THE ROLE OF TACHYKININS IN DEPRESSION, MOOD DISORDERS AND EPILEPSY.

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

Lynn McLaughlin

July 2011

ACKNOWLEDGEMENTS

I would like to express special thanks to my supervisors, Professor John Quinn and Dr. Jill Bubb for all their endless help, support and patience during my studies.

I am very grateful for all the help, support and advice given to me over the years by all the members of the laboratory past and present who, due to my part time status, are too numerous to list. Exceptional thanks must go to Dr. Kate Haddley, Dr. Sylvia Vasiliou, Dr. Mark Howard, Dr. Ursula Paredes and Dr. Fahad Ali for making the time I spent doing my PhD the most enjoyable and rewarding experience of my working life.

Additional thanks to Professor Anja Kipar for help with immunohistology, Dr. Thippeswamy and Mr. Iain Kirk.

Exceptional thanks must also go to my friends and family who make up the most important and rewarding aspect of my life and have encouraged me to continue when I have felt like giving up, have always believed in me and have given constant help and support.

Finally, thanks must go to my partner Valmai Roberts and our sons, Ben and Connor McLaughlin, for all their love and support.

I would like to dedicate my thesis to my Mum and Dad (Irene and Peter McLaughlin) in acknowledgement for all their unconditional love and support.

ABSTRACT

Transgenic and knockout mouse models of disease and gene function have revolutionised the field of biomedical research and the targeted mutations of genes expressed in the brain are revealing the mechanisms underlying normal behaviour and behavioural abnormalities which has led to the development of behavioural neuroscience. However, it is also clear that the phenomena know as "redundancy" can limit the effectiveness of traditional knockout models by the effects of compensatory mechanisms. This thesis utilises transgenic and knockout mouse models to explore the role of the tachykinins in the pathogenesis of anxiety, depression and epilepsy focusing on the *TAC1* gene products and their functionality via the *TACR1 (NK1)* receptor.

The generation of a novel double knockout line via the cross breeding of single knockout models relevant for the tachykinin signalling pathways circumnavigates the problems associated with redundancy and also reveals that this phenomena is contributing to the data generated using the single knockouts. The use of these animals in a variety of experimental models to compare their function with the original "parental" knockouts and their corresponding wildtype controls reveals novel models for the action of the tachykinins in epilepsy and suggests a correlation with serotonin levels with an ultimate effect on the behavioural responses observed.

Interestingly, during analysis and experimental design it became clear that the sex of the animal was a parameter that had a statistically significant effect on the results. This was further investigated and the data alludes to a sex dependent level of control of the tachykinins and their involvement in the aetiology of epilepsy. Models generated as

part of this thesis were also analysed and utilised in virus infection experiments were it became apparent that the genetic background of the mouse model is also a determining parameter.

The data presented in this thesis clearly demonstrates that the double knockout of both the *Tac1* gene and the high affinity receptor *Tacr1* has a distinct behavioural response that is unique to either individual knockout for certain neurological disorders but displays a phenotype that is consistent with that of the single knockouts for other disorders of neurological origin.

This demonstrates the complex regulatory mechanisms involved in the mood disorders and epilepsy and also supports a differential functional response of the *Tacr1* mediate by various tachykinin activation of the receptor.

Contents

List of Figures

Chapter one

Chapter Three

List of Abbreviations

CNS	Central nervous system
dATP	deoxyadenosine trisphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
HEPES	4-(2-hydroxyethyl)-1-poperazineethanesulfonic acid
Hox	Homeotic box
Kb	Kilobases
l-allele	Long variants of the 5' promoter VNTR of the SLC6A4 gene
LH	Luteinizing hormone
LPR	Linked polymorphic region
Luc	Luciferase (firefly)
µg	Micro-grams
µM	Micro-molar
µl	Micro-litre
ml	Milli-litres

mM	Mill-molar
mRNA	Messenger ribonucleic acid
NCBI	National centre for biotechnology information
NK1	Neurokinin 1 gene
NK1R	Neurokinin 1 receptor
Ng	nano-grams
Nm	nanometer
ORF	Open reading frame
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PFC	Prefrontal cortex
PIC	Preinitiation complex
PKA	Protein kinase A
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
s-allele	Short variants of the 5'promoter VNTR of the SLC6A4 gene

SERT	Serotonin transporter
SLC6A4	Serotonin transporter gene
SNP	Single nucleotide polymorphisms
Stin2	Intron 2 VNTR
SSRI	Selective serotonin reuptake inhibitors
SV40	Simian virus 40
Tac1	Tachykinin 1 gene
Tacr1	Neurokinin 1 receptor
TBP	TATA binding protein
TCA	Tricyclic antidepressants
TF	Transcription factor
VNTR	Variable number of tandem repeat
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside
YB-1	Y-Box binding protein
5HT	Serotonin
5HTT	Serotonin transporter protein

Chapter 1. General introduction.

1: Introduction

1.1 Overview

The study described in the following thesis has generated and utilised mouse models to examine and explore the role of the tachykinin genes involved in the mood disorders and epilepsy *in-vivo*. In addition, an important part of this thesis was to explore the transcription factors implicated in the neurological disorder that is epilepsy and its association with the mood disorders suggesting a regulatory link of the tachykinins on serotonin levels.

Genes of particular interest in the mood disorders and epilepsy are explored including the *TAC1* gene (originally known as Preprotachykinin-A, PPTA) which encodes several tachykinins including Substance P (SP) and Neurokinin A (NKA) and the *TACR1* gene (also known as Neurokinin 1 gene, NK1) which is SP's preferred receptor in the central nervous system (CNS), as well as the *SLC6A4* gene (producing the Serotonin Transporter protein, 5HTT or SERT). Recently, another tachykinin peptide produced in non neuronal cells and termed hemokinin (encoded by the *TAC4* gene) has extended the tachykinin family. Hemokinin also shows a preference for the receptor *TACR1* and the importance of this is explored and discussed.

Both the human and rodent genes and gene products are studied, compared and contrasted during the course of the thesis. The objective, to contribute to the knowledge of the regulation and role these genes play in the mood disorders and epilepsy, is discussed and summarised during the course of the thesis.

1.2 Human verses mouse genome

Humans and mice diverged an estimated 75 million years ago. Although this has meant that evolution has changed their comparative genomes via substitution, deletion and insertion of nucleotides it has also resulted in regions that can be recognised as being functionally important due to the high level of conservation of the genetic sequences. Biologically, humans and mice are closely related and have almost all the same organs. In fact, mice are at least as closely related biologically to humans as any of the familiar agricultural and domestic mammalian species. Only primates are closer evolutionarily. The human genome mapping project (HGMP) completed a working draft of the human genome in 2001 with a complete sequence in 2003. Recognising the importance of mice in research the sequencing of the mouse genome was quickly completed and published using the most widely used inbred strain, the C57BL/6J mouse in 2002 by the Mouse Genome Sequencing Consortium (only females were used due to the highly repetitive regions in the Y chromosome, 99.9% sequence homology). Comparison between the genomes provided the following key points (taken from nature article *"Initial sequencing and comparative analysis of the mouse genome"*; Waterston *et al*, 2002):-

- The mouse genome is 14% smaller than the humans (2.5 GB compared to 2.9 GB).
- More than 90% of the human and mouse genomes can be partitioned into corresponding regions of conserved synteny showing that the gene order from our common shared ancestor has been conserved in both species.
- On a nucleotide level, approximately 40% of the human genome can be directly aligned to the mouse genome.

- Comparison of the genomes found a level of conservation that cannot be explained by protein-coding sequencing alone, indicating that a larger proportion of conservation has occurred in regulatory regions, non-protein coding genes and chromosomal structural elements.

- The level of the genomes that is protein-coding genes is comparable with an estimated 30,000 protein-coding genes in each species.

The mouse genome consortium evaluated the conserved synteny between the human and mouse genome across the species chromosomes (figure 1.1) and provided knowledge with regards some of the problems that can be encountered when trying to generate humanised mouse models because genes that are conserved between the species have been subjected to translocations, chromosomal breaks and chromosomal rearrangements. Thus, even if the protein-coding regions of an individual gene are conserved between the two species the nearby genes and regulatory regions may not be and this must be considered when making mouse models as it may not be the final translated protein that differs between the species but rather its temporal and spatial expression pattern which will affect its ultimate functionality. The similarity of the protein-coding regions across the two species can also lead to problems during the analysis of the model and this must also be considered when generating mouse models. Both of these points will be discussed further in the relevant sections of this thesis.

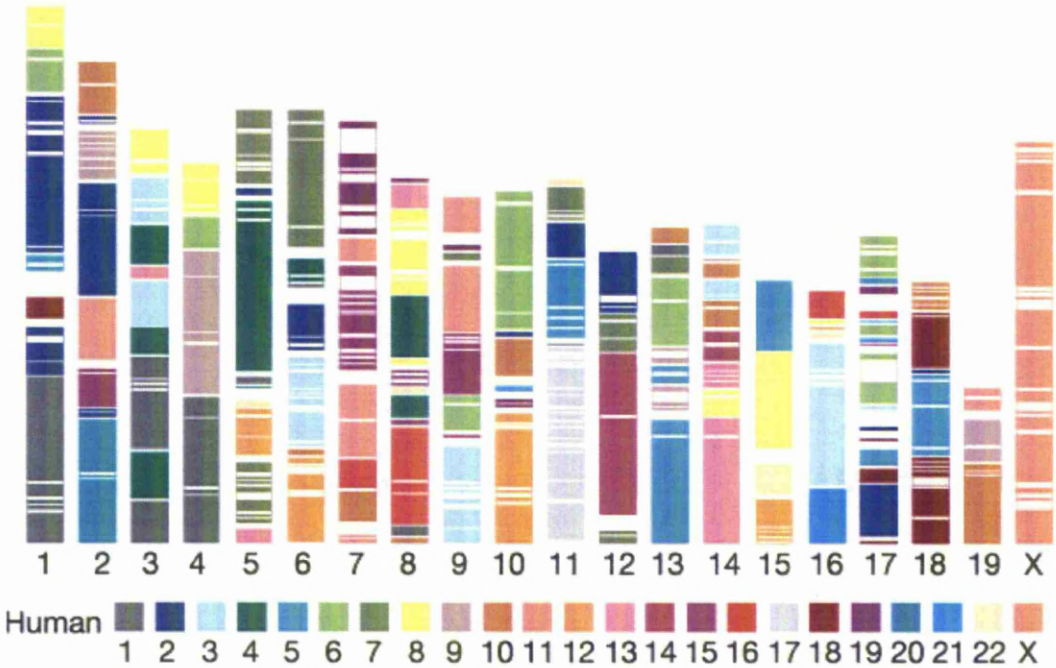

Figure 1.1 *reproduced from mouse genome sequencing consortium. Each colour corresponds to a particular human chromosome. The bottom boxes indicated a colour for each of the human chromosomes. This colour guide is used to illustrate the corresponding position of the genes found on human chromosomes with regards their position on the mouse chromosomes. For example, the genes on human chromosome 1 represented in grey are present on mouse chromosomes but distributed across mouse chromosomes 1, 3 and 4. In contrast the human and mouse X chromosome has almost perfect synteny.*

The fact that protein-coding coding sequences and an even higher proportion of non-protein coding sequences have been conserved across the two species explains why the mouse has been such a valuable tool in *in-vivo* research even before the use of recombinant DNA technology. Like humans and many other mammals, mice naturally develop diseases that affect physiological systems, including cancer,

atherosclerosis, hypertension, diabetes, osteoporosis and glaucoma. Early work done on the first laboratory strains of mice has laid the foundation for current knowledge on cancer and tumour growth and led to the discovery and elucidation of the role of the TP53 gene that expresses the tumour suppressor p53 as well as numerous other oncogenes (review, Goldstein *et al*, 2011). The discovery and use of recombinant DNA technology has helped with diseases that afflict humans but are not usually found within the murine population, such as cystic fibrosis and Alzheimer's disease, as they can be induced by manipulating the mouse genome and environment. There are also behavioural test analogues for the mood disorders in humans that can be applied to mice which have been particularly useful in mouse models for studying behaviour disorders and antidepressant drugs and these have been applied and used within this thesis. It is worth noting, however, that the use of mouse models does have limitations and drawbacks such as, for example, the absence of many of the human polymorphic domains associated with the progression of neurological disorders. In particular they lack the VNTR region (variable number tandem repeats) found in the *SLC6A4* gene (Holmes *et al*, 2003) and this will also be considered and discussed further in the introduction of this thesis.

1.3 Knockout mouse lines

The use of recombinant DNA techniques and embryonic stem cell technology in the 1980s resulted in knockout mouse models for human diseases via homologous recombination in embryonic stem cells of an artificial targeting vector and the endogenous gene of interest (Crawley, 2008; Muller, 1999, reviewed in Brandon *et al*, 1995). Generally, knockout defines a mouse model that has had an endogenous

gene disrupted or partially deleted. It is specifically defined as a targeted mutation (tm) in the nomenclature and knowledge of the gene sequence to be disrupted must be known in order to construct the targeting vector. In the vast majority of cases knockouts have a mutation introduced into an exon of the genes reading frame that is critical for the expression of the gene product of interest. The mutation usually results in deletion of a portion of DNA but can also shift the reading frame for the DNA resulting in incorrect reading of the triplet base pair codes compromising the gene product. It is often thought that the resultant knockout will have a phenotype that has a complete absence of the protein of interest. While this is sometimes the case it is also possible to have a truncated form of the gene product or an inactivated form of the gene product due to incorrect translation or incorrect folding of the protein post translational. The targeting vector that achieves this disruption often has the neomycin resistance gene (Neo) added as a selectable marker along with sequences homologous to the gene of interest for the recombination event (Figure 1.2).

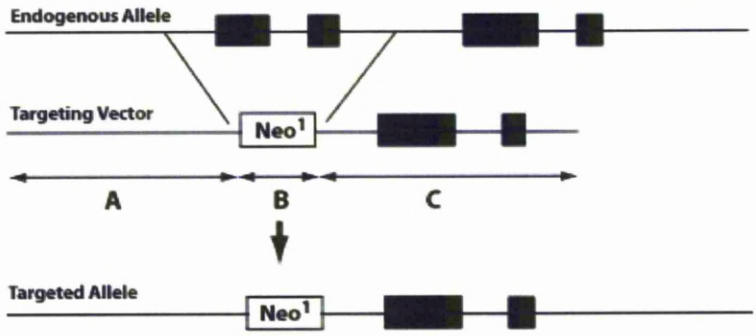

Figure 1.2. *A typical targeting vector reproduced from transgenic mouse core facility. A targeting vector contains three basic units consisting of A) a fragment of DNA homologous to the 5' end of the gene (the 5' arm), B) a selectable gene marker, most commonly the neomycin resistance gene, and C) a fragment of DNA homologous to the 3' end of the gene (the 3' arm). In general, the targeting vector is designed such that some portion of the endogenous gene is replaced by the neomycin cassette in the targeted allele. Deletion of the initial coding exon can ensure that a truncated protein is not produced.*

1.4 TAC1 and TACR 1

Substance P (SP) was discovered in 1931 (Von Euler and Gaddum, 1931) and has been extensively studied along with the ever growing numbers of identified neuropeptides. Neuropeptides are small proteins that serve neurotransmitter-like roles in the nervous system. Similar signalling peptides (growth factors and cytokines) are considered to be a distinct class even though they may have some overlapping functions. SP has been of particular interest because it is implicated in a multitude of functions including pain perception, mood, fear and anxiety, inflammation, blood pressure, appetite and digestion (review, MacKenzie and

Quinn, 2004). It is SPs actions in the regulation of mood and in particular the control of fear and anxiety that this thesis has focused on and the initiation of its functions via its preferred receptor in CNS, *TACR1* (previously known as Neurokinin-1 receptor).

SP is coded for by the *TAC1* gene which is sometimes referred to as the PPTA gene. The *TAC1* gene encodes four products of the tachykinin peptide family, SP and neurokinin A (NKA, previously known as substance K), as well as related peptides, neuropeptide K and neuropeptide gamma (neuropeptide-γ) (Bannon *et al*, 1992).

The human *TAC1* gene is 8kb long located on chromosome 7 (7q21-q22) and consists of 7 exons which give rise to 4 alternatively spliced mRNA transcripts (prepropeptides) which are termed α, β, γ, and δ (Figure 1.3). Each prepropeptide differs in its exon composition: the β utilises all 7 exons, the α lacks exon 6, the γ lacks exon 4 and the δ lacks both exons 4 and 6. Polypeptides produced from these splice variants allow cells to produce SP encoded from exon 3 present in all splice variants. NKA is encoded from exon 6 and is therefore only produced from β and γ mRNAs. NPK and NPγ are N-terminal extended forms of NKA and are produced from β and γ PPT-A mRNA respectively. Alternative splicing of these 4 mRNA transcripts and post translation modification of the precursor polypeptide results in the production of the active neuropeptides (Satake *et al*, 2004).

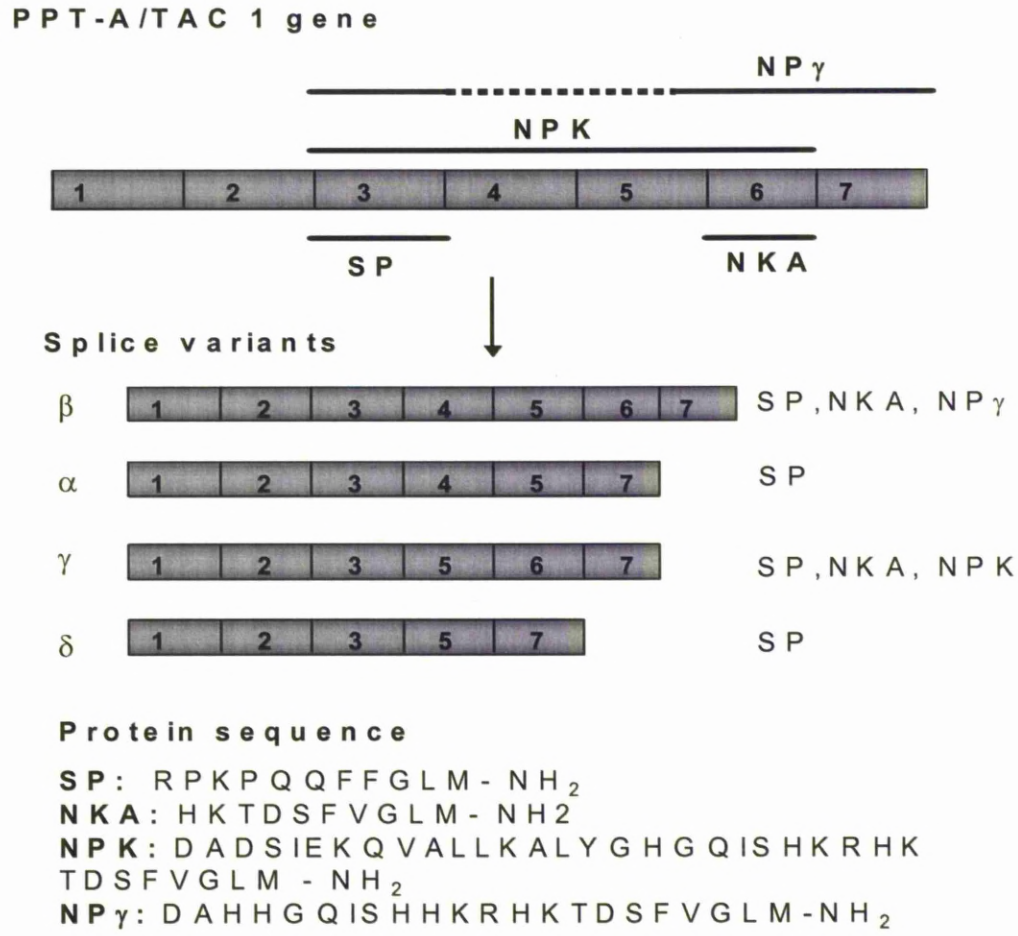

Figure 1.3: Schematic representation of biosynthesis of tachykinin products from humanTAC1. *The TAC1 gene encodes 4 splice variants α, β, δ, γ which have different combinations of exons. Exon 3 is required for the production of SP, exon 6 is required to encode NKA. NPK requires exons 5 and 6 and NPγ requires exons 6 and 7. Transcription and translation products are shown.*

In contrast, the mouse *Tac1* gene is located on chromosome 6 (chr6:7505071-7512973bp) and is also alternatively spliced and undergoes post-translational processing to produce SP, NKA, NPK and NPγ. The related tachykinin peptide neurokinin B (NKB) is coded by the *TAC3* gene in humans and the *Tac2* gene in mice.

TACR1 (often referred to as NK1 receptor) is expressed by a single-copy *TACR1* gene found on chromosome 2 in humans (2p13.1-p12), consists of 407 amino acid residues and has a molecular weight of 58u. SP is selective for the TACR1 receptor and has a high affinity for it, the affinity of NKA, for comparison, is 100 fold lower (Gerard *et al*, 1991). The order of affinity for the TACR1 is SP>NKA>NKB. NKA has a preference for TACR2. The *TACR1* gene belongs to a gene family of tachykinin receptors that are characterized by their interactions with G proteins and contain seven hydrophobic transmembrane regions and also includes NK2 and NK3 receptors. TACR1 is localized in high concentrations in the CNS (particularly the striatum, amygdala and some thalamic nuclei) and peripheral tissue where they mediate their control of physiological functions including stress reactions, mood, immune modulation, excitatory neurotransmission and neurogenic inflammation. However, TACR1 is also found in epithelium and many other cells.

The mouse Tacr1 is coded by the *Tacr1* gene located on chromosome 6 (chr6:82352469-82510093bp) and is also a single-copy *Tacr1* gene that is also expressed and localised in high concentrations in the CNS and peripheral tissues. Further human and mouse expression patterns will be discussed in more detail in the relevant chapters of this thesis.

As mentioned previously SP belongs to a family of neuropeptides known as the tachykinins found in the CNS as well as the PNS and have neurotransmitter like properties. They share the same hydrophobic C-terminal region with the amino acid sequence Phe-X-Gly-Leu-Met-NH2 where X represents a hydrophobic residue that is either aromatic or aliphatic (ringed or non-ringed carbon atoms). They can initiate

neurotransmission by themselves or synergistically with classic neurotransmitters such as the monoamines, acetylcholine and glutamate at the nerve terminals of neurons (Sergeyev *et al*, 1999). However, the production, synaptic storage and regulation of neuropeptides is distinct from the classic neurotransmitters which are synthesised in the presynaptic terminal and stored in small synaptic vesicles that are constructed in the terminal region. In contrast, the tachykinins are expressed by endogenous genes and their complexity and numbers are obtained via transcriptional, translational and posttranslational modification in the cell body and axon regions. They are then processed and packaged into large dense core vesicles that are assembled in the Golgi apparatus and transported to the synapse. The tachykinins and classic neurotransmitters do share a common feature however, and that is the co-localisation of their vesicles at the nerve terminals of neuronal cell types. In fact, this co-localisation of vesicle storage is a defining feature of all the neuropeptides (figure 1.4).

SP is released through the depolarising action of calcium dependent mechanisms and stimulates the membrane bound TACR1 to generate second messengers that can trigger a wide range of effecter mechanisms that regulate cellular excitability and function.

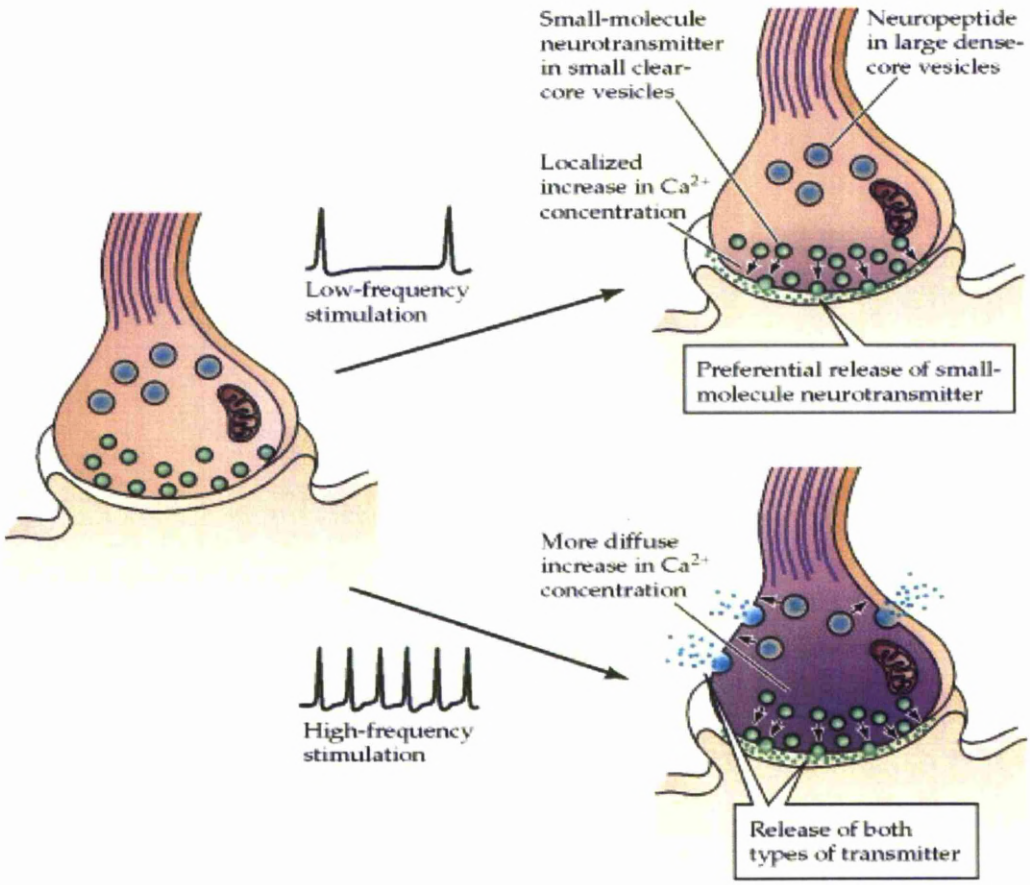

Figure 1.4. *Reproduced from the NCBI website. Co-localisation of classic neurotransmitters in small clear core vesicles near the synaptic terminal along with the large dense core vesicles containing the neuropeptides that are transported to the membrane for release on stimulation.*

Originally, it was thought that SP and its preferred receptor, TACR1, existed primarily within the neuronal synapse, this was the assumption for many neuropeptides and their receptors. With research it has been discovered that this is clearly not the case especially with regards the TACR1 which has been found all over the cell body of neurons as well as in the brainstem, on the surface of vascular

endothelial cells, in muscle, gastrointestinal tracts, pulmonary tissue, thyroid gland and immune cells.

