CCA CCT CCA CCA TCA TCA G</td>
</tr>
<tr>
<td>ApN RT R</td>
<td>TCC TCC ACC GTA ATT CTC AAA CAG</td>
</tr>
<tr>
<td>IRAP RT F</td>
<td>CAC ATT TAT CTG AGG TCC AGG C</td>
</tr>
<tr>
<td>IRAP RT R</td>
<td>CCT TCA CTC AAC CTT TGC GA</td>
</tr>
</tbody>
</table>

Table 2.8. List of RT-qPCR primers.
2.8.4 DNA Extraction using QIAGEN DNeasy kit

For the extraction of DNA from mice the manufactures protocol was followed: DNA from mice was obtained by taking 10-20mm of tail tissue which was then added to 180µl of buffer ATL with 20µl of protinase K (Qiagen UK), mixed and incubated overnight at 56°C. DNA was extracted using a Qiagen DNAeasy kit (Qiagen UK -69504). The samples were briefly vortexed and added to 200µl of buffer AL. The samples were then vortexed followed by the addition of 200µl of 96-100% ethanol. The mixture was then added to a DNeasy spin column and centrifuged (Eppendorf 5424 –Eppendorf UK) at 6000g for 1 minute. The flow through was discarded and 500µl of buffer AW1 added to the column and re-centrifuged at 6000g for 1 minute. Flow through was again discarded and 500µl of buffer AW2 was added to the column that was then centrifuged for 3 minutes at 20,000g. The column was then placed in a 1.5ml microcentrifuge tube and 200µl of buffer AE was added directly to the DNeasy membrane, incubated at 1 minute at room temperature before centrifuging at 6000g for 1 minute. The sample was then stored at 4°C until required. DNA was quantified on a spectrophotometer (Eppendorf biophotometer) and confirmed by running DNA samples against known Lambda DNA standards on a 2% agarose gel.

2.8.5 PCR -General

PCR conditions were as follows: 1 unit of Invitrogen platinum Taq polymerase, 10ng of purified DNA, 1X PCR buffer (20mM Tris-HCL (pH 8.0) 0.1nM EDTA, 1mM DTT, Stabilizers and 50% w/v glycerol), 200-400nm primer, 1.5mM Mg²⁺, 0.2 mM of each dNTP made up to a final 50µl reaction volume with PCR grade water. Samples then underwent the following thermal cycling conditions in a Hybaid touchdown thermal cycler:

<table>
<thead>
<tr>
<th>Stage 1:</th>
<th>94°C</th>
<th>2 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 2:</td>
<td>94°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>Step 1:</td>
<td>55-62°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>Step 2:</td>
<td>72°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>Stage 3:</td>
<td>72°C</td>
<td>1 minute</td>
</tr>
</tbody>
</table>

x 40 Cycles
* Annealing temperatures vary according to primers. PCR products were electrophoresed on a 2% agarose gel (Fisher UK) for 1 hour at 80V and stained using ethidium bromide.

2.8.6 Extraction and cleanup of PCR product for sequencing

Manufactures protocol followed: single DNA bands (Figure 2.3) were excised from the agarose and cleaned through a Qiagen PCR cleanup kit (Qiagen UK - 28104). DNA fragments were excised with a clean scalpel and the fragments weighed. 3 volumes of buffer QG were added to 1 volume of gel (where 100mg ~100µl). Samples were incubated at 50°C for 10 minutes and vortexed every 2-3 minutes. 1 volume of isopropanol (Fisher UK) was added to the sample and mixed before being transferred to a QIAquick spin column and centrifuged at 10,000g for 1 minute. Flow through was discarded and 500µl of buffer QG was added and re-centrifuged. 750 µl of buffer PE was added to the column and allowed to stand for 5 minutes before centrifugation 10,000g for 1 minute. The flow through was again discarded and the tube centrifuged 10,000g for 1 minute to dry the column. DNA was eluted into a 1.5ml centrifuge tube by adding 30ul of water to the membrane incubating at room temperature for 1 minute and centrifuging at 10,000g for 1 minute.

![Figure 2.3. Example of PCR products for sequencing.](image)

Representative example of specific 400bp PCR products for sequencing. Products were run on a 2% Agarose gel and stained with Ethidium bromide. Bands were excised and cleaned before sequencing. Ladder =1000bp
2.8.7 Precipitation of DNA

Purified DNA samples were ethanol precipitated in 2 volumes of ice-cold absolute ethanol and 1/10 volume 3M Sodium Acetate and stored overnight at −20°C. Samples were then centrifuged at 10,000 g for 15 minutes at 4°C. The supernatant was then removed and the pellet washed twice with 75% ethanol. Samples were vortexed briefly and centrifuged at 10,000 g for 3 minutes between each wash. The sample was then centrifuged at 10,000 g for 15 minutes at 4°C. The supernatant was aspirated using a fine needle and the sample air dried at room temperature. Samples were re-suspended at a concentration of 20ng/100bp in PCR grade water. Samples were then sequenced, using the same forward primer used for the initial PCR, by MWG biotech (Eurofins) and Cogenics UK. Mouse numbers: n=6 for BKW/CD n=3 for C57/DBA2.

2.8.8 Microsatellite detection

Simple sequence repeats (SSR) or Microsatellites are highly polymorphic DNA tracts composed of repetitions of short motifs which when located in gene promoter regions have been shown to influence gene expression.

2.8.9 Metaphor Agarose

Metaphor agarose is a high resolution agarose used in genotyping analysis. To prepare, Metaphor agarose is slowly added to chilled 1X TBE buffer and stirred using a Teflon coated bead. The agarose is allowed to soak in buffer for 15 minutes on ice. The mixture is then weighed and microwaved for 1 minute on full power, allowed to cool and re-heated on medium power for 2 minutes. The solution is removed from the microwave and swirled to mix before being re-heated on high power until the solution boils. The mixture is then held at boiling point for 1 minute or until all the particles are dissolved. Hot water is then added to return the beaker and solution to the original concentration. The mixture is then allowed to cool and ethidium bromide added (7µl/40ml) before being cast. The gel is then allowed to set at 4°C to obtain optimal resolving.
2.8.10 Polyacrylamide Gel.

The resolving capabilities of Metaphor agarose is limited, to detect small differences in microsatellite size polyacrylamide gels, which offer a higher degree of detection (2-3bp differences) over Metaphor, was used.

Polyacrylamide gels for microsatellite analysis were made using Ready to pour Sequagel 4.5 (National Diagnostics). 100ml of polyacrylamide was prepared using 80ml of Sequagel monomer solution (Urea, bisacrylamide and acrylamide (19:1 w/w) and 20ml of complete Sequagel buffer solution (5X TBE and Tetramethylethylenediamine (TEMED in deionised water). To allow polymerisation 800µl of 10% ammonium persulfate (Sigma UK) was added to the mixed monomer and buffer solution, poured between 0.1mm spaced gel plates and allowed to polymerise for 2-3 hours. Bands were then stained using Silver Stain kit (Bio-Rad 161-0449EDU). Gel staining was performed as described in the Amersham manufactures protocol: 400ml of fixative (40% methanol/10% acetic acid v/v) was added to the gel for 30 minutes followed by two washes of a second 400ml fixative solution (10% ethanol/5% acetic acid) for 15 minutes. 200ml of oxidiser was added for 3 minutes followed by three washes of deionised water. This was followed by 200ml of silver reagent added for 15 minutes followed by a wash of deionised water. Finally three washes of 200ml of developer were added for 30 seconds followed by 400ml of stop solution (5% acetic acid) for 5 minutes. Gels were then imaged in a chemi-imager under white light conditions.

2.8.11 RNA isolation for RT PCR assay

Mice were sacrificed, the brains immediately removed, and 50mg of hypothalamic brain tissue was used to isolate total RNA using the guanidine isothiocyanate enhanced TRIzol method (Invitrogen) to isolate RNA from lipid dense tissue. 50mg of brain tissue was added to a centrifuge tube containing 1.0ml of TRIzol per 50mg of tissue and homogenised at full speed for 30 seconds on a Ystral homogeniser. 200µl of chloroform for each 1.0ml of TRIzol was added and mixed for 15seconds followed by incubation at room temperature for 15minutes. A heavy phase lock gel was prepared by centrifuging at 10,000g for 3 minutes. The homogenate was transferred to the phase lock tube and centrifuged at 12,000g for 15 minutes at 4°C. The colourless upper aqueous phase
was removed and transferred to a clean micro centrifuge tube and 0.5ml of isopropanol added per 1.0ml of TRIzol. The solution was incubated at room temperature for 15minutes to allow the RNA to precipitate. The sample was then centrifuged at 12,000g for 15minutes at 4°C, the supernatant removed and the RNA pellet washed twice using 1.0ml of 75% ethanol. The samples were vortexed after the addition of each ethanol wash and centrifuged at 7500g for 3 minutes at 4°C to ensure the pellet is sedimented before alcohol removal. The RNA was allowed to dry for 10 minutes at room temperature and then resuspended in 1μl of ddH2O per 1mg of tissue, vortexed, placed on ice for 10 minutes and then heated at 65°C for 10 minutes before storage at -80°C. The integrity of the RNA was determined spectrophotometrically and only RNA with a A260/A280 ratio above 1.8 was used for reverse transcription. 1ug of RNA was treated with Turbo DNase I (Ambion) before being reverse transcribed using BIO-RAD Iscript RT PCR kit with random oligo(dT) primers. The cycling parameters were 1 cycle at 65 ° C for 5 minutes, 1 cycle at 42° C for 90 minutes and a final step at 85°C for 5 minutes.

2.8.12 RT-qPCR Protocol for Probe Assay.

To examine potential differences in basal levels of the AT1 gene expression across strains, an RT-qPCR method was developed. Any differences were calibrated against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression, which acted as an endogenous control using the 2^\text{-ΔΔCt} method (Livak et al. 2001). For analysis of both genes 2 μl of cDNA template from the reverse transcription reaction was mixed with IQ supermix (100mM KCl, 40mM Tris-HCL, pH 8.4, 0.4mM each dNTP (dATP, dCTP, dGTP, dTTP, 50 U/ml iTaq DNA polymerase, 6mM MgCl2 and stabilisers). This was used for RT-qPCR analysis using a 2 step RT-qPCR cycling parameter optimised for the primer and probe sets to ensure primer efficiency (90-105%) using a BIO-RAD icycler iQ real-time PCR system (Biorad UK). Stage 1: 95°C for 3 minutes; stage 2: 95 ° C 30 seconds, 57 ° C for 45 seconds x35 cycles; stage 3: 72 ° C for 10min. Primers and probes were designed using Perlprimer (Marshall 2004) and were developed to span Intron/Exon boundaries with base overlap. The primers and probe were synthesised by MWG Biotech. The AT1 probe was labelled with a FAM fluorescent label and GAPDH with a HEX fluorescent label (Figure 2.4a). The primers and probe were also designed to be specific to AT1a by avoiding regions of high similarity elsewhere in the genome and designing primers in the 5'
untranslated region (Figure 2.4). Due to the probe chemistries, melt curve analysis is not possible as the probe is not an intercalating dye. Agarose gel analysis of single products was also carried out which show single PCR products GAPDH 133 base pairs and AT, 147 base pairs (Figure 2.5). \textit{n=3 in triplicate in all cases.}
Figure 2.4. GAPDH and AT₁ RT-qPCR data.

a. Representative example of RT-qPCR amplification plot analysis using BIO-RAD Icycler 4 thermocycler for HEX (GAPDH) probe.

b. Representative example of RT-qPCR amplification plot analysis using BIO-RAD Icycler 4 thermocycler for FAM (AT₁) probe.

Due to the probe chemistry, no melt curve analysis is available.