SP and TACR1 have been implicated in a wide range of functions including pain perception and neurogenic inflammation. Since inflammation and pain is involved in the vast majority of disease states and tissue injury it is no surprise that SP has been implicated in the pathophysiology of many abnormal states ranging from stress, anxiety, infection and the immune response, mood, fibromyalgia, depression and epileptic seizures. Once an inflammatory state is induced the levels of SP and TACR1 increase and SP is able to diffuse even deeper across more layers of tissue and bind receptors in the surrounding neurons creating a cascade effect (Brown *et al*, 1995). As a result both SP and TACR1 are often the targets for preclinical trials of antagonists for a variety of conditions.

For the purposes of this thesis we are particularly interested in the role SP and TACR1 plays in the CNS with regards anxiety, depression and epilepsy and this will be discussed and documented further in the chapters.

SP and TACR1 are particularly prevalent in the areas associated with the control of fear and anxiety such as the amygdala, hypothalamus and periaqueductal gray (Davidson *et al* 2006, Riberiro-da-Silva *et al* 2000) and also in the hippocampus which is the main area of the brain associated with epileptic seizure activity (figure 1.5).

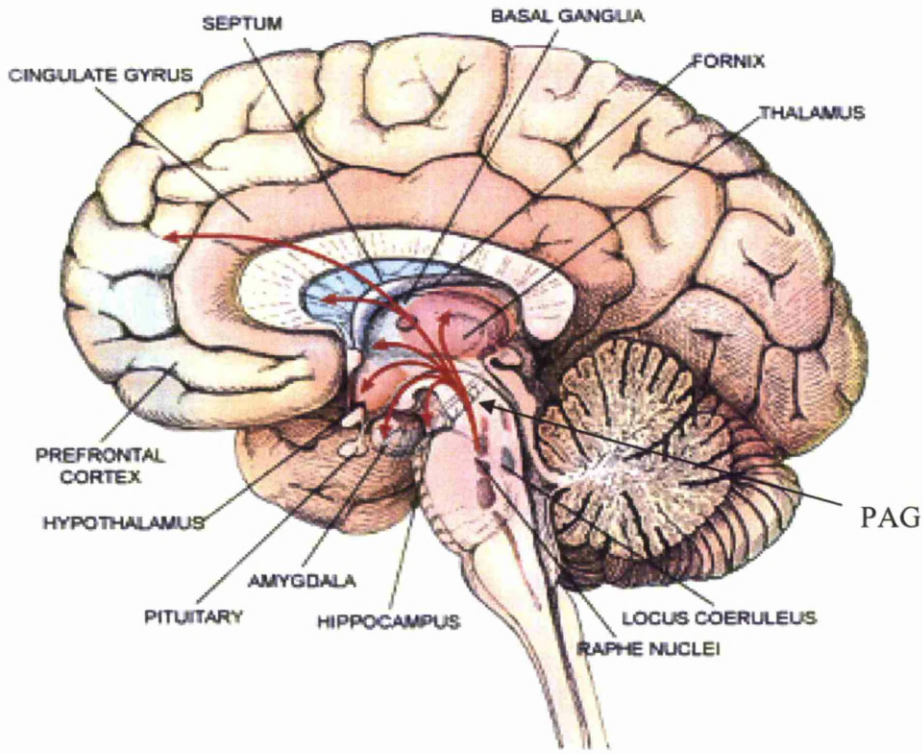

Figure 1.5. *Reproduced and modified from mindblog. The periaqueductal gray (PAG) can only be seen through sections of the brain but is located in close proximity to the amygdala and hypothalamus.*

As a result many TACR1 antagonists have been deployed and used in animal research and have been shown to display anxiolytic and antidepressant like effects (Vendruscolo *et al* 2003, Rodgers *et al* 2004). Support for the involvement of the tachykinin system in the pathophysiology of mood disorders was further demonstrated by the use of the TACR1 receptor antagonist, MK869, in a placebo-controlled clinical trial (Czeh *et al* 2006, Kramer *et al* 1998).

1.5.1 Tac1 and Tacr1 knockout mouse models

The research into SP function and regulation via its preferred receptor TACR1 has been aided by the creation of genetically altered mice that have had their *Tac1* and *Tacr1* genes disrupted resulting in strains of "knockout" mice that lack the gene products SP and Tacr1 respectively.

There are currently two knockout models for *Tac1* gene. The first, and the one used in this thesis is the "Zimmer" model and is discussed in more depth further on. The correct nomenclature for this knockout is B6.Cg-*Tac1* ^{tm1nimh}/J. For this strain a targeting vector containing the neomycin and thymidine kinase resistance genes was used to disrupt the *Tac1* gene by replacing parts of exons 2 and 3 using MP12 embryonic stem cells for homologous recombination. The neomycin gene allows for positive selection of recombinant embryonic stem cells and the thymidine kinase resistance gene is used to check for non-specific recombination events. Zimmer then backcrossed his mice for 10 generations onto C57BL/6J mice before inbreeding to generate a null model that does not produce any detectable levels of SP or NKA but does express the other gene product peptides. It is not clear from the literature but the partial removal of exon 3 may produce a detectable but possibly inactive or truncated form of NPK (neuropeptide K).

The second is the "Basbaum" model. Correct nomenclature is B6.Cg-*Tac1*^{tm1Bbm}/J. For this strain a targeting vector containing the neomycin resistance and thymidine kinase genes was used to disrupt the *Tac1* gene by replacing coding exon 3 and deleting the NKA coding region in exon 6 using CB1-4 embryonic stem cells for homologous recombination. Basbaum then backcrossed the mice for 7 generations onto C57BL/6J before sending them to the Jax labs who then backcrossed them for

another 5 generations onto C57BL/6J before inbreeding the homozygous null line which is currently up to generation 6 of filial inbreeding (sister x brother mating) as of January 2011. This null model also does not produce any SP or NKA but may have a very different flanking sequence to the B6.Cg-*Tac1* ^{tm1nimh}/J model. The importance of the flanking gene sequence is discussed further in the relevant chapters of this thesis.

There are currently two knockout models for the *Tacr1* gene. Since the *Tacr1* gene has no alternative splicing and therefore only produces Tacr1, different models are less of an issue because they will all essentially be null for the Tacr1. What is a big issue for these (and all mouse models) is the background strain they are on and the two that are available are on different backgrounds and have produced very different and contrasting results which demonstrates the importance of background (Bilkei-Gorzo *et al* paper in 2002).

The first Tacr1 null (B6.Cg-*Tacr1*^{tm1ucl}/J), and the one used in this thesis, is known as the "Hunt" model and is discussed in more detail further on in this thesis (Carmen De Felipe *et al*, 1998). The recombination event deletes exon 1 which ensures that there will be no truncated product produced.

The second Tacr1 null (not used in this thesis) is documented in Santarelli *et al*, 2001 and uses a PGK-neo targeting cassette which also replaces exon 1 resulting in the deletion of the first two transmembrane domains of the Tacr1 generating a mouse model null for the receptor as confirmed by in situ hybridisation and immunocytochemistry. In this model, however, they used only 129/Sv mice generating a pure inbred background but this strategy does have its drawbacks which will be discussed further on in this chapter.

The two "knockout" mice strains used in this thesis (B6.Cg-*Tacr1*^{tm1ucl}/J and B6.Cg-*Tac1* ^{tm1nimh}/J models) have been extensively studied in behavioural testing and have been found to have common phenotypes including lowered anxiety levels, reduced fear responses, higher pain thresholds and lowered inflammatory response (Bilkei-Gorzo *et al*, 2002; Zimmer *et al* 1998). These models were also subjected to various behavioural testing as part of this thesis and the relevance of the tests used as well as the results from this thesis and other peer reviewed papers will be compared and contrasted in the relevant chapters of this thesis.

1.5.2 Tac1 knockout mouse model used in this thesis

Since it has already been stated that the background strain of mouse used for a knockout line makes a difference further details on the generation and breeding of knockout mice used in this thesis are now given. The first was the Tac1 knockout (B6.Cg-*Tac1* ^{tm1nimh}/J) generated by selective deletion of the SP coding region by homologous recombination of a targeting vector with parts of exons 2 and 3 in ES cell lines which are derived from the 129SV/J strain (see figure 1.6 for targeted deletion).

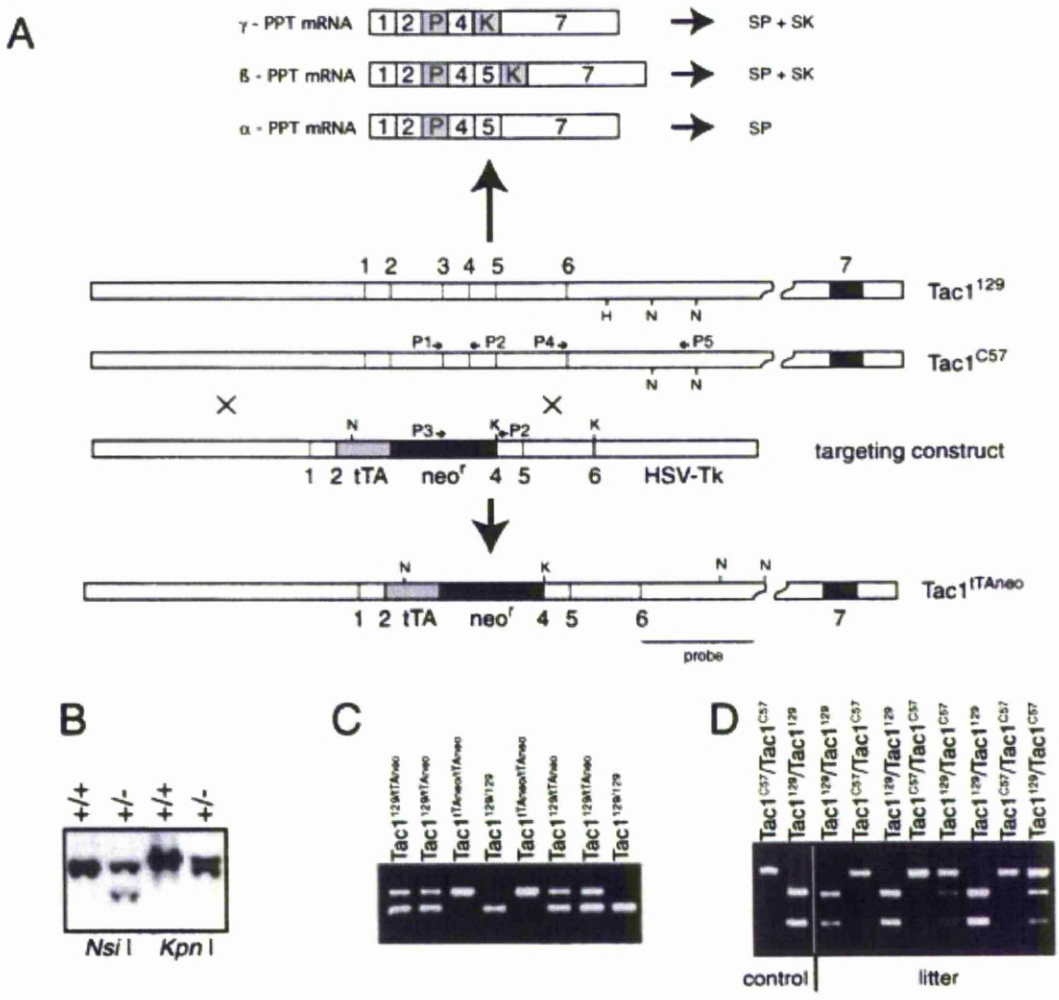

Figure 1.6 Reproduced from Zimmer et al, 1998 to show targeted deletion on endogenous mouse PPT-A gene for generation of knockout mice. *Targeted mutagenesis of the Tac1 gene. (A), shows the prepropeptide mRNA transcripts and also the different cDNA from the mouse strains 129SV/J ($Tac1^{129}$) and the C57BL/6J ($Tac1^{C57}$), the targeting construct used with the neomycin resistance gene, and the recombinant locus ($Tac1^{tTAneo}$). (B) Shows Southern blot analysis to identify recombinant ES cell. (C) Shows PCR genotyping of offspring from $Tac1^{tTAneo/C57}$ × $Tac1^{tTAneo/C57}$ matings (D), Zimmer used probes to differentiate between the genotypes of offspring from $Tac1^{129/C57}$ × $Tac1^{129/C57}$ matings by PCR amplification with primers P4 and P5, and subsequent digestion with HpaII. Note that HpaII cuts only the PCR fragment derived from the 129 allele, but not the C57BL/6 allele. tTA (tetracycline transactivator protein).*

In the original paper problems associated with the allele distribution within the offspring of his F1 progeny from the germ-line chimera to C57BL/6J matings was recognised (Zimmer *et al*, 1998). To address this issue the *hpa*II polymorphism was used as a unique DNA marker sequence as it is only present in intron 6 of the 129SV/J mice so it is possible to distinguish the wildtype alleles donated by either the 129 or C57BL/6J line. Zimmer then interbred his F1 progeny together to produce two lines, a knockout line for the Tac1gene and a suitable wildtype control line whose Tac1 locus is derived only from 129SV/J mice. However, whilst they are suitable controls and knockouts for the Tac1 locus they are now on a mixed background of 129 x C57BL/6 strains so they are a heterogeneous population with increased variability. Never the less Zimmer has effectively addressed the control animal for the area of modification he is looking at, in other words comparing the 129 Tac1 locus intact with the 129 Tac1 locus disrupted by the targeting vector.

The B6.Cg-*Tac1* ^{tm1nimh}/J mice were then used for behavioural testing. For certain aspects of behavioural testing the 129SV line of laboratory mice makes a poor model due to a lack of communication between the two cerebral hemispheres caused by congenital agenesis of the corpus callosum (Magara *et al*, 1999; Trillat *et al*, 1998). The corpus callosum is a large bundle of axons that interconnect neurons in the two hemispheres. The developmental mechanisms causing the defects of the corpus callosum are not completely understood but research indicates the problem is in the substrates of axon guidance at the midline structure and the BALB/c inbred strain also suffers from this phenomenon (Ozaki and Wahlsten, 1993). In a paper by Gardier and Bourin, 2001, demonstrated the problem of background strain is noted as an issue particularly when testing knockout mice as models of depression or using them to test the efficacy of antidepressants. Unfortunately this is quite a common

problem as many existing models are from a 129 lineage as discussed earlier with regards availability of ES cells lines.

To address the problem of background Zimmer went on to backcross his Tac1 knockout mice for 10 consecutive generations to a C57BL/6J strain. In a paper in 2002 he subjected the mice to various behavioural testing to evaluate their responses with confidence (Bilkei-Gorzo et al, 2002). The B6.Cg-*Tac1* ^{tm1nimh}/J knockout on the C57BL/6J background is the one that was used throughout this thesis therefore the problem of 129SV lineage will be effectively dismissed but the DNA sequences flanking the Tac1 open reading frame may still be an issue with regards this strain and this will be discussed in chapter 3 of this thesis.

1.5.3 Tacr1 knockout mouse model used in this thesis

The other knockout used in this thesis was the B6.Cg-*Tacr1*^{tm1ucl}/J line (Carmen De Felipe et al, 1998). Here, the ES cell line used was the HM1 line which is derived from the 129P2/OlaHsd line (Jax labs). The authors, however, quote a 129/Sv parentage. The incorrect use of mouse nomenclature is a common problem in many publications and has ramifications with regards replication of results. Whilst the problem of defective corpus callosum is not reported as frequently with the 129P2/OlaHsd lineage of 129 mice they are still related to the Sv substrain and should still be considered a poor model background strain for behavioural studies and testing of antidepressant drugs. In the original work there was no provision made to distinguish between the wildtype allele originating from the 129 line and the one originating from the C57BL/6 line used to produce the F1 generation. Mice heterozygous for the disrupted allele were interbred to produce the null Tacr1 mice and presumably the wildtype controls that were used to confirm successful disruption of the *Tacr1* gene via auto radiographic mapping of the SP binding sites in the forebrain and spinal cord (see figure 1.7).

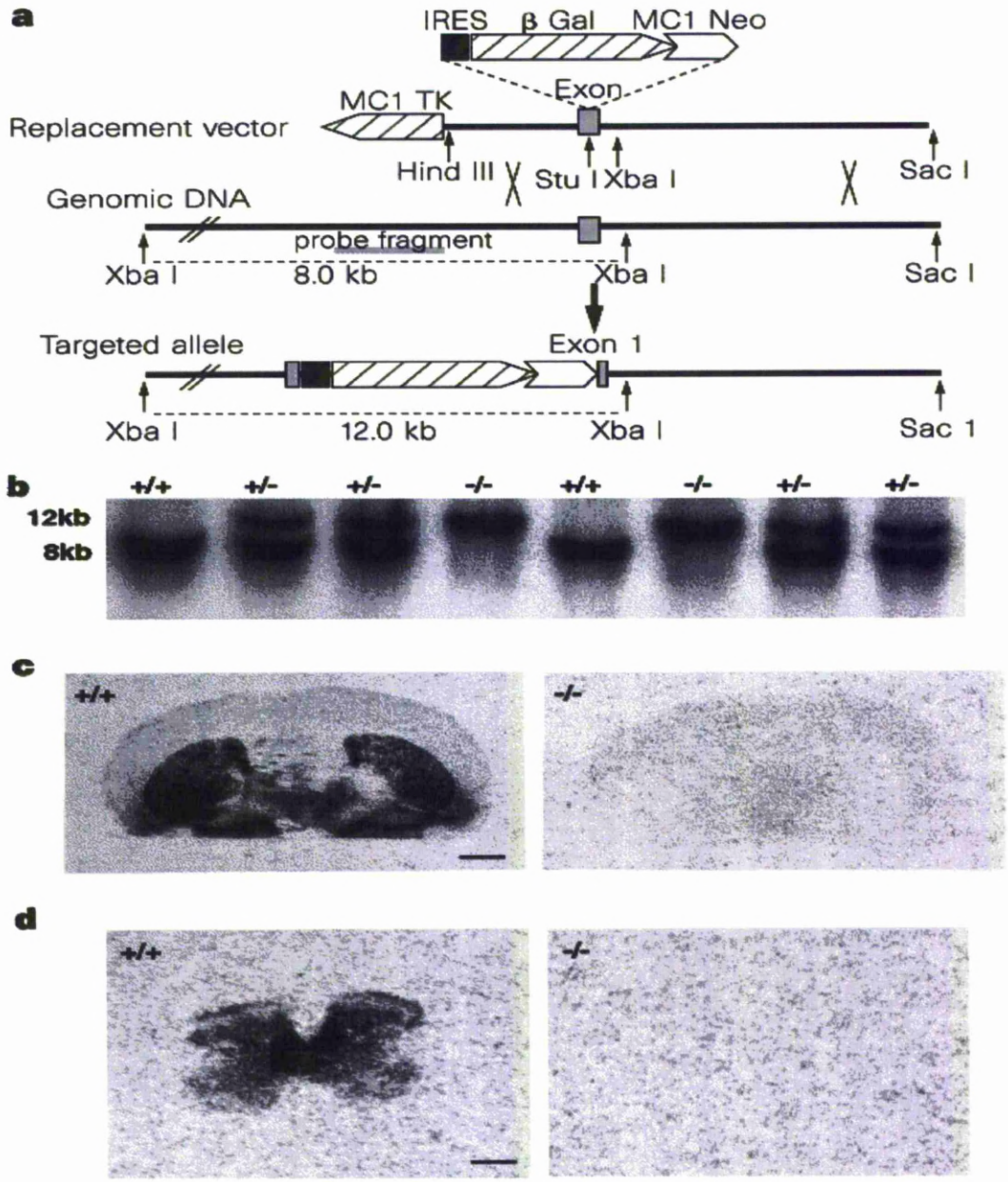

Figure 1.7 Reproduced from Carmen De Felipe et al paper to show targeting construct in the B6.Cg-Tacr1^{tm1ucl}/J mice. ***A****, Drawing of the region of the wild-type NK-1 locus containing exon 1 and the recombination event with the targeting vector.* ***B****, Southern blot analysis of offspring from an NK1$^{+/-}$ intercross to detect nulls. Homozygous (12 kb), heterozygous (12 kb and 8 kb) and wild-type (8 kb) mice were generated at a ratio of 1:2:1.* ***c****,* ***d****, Auto radiographic mapping of SP binding sites in forebrain (**c**) and spinal cord (**d**) sections of NK1$^{-/-}$ and NK1$^{+/+}$ mice.*

These two lines of mice (nulls versus controls) were then subjected to various nociceptive and behavioural testing. Whilst the assessment of the null *Tacr1* locus is homozygous (both alleles disrupted) and 129 in origin, the testing and assessment of the wildtype locus could potentially be very mixed due to flanking sequences originating from both 129 and C57BL/6J (BL6) background strains. In the F2 and F3 generations there is the statistically likelihood that controls with the following wildtype *Tacr1* locus origins will have been used; 129/129; BL6/BL6 and 129/BL6, in other words not just variation on the background strain but also variation at the locus of interest. As with the B6.Cg-*Tac1* ^{tm1nimh}/J knockout line this variation and lack of suitable controls was addressed with the backcrossing of the B6.Cg-*Tacr1*$_{tm1ucl}$/J mice for 10 consecutive generations onto the C57BL/6J background and it was this line that we received and used as part of this thesis. For that reason we will again dismiss the problem of the 129P2/OlaHsd lineage but consider the flanking gene issue in our discussion of the results from experiments using this line.

1.6 Role of serotonin (5HT) in behaviour disorders

Since the data presented in this thesis focuses on the role of the tachykinins in anxiety, depression and epilepsy it is appropriate to mention and give an overview of the neurotransmitter serotonin (5HT) as it is often the monoamine that is the focus for all the mood disorders.

Serotonin is one of the most extensively studied neurotransmitters and yet its role in the brain remains poorly understood. This is no doubt in part due to the fact that it seems to be involved in virtually everything from mood to sleep, human behaviour and mental disorders, circadian rhymes, smooth muscle contractions and recently implicated in epilepsy as well. There is also evidence to suggest that serotonin (also known as 5-hydroxytryptamine, 5HT) acts as maternal "morphogen" in conjunction with the foetal serotonin transporter protein (SLC6A4 also known as 5HTT and SERT) to direct and "orchestrate" the complex neuronal arrangement of not only the central nervous system (CNS) but also the enteric nervous system (ENS), (Zhou *et al*, 2000). It is also one of the most ancient neurotransmitters involved in life on earth with its functional use dating back in evolutionary terms to jellyfish with a diversification timeline of 800 million years ago. 5HT interacts with a multitude of genes and has many regulators but without a doubt the serotonin transporter protein (SLC6A4, SERT) is one of the most critical regulators of serotonin through removal of extracellular 5HT, to fine-tune serotonin signalling (figure 1.8).

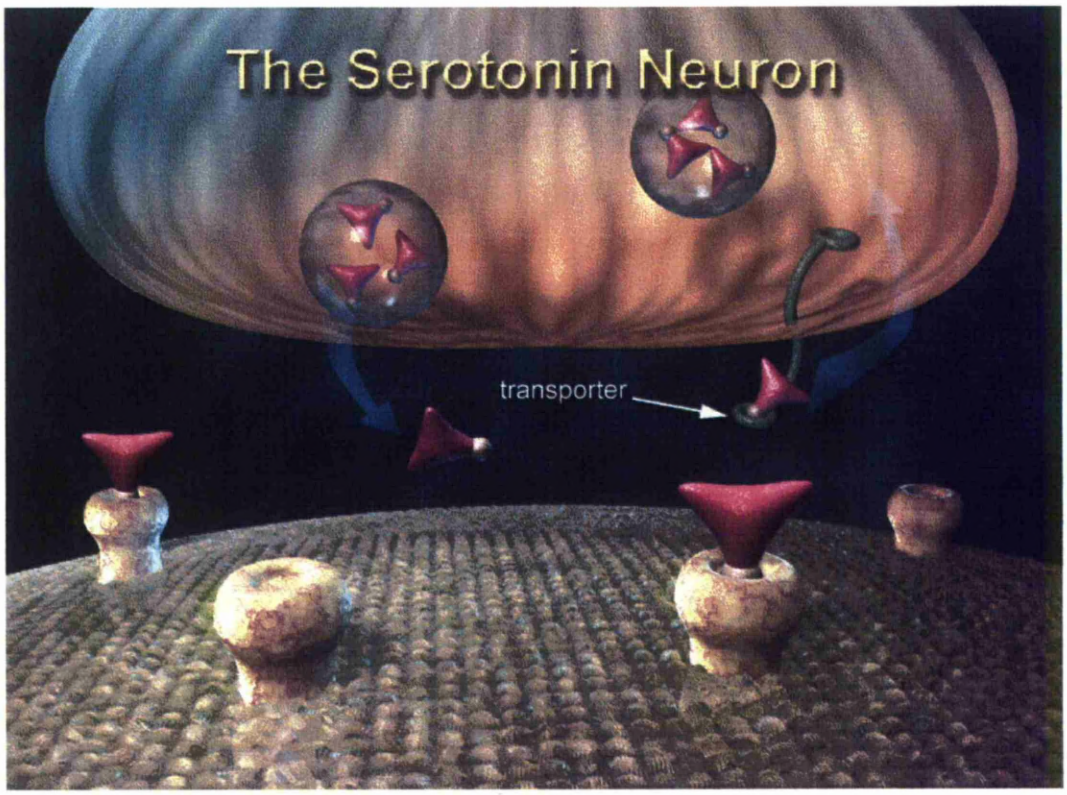

Figure 1.8 Image reproduced from NIDA website. *Serotonin is stored in small vesicles near the synaptic cleft of a neuron unlike the tachykinins that are stored in large dense core vesicles of the neuron cell body. It acts as a chemical messenger across the cleft to signal on the post synaptic neuron via various receptors. In this picture the serotonin transporter protein (*SLC6A4*) is illustrated on the membrane of the pre-synaptic neuron actively taking up the serotonin and in so doing it regulates the amount of serotonin signalling across the cleft along with various other mechanisms including a serotonin inhibitory feedback loop.*

Since the neurotransmitter serotonin (5HT), and its main regulator the SLC6A4 protein, are historically well documented to be pivotal in the control of mood and behavioural disorders a lot of research has focused on a regulatory link between 5HT, SLC6A4, SP and its preferred receptor TACR1. This is confirmed by a number of

papers that show the presence of the TACR1 receptor on 5HT neurons of the Dorsal raphe nucleus, a major source of serotonergic input to the forebrain (Lacoste *et al,* 2006), and further substantiated by the findings that disruption and drug blockage of the TACR1 receptor reduces anxiety and stress in conjunction with an increased firing rate of 5HT neurons in the dorsal raphe nucleus (Gobbi *et al*, 2005, Blier *et al,* 2004). In addition it has been documented that depressed patients have a higher level of SP in their cerebral spinal fluid (CSF) (Bilkei-Gorzo *et al*, 2002; Santarelli *et al*, 2001). The hypothesis that the TACR1 receptor can modulate the levels of 5HT signalling and extracellular levels via secondary messenger mechanisms is also supported by the findings that disruption and drug blockage of the Tacr1 has no effect on transcription regulation or binding potential of the SLC6A4 protein (David *et al*, 2004).

A regulatory link between SP, Tacr1 and 5HT levels is discussed further in the relevant chapters of this thesis.

1.7 Anxiety and depression

Anxiety is experienced by the majority of people at some point in their lives, however, whilst it can be a transient experience it can also present itself as a psychiatric disorder that is debilitating for the person concerned. The term anxiety disorder covers several different forms of abnormal and pathological fear and anxiety. While some categories are well known and may only manifest their effects in response to specific stressors (e.g. the phobias), in reality any of the anxiety disorders can be chronic in nature and cause physiological responses that are distressing and potentially debilitating (difficulty breathing, dizziness, headache, sweating, muscles spasms, palpitations and hypertension, nausea, vomiting and diarrhoea, sleep problems and cognitive disruptions).

The biological contributors of anxiety disorders are thought to be low levels of neurotransmitters specifically, serotonin (5HT) and gamma amino butyric acid (GABA), and treatment usually involves the use of the selective serotonin reuptake inhibitors (SSRIs) that target the SLC6A4 protein with varying levels of success (review, Baldwin *et al*, 2010).

Anxiety disorders are often accompanied with other mood disorders, particularly clinical depression. Research suggests that 60% of people with anxiety disorder will also be diagnosed with clinical depression (Cameron, O.G, 2007). This co-morbidity may be due to the fact that there is considerable overlap between the symptoms of both disorders and the environmental triggers are common risk factors for development of these mood disorders (stress, alcohol abuse, substance abuse and traumatic events). However, while they share some similar symptoms, such as disruption of sleep patterns and reduced sex drive, the behavioural basis of the

disorders are contrasting. Anxiety disorders have a behavioural basis associated with the fear response whereas clinical depression manifests itself with a disruption of normal motivations and an inability to experience pleasure. Not surprisingly research has focused on the neurotransmitters serotonin and dopamine in the search for a biological basis for the progression of, and susceptibility to, depressive disorder.

Depressive disorder is a serious disabling condition that can adversely affect a person's life and general health. Depressive disorder is one of the oldest recognised mood disorders and is classified in both the International Classification of Diseases and the American Psychiatric Associations diagnostic manual. It is also known as unipolar depression (or disorder), clinical depression, major depression and recurrent depressive disorder. Entire regulatory systems can become dysfunctional and sufferers report disruptive sleep patterns, loss of appetite and disruptive eating patterns, low self-esteem, lack of interest in previous pleasure activities, low mood and withdrawal and isolation from family, friends and work. Since mood disorders are highly influenced by both genes and environment research has focused on finding the underlying genetic factors that contributed to the development of the disorders.

1.8. Mouse models of anxiety and depression like states

The use of mice in *in-vivo* research usually invokes thoughts of pharmacological drug testing or infection with pathogens. What is probably less appreciated is that with the right parameters mice can be used as models to study the neurobiological mechanisms of depression and related mood disorders. While some depressive

symptoms are irreproducible in mice (e.g. thoughts of suicide) there are analogues to the human symptoms that can be monitored objectively and even be treated and reversed by the same drug treatments used for humans. Mice, in particular, exhibit anxiety-like behaviours when exposed to certain stressors (new environments, bright light, territory intruders). Many of the anxiety behaviours exhibited by mice have been improved and even reversed by the same classes of drugs used to treat humans and the results are well documented in the literature (Hagenbuch *et al*, 2006; Gobbi and Blier, 2005; Santarelli *et al*, 2001). The C57BL/6J strain is often used for behavioural testing and is well documented to have the most stable and "normal" behaviour with a low level of anxiety when compared with other inbred strains (Mouse Phenome Database MPD; Festings, 1979).

Regardless of whether the researcher intends to use genetically modified or conventional mouse strains it is vitally important to "know thy strain" before any behavioural testing is conducted. The Jax lab website has a detailed wealth of knowledge on every inbred background of conventional mouse and if the transgenic or knockout model is available from the Jax lab they will have an equally impressive list of data on these models too.

(**www.jax.org/phenome**; **www.informatics.jax.org**)

It is important to know what the normal behaviour of the strain is prior to the behavioural testing or stressor tests. If a strain that is high for anxiety is used then this will just mask the effects of subtle changes due to stressors, genetic modifications or the use of pharmacological interventions. In addition, strains with poor eyesight are unsuitable for some experiments such as the C3H strain that carries the *rd* (retinal degeneration) gene and is blind after about 6 weeks of age.

Albino strains too (Balb/C, FVB) all have poor eyesight making them strains to be used with care in behavioural studies.

It should also be remembered that other factors such as age, weight, sex, stage of oestrous cycle, diet, environmental enrichment and the housing of animals prior to an experiment will all have an effect on studies into anxiety and depression like behaviours. In particular the females will have a different response to the males and are an unsuitable choice for some experiments such as the intruder test or social dominance since they are evolutionarily designed to live and breed in large co-operative groups and very rarely display any intruder or intergroup aggressions and anxiety like behaviours.

Observational data can also prove to be valuable such as mouse coat state assessment. This can be a quick and qualitative method of assessing mouse depression like states as rodents tend to quickly lose coat quality appearance when stressed. Part of rodents normal routine is frequent coat grooming both personal and intergroup social grooming, stress and anxiety quickly lead to a change in normal behaviour with grooming being neglected in a similar manner to the poor hygiene that can be exhibited in depressed patients. The use of antidepressants can reverse this abnormal behaviour and quickly improve coat condition (Yalcin *et al*, 2006). Studies have linked a reduction in corticotrophin-releasing factor (CRF) with an improvement in coat condition which is relevant to depression like states as antidepressants (imipramine) and anxiolytic (chlordiazepoxide) interact with CRF. Therefore, using a scoring system (e.g. Irwin scale) a mouse can have a full observational medical prior, during, and after the experimental study which can contribute to the experimental data gathered.

1.8.1 Rodent behavioural tests for anxiety

Rodent tests for anxiety related behaviours are usually based on the conflict inherent in approach-avoidance situations. For example, a mouse will have a natural desire to explore a new environment for a potential new food source or, even better, a potential mate. However, being a highly preyed upon mammal with many predators it is reluctant to venture out into open exposed spaces during daylight hours.

To evaluate anxiety levels of mouse strains and knockout lines the light/dark box test can be employed which contrasts the animal's natural instinct to explore its surroundings and environment and its reservation to enter a light illuminated field. Animals that spend more time in the dark boxed area compared with the open light illuminated area are considered more anxious as they are reluctant to leave the security of the dark box (Karl *et al* 2008, Mathis *et al* 1994; Crawley and Goodwin 1980). Mice with lower anxiety levels spend more time in the light area exploring the environment in comparison to the mice with high anxiety levels that are reluctant to leave the safe dark area of the box. Figure 1.9.

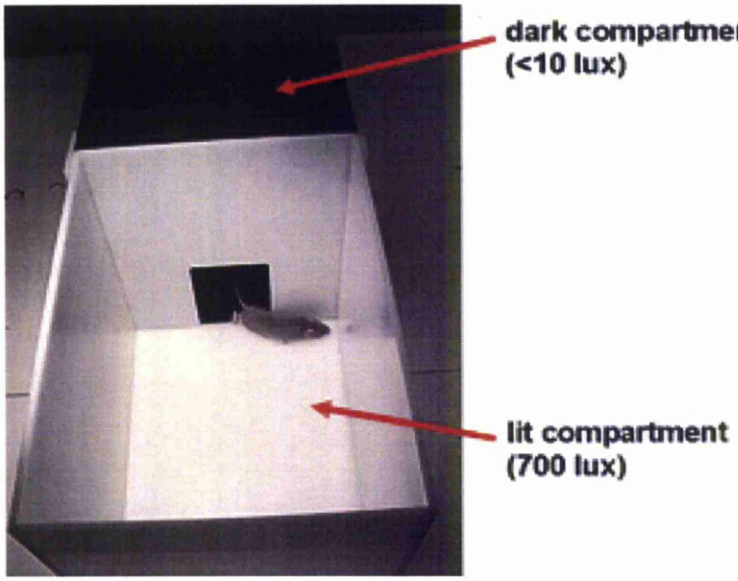

Figure 1.9. *Image reproduced from UCLA behavioural core. The light/dark box test evaluates the conflict between the animals instinct to explore with the desire for safe refuge and is a standard behavioural test for anxiety.*

1.8.2. Rodent behavioural tests for depression

There are a number of tests for depression in rodents including the tail suspension test but the most widely used one is the Porsolt forced swim test. These types of tests are based on an inescapable aversive situation and measure the animal's behavioural despair. The animal's early cessation of attempts to escape the situation it finds itself in is considered a rodent analogue of stress induced depression (Drugan *et al* 1989; Porsolt *et al* 1977, 1978). Again, the validity of these tests are reinforced by the use of antidepressant drugs that increase the time the animal in question spends looking for a means of escape. In particular, in the Porsolt forced swim test the time spent swimming (looking for escape) is greatly increased in animals that are administered antidepressant drugs (Bergner *et al*, 2010; Holmes *et al*, 2003). Figure 1.10.

Figure 1.10. *Image reproduced from UCLA behavioural core. The forced swim test evaluates the animal's response to an inescapable aversive situation. Mice are placed into a cylinder of water at room temperature and a depth of 50cm from which they cannot escape. The time they spend floating as opposed to swimming (looking for an escape) is measured over 10 minutes duration.*

1.9. Epilepsy and status epilepticus

In recent years there has been a focus on the correlation between depression and epilepsy (Chungani et al 2005, Bagdy et al 2006) with particular interest focusing on the role of the tachykinins and the NK1 receptors and, more recently, the regulation of serotonin levels in the aetiology of epilepsy. Epilepsy is one of the most common neurological disorders and is associated with increased hippocampal excitability.

There are over 40 types of epilepsies known (Engel J, 2001) but the most common single form of epilepsy is temporal lobe epilepsy (TLE). TLE affects both humans and

animals and the recurrent epileptic seizures arise in the hippocampus, parahippocampal gyrus and amygdala (mesial temporal lobe epilepsy, MTLE).

Once a patient develops epilepsy one of the most distressing symptoms reported is the loss of memory and the inability to consolidate short term memory into long term (Gaitatzis *et al,* 2004; Johnson *et al*, 2004). This is hypothesised to be due to neuronal damage in the hippocampus since this is the region of the brain that is vital to memory formation. The hippocampus is also the major brain region associated with TLE. It can be divided into four fields, CA1 to CA3 and the dentate gyrus which is sometimes referred to as CA4 (figure 1.11). KA is known to effect mostly the CA3 region of the hippocampus and illicit its effects in the mossy fibres of this area.

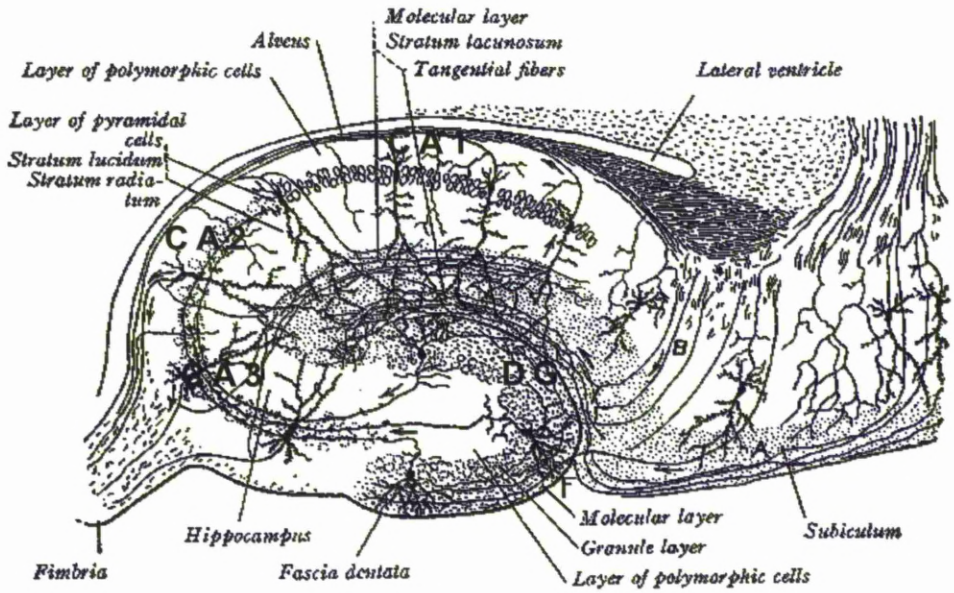

Figure 1.11. *Modified from Ramon and Cajal, 1911. The hippocampus regions CA1 to 3 and the dentate gyrus (DG) are identified. KA effects mossy fibres in CA3 region and generates seizures.*

The term Status epilepticus (SE) is often used and refers to a state of severe, repetitive seizure activity which often results in neuronal death (De Lorenzo, 1997). Research suggests that SE results from excessive stimulation of neurons by the excitatory amino acid glutamate but the mechanisms that cause excessive glutamate release still remain poorly understood. With this in mind, the neurotoxicant Kainic acid (KA) is frequently used as a chemical convulsant in epilepsy studies because it is a glutamate receptor agonist and "mimics" the amino acid neurotransmitter glutamate and activates its receptors causing a cascade of intracellular events such as, dysregulation of glutamate neurotransmissions, alteration of membrane electrophysiology and disruption of calcium signalling (Wang *et al*, 2005; Ben-Ari *et al*, 2000).

Since the pathological changes in neurotransmitter mediated excitability have been correlated to an increase in SP and NKB expression which in turn leads to long lasting changes in excitability by further potentiating glutamate release, research has focused on the transcriptional regulation of the *Tac1* gene (Wasterlain *et al.*, 2002).

1.10. Role of transcription factors

Transcription factors are proteins that bind specific DNA sequences and contribute to the transcriptional regulation of target genes. Whilst many proteins can play crucial roles in gene regulation (co activators, chromatin remodelers, histone acetylases, deacetylases, kinases and methylases) the transcription factors have the defining feature of DNA-binding domains (DBDs) which bind specific sequences of DNA adjacent to the genes they regulate (Mitchell and Tjian, 1989). It is estimated

that 10% of genes in the human genome code for transcription factors and further complexity is added by the fact that genes are often flanked by several binding sites for distinct transcription factors. Therefore, unique regulation of every gene can be achieved by combinational use of the estimated 2000 transcription factors.

Not surprisingly, transcription factors can be highly evolutionarily conserved even across distance related organisms. For example the Hox transcription factor family is important for determining correct body pattern formation and has been conserved from fruit flies to humans (Lemons *et al*, 2006). Transcription factors can regulate the communication between cells by up regulating or down regulating the production of signalling molecules. An example of this is the short signalling cascade involving oestrogen signalling via the oestrogen receptor transcription factor. Oestrogen is secreted by the ovaries and placenta and crosses the cell membrane of the recipient cell where it is bound by the oestrogen receptor in the cytoplasm. Once activated the oestrogen receptor then enters the cell's nucleus and binds to its specific DNA binding sites and changes the transcriptional regulation of the associated genes (Osborne *et al*, 2001). Transcription factors can also help in the response to environmental stimuli such as heat shock factor (HSF) which up regulates genes involved in survival at higher temperatures and hypoxia inducible factor (HIF) which up regulates genes for cell survival when in low-oxygen environments. Transcription factors also regulate the internal cell cycle and when they go wrong the results can be disastrous as in the case of oncogenes or tumour suppressors.

The transcription factors of particular interest to this thesis are the ones associated with the regulation of the *Tac1* gene expression, the repressor element-1 silencing transcription factor (REST), a truncated version of REST discovered in rodents

known as REST4 and the highly conserved activity-dependent neuroprotective protein known as ADNP which has recently been suggested as a nuclear transcription factor with neuroprotective properties.

1.10.1 REST and REST4

Zinc finger proteins are the most common DNA binding proteins in humans and the genes encoding them represent approximately 3% of the genome. These proteins contain repeating "finger like" structures formed by the co-ordinate bonding of two cysteines and two histidines (C_2H_2), separated by linker regions of hydrophobic amino acids that form positively charged loops that interact with DNA (Wolfe *et al.*, 2000). REST (also termed neuron restrictive silencing factor, NRSF) is a zinc finger protein that is encoded by the *REST* gene and belongs to the Kruppel-type zinc finger transcription factor family. It binds to a conserved 23 bp consensus sequence termed neuron restrictive silencer element (NRSE) and it has been observed to be dynamically regulated during seizure (Palm *et al*; 1998).

REST was originally thought to be a repressor of neuronal genes in non neuronal cells (Mori *et al.*, 1992; Kraner *et al.*, 1992), however, functional NRSE sites have been identified in several genes involved in neuronal function suggesting REST may also regulate expression of genes in neurons. REST is also found in undifferentiated neuronal progenitor cells and it is thought that it may act as a master negative regulator of neurogenesis. REST mediates gene repression by recruiting several chromatin-modifying enzymes, decreasing histone acetylation and increasing DNA methylation (Ooi and Wood, 2007). Interestingly, splicing variants have been observed which may have functional differences from the full length REST protein,

in particular rodent REST4 and the analogous human sNRSF have been identified which contain only five of the eight zinc fingers found in the full length REST and as a result they lack the C-terminal zinc finger repression domain which is responsible for recruiting a HDAC complex (histone deacetylase complex) via its interaction with CoREST (Ballas *et al*, 2001). It is possible that these variants may bind different cofactors to regulate gene expression in a manner that is different from the full length REST protein.

The role of REST4 remains debatable, however, there is evidence of it possessing anti-silencer function as it is able to interact with REST to antagonise its repression activity of the gene encoding choline acetyltransferase in PC12 cells (Roopra *et al.*, 2001; Shimojo *et al.*, 1999). EMSA has shown that REST4 can interact with NRSE domains but with a 10-20 fold lower interaction than the full length REST, however, overexpression of REST4 resulted in alleviated repression of BDNF (brain derived neurotrophic factor) in cortical neurons (Tabuchi *et al.*, 2002; Palm *et al.*, 1998).

The *TAC1* promoter contains a NRSE consensus sequence adjacent to the start of transcription and REST has been previously shown to regulate the *Tac1* gene *in vitro* models (Quinn *et al*, 2002). Particularly relevant to this thesis is the fact that two REST isoforms are induced and differentially regulated in response to seizures in rodents (Palm *et al*, 1998) and our group have previously postulated that REST is a key candidate to regulate *Tac1* gene expression during epilepsy with an interplay between REST and REST4 (Spencer *et al*, 2006; Quinn *et al*, 2002).

1.10.2 ADNP

ADNP is regulated by the 28 amino acid peptide VIP (vasoactive intestinal peptide). VIP was first isolated from the small intestine of pigs because of its ability to cause vasodilation (Said and Mutt, 1970) but despite its name it is also expressed in the brain. ADNP is known to be essential for accurate brain formation during development and is abundantly expressed in the adult brain suggesting a continuing important role post gestational (Gennet *et al*, 2008).

ADNP has been hypothesised to mediate VIP induced neuroprotection due to the fact that a synthetic peptide (NAP, eight amino acids in length) derived from the ADNP protein sequence has been shown to have highly neuroprotective effects *in vitro* (Gozes *et al*, 2000).

As well as a hypothesised role in neuronal protection as a result of trauma or insult, ADNP has been shown to protect against some forms of excitotoxicity which is a term given to the neuronal damage that results from over stimulation of excitatory transmitters such as glutamate (Gozes *et al*, 2000; Bassan *et al*, 1999). Since excitotoxicity is implicated in epilepsy the dynamic regulation of ADNP in our mouse models was of particular interest to this thesis and this is explored further in Chapter three.

1.10.3 Thesis hypothesis

Previous studies have revealed a role for the tachykinins in anxiety, depression and also epilepsy, however, conflicting publications suggest that the redundancy phenomena may be an issue when using the single knockouts for this area of research. It was hypothesised that the generation of a "double knockout" mouse model with a disruption of the preferred receptor/ligand pathway of B6.Cg-*Tac1* ^{tm1nimh}/J X B6.Cg-

Tacr1$_{tm1ucl}$/J would reveal that a level of redundancy is present in the contributing parental single knockout lines of B6.Cg-*Tac1* ^{tm1nimh}/J and B6.Cg-*Tacr1*$_{tm1ucl}$/J when subjected to behavioural and pharmacological testing.

The use of the single knockouts in previous studies in our laboratory had also revealed a potential model of seizure resistance with modulation of the *Tac1* gene via the transcription factors REST and REST4 with ADNP being implicated as having a neuroprotective role in the experimental models used.

The generation and use of the mouse models in this thesis intended to address the hypothesis that the transcription factors REST and REST4 are differentially modulated in the double knockout and single knockout lines of B6.Cg-*Tac1* ^{tm1nimh}/J and B6.Cg-*Tacr1*$_{tm1ucl}$/J in an experimental mouse model for TLE using the neurotoxicant Kainic acid (KA).

The same models would be utilised to explore the role of ADNP and address the hypothesis that it is differentially modulated in the double knockout and single knockout lines of B6.Cg-*Tac1* ^{tm1nimh}/J and B6.Cg-*Tacr1*$_{tm1ucl}$/J in an experimental mouse model for TLE using the neurotoxicant Kainic acid (KA) that may allude to its neuroprotective properties against excitotoxicity.

1.11 Mouse nomenclature

In conclusion for the introduction chapter of this thesis I would like to give a very brief overview of mouse nomenclature. As mentioned previously, nomenclature is vitally important when using mouse strains for their correct identification but also for replication studies. For this reason it is appropriate to review the rules and meaning behind the internationally standardised nomenclature that is used not only for existing mouse strains but also for the generation of new and novel mouse models.

Inbred strains of mice are ones that have been inbred by filial mating (brother x sister) for at least 20 consecutive generations. They were originally created for an expression of a trait with the aim of making the trait as consistent as possible by removing genetic variance.

Most commercial laboratories originally obtained their mice from Jax labs but then continued to maintain the line themselves with brother x sister matings. Since it is estimated that a spontaneous mutation can arise on average every 7 generations (Drake *et al*, 1998) the vast majority of commercial suppliers have a unique genetic and phenotypic substrain of the original Jax lab strain. Thus it is very important to put in the nomenclature for the lab maintaining the strain as this can tell the researcher the exact substrain of mouse used in studies (figure 1.12).

A). Typical inbred line =

Miss Abbie Lathrop's "pet shop" stock C.C. Little (1921) mating of female 57 C57BL (Black)/C57BR (Brown)/C57L (Leaden).

C57BL/6J (6 is substrain of original C57BL line and J denotes Jax labs)

B). Spontaneous mutation = **129P3/J-*Lepr^{db-3J}*/J** =

(Background Strain) (Gene Affected) (Allele designation) (Lab Maintaining Strain)

C). Knockout mouse line = **B6;129P2-*Il2^{tm1Tior}*/J** =

(Background (mixed) (Targeted gene) (Targeted mutation) (Allele designation) (Lab registration code) (Lab maintaining strain)

D). Transgenic mouse line = **C57BL/6-Tg(CAG-EGFP)1Osb/J** =

(Background Strain) (Transgenic) (Promoter) (Gene expressed) (Founder line number) (Lab registration code) (Lab Maintaining Strain)

Figure 1.12. *A). Shows the origin of the letters and numbers used in a common inbred line. What is important is the letters after the C57BL/6 as these will indicate the laboratory maintaining the line and therefore the genetics. B). Shows correct nomenclature to be used (colour coded) when spontaneous mutation has arisen and is bred for its phenotype. C). Correct nomenclature for knockout (tm = targeted mutation) again colour coded. D). Correct nomenclature for transgenic line (Tg) all colour coded.*

There are numerous examples on the Jax lab website and MGI website on the genetic and phenotypic differences between the substrains of mice that originally came from one line. To give an example of how this can impact a researcher's work we will look at two lines of C57BL/6J.

C57BL/6J = maintained by Jax labs

C57BL/6JOlaHsd = maintained by Harlan UK

On the face of it they appear to be both C57BL/6J but the second strain has been maintained at Harlan for over 20 generations and this strain has developed a mutation which has resulted in a deletion of *Snca* gene that is now homozygous throughout the entire colony. The Jax lab BL6J remains wildtype for the *Snca* gene. The SNCA protein (presynaptic protein α-synuclein) is implicated in a range of neurodegenerative diseases and is the primary structural component of Lewy bodies found in Parkinson's disease brains. Therefore, if these two strains of mice were used in neurological behavioural studies, or were used for backcrossing of a knockout or transgenic line onto C57BL/6J they would have very different genetics and potentially very different phenotypes as it has been documented that the Harlan BL6 strain has an alteration of dopamine release in the nigrostriatal system (Specht and Schoepfer, 2001).

Although descended from the same inbred strain the substrains that exist may have very different phenotypes. The longer the strain has been maintained without replenishing the genetics the further apart from the original strain it will genetically drift. This is especially important for knockout and transgenic strains that have the further complication and variability with the flanking gene sequences around the locus of the genetic modification.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Commonly used solutions and reagents

Luria broth (LB) media

10.0g/l peptone, 5.0g/l yeast extract, and 10.0g/l NaCl

LB Agar

10.0g/l peptone, 5.0g/l yeast extract, and 12g/l agar

5x TBE buffer

54g Tris(hydroxymethyl)methylamine (Tris) (BDH), 27.5g boric acid (VWR International), 20ml 0.5M Ethylenediamine Tetraacetic Acid (EDTA) (Sigma) and dH_2O up to 1 litre

Tris-buffered saline (TBS)

137mM NaCl (BDH), 27mM KCl (Sigma) and 25mM Tris (BDH) to a total volume of 1 litre. The pH is adjusted to pH7.4

6x agarose gel loading buffer

0.25% (w/v) Bromophenol blue, 0.25% (w/v) xylene cyanol, 1mM EDTA, 30% glycerol and 70% water.

Selective antibiotics

Ampicillin. Ampicillin sodium salt dissolved in water as 1000x stock (concentration 100mg/ml) and stored at -20°C. For positive selection of bacteria containing a plasmid with ampicillin resistance gene β-lactamase, growth media or plates supplemented with 1μl/ml of ampicillin stock to give a concentration of 100μg/ml.

Kanamycin. Kanamycin monosodium salt dissolved in water as 1000x stock (concentration 25mg/ml) and stored at -20°C. 1μl/ml of kanamycin stock was added to growth media or plates to give a final concentration of 25μg/ml.