*Original in colour.*
To examine potential differences in basal levels of the IRAP and ApN gene expression across mouse strains, an RT-qPCR method was developed. Again, differences were calibrated against the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene acting as an endogenous control using the $2^{(\Delta \Delta Ct)}$ method (Livak et al. 2001). For the analysis of IRAP, ApN and GAPDH genes, 2 μl of cDNA template from the reverse transcription reaction was mixed with IQ SYBR green supermix (100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM of each dNTP (dATP, dCTP, dGTP, and dTTP), iTaq DNA polymerase, 50U/ml, 6 mM MgCl$_2$, SYBR Green I 20 nM fluoresein, and stabilizers. This was used for RT-qPCR analysis using a 2 step RT-qPCR (one step denaturing and a combined annealing-extension) cycling parameter as described previously for AT$_{1A}$ gene. Primers were designed using Perlprimer (Marshall 2004) and were developed to span Intron/Exon boundaries with base

![GAPDH 133bp AT1 147bp](image)

**Figure 2.5. Agarose gel analysis of PCR Products.**

Representative example of RT-qPCR amplification products showing single products for GAPDH (133bp) and AT1 (147bp) primer pairs. PCR products were run on a 2% Agarose gel and stained with ethidium bromide. Specific products size was compared to a 100bp ladder. **Original in colour.**

### 2.8.13 RT-qPCR Protocol for SYBR green assay.

To examine potential differences in basal levels of the IRAP and ApN gene expression across mouse strains, an RT-qPCR method was developed. Again, differences were calibrated against the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene acting as an endogenous control using the $2^{(\Delta \Delta Ct)}$ method (Livak et al. 2001). For the analysis of IRAP, ApN and GAPDH genes, 2 μl of cDNA template from the reverse transcription reaction was mixed with IQ SYBR green supermix (100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM of each dNTP (dATP, dCTP, dGTP, and dTTP), iTaq DNA polymerase, 50U/ml, 6 mM MgCl$_2$, SYBR Green I 20 nM fluoresein, and stabilizers. This was used for RT-qPCR analysis using a 2 step RT-qPCR (one step denaturing and a combined annealing-extension) cycling parameter as described previously for AT$_{1A}$ gene. Primers were designed using Perlprimer (Marshall 2004) and were developed to span Intron/Exon boundaries with base
overlap. These primers were also designed to be IRAP and ApN specific by avoiding regions of high homology across aminopeptidases of the same family (Table 4.0).

Confirmation of product specificity by size was established by melt curve analysis (IRAP Figure 2.6b, ApN Figure 2.7b) and agarose gel electrophoresis (BIORAD- UK) revealed single specific PCR products of 133bp, 206bp and 110bp (Figure 2.8), corresponding with GAPDH, IRAP and ApN products respectively. *n=3 in triplicate.*
Figure 2.6. IRAP RT-qPCR data.

a. Representative example of RT-qPCR amplification plot analysis using BIO-RAD iCycler 4 thermocycler and SYBR Green as a fluorophore for IRAP.
b. Melt curve analysis (multiple traces and two individual traces) shows discrete peaks representing specific product amplification. **Original in colour.**
Figure 2.7. ApN RT-qPCR data.

a. Representative example of RT-qPCR amplification plot analysis using BIO-RAD iCycler 4 thermocycler and SYBR Green as a fluorophore for ApN.

b. Melt curve (multiple traces and two individual traces) show 2 discrete peaks representing specific product amplification. Original in colour.
2.9 Statistical Analysis.

Graphpad prism (v4.0 GraphPad Software, San Diego California USA, www.graphpad.com) was used for radioligand binding using an iterative nonlinear regression one-site competitive binding equation.

Normalisation according to an internal reference gene, using the delta delta C\textsubscript{t} method of Livak \textit{et al.} (2001): $\Delta C_t = C_t_{\text{target gene}} - C_t_{\text{reference gene}}$ expression ratio $= 2^{-\Delta \Delta C_t}$.

$\Delta \Delta C_t = \Delta C_t_{\text{target}} - \Delta C_t_{\text{reference}}$. $C_t$ represents the cycle threshold, the point at which fluorescence values increase over basal levels. The data are presented as the fold.

Figure 2.8. Agarose gel analysis of PCR products.

Representative example of RT-qPCR amplification products showing single products for GAPDH (133bp), IRAP (206bp) and ApN (110bp) primer pairs. PCR products were run on a 2% Agarose gel and stained with ethidium bromide. Specific products size was compared to a 100bp ladder. \textit{Original in colour}. 

![Figure 2.8. Agarose gel analysis of PCR products.](image)
change in gene expression normalised to the internal control RNA, in this case GAPDH.

2.9.1 Bayesian Analysis for RT-qPCR.

In this study, additional expression data was used to update prior results using a Bayesian approach. The initial dataset (n=3) is used as a prior distribution of ΔΔCt values that are assumed to be normally distributed with mean and standard deviation. The additional dataset of (n=3) is considered the likelihood of the ΔΔCt values, which are also considered to be normally distributed with mean and standard deviation.

2.10 Behavioural studies

The novel object recognition (NOR) test was selected as a measure of learning and memory as it is not confounded by any effects of reward or punishment and investigates selected drug effects on memory consolidation. Object recognition was determined in mice using a modification of the method previously described for rats by Braszko et al (1997). For testing, mice were placed into an open field (34 x 60 cm), the floor of which was marked with a 3 x 6 grid and in which two identical solid, impermeable objects were positioned at one end of the field, each 10 cm from the adjacent walls. Mice were allowed to habituate for three minutes. One hour later they were again exposed to the same open field and objects, which had been cleaned thoroughly with 50% ethanol, for three minutes. The mice do not receive any drug treatment until after the second exposure. 24 hours later the mice were again exposed to the cleaned field now containing one of the original objects and a novel (different size, shape and colour) object. The position of the novel object alternated left or right but using the same locations to counter-balance any place preference. Final trials were video recorded and time spent exploring each of the objects was recorded manually over three minutes, as was locomotor activity (line crossing). Exploration of the object was defined as sniffing, or approaching within 5mm. Climbing or sitting on the object was not considered as exploration. Learning (Discrimination (D) score) was quantified as the proportion of time spent exploring the novel object compared with the familiar object, taking into account possible confounding effects of changes in locomotor activity. The D score was
calculated as \( \frac{(A-B)}{(A+B)} \) where A is the time spent exploring the novel object and B is the time spent exploring the familiar object.

The effects of Ang IV (4.7, 47 or 470 \( \mu \text{g kg}^{-1} \), s.c. in 10 ml kg\(^{-1}\)) administered immediately after the second training trial, was assessed in comparison to saline controls; \( n=8 \) in all cases.
Chapter 3

Results
Results 3.1

Angiotensin II Radioligand binding.
3.1.1 Introduction

Peripheral administration of the AT\textsubscript{1} antagonist losartan to mice elicits an anxiolytic response when tested with the EPM behavioural paradigm (Barnes et al. 1990; Gard et al. 2001). In rats, this effect is attributed to antagonism of the AT\textsubscript{1} receptor (Saavedra et al. 2005); and in mice the response is strain specific, occurring only in BKW animals (Gard et al. 2001). BKW mice that display an anxiolytic response also show reduced AT\textsubscript{1} receptor signalling. To investigate the strain differences and further define the role of AT\textsubscript{1} receptors and losartan in anxiety and their strain specific effects, a method was developed to explore ligand-receptor interactions of the AT\textsubscript{1} receptor.

A competitive radioligand binding assay allows the investigation of receptor ligand interactions and provides empirical values for total receptor numbers (B\textsubscript{max}) and drug binding affinity (IC\textsubscript{50}). A one site competitive binding assay utilises a single concentration of radiolabeled ligand, in this instance tritiated Angiotensin II, that is displaced with increasing concentrations of an unlabeled antagonist: losartan (Figure 3.1.) (Hulme 1992). This method allows subtle differences in losartan and Angiotensin II interactions with the AT\textsubscript{1} receptor to be explored between strains.
Example of theoretical one site competitive radioligand binding data

3.1.2 Method

Radioligand binding experiments were conducted as described in Chapter 2.6.1 using tritiated Angiotensin II with increasing concentrations of losartan. Liver tissue from C57, DBA₂ and BKW mice were compared (n=3 in triplicate) for each strain. Data analysis was performed using iterative non-linear one site competition analysis: \( Y = \text{bottom} + \frac{(\text{Top-Bottom})}{(1+10^{(X-\text{LogEC50})})} \) using Graph Pad prism 4.0. (GraphPad Software, San Diego California USA, www.graphpad.com)

3.1.3 Results
Three strain one site competitive [3H] Radioligand binding

Figure 3.2. Comparison of Radioligand binding of \(^{3}\)H-Angiotensin II and losartan between strains.

One site competitive binding of tritiated Angiotensin II in the presence of losartan in C57, DBA\(_2\) and BKW mouse strains using a fixed concentration of \(^{3}\)H-Angiotensin II (6 µM) and increasing concentrations of losartan 1pm to 1 µM. \(B_{max}\) of BKW 662, DBA\(_2\) 718 and BKW 782. \(EC_{50}\) BKW 5E\(^{-9}\), DBA\(_2\) 8.3E\(^{-9}\) and C57 7.4E\(^{-9}\). There was no significant difference between strains (P>0.05 F=2.11 2 way -ANOVA). n=3, tested in triplicate. For each mouse the triplicate values are averaged ± S.E.Mean.
3.1.4 Discussion

One site competitive binding analysis shows no significant difference between strains \( P>0.05 \) \( F=2.11 \) and all three strains display similar IC\(_{50}\) values in the nanomolar range. The Bmax values do however vary, with C57 showing the highest total binding (782 dpm) followed by DBA2 (718dpm) and BKW (662 dpm) mice although there was no statistically significant difference (Figure 3.2.). The similarity in the IC\(_{50}\) of losartan between the strains suggests that the differences in the binding of losartan and therefore the ability to displace Angiotensin II from AT\(_1\) R does not account for the observed strain differences in the behavioural response to losartan.

AT\(_1\) receptor expression in mouse brain is low, highlighted by the 4 week duration required for resolving receptors by in-situ hybridisation using a \(^{14}\)C label (Chen et al. 2002). The use of tritium as a radiolabel, was chosen for its safety in handling; yet, while the alternative \(^{14}\)C has a longer \( T_{1/2} \), neither tritium nor \(^{14}\)C provides sufficient sensitivity for detection of AT\(_1\) receptors in the brain. Liver tissue was therefore selected as an alternative due to the higher levels of AT\(_1\) expression (Grove et al. 1993) coupled with the abundance of tissue. Although expression of AT\(_1\) receptors in liver tissue is not representative of brain expression, the data could highlight significant differences in receptor function/structure that are derived from the same encoding gene sequence found in brain receptor genes. It is therefore possible to relate ligand-receptor interactions between locations but this does assume similar post-translational modifications (PTM) such as: \( N \)-glycosylation targeting to cell surface, phosphorylation and internalisation of receptor and \( S \)-nitrosylation. \( S \)-nitrosylation is a process that modifies cysteine residues by nitric oxide and can modulate protein function. This can occur in AT\(_1\) receptors at cysteine 289 located in the transmembrane domain and can modify the affinity of Angiotensin II (Leclerc et al. 2006) and can provide site-specific regulation of the receptor. Although differences across strains are feasible, this has not been explored here due to the lack of purified receptor protein.

The presence of sodium chloride (NaCl) in the buffer may also have an effect on binding data as sodium ions are reported to cause a shift in the conformation from agonist-preferring to antagonist-preferring binding sites (Widdowson et al. 1992). In binding experiments using \(^3\)H Angiotensin II these authors report
higher binding in the absence of NaCl. The buffer used in these experiments used NaCl in relatively high concentrations (150mM), but its effect would also be expected to manifest in the rat ligand binding data. Losartan also recognizes a large population of non-angiotensin binding sites in hepatic membranes that are not found in adrenal membranes (Widdowson *et al.* 1992) and other groups have reported that losartan binds to three or more different sites in the liver (Grove *et al.* 1993), possibly a prostaglandin recognition site. If the ratio of these other non angiotensin binding sites are greater in the mouse compared to the rat, there is the potential for a reduction in the amount of free losartan for the displacement of Angiotensin II in mice. This does not however explain the low $B_{\text{max}}$ values.

The use of $^{125}$I-Angiotensin II (which is able to detect much lower receptor numbers due to increased sensitivity in detection) and the use of brain sections should be considered as an alternative method for detection, which would also highlight any regional and/or density differences.

Subtle differences in receptor structure conferred by differences in amino sequences are only detected by ligand binding when changes result in large differences in $K_m$. Mutational studies specifically targeted at key amino acids in the transmembrane binding domains show only small differences (Hjoth *et al.*, 1996). It is plausible that more subtle differences may not be detected by this method. A more sensitive approach would be the use of proteomics, applying mass spectrometry, matrix-assisted laser desorption/ionization–time of flight (MALDI-ToF) detection (Garvin *et al.* 2000). Although economically more demanding, this method is able to detect mutations in protein structure and can potentially highlight any inter-strain differences in receptor structure.
Results Chapter 3.2

AT₁R sequencing
3.2.1 Introduction

Radioligand binding assays can highlight differences in affinity that may arise from potential differences in key binding domains; however, they do not provide data on potential differences in domains responsible for cell signalling, receptor structure/PTM differences or the domains required for interaction with receptor associated proteins. Therefore, to investigate whether structural differences in the AT1 receptor are responsible for the observed strain differences in the behavioural response to Losartan and to further explore the findings from the ligand binding data, the AT1 receptor gene was sequenced.