2.2. Animals

Animals were utilised from Biomedical Services Unit, University of Liverpool and culled under local and national schedule one guidelines. All procedures were carried out according to home office licences and regulations. Transgenic mice analysis involved the use of Tac1 and Tacr1 knockout transgenic lines on a C57BL/6J background. Transient transgenics were generated as described in 2.3 of this chapter. All animals were bred in house under licence procedures except for the wildtype C57BL/6J controls that were purchased from Charles River UK and allowed to settle for a minimum 7 day period prior to use.

Mice were weaned at 19 to 21 days and group housed in their litter mate pairs males and females up to 6 in a cage. Light/dark cycle was summer time setting 12 hours light 12 hours dark on a 7am to 7pm cycle. Relative humidity was 45 to 55% and food and water was provided ad libitum. Food was standard chow (SDS pellet CRM). All animals used were adult and sexually mature with the exception of the transient transgenic embryos.

2.2.1. Removal of tail tips for genotyping

Removal of no more than 0.5cm of tail tip was done under general anaesthesia via gas anaesthetics trolley using Isoflurane and oxygen mix at 4-5% induction and 3-4% maintenance (1:4 millilitres per minute concentration respectfully). Ear punching identification was applied whilst still under anaesthesia (3Rs = refinement). Tail tips were frozen in 1ml ependoffs at -20°C and individually identified.

2.2.2. Isolation of genomic DNA from tail tips

Add 500µl of tail tip buffer plus 200µl of Proteinase K to each tail tip and incubate overnight at 55°C in water bath.

Freeze at -20°C for 20-30 minutes.

Spin down in cold room at 13,000 rpm for 10-15 minutes (keep on ice).

DNA precipitation. Transfer 360µl of supernatant to fresh eppendoff (freeze remaining tail tip sample).

Add 40µl of 3M sodium acetate pH7.

Add 40µl of isopropanol.

Mix (DNA should now be seen as white precipitate).

Spin briefly (30 secs) at 13,000 rpm.

Remove supernatant and gently re-suspend DNA pellet in 80% alcohol (500µl).

Spin briefly (30 secs) at 13,000 rpm and remove supernatant.

Air dry pellet at room temperature (do not allow DNA pellet to dry out completely or it will be difficult to re-suspend it).

Re-suspend in 200µl TE buffer pH8.

The DNA can now be analyzed or frozen at -20°C (can be defrosted and re-frozen several times).

2.2.3. Stimuli

5mM Kainic acid

10 mg kainic acid (Sigma) in 10mls dH_2O, dissolve by gentle mixing and warming. Kainic acid very difficult to dissolve and is aided by heating to 37°C in incubator and use immediately. Discard any unused solution. Weigh animals and dose at selected concentration.

2.3 Methods

2.3.1 Kainic acid Induction of Seizures in mice

Transgenic and knockout mice were used as described in chapter five (Tac1 null, Tacr1 null, double knockouts null for both Tac1 and Tacr1, and wildtype C57BL/6J), encoding regions were generated previously and backcrossed onto C57BL/6J background (Cao *et al.*, 1998; Zimmer *et al.*, 1998; Carmen De Felipe *et al,* 1998).

Adult mice were administered with 17mg/kg i.p of kainic acid in saline or saline alone as a control. Behavioural responses to KA were scored by means of elaboration of the methods described by (Morrison *et al.*, 1996). Seizure severity was recorded over a period of 90 minutes using an extended version of the Racine scale as follows. 0 = normal, 2 = staring, panting, frozen, 4 = head tossing, wet dog shakes, 6 = short myoclonic jerks, excessive scratching, tail rigidity, 8 = short forelimb clonus,

hunchback posture, 10 = loss of balance, hindlimb clonus, rearing, 12 = continuous forelimb clonus, discrete rearing and falling episodes, 14 = continuous rearing and falling, barrel rotations (full seizure), 16 = death. Following 90 minutes mice were administered with 10mg/kg i.p diazepam to arrest seizure activity. At 3 hours, 24 hours, 4 days and 7 days post SE mice were euthanased and the right hippocampus was removed from the right hemisphere and snap frozen in liquid nitrogen for RNA extraction.

2.3.2 The Racine scale of seizures (Racine, 1972)

Stage	Seizure type
1	Mouth and facial movements
2	Head nodding
3	Forelimb clonus
4	Rearing
5	Rearing and falling

Extended Racine scale for behavioural scoring:-

0 = normal,

2 = staring, panting, frozen,

4 = head tossing, wet dog shakes,

6 = short myoclonic jerks, excessive scratching, tail rigidity,

8 = short forelimb clonus, hunchback posture,

10 = loss of balance, hindlimb clonus, rearing,

12 = continuous forelimb clonus, discrete rearing and falling episodes,

14 = continuous rearing and falling, barrel rotations (full seizure),

16=death

2.3.3 Anaesthesia

Injectable for oviduct transfer (hypnorm and valium). Hypnorm injected intraperitoneally at 0.01ml/30g mouse, diazepam (valium) injected intra muscularly at 5mg/Kg body weight. Mouse remains anaesthetised for 30 to 40 minutes but has a sleep time of up to 2 hours during which time they must be kept warm using heat pad or heated recovery room.

Gaseous for tail tip amputation (Isoflurane and oxygen). Isoflurane anaesthesia induction at 4-5% mix and maintenance at 3-4% mix with oxygen.

2.3.4 Analgesia

Buprenorphine (temgesic). Dose for mice is 0.5-1mg/Kg. Weigh mouse and inject appropriate dose sub-cutaneously during anaesthesia sleep time. This may prolong the sleep time of the mouse. Check mouse regularly and dose every 12 hours if required.

2.4 Cloning of standards

2.4.1 Analysis of DNA using Agarose Gel-Electrophoresis

For the analysis of PCR products from reactions (section 2.2.10) or fragments generated by restriction digests (section 2.2.14), agarose gel-electrophoresis was employed. 1% agarose (multi-purpose agarose, Bioline) was melted in 0.5x TBE buffer and supplemented with 2-4µl ethidium bromide (10mg/ml aqueous solution, Sigma). Gels of 150ml were cast in 12x14cm or 20.5x10cm trays; 50ml gels were cast in 7x10cm trays, and appropriate combs inserted. After allowing the gels to set for 1 hour at RT, the gels were submerged in horizontal gel electrophoresis tanks (Hybaid turn and cast submarine gel system, Hybaid, or Savant HG 350 tank) containing 0.5x TBE buffer. Samples were mixed with 6x loading buffer and loaded into the wells. A DNA ladder (typically mass ruler, Fermentas) was also loaded, to size the products or fragments of the samples. 150ml gels were generally run at 120-150V/cm (Hybaid). The electrophoretically separated samples were visualised with an Evenscan broadband dual wavelength transluminator in a MultiImageII Light Cabinet (both Alpha Innotech Corporation) at a wavelength of 302nm. Permanent records were taken with a CCD camera (Alpha Innotech Corporation) and stored electronically.

2.5. Restriction endonuclease digest

Restriction enzyme digests were carried out in 1x restriction enzyme buffer with the addition of BSA. The digests were carried out at the appropriate temperature for the respective enzyme for a minimum of 90 minutes. For double digests with enzymes requiring distinct buffers, the DNA was purified from the first reaction using a Qiaquick Spin Column (Qiagen) according to the manufacturer's protocol after the first digest. The second digest was set up adjusted to the volume of the eluate. Enzymes were mostly obtained from Promega, or alternatively from New England Biolabs.

2.6 Sequencing

DNA sequencing was performed by The Sequencing Service (School of Life Sciences, University of Dundee, Scotland); using Applied Biosystems Big-Dye Ver3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer.

2.7 Immunohistology

2.7.1 Processing of tissue from animal model

Brains were extracted manually and fixed in 4% buffered PFA as indicated. The brains were cut into slices of 2 mm thickness with the aid of a rat brain blocker, and were embedded in paraffin wax. 3-5μm thick, consecutive sections were cut and placed on polylysine-coated slides (BDH Laboratory Supplies, Lutterworth, UK). Brain sections were dried onto the slides at 37°C overnight, and deparaffinised for 2 x 5 minutes in 100% xylene. Slides were re-hydrated for 2 x 5 minutes in 100% isopropanol and 5 minutes in 96% ethanol. Slides used for immunohistological analysis were then stained with Papanicolaou's haematoxylin-eosin followed by blueing in tap-water, and were then preserved with DPX and cover slipped. Consecutive sections were deparaffinised and rehydrated in the same way, but instead of haematoxylin-eosin staining, they were used for immunostaining as described below.

2.7.2. Immunohistological staining of paraffin-embedded tissue sections

Immunohistology for ADNP was performed, using rabbit anti-ADNP Ig (Chemicon) and the peroxidise anti-peroxidase (PAP) method, adapting previously published protocols for polyclonal antibodies (Kipar *et al.*, 1998).

Immunohistology for REST and REST4 was performed using the rabbit antirat antibodies (Sigma) and peroxidise antiperoxidase (PAP) method.

Briefly, after the rehydration step in 96% ethanol, endogenous peroxidase activity was blocked for 30 minutes with 0.5% H_2O_2 in 100% methanol. Slides were then washed with TBS and inserted into Sequenza-immunostaining racks, followed by a further wash with TBS. Sections were then blocked with 1:2 non-specific swine serum in TBS, 100µl per slide. REST, REST 4 and ADNP antibodies were prepared in TBS with 20% swine serum, at a dilution of 1:100. 100µl of diluted antibody were added to each racked slide, and left to incubate at 4°C overnight. The following day, the slides were washed for 5 minutes, 3 times in TBS, and then incubated for 30 minutes in swine anti-rabbit IgG at a dilution of 1:100, prepared in TBS with 20% swine serum. The slides were washed again for 5 minutes in changes of TBS, and incubated for 30 minutes in PAP (peroxidase-anti-peroxidase) rabbit complex, diluted 1:100 in TBS with 20% swine serum. After 5 minutes of washes in TBS, the slides were removed from the immunostaining-racks, and placed in water. The slides

were then incubated for 5 minutes with stirring in freshly prepared diaminobenzidin (DAB) solution with 0.01% H_2O_2. Finally, the slides were then transferred back into water, followed by 3 washes of 5 minutes in TBS, and one in water. The slides were then dehydrated in ascending concentrations of ethanol (70%-96%), followed by three washes in xylene. Sections were preserved in DPX and cover slipped. Consecutive sections incubated with normal rabbit serum instead of the primary antibody served as negative controls.

2.8. Standard Polymerase Chain Reaction (PCR)

PCR was used as a method for amplifying DNA fragments for use in molecular cloning. PCR was performed in a PxE thermal cycler (Thermo Electron Corporation) or Hybrid Sprint thermal cycler. 50µl PCR reactions included 10-100ng DNA template, 2mM $MgCl_2$, 0.2 mM of each dNTP, 0.2µl of each primer, 1x diamond buffer and 1.5 units of diamond DNA polymerase.

2.8.1 Annealing temperature (Tm)

Tm is the most critical factor governing the specificity of a PCR reaction and was calculated according to the formula:

60 + (0.41 x %GC content) – 675/n (were n = number of nucleotides).

Concentration was determined using spectrophotometer at 260nm:

μg/ml = A_{260} x μg per OD x dilution factor.

Total μg was then used to calculate μmoles:

μmoles = total μg/MWT.

2.8.2 PCR primer design

Primers were designed, in general, to be between 20 to 25bp long, melting temperatures of 50-65°C, with GC content between 40-60%.

2.8.3 PCR purification

To purify the double stranded DNA fragments from PCR and other enzymatic reactions the QIAquick PCR purification kit was used following manufacturer's instructions.

2.9 Statistics

All results are presented as mean ± standard error of the mean (standard deviation) were appropriate; comparisons were made as appropriate using the Student t-test in Microsoft Excel. Significant differences were identified at a value of $p<0.05$ significant, $p<0.01$ highly significant and $p<0.005$ very highly.

Chapter Three: Generation of double knockout for the Tac1/ Tacr1 pathway and subsequent phenotyping of the model.

3.1 Introduction

Pre-existing knockout mouse models described previously were used to generate a double knockout mouse model that had a preferred ligand and receptor pathway disrupted. This double knockout was generated in order to investigate the issue of redundancy in the contributing parental single knockout lines, the rationale being that a mouse line whose endogenous signalling pathway of SP and Tacr1 was disrupted simultaneously would display a more substantial phenotype in comparison to the single knockout lines.

During its generation it was hypothesised that there may be an obvious phenotype due to the abolition of a preferred ligand and receptor pathway. It was even considered that the double knockout may be embryonic lethal and alternative breeding strategies were contemplated for generation of the model in the eventuality of such an outcome. The double knockout was generated and its statistical occurrence was compliant with Mendelian trait predictions, however, it soon became apparent that the double knockout

model was displaying an interesting phenotype. The double knockout of Tac1-/- x Tacr1-/- was on a C57BL/6J background as were the single knockouts that had previously been well characterised with regards anxiety and depression like behaviours (Bilkei-Gorzo *et al*, 2002; Zimmer *et al*, 1998; Carmen De Felipe *et al*, 1998) as well as a resistance to chemically induced seizure (Liu *et al,* 1999a; Cao *et al,* 1998). Therefore, there was an opportunity to characterise and validate this double knockout model especially in light of the redundancy issue discussed in the introduction (1.5.8) and consequently this model was subjected to various behavioural tests and ultimately pharmacological experimentation.

As previously mentioned in this chapter introduction, TACR1 antagonists showed promise as an intermittent drug treatment for anxiety and depression to increase 5HT levels on a short term basis while waiting for the effects of widely used medications, like the SSRIs or tricyclics, to commence (Gobbi and Blier, 2005; Kramer *et al*, 1998). The double knockouts behavioural response to anxiety and depression like states was assessed in comparison to the individual knockouts for Tacr1 and Tac1. Animal models are widely used to study the neurobiological mechanisms of depression and anxiety. Mice, in particular, exhibit robust anxiety like behaviours in the appropriate field tests. Indeed, the pharmacological mechanisms of psychoactive drug responses are similar across many mammalian species confirming a common conserved behavioural response

to certain stimuli (review, Bergner *et al*, 2010). The double knockout was compared and contrasted with the single knockouts in a behavioural test for anxiety (light/dark box) and a behavioural test for an animal analogue of depression (Porsolt forced swim test).

3.2 Hypothesis

The double knockout would display a more substantial phenotype in the behavioural tests in comparison to the single knockouts which may be attributed to redundancy effects in the single knockouts given the level of interaction that is evident across related neuropeptides and preferred receptors.

The double knockout would display a distinct phenotype that is in contrast to the single knockouts in a pharmacological experimental model for TLE with the use of the neurotoxicant Kainic acid.

3.3 Aims

To generate a double knockout mouse model that had the endogenous signalling pathway of SP from the Tac1 gene and its preferred receptor Tacr1 disrupted to investigate the issue of redundancy in the parental single knockout lines.

To validate the double knockout model in comparison to the single knockout lines that were utilised for its generation as well as the wildtype

background strain (C57BL/6J) in behavioural studies for anxiety and depression in order to assess level of redundancy (if any) and also the hypothesised regulatory link between the SP/Tacr1 pathway and 5HT.

To validate the double knockout model in comparison to the single knockout lines which have been documented to be resistant to chemically induced seizures using the neurotoxicant kainic acid.

To investigate the expression of transcription factors post kainic acid treatment in the double knockout and single knockout mouse models. The transcription factors REST and REST4 are known to be dynamically regulated in seizure activity and also modulate neuropeptide expression.

To investigate the expression of a protein in the double and single knockout mouse models post kainic acid treatment. The protein, ADNP, has been hypothesised to have neuronal protective properties during seizure.

To investigate the sex dependant differences that became apparent during the kainic acid experiments.

To ultimately contribute knowledge to the tachykinin signalling pathway with regards the mood disorders and contribute an explanation for the conflicting results in peer reviewed literature.

To ultimately contribute knowledge to the regulatory role SP plays in the well documented neurological disorder, epilepsy, which is correlated with the mood disorders.

3.4 Methods

Animals.

All mice used were C57BL/6J wild types or GA mice whose background strain is C57BL/6J (BL6). All mice where aged 12 to 16 weeks old (adult and sexually mature) and were group housed on a 12/12 light dark cycle with access to food and water ad libitum, relative humidity 40-55%.

Strains used.

Tac1-/- mouse kindly donated by Andreas Zimmer, University of Bonn (Zimmer et al 1998). Tacr1-/- mouse kindly donated by Steve Hunt, UCL (Laird *et al* 2000; Carmen De Felipe *et al*, 1998). C57BL/6J wild types purchased from Charles River LTD.

GA mice generated. Tac1-/- x Tacr1-/- double knockout generated by selective cross breeding of the above strains to produce a mouse that is null for the SP/Tacr1 pathway.

Screening for double knockout.

Tac1 knockout males and Tacr1 knockout females were bred together to produce an F1 generation that carried each of the deletions heterozygously. The F1s were then backcrossed to Tac1 knockout mice to produce an F2 generation 25% of which were homozygous for the Tac1 deletion and heterozygous for the Tacr1 deletion. Males and females of this genotype were then bred together and 25% of their offspring were the double knockout genotype that we were looking for. This method of breeding greatly reduces the number of mice needed to achieve generation of a double knockout and reduces waste of genotypes that are non-desirable. This method also enhances the chances of generating a double knockout that may be physiologically challenged as the ratio is always 1 in 4 for the desired genotype as opposed to 1 in 16 if the heterozygous F1 generation where bred together. This is consistent with the aims of the 3Rs *(replacement, reduction, refinement).* All offspring had less than 0.5cm of their tail removed under general gaseous anaesthetic using Isoflurane at 3-4% mix with oxygen, bleeding was controlled by cauterisation and mice were ear marked with unique identifications all while still under

anaesthesia (*replacement, reduction, refinement;* Russell and Burch, 1959). DNA was extracted from the tail samples by overnight digestion in tail tip buffer and PCR performed for Tac1 and Tacr1 separately on all samples using the following primers;

Tacr1 primers,

NK-1Fwd 5'-CTGTGGACTCTGATCTCTTCC -3',

NK-1Rev 5'-ACAGCTGTCATGGAGTAGATAC -3',

NeoFwd 5'- GCAGCGCATCGCCTTCTATC -3';

Tac1 primers,

Tac1 common (AZ95) 5'-GCC TTT AAC AGG GCC ACT TGT TTT TCA ATT-3',

Tac1 knockout (CNKO4) 5'-ACT GTG GTT TCC AAA TGT GTC AGT T-3',

Tac1 wildtype (AZ99) 5'-AGA CCC AAG CCT CAG CAG TTC TTT GGA TTA ATG-3'.

Behavioural testing.

All behavioural testing was preformed blind. The four lines of mice were identified only by numbers 1 to 4 and behavioural testing conducted without the knowledge of which line were the wildtypes, single knockouts or double knockouts.

All lines were subjected to Porsolt forced swim test (Drugan *et al* 1989; Porsolt *et al* 1977, 1978) to evaluate stress induced depression, and light/dark box exploratory test (Mathis *et al* 1994; Crawley and Goodwin 1980) to evaluate anxiety levels. As previously described, mice from all the strains were placed into a large open cage half of which was illuminated with an overhead lamp, the other half had a box covering it so it was dark inside with a small opening for the mice to exit in and out of the box.

Statistical analysis was done in excel using the students t-test for all the lines in comparison to the wildtype control.

Chemically induced seizures.

All testing was preformed blind. The four lines of mice were identified only by numbers 1 to 4 and chemically induced seizures evaluated without the knowledge of which line were the wildtypes, single knockouts or double knockouts.

A dose-response curve was constructed for BL6 wild type mice to ascertain what dose of kainic acid (KA) would not cause a full seizure but would induce mild seizures and behavioural changes that could be evaluated in all the strains. Males and females were paired together in batches, the dosage range was: 15mg/Kg, 25mg/Kg and 35mg/Kg. Following seizure severity scoring a dose of 17mg/Kg was chosen, however, it became clear when assessing the dose-response curve that there were distinct gender differences in the behavioural response and sensitivity to KA injection between the sexes. The decision was taken to expand the study and analyse the males and females separately but using the same 17mg/Kg dose.

All strains were then injected intraperitoneally (IP) with 17mg/Kg of KA and seizure activity was scored using the Racine scale for seizure severity (Racine 1972). Seizure severity was recorded over a period of 90 minutes post injection using an extended version of the Racine scale as follows:-

0 = normal,

2 = staring, panting, frozen,

4 = head tossing, wet dog shakes,

6 = short myoclonic jerks, excessive scratching, tail rigidity,

8 = short forelimb clonus, hunchback posture,

10 = loss of balance, hindlimb clonus, rearing,

12 = continuous forelimb clonus, discrete rearing and falling episodes,

14 = continuous rearing and falling, barrel rotations (full seizure),

16 = death.

Statistical analysis was done in excel using the students t-test for all the lines in comparison to the wildtype control

Tissue collection and processing.

All mice were allowed to recover from seizures and were humanely culled via a schedule one technique (Sch1) and brains removed at the following time points post injection: 3 hours, 24 hours, 4 days and 7 days (n=3-4 per group) time course intervals for histology were based on previous research which revealed KA induced damage in adult C57BL/6J mice by 12 hours following treatment (Benkovic *et al,* 2006). The brains were bisected and one half fixed in 4% paraformaldehyde, embedded in wax, sections taken of hippocampus and every other stained with haematoxylin and eosin (HE) and the rest used in immunohistology. The other half was snap frozen in liquid nitrogen and stored in -80 freezers for future rtPCR. See methods and materials section for protocols.

Immunohistology.

Sections from the above time point (n=3-4 per group) were immunostained (see methods and materials) for the following; REST, REST4 and ADNP (activity-dependent neuroprotective protein).

Statistical analysis.

Differences between means were analysed in excel using the Students t-test and were considered different when probability values were less than 0.05.

3.5 Results

3.5.1 The light/dark box test for anxiety

The strain showing the highest anxiety levels were the C57BL/6J wildtypes. The strain with the lowest anxiety score by far was the Tacr1-/- mice with statistical significance in comparison to the wildtype controls (see figure 3.1), which is consistent with the published literature (Rodgers *et al,* 2004; Santarelli *et al,* 2001).

The double knockout also demonstrated a lower level of anxiety in comparison to the wildtype controls that was statistically significant but not to the extent of the Tacr1 single knockout (figure 3.1).

The Tac1-/- mice displayed lower anxiety levels compared to wild type figure 3.1) but it was not statistically significant, however it should be noted that this model has documented lower levels of anxiety in the literature (Zimmer et al, 1998) and it may be that increasing the n number would show a statistical difference in comparison to the wildtype strain.

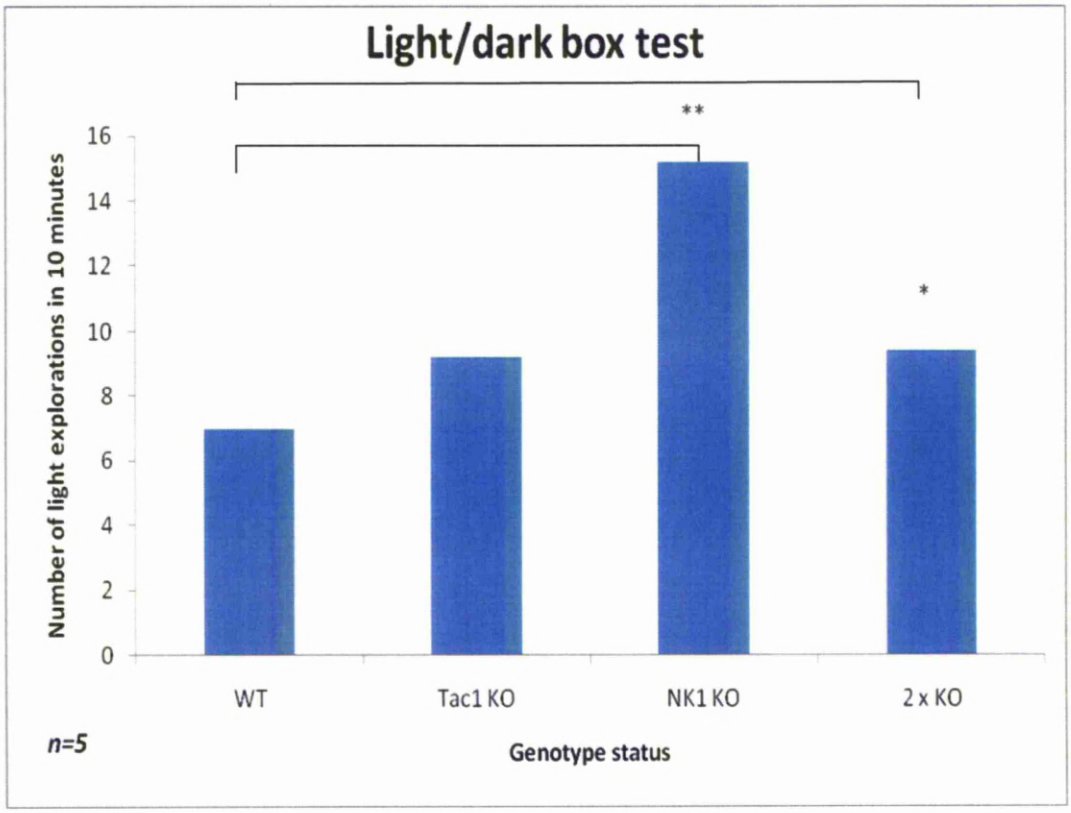

Figure 3.1 Light-Dark Box Analysis (n=5).

*The Tacr1 (NK1) knockouts and the double knockouts showed lowered anxiety levels in comparison to the wildtype controls. The TAC1KO were not statistically different to wild type animals. Student t-test used were *=$p<0.05$ considered significant and **=$p<0.01$ considered highly significant.*

3.5.2 The Porsolt forced swim test for stress induced depression

Consistent with previous data on individual Tac1-/- or Tacr1-/- , the single knockout animals tested swam for a greater duration of time than their wild type controls indicating a resistance to stress induced depression (Bilkei-Gorzo *et al*, 2002). However, the double knockout strain was surprisingly the least active suggesting that this strain is prone to stress induced depression (figure 3.2).