In mice the AT1 receptor gene is comprised of 3 exons with the entire open reading frame located on the third exon. Of particular interest were the key peptide and non-peptide binding domains that confer ligand-receptor interactions in transmembrane domains I-V. The receptor gene was sequenced in three sections in order to keep sequenced regions below 500bp to ensure high quality sequencing throughout. These three regions comprised the extracellular tail, the central coding region and the intracellular tail. Two isoforms of the AT1 receptor exist in rodents, AT1A and AT1B, which show 96% homology. To ensure the AT1A isoform was amplified, which is more representative of the human variant, although pharmacologically indistinguishable from the AT1B isoform, primers were designed within the intron that flanks exons 2 and 3 as this region does not show such high degrees of homology between the isoforms and allows the AT1A isoform to be targeted specifically. The high degree of homology in the central coding region does not allow the design of discrete primers and in some samples the sequencing of both the AT1A and AT1B variants were identified as heterozygous bases in the sequence. These variants were matched with both A and B isoforms in the C57 reference sequence.

3.2.2 Method

DNA was extracted as described in Chapter 2.7.4 using a Qiagen DNeasy extraction kit, followed by PCR using the specified reaction conditions and the following primer pairs listed in table 2. Following PCR, products were electrophoresed, stained and the DNA bands excised and cleaned through a
Qiagen PCR cleanup kit and ethanol precipitated before sequencing. To ensure sequencing did not represent PCR errors six different individuals were used and any detected polymorphisms were re-sequenced to safeguard against potential error. The sequence data were then compared with the corresponding genomic sequence online: (AGTR1A: MGI:87964 http://www.informatics.jax.org 2008). 

$\text{n}=6$ for BKW $\text{n}=3$ for C57/DBA2. Sequence data were analysed using Chromas lite and clustal multiple alignment.

### 3.2.3 Results

Sequencing revealed no differences between AT$_{1a}$ genomic sequences between strains. $\text{n}=6$ BKW $\text{n}=3$ C57/DBA2. MGI reference MGI:87964.

![Figure 3.3. Representative sequence data from AT$_{1a}$ receptor gene. (original in colour).](image)

Representative genomic sequencing of AT$_1$ exon 3 gene showing bases 1421-1458. No differences were seen between BKW, C57 and DBA$_2$ mouse strains across the entire AT$_1$ ORF (2284bp). $\text{n}=6$ BKW, $\text{n}=3$ C57 and DBA$_2$. 
3.2.4 Discussion

No differences were observed in the sequences between BKW mice and the reference C57 strain. Comparisons of the sequencing data using ClustalW alignment showed that AT₁ R sequence was identical across strains. The data are in agreement with the radioligand binding data that also show no significant differences in IC₅₀ values between strains. The sequence homology across strains confirms that any strain differences in behaviour are not therefore attributable to ligand-receptor interactions due to variation in the encoding gene. This lack of variation in the AT₁A receptor sequence is therefore suggestive of an alternative mechanism underlying the different response to losartan between strains.

The AT₁ subtypes are however, highly homologous with pharmacologically indistinguishable binding profiles. Brain AT₁A receptor expression exceeds that of AT₁B (except in the pituitary gland); but due to the fact that pharmacological AT₁ receptor antagonists cause equivalent inhibition of both subtypes (Davison et al., 2000), the behavioural effects of the AT₁B subtype cannot be ruled out and indeed the AT₁B receptors may play a role in behaviour.

It would be interesting therefore, to explore the expression of both subtypes to see if strain differences in expression exist, indeed it is possible to design specific 1A and 1B primers, but due to the more restricted expression of AT₁A in the hypothalamus and the possible influence on the HPA axis, the AT₁A subtype was chosen. A more succinct method utilising AT1A and AT1B knockout mouse lines would highlight any phenotypic behavioural differences and further, the effects of AT₁ antagonists in modifying behaviour could be elucidated (Swafford et al. 2006; Cervenka et al. 1996).
Results Chapter 3.3

Cytochrome P450 enzyme polymorphisms
3.3.1 Introduction

Cytochrome P450 (CYP P450) enzymes are responsible for the first step of metabolism of many drugs. In humans, CYP2C9 is the CYP enzyme responsible for the metabolism of Losartan (Yasar et al. 2001). Mice do not possess a direct CYP2C variant, but CYP2C55 is thought to represent its direct homologue and shows ~79% homology when comparing protein sequences using Clustal alignment. The first stage of losartan catabolism results in the formation of a more potent intermediate (EXP3174) than the parent compound (Stearns et al. 1992). Rapid metabolisers may demonstrate increased receptor antagonism compared to that of intermediate or slow variants due to the production of this more potent intermediate.

Two non-synonymous SNP variants are present in the murine CYP2C55 which are located on exon 7 (rs51596326) and exon 8 (rs51015455). The latter SNP resides adjacent to a substrate recognition site (SRS) and is therefore more likely to affect enzyme function (Figure 3.4.). To identify any potential for high or low metabolism variants present in the BKW mouse strain, sequencing of these variants was conducted.
CYP2C55 protein sequence of Exon 7 and 8 showing SNP and SRS.

Exon 7
KVQAEIDHVIGHRSPCMQDRT[H/R]MPYTDAMVHEIQRYID\IPNNVPHAA
TCNVRFRSYFIPK

Exon 8
GT[D/E]LVTSLLTSVLHDDKEFPNPVFDPGHDENGNFKNKSDYFMPF5I

Figure 3.4. CYP2C55 Protein coding sequence.

Schematic representation of the protein coding sequence on exons 7 and 8 of CYP2C55 showing the locations of the non-synonymous SNPs rs51596326 resulting in a Histidine to Arginine and rs51015455 aspartic acid to glutamic acid. The substrate recognition site (SRS) is underlined. Mouse C57/BL6j.

3.3.2 Method

DNA was extracted as described in Chapter 2.7.4 using a DNeasy extraction kit, followed by PCR using the specified reaction conditions and the primer pairs listed in table 2. Following PCR, products were electrophoresed and the DNA bands excised and cleaned through a Qiagen PCR cleanup kit and ethanol precipitated before sequencing. Sequence data were analysed using Chromas lite and clustal multiple alignment. Primers were designed using Perlprimer using Genomic sequence: (CYP2C55: MGI:1919332 http://www.informatics.jax.org 2008). Sequencing by Cogenics UK. n=3 for BKW and compared to C57 reference strain.

3.3.3 Results

No variation was identified in the BKW strains in either of the two CYP2C55 Exons that show non-synonymous SNPs in other strains (n=3). Both Exon 7 (rs51015455) and Exon 8 (rs51596326) show the wild type H variant (Figure 3.5 and 3.6).
Sequence data of rs51596326 SNP: CYP2C55 Exon 7.

Figure 3.5. Representative sequence data from CYP2C55 exon 7.

Representative sequence of CYP2C55, exon 7 showing rs51596326 SNP. Arrow marks the location of the SNP location and shows wild type C variant. BKW, C57 and DBA\(_2\) \(n=3\) (original in colour).

Sequence data of rs51015455 SNP: CYP2C55 Exon 8.

Figure 3.6. Representative sequence data from CYP2C55 exon 8.

Representative sequence of CYP2C55, exon 8 showing rs51015455 SNP. Arrow marks the location of the SNP location and shows wild type G variant. BKW, C57 and DBA\(_2\) \(n=3\) (original in colour).
3.3.4 Discussion

The two non-synonymous SNP variants are not present within the BKW mouse strain. While only looking at two Exons does not rule out variation/mutations in other domains, these regions have been shown to be variable in other mouse strains (dbSNP) and owing to the location in, or adjacent to, a SRS would provide a novel line of investigation and possibly identify high or low CYP450 variants.

Polymorphisms are not always indicative of a functional effect or physiological differences and a more thorough analysis of these enzymes would involve an assay of liver microsomes from each strain. Analysis of Losartan metabolism by CYP enzymes and compound detection by HPLC analysis would identify rapid or slow metabolisms between strains.

Complications now exist however in the true murine orthologue of CYP2C9. This is because the orthologues of 2C9 in mice are difficult to identify as there are 15 CYP2C genes in mice and none of them cluster closer to a single human P450 than any of the others. The most likely result is that the human genes are just paralogs of the 15 mouse genes (David Nelson. 2007, Personal communication). A number of sources also show different variants: Nelson et al (2004) identifies CYP2C37 as the murine 2C9 homologue while other sources suggest CYP2C65 and CYP2C29 (Guo et al. 2006) (Data sourced from www.genecards.org and www.ihop.net). It is therefore difficult to identify which CYP gene to target for Losartan metabolism; even though 80% of mouse genes have a 1:1 orthologus relationship with human genes, there are 147 gene clusters within the genome which represent gene duplications in the mouse.
To identify potential strain differences in the metabolism profiles of losartan and any differences in the production of the intermediate EXP3174, high performance liquid chromatography (HPLC) could be implemented. Indeed HPLC coupled with UV detection can determine the levels of losartan and its active metabolite in plasma in rats (Solner et al, 1998). Administration of losartan across strains followed by the collection and subsequent analysis of plasma would allow area under curve (AUC) clearance, the concentration of a drug in plasma as a function of time, to be calculated between strains. Any differences in the relative conversion of the parent compound losartan, to the intermediate and the subsequent rate of clearance of EXP3174 could be readily identified.

1 Genes in different species evolved from a common ancestor that retains the same function.
2 Genes related by duplication in a genome but evolve new functions.
Results Chapter 3.4

AT₁ R gene Expression
3.4.1 Introduction

The anxiolytic effect of Losartan has previously been demonstrated in BKW mice (Barnes et al. 1990; Gard et al. 2001). The specific importance of AT$_1$ receptors and their role in anxiety is highlighted by Saavedra et al (2005) who proposed that the anxiolytic effect of AT$_1$ antagonism results from the blocking of stress-induced changes in corticotrophin releasing hormone (CRH$_1$) and benzodiazepine (BZ) binding. Variation in the expression of AT$_1$ receptors may therefore be expected to result in different phenotypes of anxiety, whereby more anxious strains would show higher AT$_1$ receptor expression. This would result in increased AT$_1$ receptor stimulation of CRH$_1$ and BZ binding in the hypothalamic-pituitary axis (HPA) and higher brain centres.

Strain differences in anxiety were explored in relation to their expression of AT$_1$ receptor using a RT-qPCR assay. The use of RT-qPCR methods over in situ hybridisation (ISH), which also allows the detection of RNA, was chosen due to the greater degree of sensitivity that RT-qPCR offers. This method also offers greater sensitivity over immunohistochemical techniques, with the ability of RT-qPCR to detect much lower copy numbers per cell.

The subcutaneous (Gard et al. 2001) and oral (Barnes et al. 1990) administration routes of Losartan both result in anxiolytic effects. The circumventricular organs are unique as they lack an effective blood brain barrier and are therefore susceptible to peripheral drug administration. This region also displays the highest number of AT$_1$ receptors in the brain in a discrete subsection - the parvocellular proportion of the para ventricular nucleus, which has efferent connections to the amygdala, an area of importance in anxiety in its own right. It is also directly implicated in anxiety forming a constituent part of the hypothalamic-pituitary-adrenal axis (Saavedra et al, 2006).

3.4.2 Method

A RT-qPCR method was developed to investigate AT$_1$ receptor expression in hypothalamic brain tissue using the method described previously (Methods Section 2.7.12). Initially the use of conventional RT-qPCR intercalating dye chemistries (SYBR green) was problematic due to the non-specific nature of the
dye. The AT\textsubscript{1a} and AT\textsubscript{1b} receptors show high sequence homology in translated domains but lower homology in intronic flanking regions. Careful primer positioning within the 5’ untranslated regions still resulted in some minor non-specific amplification events, even at annealing temperatures above 60°C. The use of probe based chemistries allowed precise amplification of only the AT\textsubscript{1a} isoform by placing one of the primers in the 5’UTR and the probe across the 5’ UTR/exon boundary that displays low homology with the other isoform. This ensured that only amplification of AT\textsubscript{1a} isoform occurred. The relatively low amplification levels of AT\textsubscript{1} receptors in the brain make amplification difficult and probe based detection can also provide higher specificity. Initial expression studies were suggestive of a trend in expression of AT\textsubscript{1} receptor with BKW strains having higher expression levels in comparison to C57 mice, although high amounts of variation confounded analysis.