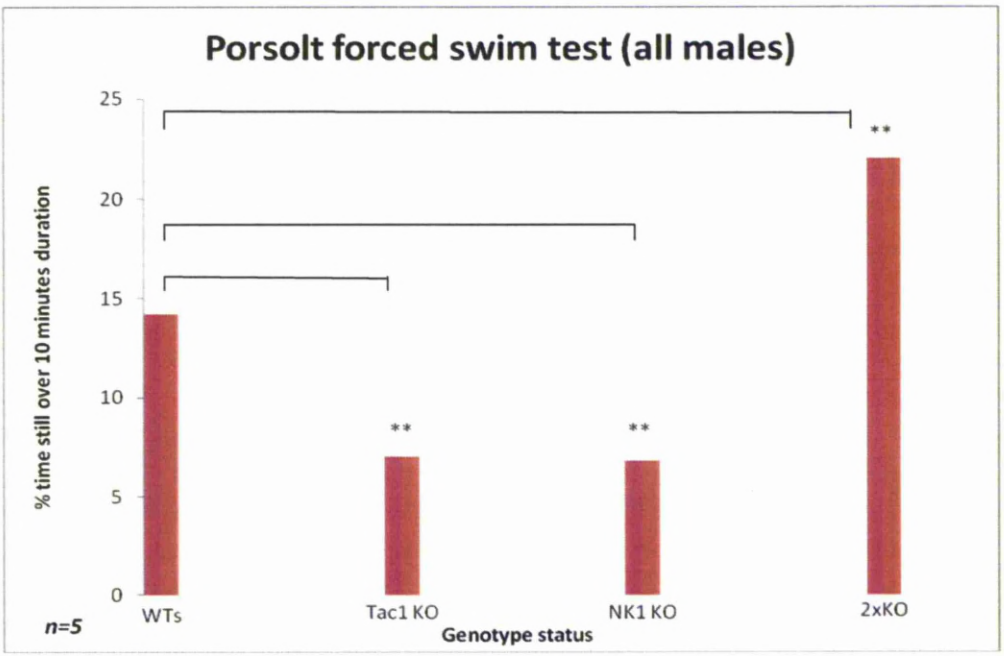

Figure 3.2. Porsolt Forced Swim test (n=5).

*Transgenic strains where compared to the C57BL6 wild type (WTs). The Tac1 and Tacr1 (NK1) knockouts were the most active compared to the wildtypes with a high significance. Surprisingly the double knockout mice were the least active and showed a statistical significant tendency to stress induced depression. Student t-test used were *=$p<0.05$ considered significant and **=$p<0.01$ considered highly significant.*

3.5.3 Pharmacological model of TLE (temporal lobe epilepsy)

The final test was to address the response of all the strains of animals to chemically induced epileptic seizures initiated by Kainic acid (Benkovic *et al*, 2006). Males and females were paired in batches together for the dose range study to determine an appropriate dose of Kainic acid (KA) for this study; however it became very clear that there were distinct gender differences in the behavioural response and sensitivity to KA injection between the sexes (figure 3.6). Therefore we expanded on the experimental design in order to address the gender differences by separating the males and females. To try and observe differences in the behavioural response of the lines we used the lowest range of KA, from figure 3.3, to highlight any increased susceptibility. Under these conditions for the males we saw increased susceptibility only the double KO line (figure 3.4). For the females, however, all the lines showed an increased susceptibility at this low dose of KA when compared to their male counterparts (figure 3.6).

3.5.4 Chemically induced seizures using KA

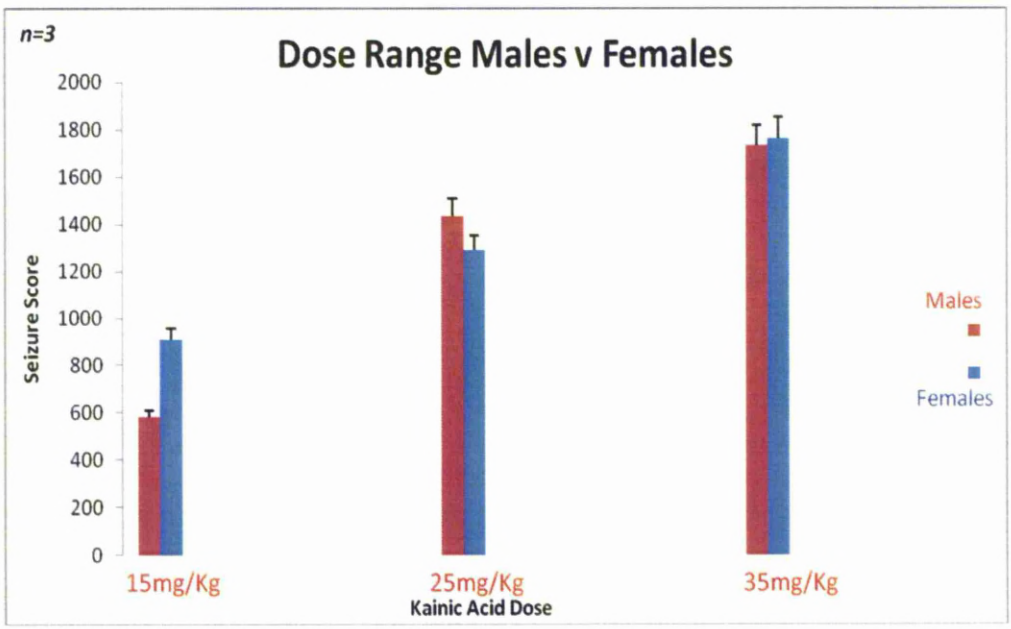

Figure 3.3. Dose range study (n=3).

Demonstrates the difference between the sexes at the lower dose but this difference disappears at higher doses. Statistical analysis not appropriate as this was a dose range study. 17mg/Kg chosen on observations of behaviour as 15mg/Kg was considered too low but 25mg/Kg was too high.

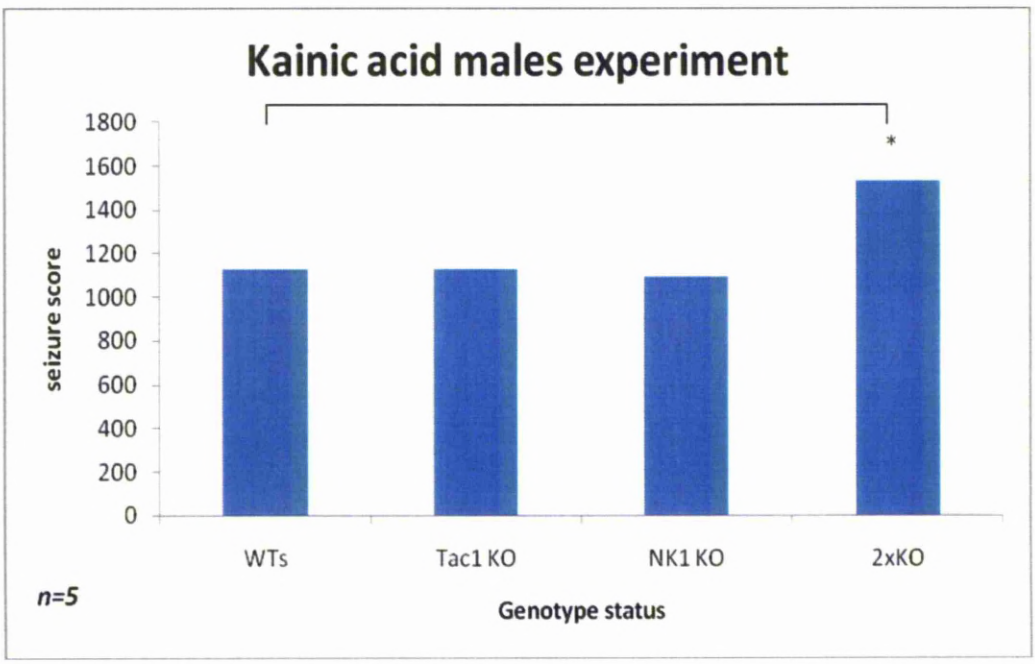

Figure 3.4. KA induced SSSE in males, dose 17mg/Kg (n=5).

All mice injected IP with 17mg/Kg KA and severity was scored for 90 minutes post injection using the Racine scale. The Tacr1 (NK1) and Tac1 knockouts have a score comparable to their wild type controls (WTs), but the double knockouts had an unexpected full seizure even at this low dose that is statistically significant in comparison to their WT controls. Student t-test used were $*=p<0.05$ *considered significant and* $**=p<0.01$ *considered highly significant.*

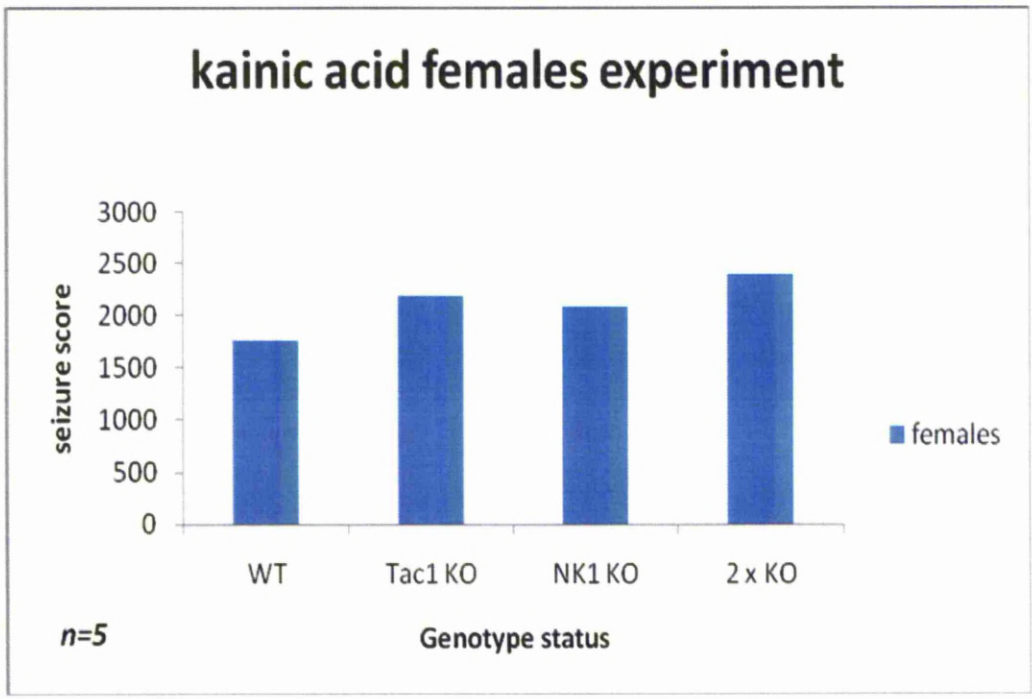

Figure 3.5. KA induced SSSE in females, dose 17mg/Kg (n=5).

*Seizure severity was scored for 90 minutes post IP injection using the Racine scale. The females of all the strains, including wild types, showed a greater sensitivity to KA injection. Although the double knockout was the most sensitive in comparison to its WT controls it was not statistically significant. Student t-test used were *=$p<0.05$ considered significant and **=$p<0.01$ considered highly significant.*

The females of all the lines showed a greater sensitivity to the KA induced seizures (see figure 3.5). This became more apparent when the males were compared to the females on the same graph and analysed statistically using the student t-test to compare the sexes of each strain (figure 3.6).

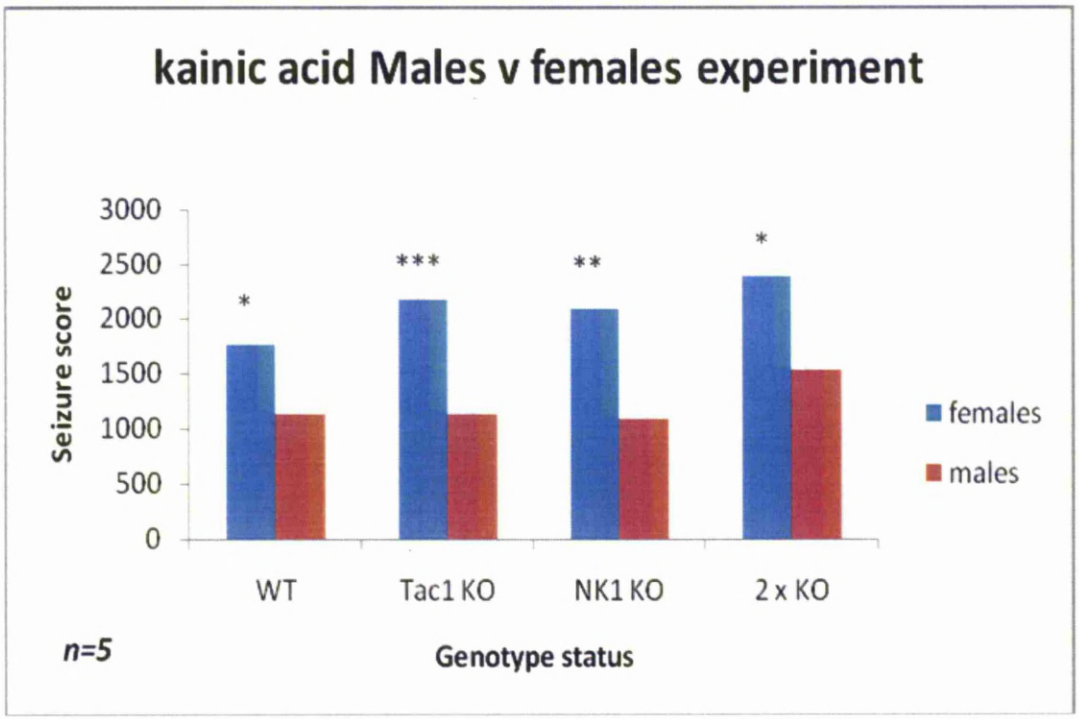

Figure 3.6. Comparison of male versus female mice injected IP with 17mg/Kg KA (n=5).

*Seizure severity was scored for 90 minutes post injection using the Racine scale. When the males vs. females are compared together the difference can be seen with all four lines (WT, Tac1-/-, Tacr1 (NK1-/-) and double knockout) with statistical significance in comparison to their sexual counterparts. Student t-test used were *=$p<0.05$ considered significant, **=$p<0.01$ considered highly significant and ***=$p<0.001$ considered very highly significant.*

3.5.5 Immunostaining results.

All sections are from the CA3 region of hippocampus, stain is always brown and 3 mice per line for each time point were done.

3.5.6 Kainic acid (KA) injected C57BL6 wildtype mice compared with saline injected controls.

Figures A to K compare the response of the wildtype C57BL/6J background stain to KA injection for 3 hour and 24 hour time points post injection (Figures 3.7, 3.8, 3.9, 3.10 and 3.11). The 4 day and 7 day post KA injection time points showed no difference in staining or pathology in any of the strains.

At 3 hour post KA injection REST and REST4 remains cytoplasmic but both appear weaker in the KA wildtype (Figures 3.7 and 3.8). ADNP remained the same at this time point in both saline control and KA subjects and was always nuclear.

At 24 hours the ADNP staining is weaker in the KA injected mice in comparison to the saline injected (Figure 3.9). In addition, the REST and REST4 appear to be weaker in the KA mice compared with the saline controls (Figures 3.10 and 3.11).

Overall, no obvious pathology in the wildtype strain at the low dose of 17mg/Kg KA injection in comparison to the saline injected controls.

Immunostaining results.

All sections are from the CA3 region of hippocampus, stain is always brown and 3 mice per strain for each time point were done.

Kainic acid (KA) injected C57BL6 wildtype mice compared with saline injected wildtype controls.

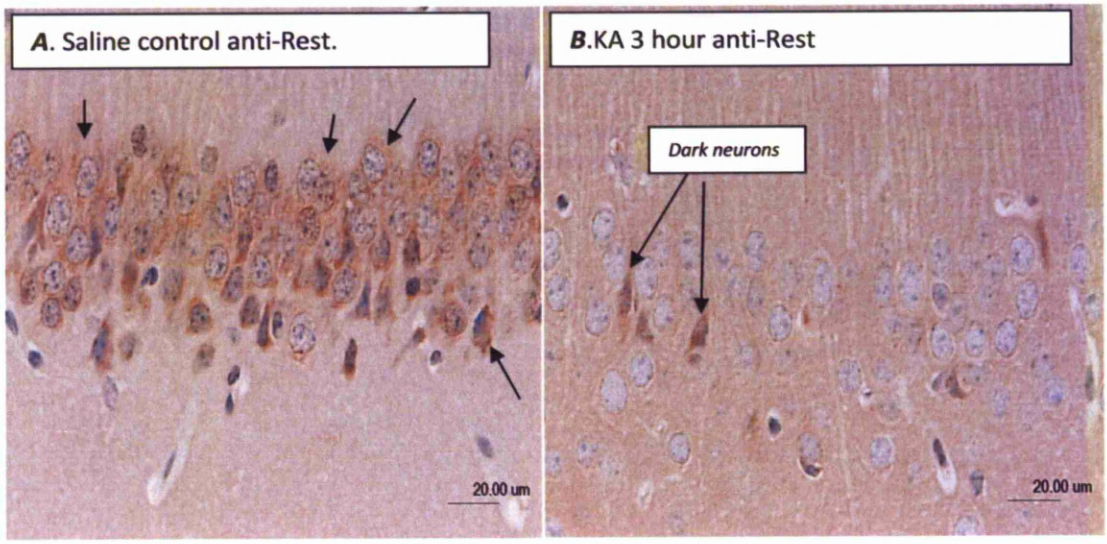

Figure 3.7 Wildtypes at 3 hours post injection looking at REST. *(A) Shows Rest staining in saline control, lots of cells are stained and it is always cytoplasmic. (B) is the 3 hour time point for Rest staining in wild type mouse, it is much weaker expect for a few "dark neurons" (damaged or dying) where the staining is stronger.*

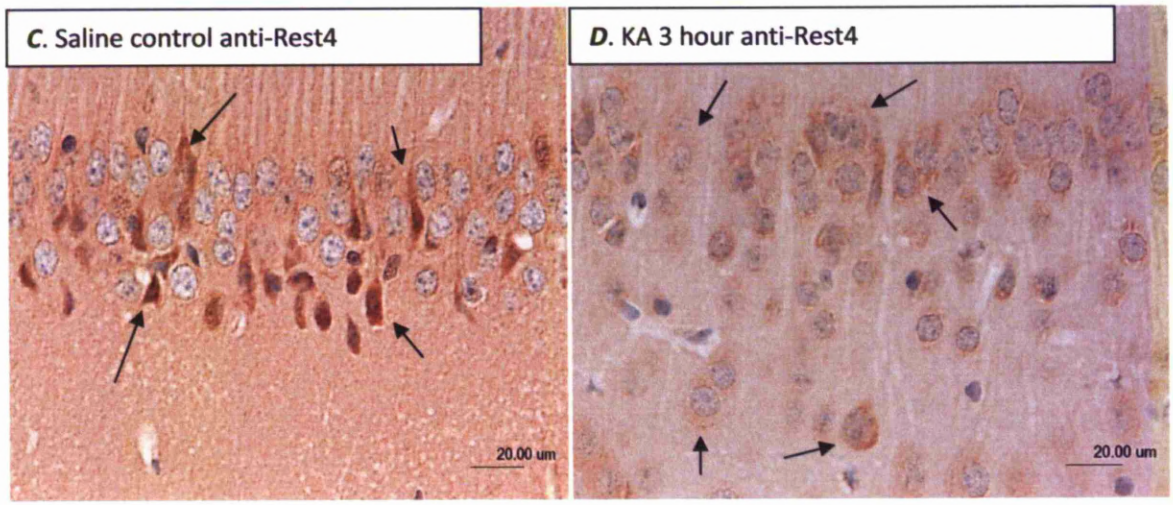

Figure 3.8 Wildtypes at 3 hours post injection looking at REST4. *(C) And (D) show Rest 4 staining which is present in some cells in control and always cytoplasmic. In the KA 3 hour time point (D) it is much weaker but remains cytoplasmic.*

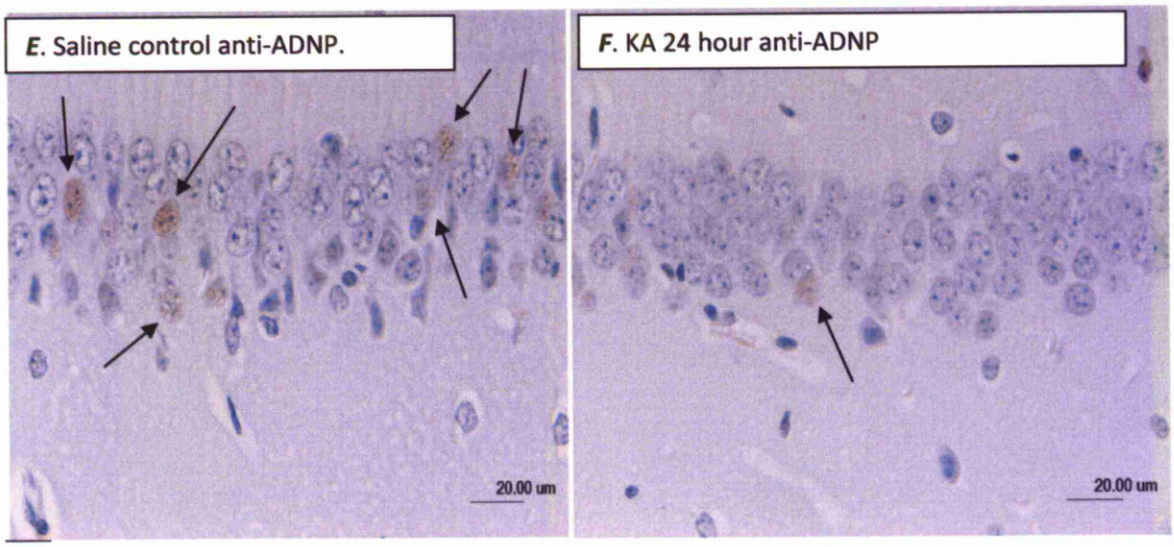

Figure 3.9 Wildtypes at 24 hour post injection looking at ADNP. *(E) and (F) show staining for ADNP which is nuclear in both the control (E) and KA 24 hour time point (F) but the KA (F) is much weaker with only one stained neuron (arrowed).*

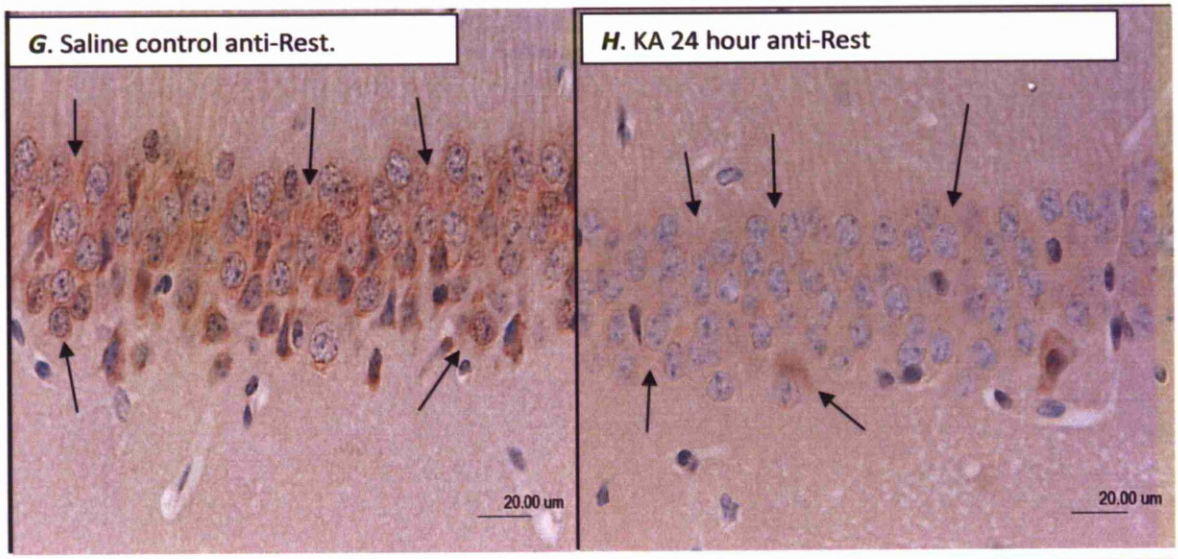

Figure 3.10 Wildtypes at 24 hour post injection looking at REST. *(G) Shows Rest staining in saline control, always cytoplasmic and in nearly all cells. (H) is KA 24 hour time point in wild type mouse, staining is much the same only weaker.*

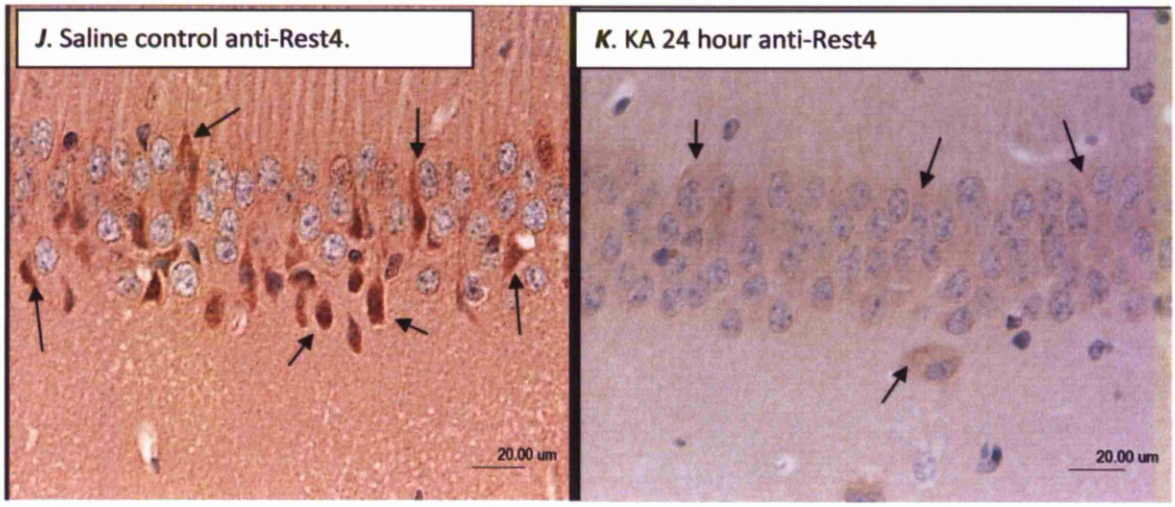

Figure 3.11 Wildtypes at 24 hour post injection looking at REST4. *(J) Shows Rest 4 staining in control, cytoplasmic and in some cells. (K) shows KA 24 hour time point, staining for Rest 4 is much weaker.*

3.5.7 KA injected double knockouts compared with KA injected wildtype controls.

At the 3 hour post KA time point REST and REST4 appears similar in double knockout compared to the wildtype except for the complete absence of REST in the necrotic neurons of the double knockout (figure M).
The most interesting observation at this time point is for ADNP which remains nuclear in the KA wildtype but has gone cytoplasmic in the double knockout with the exception of one dark neuron (figure O).

At the 24 hour post KA injection the REST staining is stronger than in the wildtype but it is completely absent in the necrotic cells (figure Q).
For REST4 at 24 hours the staining is much stronger in the double knockouts than the wildtypes (figure S).
Again, the most interesting observation is the fact the ADNP is nuclear in the wildtype but remains cytoplasmic in the double knockout as observed at the 3 hour time point (figure U).