RNA isolation, Reverse transcription and RT-qPCR was undertaken as described in Chapter 2.7.11 using the primers and probes listed in table 3 (synthesised by MWG Biotech): AT\textsubscript{1} probe labelled with FAM fluorescent label/Black Hole Quencher 1 (BHQ1) and GAPDH with a HEX fluorescent label/Black Hole Quencher 1 (BHQ1). The primers and probe were also designed to be a) cDNA specific and b) specific AT\textsubscript{1a} specific by avoiding regions of high similarity and designing primers in the 5’ untranslated region. \textit{n=3 in triplicate in all cases}

Because RT-qPCR involves comparison of fluorescence between genes of interest and house keeping genes in the same reaction, it is not straightforward to include additional samples from an independent experiment. This is because of differences in threshold levels between samples are difficult to keep constant between reactions. In this study, additional expression data was used to update prior results using a Bayesian approach. The initial dataset \((n=3)\) is used as a prior distribution of \(\Delta\Delta\text{Ct}\) values that are assumed to be normally distributed with mean and standard deviation given in table 2. The additional dataset of \(n=3\) is considered the likelihood that the \(\Delta\Delta\text{Ct}\) values, which are also considered to be normally distributed with mean and standard deviation.
Table 3.1. AT$_1$ Expression data sets

<table>
<thead>
<tr>
<th></th>
<th>C57</th>
<th>DBA$_2$</th>
<th>BKW</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean</strong></td>
<td>1.0806</td>
<td>2.023</td>
<td>2.107</td>
</tr>
<tr>
<td><strong>S.D</strong></td>
<td>0.418</td>
<td>0.951</td>
<td>0.809</td>
</tr>
<tr>
<td><strong>Var</strong></td>
<td>0.175</td>
<td>0.904</td>
<td>0.656</td>
</tr>
<tr>
<td><strong>N</strong></td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 3.2. Bayesian Analysis.

Data from 2nd experiment used to update data from 1st experiment. Comparisons made between posterior $\Delta\Delta$Ct means between strains.
3.4.3 Results

Three strain AT1 RT-qPCR Expression data.

Figure 3.7. AT1 RT-qPCR Relative Expression data between mouse strains.

RT-qPCR expression of AT1a receptors in hypothalamic tissue using a probe based RT-qPCR analysis comparing C57, DBA2 and BKW tissue. Two separate expression experiments show increased AT1 receptor expression in BKW and DBA2 strains compared to C57. GAPDH was used as reference gene. (Two separate experiments. n=3 in triplicate Error bars. ± s.e. mean.)
Bayesian Analysis of both AT₁ expression data sets.

**Figure 3.8.** Graphical representation of AT₁ RT-qPCR expression data.

Bayesian analysis of both AT₁ expression sets. Expression data was used to update prior results. The initial dataset (n=3) is used as a prior distribution of ΔCt values that are assumed to be normally distributed with mean and standard deviation. The additional dataset of n=3 is considered the likelihood that the ΔCt values, which are also considered to be normally distributed with mean and standard deviation. Solid lines represent prior probability distribution, dotted posterior probability distribution for three strains BKW,C57 and DBA₂. (n=6) in triplicate. (original in colour).

Bayesian analysis of prior and posterior expression analysis is summarised in Figure 3.8. Comparisons of C57 and BKW show significant differences between strains, represented by the non-overlapping 95% confidence intervals in Table 3.2 and Figure 3.8. In summary C57 shows significantly different AT₁ R expression levels to BKW and DBA₂ strains.
3.4.4 Discussion

AT₁ expression was variable between strains and showed an order of expression: C57 showed the lowest expression, DBA₂ an intermediate level and BKW the highest ($n=6$) (Figure 3.7 and 3.8.). It is important to note that mRNA level differences may not be indicative of protein levels due to post translational modifications, potential differences in membrane expression and the *in vivo* half lives of proteins (Greenbaum *et al.* 2003). Correlation between mRNA and protein levels has been explored previously and demonstrates a strong relationship between expression data with most permanent protein complexes (Jansen *et al.* 2002). While the data provides preliminary inferences into differences in receptor number, investigations into protein levels via western blotting or $[^{125}]$I radiolabelling of brain sections would be required to fully correlate differences in anxiety and AT₁ receptors. The downstream effect of CRH₁ and HPA effects would also need to be studied. It is also apparent that BKW mouse strain that has previously shown the least response to Ang II (Gard *et al.* 2001), shows the highest level of AT₁ receptor expression.
Results Chapter 3.5

Microsatellite / Promoter Variants
3.5.1 Introduction

Simple sequence repeats (SSR), also known as microsatellites, are mutation prone DNA tracts composed of repetitions of short motifs of 2-6 bases that can influence molecular and phenotypic effects (Kashi et al. 2006). One important example of how microsatellites can possibly influence expression of genes in the brain is observed in the differing social behaviours of voles, where differences in behaviour are linked to variation in the expression of vasopressin receptors (VPR) in the forebrain. The protein coding domain of this receptor is highly conserved between species but variation in VPR expression, and the subsequent different phenotypes in behaviour, are linked to a SSR in the 5’ regulatory region of the gene where much of the SSR is absent in the alternative phenotype (Hammock et al. 2005). Data support SSRs being functionally important and not random and showing, amongst other roles, an effect in regulation of gene activity (Li et al. 2002). Such SSRs with a putative function are commonly located in regulatory regions and many are reported in open reading frame and protein coding domains; while SSR in 5’ UTR regions have been shown to influence gene expression and protein adaptation (Li et al. 2004).

A microsatellite is present in the promoter region of the AT1 receptor gene and has shown inter-strain variation in repeat numbers possibly in concert with a variable G/C region and flanking SNP variants and is reported to influence AT1 gene expression (Wong et al. 2003). The percentage identity plot (pip) in Figure 3.9. represents regions of similarity across species in order to identify conserved segments of DNA including exons, repetitive elements and gene regulatory elements (represented as noncoding sequences between species) (Schwartz et al. 2000) - the logic being that genomic regions of importance, such as functional sites are likely to be conserved across species whereas regions of less importance are expected to show greater variability. Due to the position of the microsatellite in the promoter region, it has the potential to influence gene expression. This variable site was chosen to identify variation across the three strains in this study in order to explain the differences observed in AT1 gene expression and identify a potential marker for anxious phenotypes.
Percentage Identity Plot of AT1a receptor + 5’ 1000bp flank comparison between mouse, rat and human sequence.

Figure 3.9. Percentage identity plot (PIP).
Percentage identity plot (pip) of genomic 5’ Untranslated region (UTR) 1000bp upstream of the AT1a gene comparing mouse, rat and human. Pink shading represent Exons, Blue shading: Microsatellite upstream of Exon 1. The microsatellite is present in both rats and mice, but is absent in humans highlighted by blue shading. Simple sequence repeats are indicated by white boxes and numbered black boxes represent exons. (original in colour).
3.5.2 Method

Initial methods to identify the size of the microsatellite were inconclusive, despite using high resolution gel techniques such as metaphor and polyacrylamide (PA) gels. Although the resolving power of PA is greater than that of metaphor agarose it was still difficult to resolve any differences between amplicon size from the strains as differences have been reported to be as small as 4bp. The PA approach also proved problematic in obtaining useable gels as the transfer of thin PA gels for silver staining often resulted in torn or damaged gels. Although sequencing of complex microsatellites can be problematic due to slippage occurring in sequencing and the presence of G/C rich regions that flank the microsatellite. Slippage produces a mixture of PCR products with various numbers of repeats that can result in multiple and fuzzy bands in gel analysis. In sequencing this is observed as heterozygosity where PCR sequences do not perfectly align, this was not observed and this method proved successful in determining microsatellite size.

DNA was extracted as described in Chapter 2.7.1 using a DNeasy extraction kit, followed by PCR using the specified reaction conditions and the primer pair listed in table 2. Following PCR, products were electrophoresed and the DNA bands excised and cleaned through a Qiagen PCR cleanup kit and ethanol precipitated before sequencing by Cogenics UK. To ensure high quality sequencing, two independent rounds of PCR and sequencing were performed on large template quantity, initially \( n=3 \) followed by a second giving a final \( n=6 \) for BKW/C57/DBA\(_2\) and all sequences aligned in ClustalW. Primers designed using Perlprimer using Genomic sequence: (AGTR1A-1000bp: MGI:87964 http://www.informatics.jax.org 2008).

3.5.3 Results

Sequencing of the microsatellite domain revealed an equal size microsatellite of 260 bases across all strains.
260 base Microsatellite in 5’ promoter region of AT$_{1a}$ receptor.

Figure 3.10. Schematic representation of AGTR1$_a$, Microsatellite.

Schematic representation of 260 base pair microsatellite in AT$_{1a}$ receptor promoter region - 1000bp downstream of exon 1. The compound microsatellite present is represented:


Figure 3.11. Graphic showing 260bp microsatellite in UTR of AT$_{1a}$ gene

Schematic diagram representing the length of the 260bp microsatellite base pair microsatellite in AT$_{1a}$ receptor promoter region 1000bp upstream of exon 1 between C57, DBA$_2$ and BKW strains.
3.5.4 Discussion.

All three strains show variable AT₁ expression as shown by RT-qPCR data (Figure 3.7 and 3.8.). This does not however correlate with variation in microsatellite size as all strains show the same size microsatellite of 260bp (Figure 3.10). This mirrors the length of the microsatellite in the blood pressure high (BPH) mouse strain used by (Wong et al. 2003). While the previous study attributed no specific link between microsatellite size and gene expression levels, the current study also suggests that variable AT₁ receptor expression in the brain is not solely conferred by microsatellite size alone as inter-strain differences exist in gene expression but not microsatellite size. Perhaps the presence of SNPs found by Wong et al (1993) near the promoter region coupled with the variable G/C rich regions upstream of the TATA box are also a factor in influencing expression levels (Wong et al. 2003). The lack of a microsatellite in the AT₁ promoter region in humans questions its role in the regulation of AT₁ expression. The role of the flanking SNPs are therefore worthy of further investigation in their role in AT₁ expression, especially as the AT₁ gene is highly polymorphic between mouse strains, although the amino acid sequence specified by the open reading frame is invariant.
Results Chapter 3.6

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Angiotensin Receptor Associated Protein
3.6.1 Introduction

The AT₁ receptor is able to interact with a variety of downstream proteins including the angiotensin receptor associated protein –ATRAP (Daviet et al. 1999). This protein acts to negatively regulate AT₁ signalling by affecting internalisation of the receptor, reducing inositol lipid levels and decreasing Ang II-mediated c-fos transcription (Cui et al. 2000).

Gard et al. (2001) suggested that the strain differences in the anxiolytic response to Losartan observed in BKW mice was due to a reduction in AT₁ signaling function. The differences observed in AT₁ expression may also be as a consequence of interactions of AT₁ receptor with ATRAP to overcome increased negative regulation. ATRAP, unlike the AT₁B subtype, is present in humans and shows a high homology with murine ATRAP. Acting downstream of the receptor, it can modulate receptor signaling, can down regulate receptor levels at the cell membrane and is able to attenuate Ang II mediated effects. It may therefore provide an interesting translational gene target and a means to mediate AT₁ receptor function supplementary to the effects of Ang II. To investigate any differences in ATRAP, a search of functional SNPs in the mouse dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) was used to identify variable regions of the gene.

3.6.2 Methods

DNA was extracted as described in Chapter 2.7.1 using a DNeasy extraction kit, followed by PCR using the specified reaction conditions and the primer pairs listed in table 2. Following PCR, products were electrophoresed and the DNA bands excised and cleaned through a Qiagen PCR cleanup kit and ethanol precipitated before sequencing by Cogenics UK. Primers designed using Perlprimer using Genomic sequence: (ATRAP: MGI:1339977 http://www.informatics.jax.org 2008). To ensure SNPs were not due to PCR errors, two independent rounds of PCR and sequencing were performed, initially $n=3$ followed by a second giving a final $n=6$ for BKW/C57/DBA₂. Sequence data were analysed using Chromas lite and clustal multiple alignment.
3.6.3 Results

Sequencing of ATRAP hydrophilic domains identified a single base transversion base at position 157 in the hydrophilic tail domain (position 109-161) resulting in an Valine → Alanine (V157A) substitution in BKW and DBA₂ strains.

**ATRAP V¹⁵⁷⁻A Functional mutation Exon 5.**

<table>
<thead>
<tr>
<th>C57</th>
<th>DBA₂ /BKW</th>
</tr>
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<tbody>
<tr>
<td>ENKGQA</td>
<td>ENKGQA</td>
</tr>
<tr>
<td>V¹⁵⁷</td>
<td>A¹⁶¹</td>
</tr>
<tr>
<td>PRGY¹⁶¹</td>
<td>PRGY¹⁶¹</td>
</tr>
</tbody>
</table>

Figure 3.12. Diagrammatic representation of ATRAP V-157-A mutation

Alignment of the amino acids 157-161 of ATRAP in C57, DBA₂ and BKW mice. BKW and DBA₂ mice have a single base transversion that results in a Valine to Alanine substitution at position 157 in DBA₂ and BKW mice (underlined). (n=6).

**Representative genomic sequence data of ATRAP Exon data.**

Figure 3.13. Representative sequence data of ATRAP in C57 strain (original in colour).

Representative sequencing data of ATRAP in C57 mouse strain showing the wild type sequence, the box highlights the sequence coding for Valine.
Figure 3.14. Representative sequence data of a mutation in ATRAP.

Representative sequence data of ATRAP in DBA₂ and BKW strains. A single base transversion T/C (boxed) in BKW and DBA₂ strains compared to C57 results in a single amino acid change -Valine to Alanine. (original in colour).

ATRAP Exon 5 Clustal genomic alignment from BKW, DBA₂ and C57 mice.

<table>
<thead>
<tr>
<th></th>
<th>BKW</th>
<th>GGCCAAGCTG -CCCCCGGGGGT</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBA₂</td>
<td>GGCCAAGCTG -CCCCCGGGGGT</td>
<td></td>
</tr>
<tr>
<td>C57</td>
<td>GGCCAAGCTGTCCCCCGGGGGT</td>
<td></td>
</tr>
<tr>
<td>Alignment</td>
<td>******* - *******</td>
<td></td>
</tr>
</tbody>
</table>

Legend: * Full alignment
– Base mismatch.

Figure 3.15 Genomic alignment of ATRAP between strains
The genomic alignment of ATRAP from BKW, DBA₂ and C57 mouse strains highlighting the non-synonymous SNP in BKW and DBA₂ mouse strains resulting in the Valine to Alanine mutation at position 157 in the hydrophilic tail domain.

3.6.4 Discussion

BKW and DBA₂ mice have a non-synonymous polymorphism in the gene that codes for a protein that acts as a negative regulating element of AT₁ receptor;
and both show increased AT1R expression. Due to the relatively recent discovery of this protein, the exact functional significance of this SNP and the involvement of this domain in ATRAP-Protein function remain unclear, and may be purely coincidental. Analysis of human genomic sequence shows the presence of a similar V-A variant in the hydrophilic tail domain at position 143 (rs17875960). This human variant is located 14 residues downstream of the murine SNP, its function is also unknown, but suggests a high degree of variability at this region. This region may be worthy of further investigation to identify any possible links between ATRAP function and any influence on AT1 expression; bearing in mind that it can directly modulate receptor signalling, a functional mutation in ATRAP may effect receptor expression levels. More structural and functional information is required for the role of ATRAP and the particular function of this SNP (if any) in regulating AT1 receptor expression. A definitive link between ATRAP, its functional mutations and AT1 receptor function/expression would also be required to highlight and uncover an integrative link between the two.

To asses the phenotypic effect of the non-synonymous SNP in ATRAP, an approach utilising site directed mutagenesis (Stratgene Quickchange II site directed mutation) could be used. This approach uses selectively designed primers which induce point mutations in cDNA while keeping the sequence “in frame”. This is followed by subsequent transfection of the vector construct with a suitable promoter into a cell line such as CHO or COS-7 cells. The phenotypic effect of the mutant ATRAP on AT1 signalling and expression could be identified via luciferase expression, ligand binding and AT1a receptor-mediated activation of phospholipase C as described by Daviet et al (1999).
Results Chapter 3.7

Behavioural studies
3.7.1 Introduction

There are a multitude of proposed mechanisms of action for both IRAP and its substrates in cognition. Such mechanisms include inhibition of the enzyme by peptides and peptide mimics that allows the build up of other cognitively-active peptides (namely vasopressin and oxytocin) (McKinley et al. 2003), activation of a classical receptor signalling cascade (van Leifde et al. 2008) and/or changes in glucose uptake (Fernando et al. 2008). To investigate the behavioural effects of subcutaneous Ang IV administration on learning and memory and to identify any strain specific effects, a Novel Object Recognition (NOR) paradigm was employed. The NOR test was selected as a measure of learning and memory as it is not confounded by any effects of reward or punishment and investigates selected drug effects on memory consolidation. Object recognition was determined in mice using a modification of the method previously described for rats by Karwowska-Polecka et al (1997).

3.7.2 Method

A NOR paradigm was used to test the cognitive effect of Ang IV across three strains of mice (Methods section 2.9). A range of Ang IV concentrations were administered subcutaneously using saline as a control. Locomotor effects were also recorded to avoid the confounding effects of sedation/hyper-locomotor activity on NOR.

3.7.3 Results
Novel Object Recognition and locomotor data across three mouse strains.

Figure 3.16. Novel Object Recognition data between strains.

a. Novel object recognition data where the C57 strain showed the best recall and DBA$_2$ worst ($P<0.05$ Tukey-Kramer). In DBA$_2$, Ang IV significantly enhanced recall at 4.7µg kg$^{-1}$ and 47µg kg$^{-1}$ ($P<0.001$ & $P<0.05$ Dunnett's versus saline), whilst C57 strain showed significant improvement only at 4.7µg kg$^{-1}$ ($P<0.001$ Dunnett's versus saline). CD mice showed no significant improvement after Ang IV.

b. Analysis of locomotor activity after Ang IV treatment. Analysis of locomotor activity after Ang IV treatment shows there was a significant difference in activity between strains ($F=53.99$ $P<0.001$) and a significant effect of dose on locomotor activity ($F=3.30$ $P<0.05$), however there was no significant dose x strain interaction.

Novel object recognition data collected by Graham Brown.
Figure 3.16. shows the results from the object recognition paradigm, where significant differences in effects of Ang IV are observed between strains. Two-way ANOVA of the exploration times during the initial exposure to the objects indicates that there was a significant difference between strains with regard to locomotor activity ($F=3.67 \ P<0.05$). Further analysis of interactions and post hoc testing revealed CD mice were exploring for longer than C57 and DBA2 strains ($P<0.05$ Tukey-Kramer). There was, however, no significant difference in the time spent exploring the left or right objects, nor a significant interaction.

Analysis of locomotor activity after Ang IV treatment shows there was a significant difference in activity between strains ($F=53.99 \ P<0.001$) and a significant effect of dose on locomotor activity ($F= 3.30 \ P<0.05$), however there was no significant dose × strain interaction.

Two-Way ANOVA including strain and dose revealed that there was a significant effect of Ang IV on NOR as indicated by D-Scores, e.g. dose ($F=5.15 \ P<0.05$), but no significant difference between strain. There was, however a significant dose × strain interaction ($F=2.95 \ P<0.05$) in NOR, indicating that the different strains responded differently as the doses of Ang IV are increased. Further analysis of interactions and post-hoc comparisons using Tukey-Kramer and Dunnett’s tests indicated that there were significant differences in baseline (saline) D-scores between the strains. Mice of C57 strain showed the best recall and DBA2 worst ($P<0.05$ Tukey-Kramer). In DBA2, Ang IV significantly enhanced recall at 4.7µg kg$^{-1}$ and 47µg kg$^{-1}$ ($P<0.001$ & $P<0.05$ Dunnett’s versus saline), whilst C57 strain showed significant improvement only at 4.7µg kg$^{-1}$ ($P<0.001$ Dunnett’s versus saline). CD mice showed no significant improvement after Ang IV administration in the Novel Object Recognition.
**Figure 3.17. Novel Object Recognition data between strains.**

**a.** Novel object recognition data between CD, C57 and DBA$_2$ strains comparing saline, training only and treatment with Ang IV (4.7μg kg$^{-1}$). In the DBA$_2$ strain Ang IV had a significant affect on NOR ($F=3.50, P<0.05$) but in CD and C57 strains were refractory.

**b.** Analysis of locomotor activity revealed a significant difference in activity between strains ($F=53.99, P<0.001$) and a significant effect of dose on locomotor activity ($F=119.33, P<0.001$), and a significant dose × strain interaction $F=4.62, P<0.05$) suggesting that there is increased locomotion with Ang IV at a dose of 4.7μg kg$^{-1}$ in C57 and training in DBA$_2$
To explore any potential gene expression changes in IRAP and ApN as a result of training and Ang IV administration between strains, a pilot data study was performed. This study aimed to identify any effect of training and administration of Ang IV compared to saline controls. Two-Way ANOVA Analysis of locomotor activity revealed there were significant differences between strains ($F=119.33$, $P=<0.001$) and a significant interaction $F=4.62$, $P=<0.05$). Two-Way ANOVA including Strain and treatment revealed a significant effect of treatment on NOR, indicated by D-scores ($F=3.50, P=<0.05$) and a significant strain x treatment interaction ($F =3.57, P=<0.05$) highlighting that the different strains respond differently to treatment by Ang IV. Further Tukey-Kramer post-hoc testing indicated that in DBA2, Ang IV at a dose of 4.7µg kg$^{-1}$ and trains significantly enhanced recall ($F=6.68$, $P=<0.05$) but in CD and C57 strains did not. However, due to small sample size ($n=3$) within strains, differences may be confounded such that inferences and comparisons to previous data are difficult and would require larger samples sizes.

3.7.4 Discussion

These results indicate that Ang IV, given subcutaneously, can enhance cognition but only in C57 and DBA2 strains while CD mice are refractory. It is also apparent that in the DBA2 strain, which initially shows the poorest performance, Ang IV can improve cognition to a level in line with the other strains. It is also apparent that the effects of Ang IV are not dose dependent as in the C57 strain and to a certain extent, the CD strain, higher doses (47-470 µg Kg$^{-1}$) appear to be amnesic (Figure 3.16.a.). In this study, Ang IV is given 24 hours before the second learning trial, suggesting a role for Ang IV in memory consolidation as opposed to acquisition or recall or that the effects of Ang IV are prolonged.
Results Chapter 3.8

Aminopeptidase activity assay
3.8.1 Introduction

To explore the strain specific effects of Ang IV and the refractive response of CD mice in NOR, the activity of IRAP and the inhibitory effect of Ang IV was explored. Using a 96 well assay format, the $K_m$ and EC$_{50}$ of IRAP and Ang IV was investigated across strains. Subcutaneous administration of peptides has been effective in modifying cognition in rodents, yet Angiotensin IV does not cross the blood brain barrier Wright and Harding 2008. The Angiotensin IV mediated effects are therefore attributed to peptides having an effect on brain regions outside the BBB or in areas in which the BBB is “leaky”, notably the circumventricular organs and the hypothalamus. The hypothalamus also has connections to brain regions thought to be involved in cognition, such as the hippocampus and the perirhinal cortices (Vertes et al. 1995), and is able to directly influence the hippocampus (Woodnorth et al. 2002). For this reason the hypothalamus was selected for analysis.

3.8.2 Methods

To investigate the $K_m$ and EC$_{50}$ of aminopeptidases a 96 well assay format was developed (Chapter 2 Method section 2.7.2.). Inhibition assays were assayed using 25 µg of brain protein at a substrate concentration of 1.2mM where activity was below maximal ($V_{max}$) with varying concentrations of Ang IV. $K_m$ was assayed using varying concentrations of the substrate L-leucine-p-nitronailine. Controls of substrate and buffer, but lacking protein, and buffer alone, were run with all assays. Iterative Non-linear regression analysis using one site binding (hyperbola) ($Y=Bmax*X/(Kd+X)$) and one site competition $Y=bottom + (Top-Bottom)/(1+10^(X-LogEC50))$ was performed using Graphpad prism V4.0. Comparing the fits of two models, in this case one site competition against two site competition analysis allows confirmation that the model is correct and that the confidence intervals are not too wide. A comparison between one site competition and two site competition where the null hypothesis is one site and the alternative two site and accepting that an $F$ ratio near 1 and a $P= <0.05$ supports two site competition was performed. For all strains the preferred model is one site competition (C57, DBA$_2$ and CD $P=>0.05$) ($F= C57 n/a DBA_2$ 4.81 DFn,DFd 2.2, CD 0.582, DFn,DFd=2.2) and $R^2$ for one site (C57 0.98, DBA$_2$ 0.96, CD 0.95).
3.8.3 Results

Non linear regression analysis of aminopeptidase activity (Figure 3.17.a.) shows a significant difference between strains \((F=22.64 \quad P<0.001, \quad C57, \quad DBA_2 \quad n=3 \quad CD \quad n=6)\)(Table 3.3). The aminopeptidase \(K_m\) from Mice of CD strain differ from C57 and DBA\(_2\) counterparts at substrate concentrations greater than 1.2mM. C57 and DBA\(_2\) strains showed a higher rate of substrate catalysis at 2.2mM and 3mM of substrate compared to CD mice, which plateau at a substrate concentration of 1.5mM. At these concentrations the rate in C57 and DBA\(_2\) counterparts continued to rise (C57 0.002 mM/min and DBA\(_2\) 0.0019 mM/min) compared to CD mice (0.0015 mM/min).