The ADNP was only found totally in the cytoplasm for the double knockout strain and only at time points 3 hour and 24 hour post KA injection.

KA injected double knockouts compared with KA injected wildtype controls.

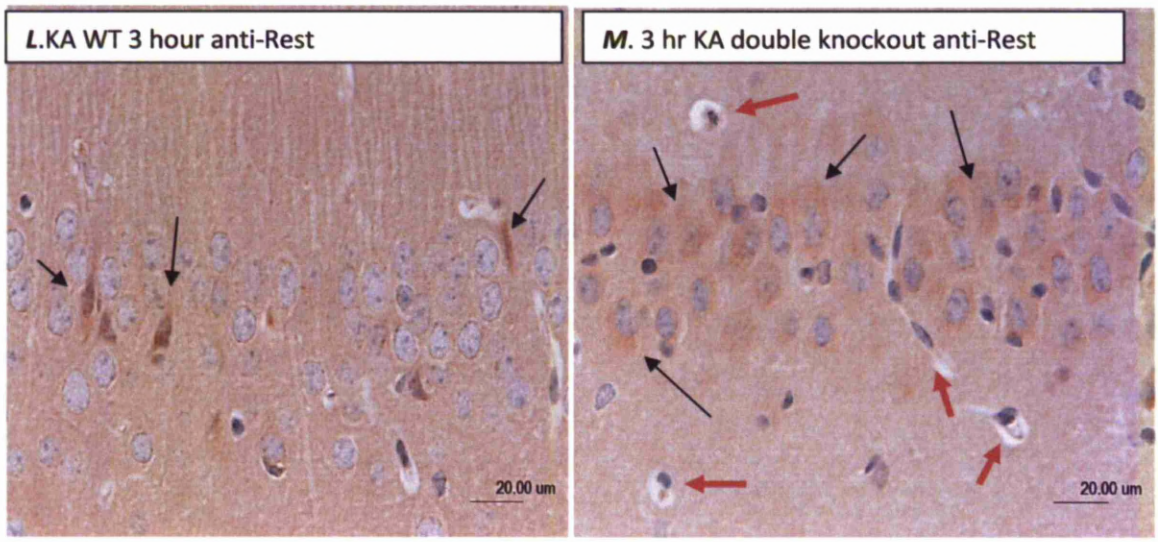

Figure 3.12 KA control WTs and double knockouts at 3 hours post injection looking at REST. *In KA 3 hour wild type time point (L) the Rest is staining strongly only in dark neurons. In the KA double KO 3 hour time point (M) there is still lots of staining for Rest but complete absence of staining in necrotic neurons (arrowed in red).*

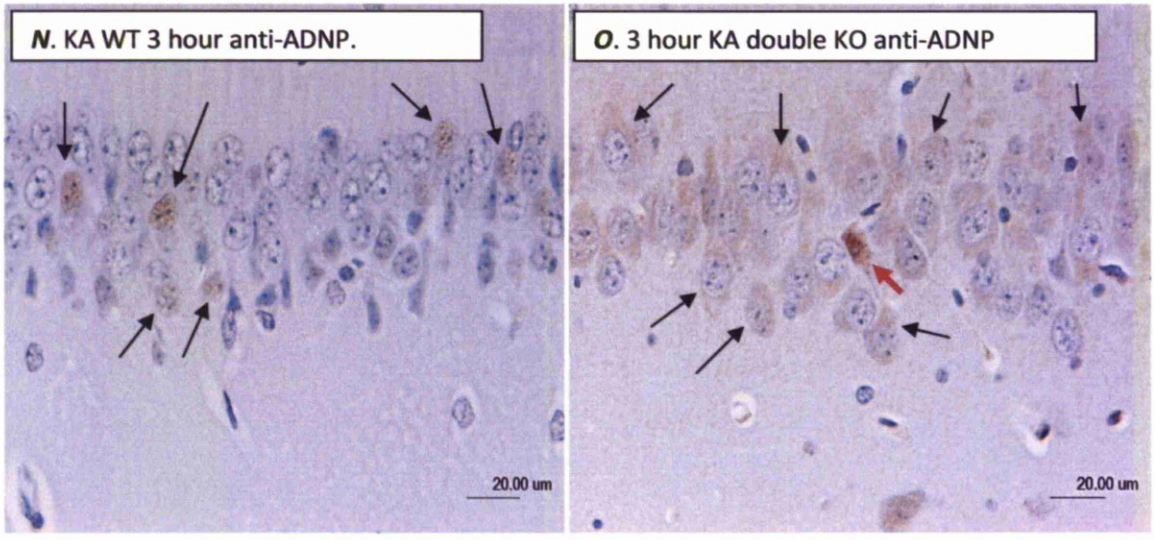

Figure 3.13 KA control WTs and double knockouts at 3 hours post injection looking at ADNP. *The KA 3 hour wildtype (N) shows ADNP staining always nuclear in some neurons. In the KA double KO 3 hour time point the ADNP has gone cytoplasmic (except for one dark neuron, red arrow). The double KO was the only strain to show this complete change in ADNP staining.*

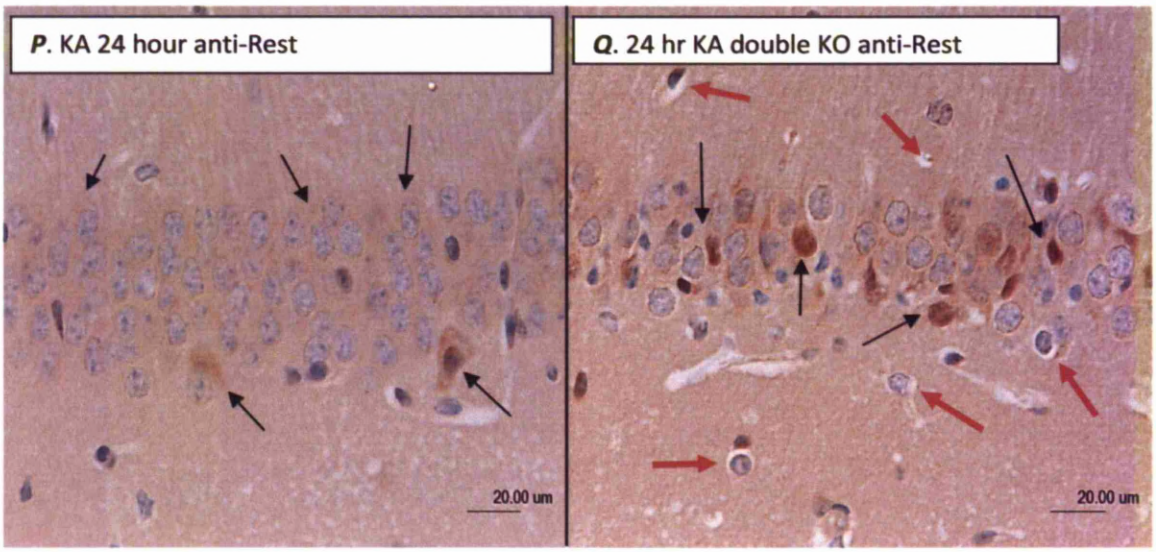

Figure 3.14 KA control WTs and double knockouts at 24 hours post injection looking at REST. *In the KA 24 hour time point for wild types (P) the Rest staining is similar to saline control (G). In the KA 24 hour time point double KO the Rest staining is strong in dark neurons but completely absent in necrotic cells (red arrows).*

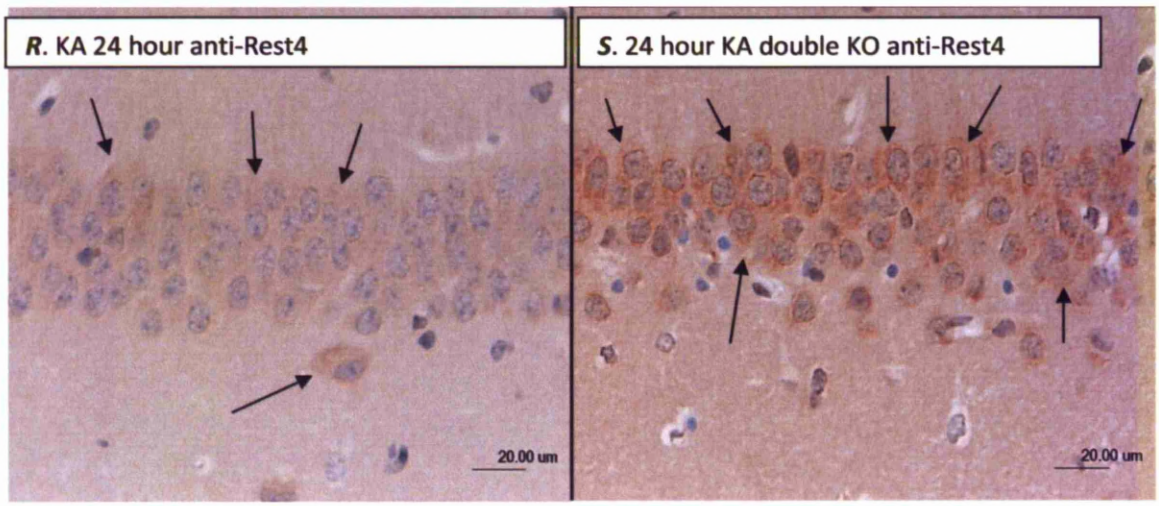

Figure 3.15 KA control WTs and double knockouts at 24 hour post injection looking at REST4. *In the KA 24 hour time point for wild types (R) the Rest 4 staining was much weaker than wild type saline control (J). In the KA 24 hour time point for the double KO the Rest 4 remained cytoplasmic but there was an increase in expression, more than both the saline control and wild type KA mice.*

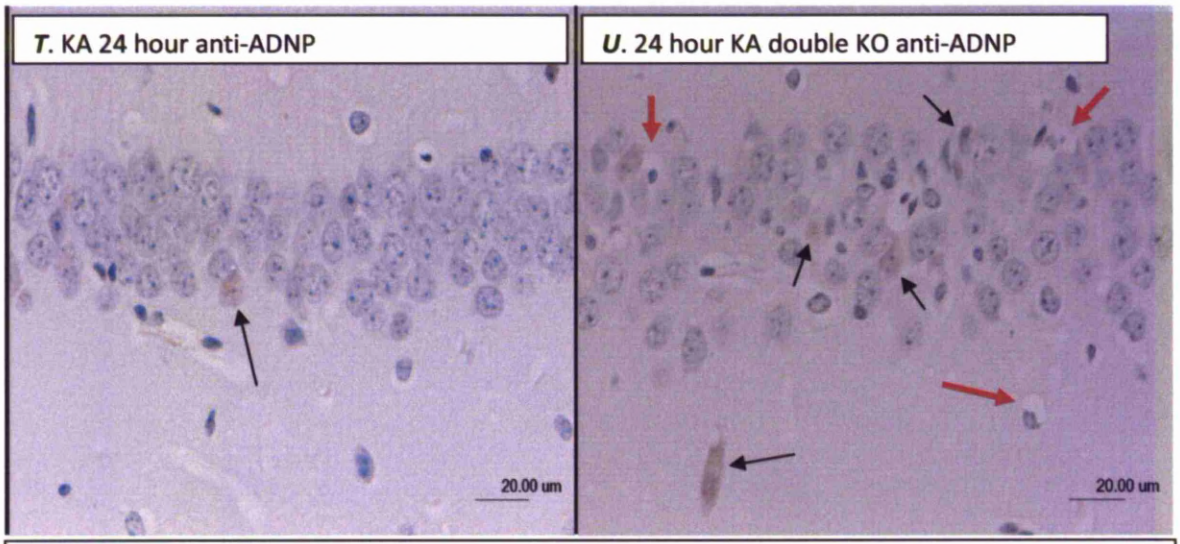

Figure 3.16 KA control wildtypes and double knockouts at 24 hours post injection looking at ADNP. *In the wild type KA 24 hour time point the ADNP is much less than saline control but remains nuclear. In the double KO KA 24 hour time point the ADNP has gone cytoplasmic (as with 3 hour time point) but remains absent from necrotic cells (red arrows).*

3.5.6 KA injected Tac1 knockout compared with KA injected wildtype controls.

At 3 hour time point the KA injected Tac1 knockouts were similar to the KA injected wildtypes.

Only at 24 hours post KA injection was any difference observed in this strain.

At 24 hours post KA injection the REST staining in the Tac1 knockout was stronger than in the wildtypes but REST4 remained similar in both.

The most interesting observation in this strain was for the ADNP which at 24 hours post KA injection was found not only in the nucleus but also in the cytoplasm. The Tac1 knockout at this time point was the only strain that had ADNP both nuclear and cytoplasmic.

The Tacr1 knockout was no different to the wildtype controls for all time points post KA injection.

KA injected Tac1 knockout compared with KA injected wildtype controls.

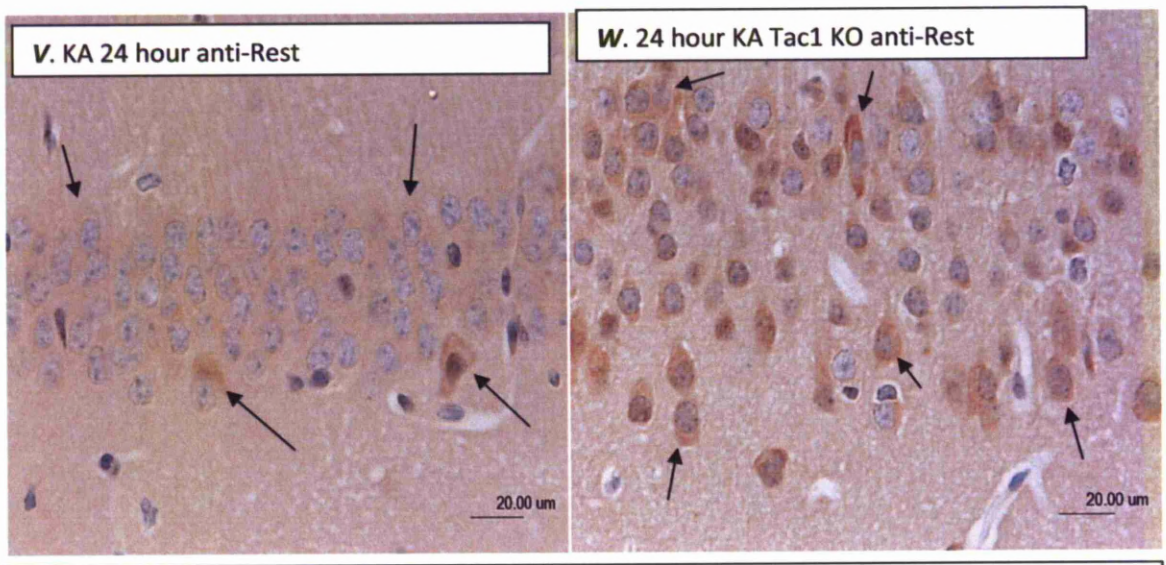

Figure 3.17 KA control wildtypes and Tac1 knockouts at 3 hours post injection looking at REST. *The wild type KA 24 hour time point (V) staining for Rest was similar to the saline control (G). The KA Tac1 KO 24 hour time point staining for Rest was more intense than both of them but remains cytoplasmic (W).*

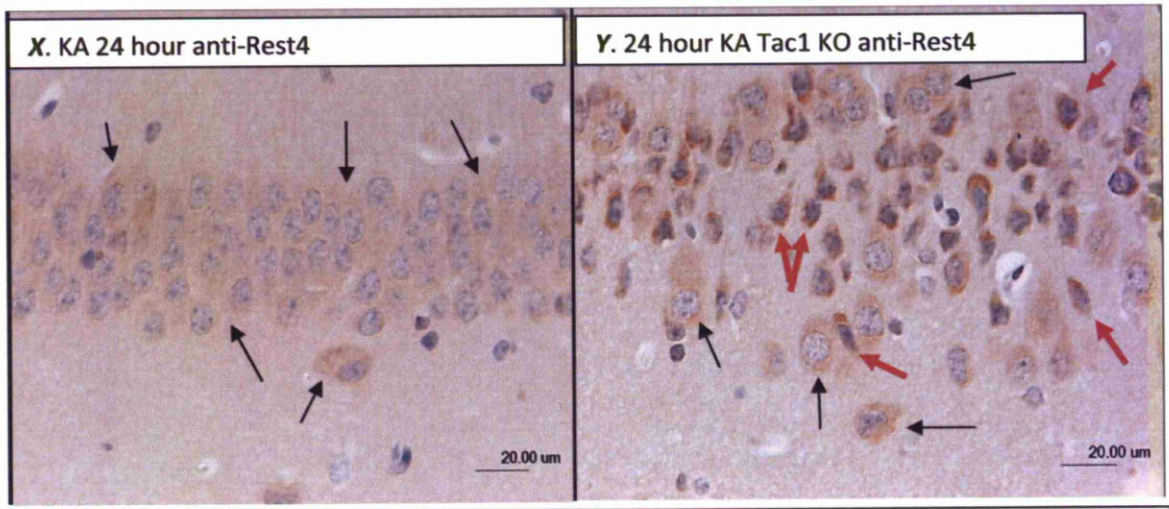

Figure 3.18 KA control wildtypes and Tac1 KOs at 24 hours post injection looking for REST4. *The Rest 4 staining in the wild type KA 24 hour time point (X) is much weaker than in saline control (J). The Rest 4 staining in the Tac1 KO KA 24 hour time point is similar to (X) except that it is more intense in the dark neurons of which there are a lot (red arrows).*

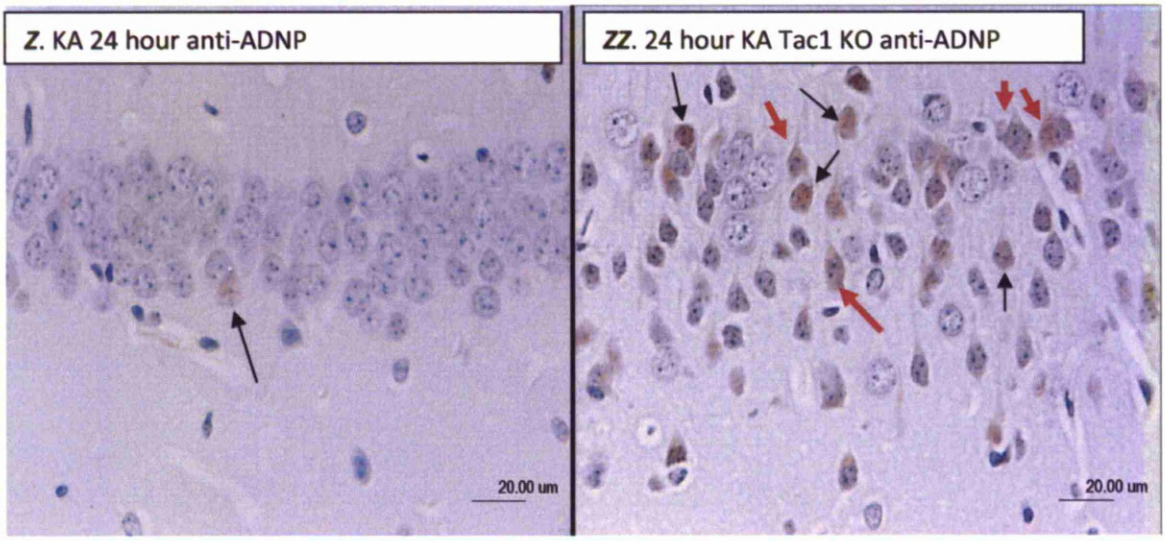

Figure 3.19 KA control wildtypes and Tac1 KOs at 24 hours post injection looking at ADNP. *In the wild type KA 24 hour time point (Z) the ADNP is much less than saline control (N) but remains nuclear. The Tac1 KO at 24 hour time point (ZZ) was most unusual as the ADNP was found both in the nucleus like controls and wild types, but also in the cytoplasm (red arrows) as with the double KO mice. This staining for ADNP in both the nucleus and cytoplasm was not found in any other strain or at any other time point.*

Table 3.1 Summary of immunostaining in positive cells 24 hours post KA injection in CA3 region of hippocampus.

Genotype status of mouse line	Expression found in cytoplasm for:-			Expression found in nucleus for:-		
	Rest	Rest4	ADNP	Rest	Rest4	ADNP
Wildtype	+++	+++	0	0	0	(+)
Tac1 knockout	+++	+++	+	0	0	+
Double knockout	+++	+++	++	0	0	0

Key. *The immunostaining was scored as follows for 400x field: 0 = no positive cells or staining reactions; (+) = very few (up to 5) positive cells or staining reactions; + = few (up to 15) positive cells or staining reactions; ++ = moderate numbers (15 or more) positive cells or staining reactions; +++ = high numbers (30 plus) positive cells or staining reactions.*

Haematoxylin and Eosin stain from 24 hour KA injected double knockout.

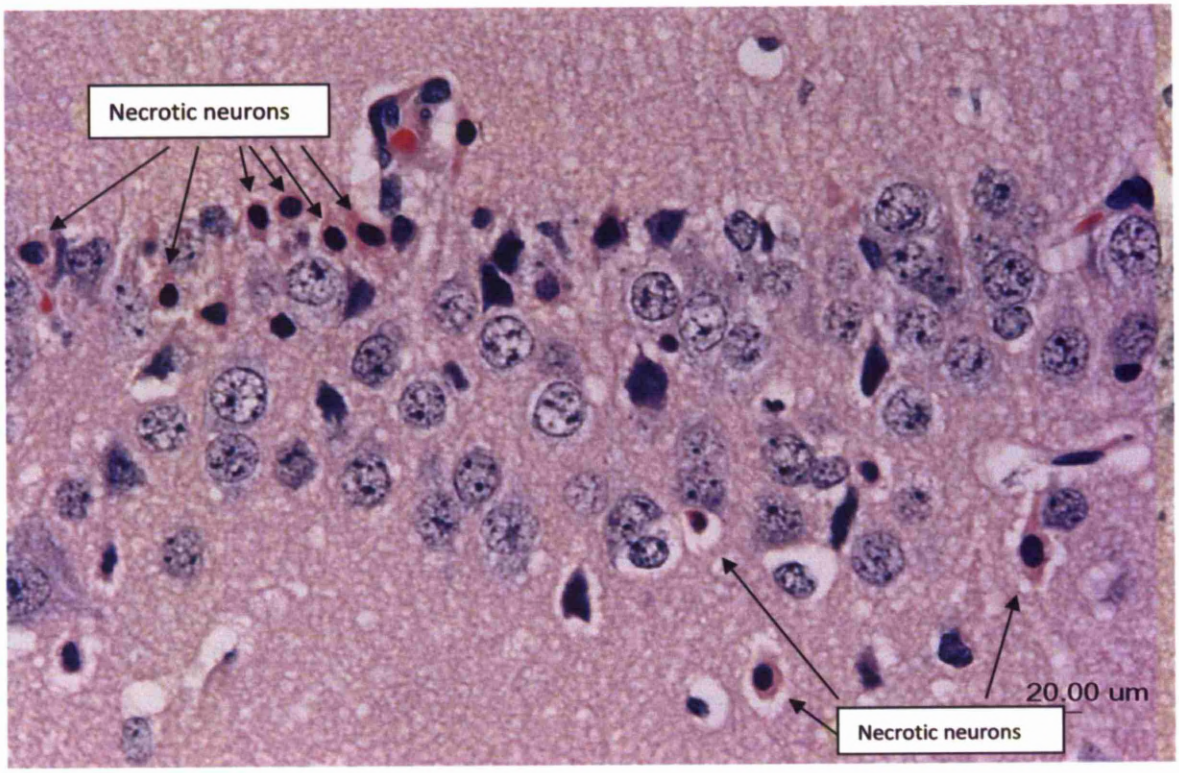

Figure 3.20 H&E staining of double knockout hippocampus CA3 region at 24 hours post injection. *Section of CA3 region of the hippocampus of a double knockout (Tac1-/- x NK1-/-) at 24 hours post KA injection. A number of necrotic neurons can be seen shrunken with pink stain round them indicating neuronal damage, this amount of necrotic neurons where not found in any other H and E staining for any other strains or time points. Necrotic neurons typically demonstrate the gradual disintegration of intracellular organelles and dispersion of their remnants into the surrounding area (Ferenc et al, 2008).*

3.6 Discussion

The data presented in this thesis indicates that the double knockout is distinct from the single knockouts and that the disruption of preferred ligand and receptor pathway has generated a unique phenotype both behaviourally and pharmacogenetically.

3.6.1 Tacr1 KO and double knockout display anxiolytic behaviours in comparison to their WT controls

As previously mentioned SP is the most abundant neurokinin in the CNS and its preferred receptor Tacr1 is highly expressed in regions involved in affective disorder and responses to stress and anxiety. In the light dark experiment, which measures anxiety, the Tacr1-/- and the double knockout were less anxious than the wildtype controls with statistical significance. The Tac1-/- was less anxious than the wild type C57BL/6J control but this was not statistically significant. This is consistent with published data and is an indication of the effect the SP/Tacr1 pathway has on anxiety control supporting the evidence for links and modulation of the serotonin (5HT) system (Santarelli *et al*, 2001) as discussed in the introduction of this thesis (chapter 1.6). This is reviewed by Blier, *et al*, 2004, were the electrophysiological, neurochemical and behavioural studies show an

effect by the TACR1 antagonists that is similar to the effect afforded by various antidepressant drugs. On a behavioural level, the SP/TACR1 pathway controls the "fight or flight" response which is emotionally mediated by the fear response. It therefore is logical that there is an interconnected neuronal network between SP, TACR1 and the release of 5HT and epinephrine (adrenaline) Blier *et al*, 2004.

This is further substantiated by the fact that SP is released in the rodent brain in areas associated with emotional behaviour such as the hippocampus and the previously mentioned amygdala, hypothalamus and PAG. In addition, SP is increased in these areas in the rodent brain in response to novelty, restraint and social isolation which are all associated with changes in serotonin levels and regulation (Rodgers *et al*, 2004).

In the case of anxiety SP may have been evolutionally selected for its role in increasing anxiety levels and ensuring behaviour that is not too bold which would inadvertently increase the risk of a prey animal (rodents) being hunted to extinction. Interestingly, some parasites actually alter the behaviour of the hosts they infect to ensure that their anxiety levels are suppressed and they become bolder in their exploratory behaviour which ensures the parasite ends up in the definitive host for its life cycle. For example, the parasite *Toxoplasma gondii* manipulates the behaviour of its intermediate host, rats, to ensure it reaches its feline definitive host. This

"manipulation mechanism" could have evolved in parasites to combat the evolving cautious behaviour that we interpret as anxiety (Berdoy *et al*, 2000). In addition, even through humans are an accidental host for *Toxoplasma gondii* and represent a dead end host the parasite is still able to alter the behaviour and brain functions of patients evidenced by reports of altered personality, deafness and lowered IQ levels in infected individuals (Sever *et al*, 1987). This indicates a common evolutionary pathway that is available for manipulation by this parasite that is conserved from rodents to man. Whilst 5HT antagonists can emulate the same response it is interesting to note that TACR1 antagonists and Tac1/Tacr1 mouse knockout models can also illicit the same behavioural response indicating a common regulatory pathway (Kramer *et al*, 2007).