Inhibition of aminopeptidase activity by Ang IV was not significantly different across strains \((F=2.10 \quad P<0.131 \quad Figure \; 3.17.b)(Table \; 3.4)\). A dose response of Ang IV results in an \(EC_{50}\) of \(1x10^{-5}\)M (substrate concentration of 1.2mM) in all strains. Aminopeptidase activity was not completely inhibited by Ang IV even at \(1x10^{-3}\)M and a degree of aminopeptidase activity remained that was not fully inhibited by Ang IV.
$K_m$ and $EC_{50}$ Aminopeptidase activity for all strains.

![Graph showing $K_m$ and $IC_{50}$ for different strains](image)

Figure 3.18. Non linear regression analysis of aminopeptidase activity.

**a.** Non linear regression analysis of aminopeptidase activity shows a significant difference between strains ($F= 22.64 \ P<0.001$, C57, DBA$_2$ $n=3$ CD $n=6$). Aminopeptidase $K_m$ from Mice of CD strain differ from C57 and DBA$_2$ counterparts at substrate concentrations greater than 1.2mM. C57 and DBA$_2$ strains showed a higher rate of substrate catalysis at 2.2mM and 3mM of substrate compared to CD mice, which plateau at a substrate concentration of 1.5mM. At these concentrations the rate in C57 and DBA$_2$ counterparts continued to rise (C57 0.002 mM/min and DBA$_2$ 0.0019 mM/min) compared to CD mice (0.0015 mM/min).

**b.** Inhibition of aminopeptidase activity by Ang IV was not significantly different across strains ($F=2.10 \ P<0.131$ Figure 3.17.b). A dose response of Ang IV results in an $EC_{50}$ of $1\times10^{-3}$M (substrate concentration of 1.2mM) in all C57, DBA$_2$ $n=3$ CD $n=6$ Error bars = ±s.e.mean.
### Table 3.3. $K_m$ Analysis of Variance for Rate, using Adjusted SS for Tests

<table>
<thead>
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<th>Source</th>
<th>DF</th>
<th>$F$</th>
<th>$P$</th>
</tr>
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<td>$&lt;0.0001$</td>
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<tr>
<td>[sub]</td>
<td>7</td>
<td>48.91</td>
<td>$&lt;0.0001$</td>
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<td>Strain*[sub]</td>
<td>14</td>
<td>1.57</td>
<td>0.110</td>
</tr>
<tr>
<td>Error</td>
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<td></td>
</tr>
<tr>
<td>Total</td>
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<td></td>
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</tbody>
</table>

### Table 3.4. $EC_{50}$ Analysis of Variance for Rate, using Adjusted SS for Tests

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<tbody>
<tr>
<td>Strain</td>
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<td>2.10</td>
<td>0.131</td>
</tr>
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<td>[sub]</td>
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<td>$&lt;0.0001$</td>
</tr>
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<td>Strain*[sub]</td>
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<td>0.57</td>
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<td>Error</td>
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<td></td>
</tr>
<tr>
<td>Total</td>
<td>83</td>
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<td></td>
</tr>
</tbody>
</table>
3.8.4 Discussion.

CD mice show no difference in the EC₅₀ of Ang IV, despite being refractive to the cognitive effects of Ang IV. Mice of this strain do however show differences in basal enzyme activity, potentially occurring as a result of defective form of the protein perhaps due to mutations in the catalytic and zinc binding domains. The use of a homogenous brain extract may contain as of yet unidentified enzymes that also demonstrate substrate specificity and may account for the difference in Kₘ observed between strains (Figure 3.17.a). It is not possible to rule out the involvement of ApN activity that shows structural homology to IRAP and at higher substrate doses may show some activity. Therefore total leucine aminopeptidase activity (encompassing IRAP and ApN) is reduced by approximately 25% in the refractive CD mouse strain.

Angiotensin IV is specific for IRAP at the concentrations used in the assay (Demaegdt et al. 2005) and therefore is indicative of the specific inhibition of IRAP in the homogenate. There were no significant differences between strains in the inhibition of IRAP (Figure 3.17.b.). This is an important facet; bearing in mind that one of the proposed mechanisms of cognitive improvement by Ang IV is by the inhibition of IRAP. It is therefore expected that CD mice, which are refractive to the effects of Ang IV would, also show cognitive improvement.
Results Chapter 3.9

IRAP/ApN Gene sequencing
3.9.1 Introduction

Aminopeptidases are characterized by specific catalytic domains: the exopeptidase motif formed of five amino acids (GAMEN) and a second zinc binding motif (HEXXH(X)_{18}E). This zinc binding motif comprises two histidine residues (H) and a second glutamate residue (E), which together form the two zinc coordinating residues (Goto et al. 2006). To investigate whether the observed differences in $K_m$ are due to mutations in the important catalytic and zinc binding domains responsible for substrate recognition and catabolism, these domains were sequenced in the reference C57 and CD mouse strains.

3.9.2 Methods

DNA was extracted as described in Chapter 2.7.1 using a DNeasy extraction kit, followed by PCR using the specified reaction conditions and the primer pair listed in table 5. Following PCR, products were electrophoresed and the DNA bands excised and cleaned through a Qiagen PCR cleanup kit and ethanol precipitated before sequencing by Cogenics UK. $n=3$ C57/DBA$_2$, $n=6$ CD. Primers designed using Perlprimer using Genomic sequence: Anpep: MGI:96749 Lnpep: MGI:2387123 [http://www.informatics.jax.org](http://www.informatics.jax.org) 2008).

3.9.3 Results

Gene sequencing of the IRAP GAMEN motif on Exon 6 and the Zinc binding motif on Exon 7 were identical between the reference C57 ([EC 3.4.11.3](https://www.chemcatalogue.com/)) and CD strains ($n=5$). Gene sequencing of ApN GAMEN motif on Exon 6 and the zinc binding motif on Exon 7 showed no difference from the reference C57 strain ([EC 3.4.11.2](https://www.chemcatalogue.com/)) ($n=5$) and no genetic polymorphisms were evident across sequenced regions. However, sequencing of the Intron (chromosome 7 location 86,983,888 – 86,984,048) between Exons 6 and 7 of the ApN gene revealed a single base deletion across C57, DBA$_2$ and CD strains that differs from the published sequence (MGI).
Representative sequence data showing single base insertion in ApN Intron between Exon 6 and 7.

![Sequence data]

Figure 3.19. Representative genomic sequence data of intron insertion.

Representative sequence data highlighting a single G base insertion in the intron between Exon 6 and 7. This insertion is identical between strains but differs from the MGI reference sequence. (original in colour).

Also of interest was a proposed juxtamembrane binding site for Ang IV that was suggested to be an allosteric mechanism of enzyme modulation (Caron et al. 2003). Located on Exon 2 of IRAP, and thought to be a secondary binding site for Ang IV, this domain was sequenced \( (n=3) \) to identify any variation in CD mouse strains that might explain their refractive response. No differences were found between mouse strains (Figure 3.18.).

### 3.9.4 Discussion

No genetic differences were found between strains neither in the important catalytic domains of IRAP nor in the proposed allosteric binding site in IRAP. Differences were however observed in basal \( K_m \). These domains only represent a few of the Exons (IRAP / ApN consists of 17 Exons) and it is conceivable that other mutations that affect IRAP/ApN activity are present in other domains that are equally likely to affect activity.

### 3.9.5 ApN Primer walk

To investigate any novel SNPs, insertions or deletions that may affect enzyme activity in ApN, a primer walk assay was designed to sequence the whole mRNA transcript using 6 primer pairs to span the entire 3367bp of ApN mRNA.
3.9.6 Method

mRNA was extracted as described previously in Chapter 2.7.11. The use of oligodT and random hex priming as well as gene specific primers was used to ensure full reverse transcription. RNA was reverse transcribed and the cDNA used for PCR using thermal cycling parameters listed in Chapter 2.7.5.

3.9.7 Results

Due to the large amplicon size (500 bp or greater) and the inability to be selective in primer placement, due the restrictions in covering the entire mRNA transcript, PCR amplification was poor and production of sufficient PCR product for sequencing was not achieved.

3.9.8 Discussion

The pooling of mRNA from a more ApN rich region or gene cloning may provide a more favourable quantity of product to allow sequencing of the transcript.
Results Chapter 3.10

Gene expression of IRAP/ApN by RT-qPCR
3.10.1 Introduction

The differences in basal aminopeptidase function ($K_m$) across strains could not be attributed to any variation in key binding domains in IRAP or ApN. To investigate whether variation in expression of these genes accounted for differences in enzyme activity a RT-qPCR method for the detection of IRAP and ApN was developed. The lack of a reliable and specific antibody for IRAP (Demaegdt H, 2007, Personal communication) confounded the identification of IRAP and ApN by western blotting method. However RT-qPCR allows more empirical quantifiable data and, by careful primer design can target both genes; this method was therefore adopted.

3.10.2 Methods

The RT-qPCR method was developed using the primer design rules described earlier in Chapter 2 Methods, using the following cDNA primers noted in Table 5.

3.10.3 Results

Figure 3.19 (a.) shows IRAP expression across strains when compared with GAPDH as a calibrator and normalised against the C57 strain ($n=3$ in triplicate). Gene expression of IRAP showed no significant differences between strains (ANOVA, $F=1.36 \ P=0.327$)(Table 3.5). In contrast gene expression of ApN varied between strains (ANOVA, $F=169.6 \ P<0.01$)(Table 3.6), with CD mice displaying 40% increased expression relative to C57 and DBA$_2$ strains (Figure 3.19.b.).
RT-qPCR data showing IRAP and ApN expression across strains.

Figure 3.20. RT-qPCR expression data of IRAP and ApN.

a. RT-qPCR expression data representing relative fold change between house keeping gene, GAPDH and gene of interest IRAP and ApN. No significant difference in relative gene expression for IRAP across strains (ANOVA, $F=1.36 \, P<0.327$).

b. RT-qPCR expression data representing fold change for ApN expression data shows 40% increase in expression of ApN in CD strain (ANOVA, $F=169.6 \, P<0.01$).

Error bars = ±s.e.mean. $n=3$ in triplicate.
### Table 3.5. Analysis of Variance GAPDH vs IRAP.

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</tr>
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<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 3.6. Analysis of variance GAPDH vs ApN.

<table>
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<th>Source</th>
<th>DF</th>
<th>F</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Strain</td>
<td>2</td>
<td>169.61</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Error</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td></td>
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</tr>
</tbody>
</table>

### 3.10.4 Discussion

The expression of IRAP is similar across strains, but the levels of ApN differ, with CD mice displaying a 40% increase in ApN expression (Figure 3.17.b.). The refractive response of CD mice to Ang IV, even at higher doses and the increase in ApN expression, an aminopeptidase responsible for both metabolism and catabolism of Ang IV is intriguing. How the increased expression of ApN correlates with the reduction observed in $K_m$ of enzyme activity is also unknown. However, the refractive CD mouse strain shows reduced enzyme activity, but not inhibition, and a 40% increase in ApN expression.
Chapter 4

Discussion
4.1 General discussion

Our understanding of the RAS has evolved far beyond the classical role of maintaining cardiovascular homeostasis and now represents discrete systemic, local and brain systems that modulate a myriad of functions. These include the actions of numerous peptides and their respective receptors along with a variety of mechanisms of action. A role for AT$_1$ receptors and their antagonists in anxiety is suggested as well as the cognitive enhancing effects of Angiotensin IV.
AT₁ Receptors and Anxiety
4.2 AT₁ Receptors and anxiety.

The anxiolytic effect of AT₁ antagonists has been demonstrated in rodents by several groups (Barnes et al. 1990; Karwowska-Polecka et al. 1997; Gard et al. 2001; Braszko 2003; Srinivasan et al. 2003; Saavedra et al. 2005). The specific targeting of AT₁ receptors is important due to their location in the HPA axis and their ability to modulate CRF, GABA_A and noradrenaline systems, all of which are implicated in anxiety.

Strain differences in the anxiolytic effects of AT₁ antagonists in mice are prevalent however as the initial effects of AT₁ antagonists identified by Barnes et al (1990) was not replicated by Sheperd et al (1996). The strain dependent nature of this anxiolytic effect was later identified by Gard et al (2001) who showed that the anxiolytic effect of AT₁ antagonists occurred only in the BKW mouse strain. Differences in receptor signalling were also observed between strains where the BKW strain showed a reduced contractile response of isolated tissue to Ang II. Interestingly, this strain displayed the lowest AT₁ receptor function, yet was the most responsive to the anxiolytic effects of losartan (Gard et al. 2001).

The results presented in Chapter 3 demonstrate that the previously reported strain dependent effects are neither due to variation in receptor sequence nor ligand receptor interactions. Differences in AT₁ expression between strains, which mirror their anxiety phenotype, are however observed and this is coupled with a functional mutation in a negative regulator of AT₁ signalling (ATRAP).

The variables that account for inter-strain differences in anxious phenotypes and the efficacy of losartan in ameliorating these effects are manifold. These include the underlying genetics of the strains leading to variation in the pharmacokinetics (PK) and pharmacodynamics (PD) of losartan, receptor-ligand interactions and receptor signalling differences, the particular test employed and variation in testing conditions / animal handling.

One explanation for the observed differences are mutations that affect ligand-receptor interactions; these would explain the reduced contractile response and indicate a possible link between strain specific effects of AT₁ antagonists and anxiety. Indeed, research into the mechanism of AT₁ antagonism using ligand binding on mutant receptors has highlighted key amino acid residues that are
paramount in conferring receptor structure and function (Hjorth et al. 1994). Mutations in these key residues directly affect agonist and antagonist binding due to the specific nature of key residues in conferring ligand binding and may also disrupt receptor signalling due to downstream interactions with signalling proteins.

Ligand binding data from this study (Chapter 3, Figure 3.2.) shows no significant difference in the IC$_{50}$ of losartan between strains. The IC$_{50}$ values for losartan are in the nanomolar range and are similar across strains and in line with other binding studies in rat liver membrane fragments (Booz et al. 1992). This suggests that the key residues that form both the peptide and non-peptide binding domains are invariant between the strains studied and indeed the coding region of the AT$_1$ receptor genes are identical between strains. This is in agreement with others who find that AT$_1$ receptors are invariant between strains and are commonly without inter-individual changes in key amino acids (Wong et al. 2003). The interaction of losartan on the AT$_1$ receptor would therefore be expected to display equi-efficacious effects between strains considering the identical genetic structure of the receptor. The strain specific anxiolytic response of BKW mice to Losartan cannot therefore be attributed to ligand-receptor interactions, nor due to any post-translational effects on the receptor. Post-translational modification (PTM) of the AT$_1$ receptor via N-glycosylation, phosphorylation and S-nitrosylation has not been explored, although differences across strains is feasible; major modifications would be expected to manifest as changes in the ligand binding experiments and are not seen. The absence of an anxiolytic effect in C57 and DBA$_2$ strains despite displaying similar binding profiles and identical receptor sequence is suggestive of another underlying and an alternative influential mechanism that allows an anxiolytic response in BKW mice.

The effect of Cytochrome P450 metabolising enzymes has been touched upon in Chapter 3, but not fully explored. The importance of these enzymes could be considered relevant in this case due to the 2-step metabolism profile of Losartan and the generation of a more potent intermediate than the parent compound. The identification of the true CYP P450 enzyme responsible for the metabolism of losartan is unclear and in mice probably involves a suite of enzyme homologues. To identify differences between strains, the use of strain specific assays of metabolism would be required. However, as the anxiolytic effect of Losartan is not reinforced with higher doses in refractive strains, the role of CYP
P450 enzymes is probably small. losartan itself also represents a rather ‘poor’ member of the sartan family, in that it forms loose receptor complexes and is surmountable (van Leifde et al. 2008). The use of candersartan, an insurmountable antagonist may provide responses in the non-responsive strains.

While no differences in ligand-receptor or AT₁ receptor sequence were observed, RT-qPCR expression analysis of hypothalamic AT₁ receptors revealed differences in expression between the strains (Chapter 3, Figures 3.7 and 3.8). BKW mice showed the highest levels of expression and DBA₂ mice an intermediate level when compared to the C57 strain. As mentioned previously (Chapter 2- Methods) investigations into protein levels and actual receptor expression would be required to fully implicate and correlate variation in AT₁ receptors with behaviour.

In previous studies by Gard et al, (2004), BKW mice showed the highest anxiety-like behaviour in the L/D and EPM test followed by DBA₂ and C57. These observations are consistent with others where the C57 strain are reported as “low anxiety” with significantly lower anxiety than DBA₂ mice (Brodkin et al. 1998; DuBois et al. 2006). The low level of anxiety in C57 mice and the higher levels of anxiety in DBA₂ and BKW mice mirrors the expression levels of AT₁ receptors in the brain where DBA₂ and BKW mice show increased expression when compared to the C57 strain; suggesting that the more anxious strains exhibit higher levels of AT₁ receptors expression.

The strain dependent effect, whereby losartan is only effective in the BKW strain, may represent an effect due to the higher levels of anxiety inherently displayed by this strain, perhaps due to this increased AT₁ expression in the PVN. Whether higher AT₁ expression in the BKW strain results in an anxious phenotype or whether increased anxiety is due to increased upregulation of AT₁ receptors through other mechanisms remains unresolved. The use of more than one strain when testing the role of AT₁ antagonists is not typical of this literature, possibly explaining why the correlation between AT₁ expression and an anxious phenotype may not have been identified previously.

The importance of AT₁ receptors in anxiety has been identified previously and shows that their location in the HPA axis supports their proposed role in mediating anxiety. Data also show that specific antagonism of AT₁ receptors
reduces anxiety and therefore suggests that levels of AT$_1$ expression in these brain regions has an important role. Whether AT$_1$ expression is the critical mediator of anxiety and whether higher AT$_1$ receptor expression is indicative of an anxious phenotype is plausible and warrants further research.

Sequencing of the microsatellite in the promoter region of the AT$_{1\alpha}$ receptor (Figures 3.10 and 3.11) as a means to identify an underlying cause of the variable expression of the AT$_1$ gene across strains revealed no differences in expression despite the identification of a microsatellite of 260bp in length. The microsatellite in this study is comparable in length to the microsatellite in the BPH mouse strain explored by Wong et al (2002). While the exact mechanism of this microsatellite is unclear, it is understood that whatever the role of a microsatellite within a gene, either in coding or noncoding regions, or within transcripts and regulatory sequences, changing the number of repeats can modulate genetic functions (Kashi et al. 2006). This is especially prevalent in this case due to its location within the promoter region. While the previous study by Wong et al (2002) highlighted variation in microsatellite size and variation of gene expression levels, this study has shown that variable AT$_1$ receptor expression in the brain is not solely conferred by microsatellite size alone. Indeed, inter-strain differences exist in gene expression but this variation is not seen in microsatellite size. Perhaps the variable SNPs also found by Wong et al between strains coupled with the variable G/C rich regions upstream of the TATAA box are also a factor in influencing expression levels. Further sequencing of the 5’ and 3’ flanking regions may highlight variation between strains that may be more indicative of receptor expression. However, it is important to note that humans do not possess this upstream microsatellite in the promoter, although SNPs linked to blood pressure regulation have been found in UTR regions – notably the A1166C variant (Bonnardeaux et al. 1994). Despite this SNP falling outside the coding region, this allele has been linked to blood pressure variations. However, no genotype effect of the AT$_1$ receptor A1166C polymorphism was detected either on the $B_{\text{max}}$ or the $K_D$ value of the Ang II receptors on platelets (Paillard et al. 1999). The most likely scenario is that the A1166C polymorphism is in linkage disequilibrium with a functional mutation further downstream that alters angiotensin II responsiveness (Peter et al. 2000), although this is not yet identified.

It is interesting to note that in the previous study by Gard et al, (2004) the C57 and DBA$_2$ strains showed similar contractile responses to Ang II, while the BKW
strain showed a significant reduction. The AT₁ receptor associated protein (ATRAP) is associated with reduced Ang II signalling and this study has identified that both DBA₂ and BKW mice possess a non-synonymous polymorphism (rs13467517) in this gene. ATRAP modifies Ang II receptor signalling in vitro and in DBA₂ and BKW mice the SNP results in an Alanine to Valine amino acid change at position 157 (A₁⁵⁷V) of the hydrophilic domain (109-161) of the protein. The precise effect of this polymorphism is unknown, but it is tempting to speculate that the higher expression of AT₁ receptors in DBA₂ and BKW, in contrast to C57 mice, is linked to this polymorphism. While this is an observational link, it is not improbable that a mutation in ATRAP, which affects AT₁ signalling, would influence expression of the receptor.

Other proteins which modify AT₁ signalling are also observed in anxiety-like behaviour, specifically the Mas protoonco gene and the product of the Mas-related gene (Mrg) (Thomas et al. 1998). These proteins enhance the effects of angiotensins on cells expressing the AT₁ subtype and specifically stimulate intracellular signalling of Ang II. Anxiety was seen to be markedly increased in knockout (Mas⁻⁻) mice and Mas⁺⁻ showed intermediate anxiety, although this did not differ significantly between both groups.

Specific modulators of AT₁ receptor function are implicated in anxiety and ATRAP, as a negative modulator of AT₁ function, may also play a role. At present the discrete localisation of ATRAP is unknown, although low levels have been identified in the brain (Laurent et al. 1998). Perhaps increased AT₁ receptor expression also results in increased ATRAP expression. Functional differences in ATRAP also provide a possible explanation of the strain differences observed in contractile effects of Ang II in isolated tissue. Despite showing no variation in receptor structure or ligand binding profile, contractile effects were reduced in BKW strains. Owing to the presence of the ATRAP-SNP in DBA₂ strains, it would be expected that the reduced contractile effect would also observed in this strain. However, DBA₂ and C57 mice show comparable contractile effects. The combined effect of increased AT₁ expression and functional mutations in ATRAP may affect signalling by another mechanism resulting in a compensational loss in signalling.

The findings in this study also show that the AT₁ receptor sequence is identical between strains, so these differences may be related to differences in
downstream receptor signalling. The level of receptor expression in the colon was not investigated and it is highly conceivable that levels of AT₁ expression differ in this tissue type and do not mirror brain expression patterns. Also, these differences may, in part, be due to the G proteins that couple to the receptor or other key parts of the signalling cascade, such as those involved in inositol phosphate (IP₃) production and subsequent signalling cascade (Hunyady et al. 2005).

In agreement with previous studies, AT₁ receptors appear to be implemented in anxiety-like behaviour. The previously identified strain differences and the “order” of anxiety seen within and between strains may be attributed to variation in AT₁ expression levels in the PVN, where more anxious animals show higher AT₁ expression. Whether these changes in AT₁ expression are mediated by the functional mutation in ATRAP or linked to SNPs in the promoter region was not specifically investigated in this study but they do suggest the existence of markers linked to altered AT₁ expression levels and anxious phenotypes.

It is conceivable; therefore, that AT₁ receptor expression in areas involved in the HPA axis may have a direct effect with anxious phenotypes. Whether high AT₁ receptor expression is fully indicative of levels of anxiety and could be used as a predicative marker is difficult to ascertain in a system that is as tightly regulated as the RAS. The use of AT₁ antagonists as a treatment of anxiety over current BZ, SSRI, MOI and heterocyclic treatments which all show limited efficacy and side effects may be warranted. Importantly, all of these treatments focus on serotonin, dopamine and noradrenaline in the brain and their efficacy and response rate has not improved despite the development of newer drugs (Moolj-van Malsen et al. 2008).