Although the Tac1 KO had lower anxiety levels than the wildtype control it was not statistically significant. Previous studies, however, have found the Tac1 KO used in this thesis to have significantly lower anxiety levels in comparison to their wild type controls (Bilkei-Gorzo *et al*, 2002). Increasing the numbers of mice used may result in statistical significance. Further testing with a variety of behavioural tests for anxiety would allow a more confident conclusion.

3.6.2 The double knockout demonstrates a phenotype in response to stress induced depression that is unique in comparison to its single KO parental strains

The Porsolt forced swim test is considered a model for measuring stress induced depression in rodents. In this test the double knockout was significantly different to the single knockouts. The single knockouts Tacr1 and Tac1 showed a resistance to stress induced depression compared to their wild type controls, this result is consistent with the published literature (Bilkei-Gorzo *et al*, 2002). The double knockout was predicted to show a resistance to stress induced depression and was speculated to be even more resistant than its single knockout contributors but surprisingly it showed a susceptibility to this kind of depressive event with statistical significance compared to the wild type control. Whilst this was unexpected it does fit with the theory of redundancy discussed earlier on in the introduction (chapter 1.5). The single knockouts have part of a preferred ligand/receptor pathway intact, the receptor Tacr1 is present in the Tac1-/- and the ligand SP is present in the Tacr1-/-. Thus, in the absence of SP it may be that another neuropeptide can bind and activate the Tacr1, e.g. NKA or NKB. Likewise, in the absence of Tacr1 it may be that SP elicits its function via another G protein coupled receptor, e.g. Tacr2 or Tacr3. However, the absence of both ligand and receptor pathway means that compensatory mechanisms are unable to play a part and a more

detrimental phenotype emerges as is the case with the double knockout and its susceptibility to stress induced depression.

Until recently it was thought that other neuropeptides could bind and illicit a biological response via the Tacr1 but it was generally accepted that SP by far demonstrated the greatest affinity for the receptor. However, it would appear that a recently discovered neuropeptide called hemokinin 1 (HK1) which is expressed from the Tac4 gene shows remarkable similarity to SP. It is 11 amino acids long and shares a 55% homology with SP and, most importantly, demonstrates the same affinity for the Tacr1 as SP. Expression of HK-1 was initially demonstrated in mouse bone marrow and thymus with additional evidence that in bone marrow, HK-1 expression was largely restricted to B cells (Zhang *et al.*, 2000). Further data, however, have illustrated moderate to strong expression in the brain, spleen, stomach, skin, lactating breast, and uterus (Kurtz *et al.,* 2002; Patak *et al.*, 2003).

It was also discovered that HK1 shares the same cell origin and is co-expressed along with SP at numerous sites and both are involved in the same immune regulatory functions (Metwali *et al*, 2011). In humans the TAC4 gene codes for a peptide with homology to the murine HK1 and in addition a truncated form of the peptide (Kurtz *et al*, 2002). What this suggests is that HK1 and SP could have evolved together in mammalian systems to control and regulate vital immune responses and also

behavioural responses. Interestingly, HK1 exerts anti-anxiety and enhanced stress coping actions which contrasts the Tac1 coded neuropeptides in behavioural responses (unpublished data from Z. Helyes, University of Pecs and A. Berger, University of Toronto). Whilst the data in this thesis supports these findings with regards anxiety related behaviours it is not substantiated in the stress induced depression model. This may be an indication that HK1 plays less of a role in this pathway than in the anxiety related response pathway where it would appear that the absence of SP and its effects via the Tacr1 allows HK1 to exert its anxiolytic properties unchallenged possibly via the proposed HK1 receptor in the Tacr1 knockout. At the time of writing this thesis a new mouse model that has its Tac4 gene disrupted resulting in mice that are null for HK1 was being utilised in experiments in both Toronto and Pecs to investigate these hypothesises further.

3.6.3 The double knockout demonstrates a lower threshold for chemically induced seizures that is in contrast to the single KOs

Besides its role in the mood disorders, SP has been associated with one of the most common neurological disorders, epilepsy. However, the literature remains conflicting with some evidence showing SP as a powerful pro-convulsant and others finding evidence to suggest it has a protective role as

an anticonvulsant via its preferred receptor Tacr1 (Toth *et al*, 2007; Maubach *et al*, 1998; Liu *et al*, 1999). More recently association between serotonin and the development and aetiology of epilepsy has been made (Stefulj *et al*, 2010; Bagdy *et al*, 2006; and Chugani *et al*, 2005). Generally speaking, agents that elevate 5HT levels tend to inhibit epileptic seizures and a depletion of brain 5HT levels confers a susceptibility to chemical and electrical evoked seizures (Bagdy *et al*, 2006). In addition, mouse knockouts for the $5HT_{1A}$ or $5HT_{2C}$ receptors display a lower seizure threshold and increased seizure activity implicating the signalling mechanism by which varying levels of 5HT contributes to seizure activity and susceptibility (Bagdy *et al,* 2006).

This is further reinforced by the receptors distribution in the brain with $5HT_{1A}$ being the major postsynaptic receptor in areas associated with seizure activity such as the hippocampal, cortical and hypothalamic regions (Sarnyai *et al*, 2000; Brennan *et al,* 1997; Tecott *et al*, 1995). Tacr1 knockouts have documented lowered anxiety levels and resistance to stress induced depression which is supported by the data in this thesis. This is in conjunction with an increased firing rate of the 5HT neurons in these mice (Gobbi *et al*, 2005). The increased firing of the 5HT neurons was also detected in wildtype mice that were acutely treated with the Tacr1 antagonist, RP67580 (Santarelli *et al*, 2001). The data presented in this thesis documents the seizure susceptibility of the double knockout that

could be due to the absence of a SP/Tacr1 pathway of regulatory control of 5HT levels.

Moreover, epilepsy is associated with a high incidence of depression (Lothe A *et al*, 2008; Bagdy *et al*, 2006)). Conferences that were run at Aintree hospital to encourage an interdisciplinary overlap between the clinical consultants treating human epilepsy patients and the scientific researchers studying the molecular and biochemical causes of epilepsy suggest a high incidence of depression in their patients. Particularly relevant to this thesis is the fact that they also associated an increased risk of seizure with women's menstruation cycle and this will be discussed further in this chapter.

We therefore tested the hypothesis that the double knockout, whose genetic and transcriptional plasticity seems to pre-dispose it to stress induced depression, would have a greater susceptibility to chemically induced epileptic seizures. Although the results are very preliminary, the double knockout (that showed a statistically significant predisposition to stress induced depression in the Porsolt forced swim test) was also the strain that had the most severe chemically induce seizures in the male mice. As mentioned previously, these results were surprising as it was originally hypothesised that the double knockout may have a phenotype similar to the two single knockouts, Tac1 and Tacr1 respectively. In fact this study found

that knocking out both ligand (SP) and preferred receptor (Tacr1) simultaneously altered the phenotype of the animal in a way that was unique from the single knockouts and gave totally different responses in experimental studies both behaviourally and pharmacologically. As mentioned the single knockouts maybe utilizing alternative ligand and receptors which, in the absence of SP and Tacr1, compensate to regulate and facilitate the system they are involved in even if it is not to the same efficiency (hence an alteration in phenotype documented in the single knockouts). This would suggest that a level of redundancy does exist in the single knockouts and the interaction of compensatory tachykinins and receptors are playing a role in the observed phenotypes.

One possible suggestion for the unexpected severity of the seizures seen in the double knockouts (even at the low dose of Kainic Acid, 17mg/Kg) is that the NK3 receptor and one of its ligands NKB may be playing more of a role in the system. NKB is a documented proconvulsant (Gillies *et al*, 2009). Another possibility, as mentioned earlier, is that serotonin is playing a role and the double knockout is having more of detrimental effect on the regulation of this neurotransmitter. As mentioned previously the neurotransmitter serotonin, and its main regulator 5HTT, are historically well documented to be pivotal in the control of mood and behavioural disorders and a regulatory link between 5HT, SP and the receptor Tacr1 is documented (Valentino & Commons, 2005; Santarelli *et al*, 2001). It is

possible that the absence of either SP or the Tacr1 receptor "dampens down" the endogenous systematic response to stress and the regulation of 5HT levels which is the organisms natural defensive mechanism to ensure survival; too prone to stress induced depression and the animal would lack the motivations to mate, seek food and shelter and fight for territory effectively resulting in its extinction. If, however, it was too resistant to stress induced depression then this would result in risky behaviour in the pursuit of its motivations and with the mouse being a heavily preyed upon animal this would also ultimately result in its extinction. This conflict of pursuit of motivations verses the survival instinct may have been responsible for the emergence of a seemingly conflicting regulatory system with regards HK1 and SP and their affinity for the Tacr1.

As previously suggested, it may be that the removal of the preferred pathway that is critical to the regulation of affective behaviour and neurochemical responses to stress has led to the double knockout showing a predisposition to stress induced depression. The removal of either SP or Tacr1 receptor merely challenges the system and the regulation of 5HT levels, resulting in a still functioning but impaired response to various stimuli, whereas the removal of the entire preferred pathway leads to a dysregulation of 5HT levels that is much more pronounced, in particular with regards the Kainic acid study. This would need further testing and measurement of 5HT neuronal firing in these models to substantiate.

3.6.4 The modulation of 5HT levels via the SP/Tacr1 pathway and its implications in epilepsy

As mentioned in the introduction of this thesis Santarelli *et al* (2001) found that SP is increased in the cerebral spinal fluid (CSF) of depressed patients. Also, as mentioned, disruption (Tacr1 knockouts) and drug blockage (MK869, NKP608) of the Tacr1 receptor reduces anxiety and stress in conjunction with an increased firing rate of serotonin neurons in the dorsal raphe nucleus of mice (Gobbi *et al*, 2005; Bilier *et al*, 2004). This fits with the theory that SP and Tacr1 play a role in regulating 5HT levels and blocking its effect removes the regulatory mechanism and may lead to aberrant 5HT levels. Depletion of brain 5HT lowers the threshold to chemical induced seizures as well as electrically evoked ones (Bagdy *et al,* 2006). If SP was acting via Tacr1 receptors as a damping mechanism then removing it (Tac1 knockouts) or blocking its effects (Tacr1 knockouts or antagonists) would result in an elevation of 5HT levels which would result in a phenotype of seizure resistance.

This is indeed what has been documented in some papers as well as a detectable increase in 5HT levels (Bagdy *et al*, 2006; Santarelli *et al*, 2001). Tacr1 antagonists also made it as far as human clinical trials as they had antidepressant effects on patients with moderate to severe depression

generally via raising 5HT levels. With this in mind then during a seizure event when the firing of excitatory neurotransmitters are increasing it could be suggested that SP may be increasing not as a pro-convulsant but in a regulatory and protective role trying to return control to the system. Its increase and apparent effects as a pro-convulsant may be masking other neuropeptides or neurotransmitters that are playing more of a role in the epileptic episode. This runs contrary to the vast majority of published literature which documents SP as a pro-convulsant often painting it as a tachykinin that needs to be controlled in order to limit the damaging effects of seizures (Liu *et al*, 1999), but could its increase in levels during seizure actually be a damage limitation attempt by a system that in evolutionary terms has developed as a protective mechanism (Maubach *et al*, 1998).

This would fit in with the many other roles SP has as a protective neuropeptide. Unfortunately an unprecedented series of events is initiated during seizure including an increase in excitatory glutamate release and levels of NKB (a hypothesised pro-convulsant) and it may take time for control to return, if during this time the levels of SP have been raised to such a level as to over compensate then this would also present problems. If 5HT levels dropped too much then the reduction of brain 5HT concentrations would lead to a susceptibility to future seizures (Chugani *et al*, 2005), and this is indeed the case in Status Epilepticus. Interestingly, a recently published article in epilepsy research alluded to the same

conclusion and found an association with the less transcriptionally efficient combined genotypes of the 5HTTLPR and 5HTTVNTR allele variants and temporal lobe epilepsy (Schenkel *et al*, 2011).

The results of the HE and histochemical staining showed that only in the double knockout strain at time point 24 hours post KA injection was there any pathology found in the neurons of the CA3 region of the hippocampus (figure 3.20). In all the other strains and time points (including 3 hour, 4 days and 7days for the double knockout) the pathology in the KA injected subjects was the same as the saline injected controls. This is consistent with the findings of the behavioural testing following KA injection whereby the double knockout showed a surprising sensitivity to the KA and had full seizures even at the low 17mg/Kg dose and also with published literature which shows that 24 hours is the time period were the majority of observed pathology occurs. In fact, the same neuronal damage has been reported in wildtype C57BL/6J mice but this was using a 35mg/Kg dose when the mice also displayed full seizures (Benkovic *et al*, 2006). However, during the dose range study the 35mg/Kg proved to be lethal in our hands. A lower dose was used in the Benkovic paper for aged C57BL/6J mice as it is reported that they have an increased sensitivity to KA and for mice in the group age 20 months plus only 20mg/Kg KA was injected. This is a very interesting observation which is of particular

relevance with regards the sex specific differences we observed in this thesis and this will be discussed further on in this section.

3.6.5 The transcription factors REST and REST4

Since previous work in our group had proposed an interplay between REST (repressor element-1 silencing transcription factor, also referred to as neuron restriction silencing factor, NRSF), REST4 (a truncated variant of REST) and expression of SP by modulation of the Tac1 gene we used immunohistochemistry to investigate the expression and presence of these transcription factors in the CA3 region of the hippocampus in control and KA injected mice (Spencer *et al*, 2006). The only time points that showed a different staining or pathology from the saline controls and wildtype KA injected controls where at 3 hours and 24 hours. Although preliminary, this is consistent with published literature and the findings in our lab with regards the critical time point for molecular changes and neurological pathology.

Spencer *et al* (2006) found an up regulation of REST4 at 3 hour through to 24 hour post KA injection in rats in the CA1 area of the hippocampus. This publication also documents a more modest increase in REST and both REST and REST4 exhibit this increase in expression only transiently up to

and including 24 hour post KA injection. These findings are partially reflected in the immunohistochemistry results in this thesis using mice and observing the CA3 region of the hippocampus. Staining for REST was stronger in the Tac1 and double knockouts at time point 24 hour post KA injection consistent with the modest up regulation found by Spencer *et al.* REST4 staining was only found to be much stronger in the double knockout strain at 24 hour post KA injection. Since the Spencer study used rats and all rats received a dose of KA that induced full seizure the results of this thesis contribute towards validating the findings in the rat model. The double knockout was the only strain to exhibit full seizure at the low dose of 17mg/Kg and it was in this strain at time point 24 hours that stronger staining for both REST and REST4 were observed alongside neuronal damage in the CA3 region. Although the Tac1 knockout also exhibited a stronger staining for REST at 24 hours without any seizures or pathology this would be expected if REST was a modulator of SP levels as there would be no elevation of SP in this null model. However, the speculation that the removal of the complete SP and Tacr1 pathway negates the redundancy effect and any compensatory mechanisms still applies and the double knockouts susceptibility to chemically induced seizures may be a result of another pathway playing a role, such as NKB and Tacr3 which accelerates the molecular dysregulation of the hippocampus leading to full seizures and ultimately neuronal damage. Likewise, this hypothesis can be reversed and it may be the loss of both

parts of a protective mechanism is what contributes to the neuronal damage and full seizures even at low doses of KA that are observed in the double knockout.

3.6.6 The double knockout and ADNP

ADNP (activity dependent neuroprotective protein) has been postulated to have neuroprotective properties due to it containing an eight amino-acid sequence (which has been termed NAP) which has been shown to have strong neuroprotective effects in a variety of models (Gennet *et al*, 2008; Steingart and Gozes, 2006). Gennet *et al* found that different localisation of ADNP correlates with different cell states and postulated that, similar to the action of some transcription factors such as NFkB, the inactive form is sequestered in the cytoplasm and only on translocation to the nucleus is it activated.

Of particular interest in the results of this thesis is the observed localisation of ADNP in the Tac1 and double knockout models at 24 hours post KA injection. Although preliminary, the double knockout line was the only one to succumb to a full seizure and additionally shows a differential expression of ADNP which is exclusively in cytoplasm in the field of study in comparison to the other lines which demonstrate expression in the

nucleus. Intriguingly, the Tac1 knockouts had an observed dual expression of ADNP in both the cytoplasm and the nucleus. Since ADNP is a VIP regulated protein and an interaction of SP and ADNP regulation is documented (Thippeswamy *et al*, 2007) it could be that disruption of SP in the Tac1 knockouts is altering the expression of ADNP upon insult and resulting in additional expression in the cytoplasm as well as nucleus. However, with complete disruption of the SP/Tacr1 pathway in the double knockouts it may be a more pronounced altered expression of ADNP is being observed exclusively in the cytoplasm, this would support the hypothesis of redundancy in the single knockouts with observed regulatory disruption of associated proteins such as ADNP being far more demonstrative in the double knockout line.

However, contrastingly, if one was to presume that ADNP has a role in neuronal protection and adaptive pathways that are activated on insult then the altered expression and translocation of ADNP to the cytoplasm in the double knockout genotype at 24 hours may be an indication of its neuroprotective actions given the double knockouts susceptibility to chemically induced seizure.

The interesting observation of ADNP in both the cytoplasm and nucleus of the Tac1 knockout at 24 hours post KA injection would suggest a "partial activation" of ADNP protective properties due to the absence of SP but

how this would correlate with the documented seizure resistance observed in this strain remains unclear.

Some proteins are inactive in the nucleus and are only activated on translocation to the cytoplasm. Therefore, if this was the case for the action of ADNP then it could indeed be postulated that the double knockouts susceptibility to KA induced seizures is the reason that this strain was the only one whose ADNP protective properties were activated explaining the total translocation of all ADNP to the cytoplasm of the neurons. The presence of ADNP in the nucleus of "dark neurons", which are known to occur as a consequence of even very subtle traumatisation of neurons (Cammermeyer, 1961), may then be observed as the inability of those neurons to adapt to the insult which ultimately is their demise. It would be interesting to observe ADNP localisation in all the lines at a line dependant dose that would induce full seizure in those animals. Whilst the observed differences in ADNP localisation in this thesis is supportive of ADNPs possible protective action during neuronal insult more research is needed to clarify its role in pathological conditions of the CNS such as epilepsy.

3.6.7 The sexual dimorphism demonstrated in response to KA induced seizure activity

The purpose of the dose range study was to evaluate a level of KA that would not induce a full seizure in wildtype C57BL/6J mice in order to study the subtle behavioural changes and the stages of seizure that precede a full seizure. The peer reviewed literature documents cases were the dose of KA always invokes a full seizure. The problem with this is that neuronal damage will inevitably ensue from such a large dose of neurotoxin leading to the inevitable dysregulation of numerous neuropeptides, neurotransmitters, transcription factors, proteins and inflammatory factors which could mask the actual pathways involved in epileptic seizures.

Experimental design recommendations often quote grouping mixed sexes together in batches as long as sex and age is balanced among the groups especially with regards the control group. In the dose range study 3 males and 3 females, all C57BL/6J and age matched, were grouped together for each of the three doses (15mg, 25mg and 35mg/Kg). Immediately it became obvious that the females showed an increased sensitivity to the effects of KA. This can be clearly seen in figure 3.5 were the females for all the strains exceeded the seizure severity compared to their male counterparts with statistical significance. In figure 3.3, the dose range male vs. female results, the clear difference in female seizure severity scoring

can be seen. However, this difference disappears at 25mg/Kg and the males show a slightly higher seizure severity score.

This highlights the point made earlier about large doses of neurotoxin such as KA masking the subtle differences in seizure activity and propagation as the threshold for full seizure is exceeded for both of the sexes. With the males the double knockout showed a statistically significant sensitivity to KA induced seizures and the Tac1 and Tacr1 knockouts showed a slightly lower seizure score compared to the wildtypes which is consistent with the published literature although this was not statistically significant in our hands. This was not seen in the females, however, all of the strains exhibited an unexpected full seizure even at the low dose of 17mg/Kg and it could be that the seizure threshold for the female sex has been exceeded and a lower dose for females may show a difference between the lines.

This thesis demonstrates why a lot of researchers tend to study one sex in their experiments. The majority of pharmacologists prefer to study males not only because they have a higher metabolic rate which favours efficient drug metabolism but also because females have variations in hormone levels during their oestrous cycle. This is of particular concern in the mouse as female's cycle every five days in this species thus presenting a problem with variables introduced from individual to individual. For translational purposes any treatments or drugs would eventually be used in

both male and female humans so it is important to remember that any analysis should take account of possible differences in response between the sexes.

Females differ from males at the cellular, molecular and whole organism level. The obvious difference is the presence of the Y chromosome in males and the fact that females have two X chromosomes. Only one X chromosome is required which is why males manage fine with XY, in fact if both X chromosomes where to express in the female the result would be very detrimental which is why there is transcriptional silencing of one X chromosome in females known as X-chromosome inactivation. The silencing, achieved through methylation and the production of a ball of inert DNA known as the Barr body, is established in development and is stable with the same X chromosome remaining inactivated in all subsequent cell generations. However, this inactivation is random and results in the female being a mosaic of cells in which either the maternally inherited or paternally inherited X chromosome is silenced thus adding another layer of variability to the research model. This, however, is less of a problem in a properly maintain inbred mouse line due to the homologous genetics but it would have consequences in an outbred colony.

A recent analysis of gene expression in mice demonstrated substantially sexual dimorphic expression in the brain, liver and adipose tissue and

whilst both sexes make the same steroid hormones they are found, of course, in different quantities (Yang *et al*, 2006). All of this can contribute to why such a pronounced difference was observed in seizure severity between the sexes after KA injection. More specifically, it may actually be the sexual differences in the SP Tacr1 pathway that is causing the contrasting sexual effects we observed. A recent study in humans found a sexual dimorphic difference in the SP/Tacr1 binding potential in males and females. The females consistently demonstrated a lower binding potential for SP with Tacr1 in numerous areas of the brain including the hippocampus. The data demonstrated that on average females have a lower brain Tacr1 binding potential (BP) with the most marked area being in the putamen where females had a 19% lower Tacr1BP compared to males (Nyman *et al*, 2006). The putamen is involved in motor control and learning and modulates neurotransmitters and neuropeptides including serotonin, glutamate and SP. Interestingly, the same study found age related changes in the SP/Tacr1 system with a decrease in the Tacr1 binding potential strongly associated with increasing age.

This could be a possible explanation as to why aged male mice show an increase in sensitivity to KA induced seizures that is also replicated in females. By contrast, serotonin (5HT1A) and GABA receptors do not appear to change with age but it is known that there are alterations in glutamate and monoamine systems with increasing age and the recent

observations of an age related decrease in Tacr1 binding potential and the consequences this disruption would have on the systems regulated by this pathway may offer an explanation. In addition, the menstrual cycle is known to disrupt Tacr1 binding and epileptic women often suffer from a seizure exacerbation aligned with their menstrual cycle. This phenomenon is termed catamenial epilepsy and is characterized by recurrent seizures, however, it is known that oestrogen can influence neuronal excitability but the disruption of the SP and Tacr1 binding potential may be a contributing factor also (Vendruscolo *et al*, 2003).

Of particular interest to this thesis are the recent publications suggesting a direct regulatory effect of oestrogen on levels of neuropeptides (Rance *et al*, 2010; Sarajari and Oblinger, 2010), in addition, oestrogen has been shown to have a negative effect on SP expression. For example, oestrogen treatment has been shown to decrease SP levels in the anterior pituitary (Brown *et al.* 1990; O'Halloran *et al.* 1990) and this may be due to Tac1 downstream events in non-coding regulatory regions. It is possible that this regulatory mechanism offers an explanation as to why the females show a susceptibility to chemically induced seizures and this would require more investigation, however, it may also be plausible that the knockout models used in this thesis and the experiments employed have merely demonstrated an evolved sexually dimorphic function of a regulatory system. As mentioned previously, the SP/Tacr1 pathway is

involved in anxiety, fight or flight response and pain perception. Males of most species (including mice) are highly motivated to seek territory and possible mating opportunities with females. The male's investment in gamete production is low and infinite, billions of sperm are produced daily, the female's investment in her gametes on the other hand is high and finite and in many species it is the female sex that provides post natal nurturing. It is in the male's interest to take risks and potentially expose themselves to situations that result in pain (confrontational competitions) because the potential rewards are so great (access to mating opportunity) in comparison to the investment in gamete production. The females contrastingly have a great deal to lose if they have risky behaviours as any injuries or confrontations could result in the loss of an entire litter and detrimentally impact any future mating opportunities. It could be that the SP/Tacr1 pathway plays a role in the management of this investment versus reward motivations and behaviours and thus the sexes have a differential response in regulating levels of expression of specific tachykinins and their preferred receptors but this would, of course, require a great deal more investigation to substantiate.

An interesting further investigation would be to lower the dose of KA to see if there is a strain dependant difference in females as demonstrated by the males. In addition, all sections in the immunohistochemistry were taken from male subjects it would be interesting to examine female CA3 regions

post KA dosage if a low enough dose could be calculated. This thesis hypothesises that there would be neuronal damage in all the females for all the strains since they all reached full seizure if the 17mg/Kg dose was repeated. If a lower dose was determined then a repeat of the above study using females may well be worthwhile but it is recommended that vagina smears are conducted to ensure all subjects are at the same stage of the oestrous cycle to eliminate the variability that this contributes to the data.

One of the aims of this thesis was to investigate and address the limitations of the use of transgenic and knockout mouse models and demonstrate some experimental designs that could overcome some of these limitations. For example, the generation of the double knockout mouse model in chapter three demonstrated how some of the issues of redundancy in knockout models can be circumnavigated with combination knockouts that can be extremely useful in research. The double knockout in this thesis, in particular, proved to be a useful and viable model in related disciplines involving tachykinin research.

The cross breeding of lines is not a new or novel concept; it has been shown to be a particularly valuable strategy when dealing with immuno-compromised models. For example, the toll like receptors (TLRs) are a class of proteins involved in the immune system response. They are single membrane bound receptors capable of recognising molecules derived from

pathogenic microbes. Whilst the single knockout models revealed their specificity for recognising certain endotoxins, for example TLR4s recognition of LPS from E.coli, the combination of the available knockout models contributed knowledge to their functional activity when they form heterodimers. TLR2 forms a heterodimer with TLR1 or TLR6 with each dimer having different ligand specificity. These double knockout models have proved so useful that Jax labs now supply them to the research community in different combinations. The same is true for the interleukin receptors (IL) which form part of a "super family" of immune receptors along with the TLRs.