Currently no clinical trials data on the effect of ACE inhibitors and AT₁ antagonists on anxiety in humans exist. Small studies in hypertensive patients who show greater anxiety scores have found that treatment with ACE inhibitors significantly reduced their anxiety; in normotensives, however, there was no effect. In the treatment of healthy volunteers with losartan, no anxiolytic effect was observed, suggesting that ACE inhibitors and AT₁ antagonists are only effective in treating anxiety in this cohort of hypertensives (Gard 2005). An alternative suggestion is that underlying genetic differences may predispose an individual to either a positive or refractory response. Indeed, angiotensin related
genes such as the insertion/deletion variant of ACE and the T/C variant in angiotensinogen has been associated with anxiety in males (Olsson et al. 2004), although there was no association between the A1166C variant of the AT\textsubscript{1} receptor gene, which is associated with hypertension and anxiety. Whether certain genotypes result in a more anxious phenotype or a predisposition to hypertension and, as a consequence, anxiety, is currently unknown. One might speculate that not all individuals would show an anxiolytic response to losartan, analogous to mouse strain differences, but that perhaps gene screening for ATRAP might be able to identify those individuals who may respond to treatment. Further research into RAS polymorphisms, their effect on anxiety and targeting of specific genotypes in its treatment may therefore be warranted.
AT$_4$ receptors and Cognition
4.3 $\text{AT}_4$ receptors and Cognition.

The positive effect of $\text{AT}_4$ (IRAP) ligands on cognition has been demonstrated in a variety of rodent paradigms and the effects are unequivocal. Data herein show that Ang IV does indeed improve learning and memory but the effects are strain dependent. What is still unknown, however, is the exact mechanism of action of these compounds in conferring a positive cognitive effect. While a few mechanisms have been postulated, none are as of yet fully defined, and in some cases the plausibility of the suggested mechanisms is questionable.

Novel object recognition was implemented to explore the effectiveness of Ang IV on improving cognition in mice. The poor (low baseline) performance of DBA$_2$ mice in the object recognition paradigm (Figure 3.16) is comparable to work by others where differences between C57 and DBA$_2$ are common in a variety of tasks. It has been observed that C57 mice outperform DBA$_2$ (specifically in object recognition tasks) and C57 mice strongly explore the displaced objects whereas DBA$_2$ mice do not (Thinus-Blanc et al. 1996). Although differences in learning and memory performance in C57 and DBA$_2$ mice are reported to depend on the task used, DBA$_2$ mice are also slower to learn than C57 mice in the water maze and object recognition tasks (Podhora et al. 2002; Voikar et al. 2005). DBA$_2$ mice also perform poorly in spatial learning tasks to an extent that they are suggested as a genetic model of hippocampal dysfunction (Ammassari-Teule et al. 1998). This highlights an important aspect in the ability of Ang IV to restore poor performers to a level comparable with other strains; in fact the poorest performers initially show the greatest level of improvement. Although a direct comparison cannot be made between tasks, male CD mice show poor performance in acquisition of a spatial working memory when tested in the 3D maze (Ennaceur et al. 2008). The basal (saline) score of CD mice observed in this study does not support this as CD mice are comparable in their object recognition to that of C57. This is the first time that CD mice have been used in the NOR task and unfortunately making comparisons with other studies is not possible.

The use of a single behavioural task in this study could be considered a limitation as NOR can be confounded by locomotor activity, although locomotor activity was recorded and no confounding effects were observed. The use of another behavioural test of learning and memory may possibly highlight Ang IV
memory-enhancing effects in the unresponsive CD mice, but this would suggest separate effects of Ang IV in different learning paradigms.

The present results indicate that peripherally administered Ang IV can enhance memory consolidation in some mouse strains. Previous workers have studied the effects of intracerebroventricular administration of Ang IV in rats; Braszko and Wright, for example, administer Ang IV before the final recall trial (Braszko et al., 1988; Wright, 1996), and as such were investigating the effects of the peptide on recall. In this and previous studies, administration occurs 24 hours earlier, after the second learning trial suggesting either a role for Ang IV in memory consolidation or that the effects of Ang IV are prolonged over a 24 hour period and still facilitate memory recall.

The strain differences in the effects on Ang IV on memory cannot be due to variation in previous experiences between strains as the animals were bred and reared under identical conditions. Similarly, the differences are unlikely to be due to pharmacokinetic differences, as altering the dose in CD mice did not alter the response. This suggests that the lack of response in this strain is neither due to bioavailability nor accelerated metabolism of the administered Ang IV. Indeed, the improved performance in the object recognition task occurs at low doses and is ablated at higher doses, with behavioural responses reported here in agreement with previous findings, where high doses (500 μg kg⁻¹) of Ang IV were shown to have no effect on object recognition in BKW mice (Gard et al. 2007). The strain differences probably lie within the processes involved in the actions of Ang IV and can therefore be used to explore the role of IRAP in the mechanism of action.

The brain areas involved in novel object recognition-memory consolidation are not clearly defined. The dorsal hippocampus, perirhinal and insular cortices and, experimentally, the basolateral amygdalae are the most commonly highlighted areas and are thought to utilise a NMDA glutamate-mediated mechanism (Dornelles et al. 2007). More recently it was suggested that the perirhinal and insular cortices are required for consolidation of familiar objects while the hippocampus is necessary for consolidation of contextual information of recognition memory (Balderas et al. 2008). In long-term object recognition memory consolidation in rats, the prefrontal cortex is obligatory for both consolidation and reconsolidation, and protein synthesis and NMDA receptor function are required for both processes (Akirav et al. 2006). While memory
consolidation occurs in the insular cortex, it is thought that cholinergic activity is critically involved in the consolidation of object familiarity underlying recognition memory (Bermudez-Rattoni et al. 2005). Some discrepancies exist however, as some authors suggest that object recognition is not a hippocampal task, as damage to this region rarely produces significant performance deficits in rats (Mumby 2001). Others suggest that a partially damaged hippocampus does, however, interfere with object recognition and this suggests that the rhinal cortex and hippocampus make different contributions to object recognition memory (Murray et al. 2001).

The present study investigated IRAP aminopeptidase activity in whole-brain homogenates but IRAP gene expression in the hypothalamus only; an area of high Ang IV concentration taken as being indicative of other brain areas. The results indicated that the structure of the GAMEN motif and Zinc binding motif of the IRAP catalytic site was identical across the strains, and that IRAP aminopeptidase activity, as defined by the aminopeptidase activity inhibited by Ang IV, was similarly equal across the strains. The only observed difference between the strains was that in the unresponsive CD mouse strain, non-IRAP aminopeptidase activity was reduced and hypothalamic ApN expression was increased. Such a finding raises the possibility of the involvement of ApN in the actions of Ang IV.

Straiger and colleagues (2007) have previously postulated the involvement of ApN in the mechanism of action of Ang IV and the observed overexpression of ApN here warrants further consideration as ApN is not only responsible for synthesis of Ang IV (Ardaillou et al. 1997) but is also capable of its metabolism (Ardaillou et al. 1998; Wallis et al. 2007). Overexpression of ApN has been reported previously in the Dahl salt-sensitive rat due to mutations within the gene promoter region (Kotlo et al. 2007). Whether the polymorphism is the cause of the increased gene expression and whether upregulation results in an increase at the protein level need to be further investigated. Importantly, the expression data for ApN indicate increased expression in the refractive strain of mouse, whereas the functional data suggest an overall decrease in aminopeptidase activity. Whether the increase in expression is a consequence of the under activity is unclear.

Interestingly, the blocking of ApN in dopamine release experiments has been seen to potentiate dopamine release, possibly by preventing the degradation of
Ang IV. Ang IV can cause dose-dependent dopamine release in the striatum and a link between the ability of dopamine, through D₁ receptors, to potentiate NMDA glutamate receptor function exists (Hallett et al. 2006). Dopamine receptor antagonists are also extremely effective in preventing memory-enhancing activity of Ang IV and des-Phe⁶ Ang IV (Braszko 2006). Given that CD mice show an upregulation of ApN, the metabolism of Ang IV could conceivably be faster in CD mice, but this speculation is not supported as even at higher doses no improvement is observed.

The data from Chapter 3 confirms that there are strain differences in the effect of peripherally-administered Ang IV on memory consolidation in the mouse. These differences do not appear to be caused by functional or genetic differences in the putative receptor for Ang IV, IRAP, questioning the role of the aminopeptidase action of IRAP in the effects of Ang IV on memory. This is further reinforced by recent findings of Albiston et al (2009) using IRAP knockout mouse models. The mechanism of IRAP inhibition, allowing the preservation of its endogenous peptide substrates is refuted by Harding and Wright who note that the dose and time parameters do not mirror that of in vitro experiments. More recent data generated using IRAP KO mouse lines has shown that in fact, instead of improving cognition as would be expected by employing an IRAP antagonist, IRAP KO mice actually show no improvement in cognitive paradigms and in fact show increased age related cognitive decline in the Y-maze paradigm. The suggested mechanism of IRAP antagonism increasing the in vivo ligands oxytocin and vasopressin is therefore questionable. Another suggested mechanism in which IRAP ligands were thought to improve cognition by mediating hippocampal glucose uptake (Fernando et al. 2008), has been tested in brain slices but has also been called into question as Ang IV and its mimic LVV hemorphin showed no change in hippocampal blood flow or glucose uptake in vivo (Bundel et al. 2009). Indeed, glucose has been demonstrated to improve cognition and the interaction of IRAP with GLUT4 via an elaborate transmembrane shuttling mechanism is suggestive of a relationship. A relationship between dopamine and Ang IV has also been suggested by Braszko as the use of selective dopamine D(4) antagonists ablated the cognitive effects of Ang IV (Braszko 2009) an effect also observed with dopamine D(1) (Braszko 2004) and D(2) (Braszko 2006) receptors, reinforcing the idea that these are required for the cognitive improvement elicited by Ang IV.
A relationship has, however, been identified between responses to Ang IV and expression of ApN, an enzyme responsible for Ang IV synthesis, capable of Ang IV metabolism, and a possible site of action for Ang IV. The suggestion that IRAP ligands inhibit the catalytic activity of IRAP is no longer tangible, due to the fact that the CD mouse strain, which is refractory to the cognitive effects of Ang IV, does not show a significant difference in the inhibition of IRAP when compared to its counterpart strains. Such a mechanism has been questioned before, where the doses that show effective improvement in cognition are much lower than the EC50 of IRAP. The timing to allow for the build-up of sufficient quantities of ligand (oxytocin/vasopressin) is also debatable. In light of the recent work showing that glucose uptake and IRAP inhibition are improbable mechanisms, the role of IRAP acting as a classical receptor and/or the role of ApN, implemented in the catabolism and metabolism of Ang IV, working as a transmembrane signalling receptor provides a new direction of research. Alternatively, the role of Angiotensin IV breakdown products may also be of interest. This is paramount as peptides as small as N-terminal tetra- and tripeptides have shown pro-cognitive effects (Wright et al. 2008a), further highlighting the importance of ApN in mediating the breakdown of Ang IV.

The mechanism by which Ang IV enhances cognition and specifically its role in acquisition, consolidation and recall, is still unclear. It is still unknown how these effects are modulated via specific IRAP inhibitors such as Ang IV and LVV hemorphin. While it is still argued that IRAP inhibition allows the preservation of neuropeptide substrates (Albiston et al. 2008) this logic is now questioned as the effects of Ang IV on memory recall are seen minutes after injection and in acquisition is given only a few minutes before training. In studies of memory consolidation, drug effects are seen a full 24 hours later. Bearing in mind that the plasma half life of Ang IV is exceptionally short and the fact that oxytocin and vasopressin are readily metabolised, this observation is paradoxical. In fact, the basal levels of Ang IV in the striatum are very low (46 pM) and it also has poor in vivo stability with a half-life of 10-20s (Katrien et al. 2007). That the HGF receptor c-met is also a receptor for Ang IV has also been suggested although the evidence seems somewhat limited and is currently unpublished.
The cognitive enhancement effect of Ang IV provides a tantalising mechanism for the treatment of cognitive decline. The mechanism of action is unclear but the AT$_4$ receptor subtype has been suggested as a new therapeutic target for the treatment of memory defects of dementia. The implementation of the neuropeptide oxytocin for the treatment of social phobia by means of a nasal spray (Baumgartner et al. 2008) has already been shown, reinforcing the idea that the AT$_4$ receptor is a viable druggable target for cognitive improvement. It just remains to be clarified which of the aminopeptidases is central to its influence and the exact mechanism of action.
Publications

Strain differences in the effects of Angiotensin IV on mouse cognition.
Submitted.

The role of IRAP in the behavioural response to Angiotensin IV.
Fundamental and Clinical Pharmacology Volume 22 Supplement 2 August 2008

Abstracts

Genetic variation between mouse strains: implications for drug discovery.
Pharmacogenomics and Family medicine, Singapore 2007.
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**Books**


**Websites**