3.6.8 Data supports the redundancy hypothesis

The data presented in this thesis supports the hypothesis that the single knockout lines of Tac1 and Tacr1 demonstrate a level of redundancy due to the compensatory interactions of related tachykinins and receptors playing a role in the absence of SP and Tacr1 respectively. The double knockout produced as part of this thesis was used to demonstrate the redundancy phenomenon in the single knockouts in a publication by Quinn *et al,* 2010. Previously, Stewart *et al,* demonstrated in a transgenic mouse model that co-expressed the human TAC1 gene with the Lac Z marker gene (MacKenzie *et al*, 2000) that non neuronal Tac1 expression early after infection may have clinical implications for the progression of lung disease apart from its well characterised later role in the inflammatory response. In

addition to this the single knockout mouse models of Tac1 null and Tacr1 null have been used to assess the tachykinins role in murine gammaherpesvirus 68 (MHV-68) infection (Payne *et al,* 2001).

Since the redundancy effect is a debated area which has been documented in this thesis an opportunity existed to address the possible redundancy effects of the single knockouts with regards a role for the tachykinins in respiratory viral infection. This was of particular relevance given the recent publications of a newly found tachykinin, hemokinin 1 (HK1) that co-localises with SP in immunoregulatory function, is expressed with SP at sites of chronic inflammation (Metwali *et al*, 2004) and also demonstrates the same affinity that SP has for the Tacr1 (Kurtz *et al,* 2002). In the 2010 publication, J.P. Quinn *et al*, confirmed previous results that showed a deletion of the Tac1 gene leads to an increased susceptibility to MHV-68 infection. In addition, it was demonstrated that the absence of the Tacr1 also showed the same results with regards increased susceptibility to MHV-68 infection.

However, as with the data presented in chapter three of this thesis, the absence of both Tac1 and Tacr1 in the double knockout model demonstrated a unique and opposite effect to the single knockouts in that it showed an increased resistance to MHV-68 infection (Quinn *et al,* 2010). Consistent with the findings and conclusions of this thesis in chapter three,

Quinn *et al,* 2010, hypothesises that the single knockouts have compensatory ligands and receptors that come into play in the absence of the preferred ones in question. Thus, as discussed in this thesis, the absence of either ligand or receptor challenges the regulatory control mechanism but in the absence of a larger preferred pathway these compensatory mechanisms cannot play a role. Quinn *et al*, suggests that the observed resistance demonstrated by the double knockout model may be due to a lack of inhibitory signalling on components of the host response mediated by the SP/Tacr1 pathway. Clearly, this publication indicates, as does the data in this thesis, the unique phenotype of the double knockout in comparison with its single knockout contributors and how it can help address the problems associated with redundancy in the single knockout models.

3.6.9 One model, two lines and differences due to hypothesised flanking gene effect

As discussed in the introduction of this thesis the flanking gene sequence of DNA around the gene of interest in knockout models should always be taken into account as it can have consequences for the mouse model in question. In conclusion for this thesis is an example of this involving the mouse lines used in this thesis and the hypothesis that the differences documented are due to differential flanking gene effects.

In March 2011 the data on the double knockout from chapter three of this thesis with regards the Porsolt forced swim test, light dark box and Kainic acid induction was presented in a poster for the winter neuropeptides conference. Another poster showcased at the conference also showed behavioural data on the Porsolt forced swim using Tac1 null mice but their data showed an opposite effect the data in this thesis. Whereas the thesis data on the Tac1 null strain showed a resistance to stress induced depression the data presented by Borbely *et al*, showed no difference in the Tac1 null in comparison with the wildtype controls in the Porsolt forced swim test. Both Tac1 nulls were documented as a C57BL/6J background and the wildtypes used were the same (C57BL/6J). Further investigation revealed that the Tac1 null used by Borbely et al, originated from Toronto and they were the "Basbum" Tac1 nulls detailed in chapter one (1.5) of this thesis. The Tac1 nulls used in this thesis for the Porsolt forced swim test were the "Zimmer" Tac1 nulls (1.5.2).

However, the Basbum Tac1 nulls (B6.Cg-*Tac1*^{tm1Bbm}/J) had been backcrossed for 10 generations onto a C57BL/6J background which is accepted in peer reviewed journals as C57BL/6J in background. The Zimmer (B6.Cg-*Tac1* ^{tm1nimh}/J) mice had also been backcrossed for 10 consecutive backcrosses onto C57BL/6J. In theory, they should be the same genetically stable null models. Interestingly, E. Borbely, who

presented the poster, worked in the Z. Helyes group in Pecs Hungary and they also had the Zimmer Tac1 nulls used in this thesis. On her return to Pecs E. Borbely repeated the forced swim test using the Basbum Tac1 nulls, the Zimmer Tac1 nulls and a wildtype control. The results were consistent with data presented in both of the posters. The Basbum Tac1 nulls showed no statistical difference to stress induced depression when compared to wildtypes in the Porsolt forced swim test. The Zimmer Tac1 nulls, however, showed a resistance to stress induced depression with statistical significance when compared with wildtype controls using the student t-test (personal communication Z. Helyes and J. Quinn). This demonstrates very different results from two knockout strains of mice that would be considered the same in the peer reviewed literature.

It is hypothesised that the differences demonstrated between the two Tac1 null models are due to the problem of the flanking genes effect (see chapter one, 1.5.1). In other words the two Tac1 null strains are congenic and retain the origin flanking sequences from the strains used in their generation (see chapter one, 1.4). As discussed previously, regulatory regions have been found up to several mega bases away from the gene they affect (Dickmeis and Muller, 2005) and regulatory effects could be different in the two Tac1 null models producing the different behavioural documented above. The MGI website (mouse genome informatics; *http://www.informatics.jax.org/genes.shtml*) documents the presence of

pseudogenes either side of the Tac1 gene in the C57BL/6J mouse genome (GM8686 and GM8676) as well as the presence of various SNPs (single nucleotide polymorphisms) that are specific to the C57BL/6J strain.

Pseudogenes are sequences that are generally non-transcribed and not translated but have an increased homology to identified genes. They are thought to be the dysfunctional relatives of their adjacent genes and often have gene like features such as promoter sequences and CpG islands, never the less they are considered inert of biological activity. However, recent publications in Nature document that some siRNAs (small interfering RNAs) are derived from pseudogenes (Tam *et al,* 2008). In addition, recent Nature publication documents the regulation of genes by their neighbouring pseudogenes (Poliseno *et al,* 2010). This could potentially, therefore, be an area of genetic variability between the two lines of Tac1 knockouts that may have consequential effects.

With regards the SNPs, any present would be specific to the BL6 strain of mice and the 129 ES cells used to generate the Zimmer and Basbaum Tac1 null models and therefore both could be different and have their own SNPs that differ from the BL6 ones. This confers additional variation that is unlikely to be changed during the backcrossing of these strains. However, since these SNPs occur in regions of DNA in between genes they are not thought to have any functional activity but they are useful for identifying

strain differences and this may be one method of answering the question of how different the flanking sequences in these two Tac1 null strains of mice really are.

However, there is another possible explanation as to why the two models are different and that is the targeting vector positioning. In the Zimmer model the targeting vector disrupted the Tac1 gene by replacing parts of exons 2 and 3. In the Basbaum model the targeting vector disrupted the Tac1 gene by replacing coding exon 3. The difference in positional disruption in the two models may have caused differences in the splice variants. Although the proteins SP and NKA were both documented as deleted in both models, other proteins or splice variants could be truncated resulting in a peptide that is detected but its functional effects could be disrupted. This may also account for the demonstrated differences.

In summary it is suggested that the single knockouts have alternative ligands and receptors that in the absence of SP and Tacr1 compensate and still regulate and facilitate the system they are involved in even if not to the same efficiency (redundancy effects), however, in the case of the double knockout when a pathway involving a known high affinity receptor and ligand is simultaneously absent then it may be that another related receptor/ligand mechanism comes into play which may help to explain why the double knockout is so different.

REFERENCES

Anand, R. (1992). Yeast artificial chromosomes (YACs) and the analysis of complex genomes. *Trends Biotechnol.* 10 (1-2): 35-40.

Antequera, F. (2003). Structure, function and evolution of CpG island promoters.*Cell Mol Life Sci* 60 (8) 1647-58.

Autry, A.E, Adachi, M, Nosyreva, E, Na, E.S, Los, M.F, Cheng, P.F, Kavalali, E.T, Monteggia, L.M. (2011). NMDA receptor blockade at rest triggers rapid behavioural antidepressant responses. *Nature,* 475(7354): 91-95.

Baby, S., Nguyen, M., Tran, D., & Raffia, R. B. (1999). Substance P antagonists: the next breakthrough in treating depression? *J.Clin.Pharm.Ther.* 24, 461-469.

Bagdy, G., Kecskemeti, V., Riba, P., and Jakus, R. (2006). Serotonin and epilepsy. *Journal of Neuroscience,* 1471-4159.

Baldwin DS, Ajel KI, Garner M. (2010). Pharmacological treatment of generalized anxiety disorder. *Curr Top Behav Neurosci.;2:453-67.*

Ballas, N., Battaglioli, E., Atouf, F., Andres, M. E., Chenoweth, J., Anderson, M. E., Burger, C., Moniwa, M., Davie, J. R., Bowers, W. J., Federoff, H. J., Rose, D. W., Rosenfeld, M. G., Brehm, P., & Mandel, G. (2001). Regulation of neuronal traits by a novel transcriptional complex. *Neuron* **31**, 353-365.

Ballas, N., Grunseich, C., Lu, D. D., Speh, J. C., & Mandel, G. (2005). REST and its corepressors mediate plasticity of neuronal gene chromatin throughout neurogenesis. *Cell* **121**, 645-657.

Banbury Conference. (1997). Banbury Report on the use of genetically modified animals and controls. *Homeoffice press.*

Bannon, M.J, Poosch, M.S, Haverstick, D.M, Mandal, A, Xue, I.C, Shibata, K.& dragovic, L.J. (1992). Preprotachyninin gene expression in the human basal ganglia; characterization of mRNAs and pre – mRNAs produced by alternate RNA splicing. *Brain Res, Mol. Brain. Res.* 12 (1-3) 225-231.

Basbaum, A. I. (1999b). Spinal mechanisms of acute and persistent pain. *Reg Anesth.Pain Med.* 24, 59-67.

Bassan M., Zamostiano R., Davidson A., Pinhasov A., Giladi E., Perl O., Bassan H., Blat C., Gibney G., Glazner G., Brenneman D.E. and Gozes I. (1999). Complete sequence of a novel protein containing a femtomolar-activity-dependent neuroprotective peptide. *J.Neurochem.* 72, 1283-1293.

Battersby, S, Ogilvie, A.D, Smith, C.A, Blackwood, D.H, Muir, W.J, Quinn, J.P, Fink, G, Goodwin, G.M. & Harmar, A.J. (1996). Structure of a variable number tandem repeat of the serotonin transporter gene and association with affective disorder. *Psychiatr Genet.* 6 (4) 177-81.

Bellivier, F, Leroux, M, Henry, C, Rayah, F, Rouillion, F, laplanche, J.L. & Leboyer, M. (2002). Serotonin transporter gene polymorphism influences age at onset in patients with bipolar affective disorder. *Neurosci Lett.*334 (1) 17-20.

Ben Ari, Y. (1985). Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy. *Neuroscience* **14**, 375-403.

Ben Ari, Y. and R. Cossart. (2000). Kainate a double agent that generates seizures: two decades of progress. *Trends Neurosci* 23 (11): 580-587.

Benkovic, S.A., O'Callaghan, J.P., and Miller, D.B. (2006). Regional neuropathology following Kainic acid intoxication in adult and aged C57BL/6J mice. *Brain Research,* 1070, 215-231.

Berdoy M, Webster JP, Macdonald DW. (2000). Fatal attraction in rats infected with Toxoplasma gondii. *Proc Biol Sci. 2000 Aug 7;267(1452):1591-4.*

Bergner, C.L, Smolinsky, A.N, Hart, P.C, Dufour, B.D, Egan, R.J, Laporte, J.L, Kalueff. A.V. (2010). Mouse Models for Studying Depression- Like States and Antidepressant Drugs. Mouse models for drug discovery. *Humana Press,* NY: 267-282.

Bhatia, M, Slavin, J, Cao, Y, Basbaum, A.I, Neoptolemos, J.P. Preprotachykinin-A gene deletion protects mice against acute pancreatitis and associated lung injury. *Am. J. Physiol Gastrointest Liver Physiol.* 284: G830-6.

Bhaumik, S, Lewis, X.Z, (2003). Optical imaging of *Renilla* luciferase, synthetic *Renilla* luciferase, and firefly luciferase reporter gene expression in living mice. *Journal of Biomedical Optics*. 9, 578-586.

Bilkei-Gorzo, A., Racz, I., Michel, K., & Zimmer, A. (2002). Diminished anxiety- and depression-related behaviors in mice with selective deletion of the Tac1 gene. *J.Neurosci.* 22, 10046-10052.

Bing, G., Wilson, B., Hudson, P., Jin, L., Feng, Z., Zhang, W., Bing, R., & Hong, J. S. (1997). A single dose of kainic acid elevates the levels of enkephalins and activator protein-1 transcription factors in the hippocampus for up to 1 year. *Proc.Natl.Acad.Sci.U.S.A* 94, 9422-9427.

Blier P, Gobbi G, Haddjeri N, Santarelli L, Mathew G, Hen R. (2004). Impact of substance P receptor antagonism on the serotonin and norepinephrine systems: relevance to the antidepressant/anxiolytic response. *J Psychiatry Neurosci. 2004 May;29(3):208-18.*

Brandon EP, Idzerda RL, McKnight GS. (1995). Targeting the mouse genome: a compendium of knockouts (Part II) *Curr Biol. Jul 1;5(7):758-65. Review.*

Brennan, T.J, Seeley, W.W, Kilgard, M, Schreiner, L.H, Tecott. (1997). Sound Induced seizures in serotonin 5-HT2c receptor mutant mice. *Nat. genet.* 16: 387 -390.

Brown ER, Harlan RE, et al. (1990). Gonadal steroid regulation of substance P (SP)-encoding messenger ribonucleic acids in the rat anterior pituitary and hypothalamus. *Endocrin* 1990;126:330–340.

Cameron OG. (2007). Delirium, depression, and other psychosocial and neurobehavioral issues in cardiovascular disease. *Crit Care Clin. Oct;23(4):881-900, viii. Review.*

Cammermeyer J. (1961). The importance of avoiding "dark" neurons in experimental neuropathology. *Acta Neuropathol.* 1, 245-270.

Cao, Y. Q., Mantyh, P. W., Carlson, E. J., Gillespie, A. M., Epstein, C. J., & Basbaum, A. I. (1998). Primary afferent tachykinins are required to experience moderate to intense pain. *Nature* **392**, 390-394.

Capsi, A, Sugden, K, Moffitt, T.E, taylor, A, Craig, I.W, Harrington, H, McClay, J, Mill, J, Martin, J, Braithwaite, A. & Poulton, R. (2003). Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science.* 301 (5631) 386-9.

Chen, Y. R., Shrivastava, A., & Tan, T. H. (2001). Down-regulation of the c-Jun N-terminal kinase (JNK) phosphatase M3/6 and activation of JNK by hydrogen peroxide and pyrrolidine dithiocarbamate. *Oncogene* **20**, 367-374.

Choi, T., Huang, M., Gorman, C., Jaenisch, R. (1991). A generic intron increases gene expression in transgenic mice. *Molecular Cellular Biology* 11, 3070-3074.

Choi, D.S, Ward, S.J, Messaddeq, N, Launay, J.M, Maroteaux, L. 5-HT2B receptor- mediated serotonin morphogenetic functions in mouse cranial neural crest and myocardiac cells. *Development. 124 1745-1755.*

Chungani, H., and Chugani, D.C. (2005). Imaging of Serotonin Mechanisms in epilepsy. *Current review in clinical science.* Vol. 5, No. 6, pp 201-206.

Clark, A.J., Archibald, A.L., McClenaghan, M., Simons, J.P., Wallace, R., Whitelaw, C.B.(1993). Enhancing the efficiency of transgene expression. *Philos Trans R Soc Lond B Biol Sci 339*, 225-232.

Collier, D.A, Arranz, M.J, Sham, P, Battersby, S, Vallada, H, Gill, P, Aitchison, K.J, Sodhi, M, Li, T, Roberts, G.W, Smith, B, Morton, J, Murray, R.M, Smith, D. & Kirov, G. (1996a). The serotonin transporter is a potential susceptibility factor for bipolar affective disorder. *Neuroreport.* 7(10) 1675 – 9.

Collier, D.A, Stober, G, Li,T, Heils,A, Catalano, M, Di Bella, D, Arranz, M.J, Murray, R.M, Vallada, H.P, Bengel, D, Muller, C.R, Roberts, G.W, Smeraldi, E, Kirov, G, Sham, P. & Lesch, K.P. (1996b). A novel functional polymorphism within the promoter of the serotonin transporter gene: possible role in susceptibility to affective disorders. *Mol Psychiatry* 1 (6) 453 – 60.

Copeland, N.G. (2001). Recombineering: A Powerful New Tool for Mouse Functional Genomics. *Nature.* 769 – 779.

Coulson, J. M., Edgson, J. L., Woll, P. J., & Quinn, J. P. (2000). A splice variant of the neuron-restrictive silencer factor repressor is expressed in small cell lung cancer: a potential role in derepression of neuroendocrine genes and a useful clinical marker. *Cancer Res.* **60**, 1840-1844

Court, D.L, Sawitzke, J.A, Thomason, L.C. (2002). Genetic Engineering Using Homologous Recombination. *Annu. Rev.Genet.* 36:361-88.

Crawley, J.N. (2000). What's wrong with my mouse? *Wiley-Liss.*

Crawley J, Goodwin FK. (1980). Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. *Pharmacol Biochem Behav. Aug;13(2):167-70.*

Crusio, W. E., Goldowitz, D., Holmes, A., and Wolfer, D. (2009). Standards for the publication of mouse mutant studies. *Genes, Brain and Behaviour.* 8:1-4.

Czeh, B, Fuchs, E, Simon, M. (2006). NK receptor antagonists under investigation for the treatment of affective disorders. *Expert Opin. Investig, Drugs.* 15 : 479-486.

David DJ, Froger N, Guiard B, Przybylski C, Jego G, Boni C, Hunt SP, De Felipe C, Hamon M, Jacquot C, Gardier AM, Lanfumey L. (2004). Serotonin transporter in substance P (neurokinin 1) receptor knock-out mice. *Eur J Pharmacol. May 10;492(1):41-8.*

Davidson, S, Miller, K.A, Doewll, A, Gildea, A, Mackenzie, A. (2006). A remote and highly conserved enhancer supports amygdala specific expression of the gene encoding the anxiogenic neuropeptide substance-P. *Mol. Psychiatry.* 11 410 – 421.

De Felipe, C., Herrero, J. F., O'Brien, J. A., Palmer, J. A., Doyle, C. A., Smith, A. J., Laird, J. M., Belmonte, C., Cervero, F., & Hunt, S. P. (1998). Altered nociception, analgesia and aggression in mice lacking the receptor for substance P. *Nature* 392, 394-397.

Dickmeis T, Müller F. (2005). The identification and functional characterisation of conserved regulatory elements in developmental genes. *Brief Funct Genomic Proteomic. 2005 Feb;3(4):332-50.*

Doherty MJ, Rostad SW, Kraemer DL, Vossler DG, Haltiner AM (August 2007). "Neocortical gliosis in temporal lobe epilepsy: gender-based differences". *Epilepsia* 48 (8): 1455–9.

Drake JW, Charlesworth B, Charlesworth D, Crow JF. (1998). Rates of spontaneous mutation. *Genetics. 1998 Apr;148(4):1667-86. Review.*

Drugan RC, Morrow AL, Weizman R, Weizman A, Deutsch SI, Crawley JN, Paul SM. (1989). Stress-induced behavioral depression in the rat is associated with a decrease in GABA receptor-mediated chloride ion flux and brain benzodiazepine receptor occupancy. *Brain Res. 1989 May 15;487(1):45-51.*

Elsawa Sherine F., William Taylor, Cynthia C. Petty, Ian Marriott, Joel V. Weinstock and Kenneth L. Bost. (2003). Reduced CTL Response and Increased Viral Burden in Substance P receptor-deficient mice infected with Murine {gamma}-Herpesvirus 68. *Journal immunology*. 170:2605-2612.

Engel J; International League Against Epilepsy (ILAE) (June 2001). "A proposed diagnostic scheme for people with epileptic seizures and with epilepsy: report of the ILAE Task Force on Classification and Terminology". *Epilepsia* 42 (6): 796–803.

Felipe, C., Herrero, J.F., O'Brien, J.A., Palmer, J., Doyle, C., Smith, A., Laird, J.M.A., Belmonte, C., Cervero, F., and Hunt S.P. (1998). Altered nociception, analgesia and aggression in mice lacking the receptor for substance P. *Nature*, Vol. 392, 394-397.

Ferenc Gallyasa, Viola Kiglicsb, Péter Baracskayb, Gábor Juhászb, András Czurkób. (2008).The mode of death of epilepsy-induced "dark" neurons is neither necrosis nor apoptosis: An electron-microscopic study. *Brain research,* 1239: 207-215.

Festing, M, Overand, P, Das, R, Borja, M.C, Berdoy, M. (2002). The Design of Animal Experiments. *Roy Soc Med Press.* 14.

Filippova, G.N, Fagerlie, S, Klenova, E.M, Myers, C, Dehner, Y, Goodwin, G, Neiman, P.E, Collins, S.J. & Lobanenkov, V.V. (1996). An exceptionally conserved transcriptional repressor, CTCF, employs different combinations of zinc fingers to bind diverged promoter sequences of avian and mammalian c-myc oncogenes. *Mol Cell Biol.* 16 (6) 2802-13.

Fiskerstrand, C.E, Lovejoy, E.A, Quinn, J.P.(1999).An intronic polymorphic domain often associated with susceptibility to affective disorders has allele dependent differential enhancer activity in embryonic stem cells. *Federation of European Biochemical Societies.* 458, 171-174.

Fiskerstrand, C. & Quinn, J. P. (1996). The molecular biology of preprotachykinin-A gene expression. *Neuropeptides* 30, 602-610.

Freeman, H.C, Hugill, A, Dear, N.T, Ashcroft, M. & Roger, D.C.(2006). A New Quantitive Trait Locus Accounting for Glucose Intolerance in C57BL/6J Mice. *Diabetes.* 55

Furman S., Hill J.M., Vulih I., Zaltzman R., Hauser J.M., Brenneman D.E. and Gozes I. (2005). Sexual dimorphism of activity-dependent neuroprotective protein in the mouse arcuate nucleus. *Neurosci. Lett.* 373, 73-78.

Gardier, A.M., Bourin, M. (2001). Appropriate use of "Knockout" mice as models of depression or models of testing the efficacy of antidepressants. *Psychopharmacology* 153:393-394.

Gaspar, P, Cases, O. & Maroteaux, L. (2003). The development role of serotonin: news from mouse molecular genetics. *Nat Rev Neurosci.* 4 (12) 1002-12.

Gaudreault, I, Guay, D. & Lebel, M. (2004). YB-1 promotes strand separation in vitro of duplex DNA containing either mispaired bases or cisplatin modifications, exhibits endnucleolytic activities and binds several DNA repair proteins. *Nucleic Acids Res.* 32 (1) 316 – 27.

Gennet, N, Herden, C, Bubb, V.J, Quinn, J.P. & Kipar. (2008). Expression of activity-dependent neuroprotective protein in the brain of adult rats. *Histol Histopathol.* 23:309 -317.

Gerard, N.P., Garraway, L.A., Eddy, R.L., Shows, T.B., Iijima, H., Paquet, J.L., Gerard, C. (1991). Human substance P receptor (NK-1): organization of the gene, chromosome localization, and functional expression of cDNA clones. *Biochemistry.* 30(44): 10640-6.

Gershon MD. (2005). Nerves, reflexes, and the enteric nervous system: pathogenesis of the irritable bowel syndrome. *J Clin Gastroenterol.* ;39(5 Suppl 3):S184-93.

Gillies S, Haddley K, Vasiliou S, Bubb VJ, Quinn JP. (2009). The human neurokinin B gene, TAC3, and its promoter are regulated by Neuron Restrictive Silencing Factor (NRSF) transcription factor family. *Neuropeptides.*Aug;43(4):333-40. Epub 2009 Jun 17.

Gobbi, G. & Blier, P. (2005). Effect of neurokinin-1 receptor antagonists on serotoninergic, noradrenergic and hippocampal neurons: Comparison with antidepressant drugs. *Peptides 26: 1383–1393.*

Goldstein I, Ezra O, Rivlin N, Molchadsky A, Madar S, Goldfinger N, Rotter V. (2011). p53, a novel regulator of lipid metabolism pathways. *J Hepatol. Mar;56(3):656-62.*

Gozes I., Bassan M., Zamostiano R., Pinhasov A., Davidson A., Giladi E., Perl O., Glazner G.W. and Brenneman D.E. (1999). A novel signaling molecule for neuropeptide action: activity-dependent neuroprotective protein. *Ann. NY Acad. Sci.* 897, 125-135.

Gozes I., Zamostiano R., Pinhasov A., Bassan M., Giladi E., Steingart R.A. and Brenneman D.E. (2000). A novel VIP responsive gene. Activity dependent neuroprotective protein. *Ann. NY Acad. Sci.* 921,115-118.

Gozes I. (2007). Activity-dependent neuroprotective protein: from gene to drug candidate. *Pharmacol Ther. May;114(2):146-54.*

Guiard, B.P, Froger, N, Hamon, M, Gardier, A and Lanfumey, L. (2005). Sustained pharmacological blockade of NK1 substance P receptors causes functional desensitization of dorsal raphe 5-HT1A autoreceptors in mice. *Journal of Neurochemistry, 95, 1713–1723*

Hagenbuch N, Feldon J, Yee BK. (2006). Use of the elevated plus-maze test with opaque or transparent walls in the detection of mouse strain differences and the anxiolytic effects of diazepam. *Behav Pharmacol. 2006 Feb;17(1):31-41.*

Hammen, C, Brennan, P.A, Keenan-Miller, D, Hazel, N.A, Najman, J.M. (2009). Chronic and acute stress, gender, and serotonin transporter gene-environment interactions predicting depression symptoms in youth. *Journal of Child Psychology and Psychiatry.*

Haneda, E, Higuchi, M, Maeda, J, Inaji, M, Okauchi, T, Ando, K, Obayashi, S, Nagai, Y, Narazaki, M, Ikehira, H, Nakao, R, Zhang, M, Suzuki, K, Suzuki, H, Suhara, T. (2006). In Vivo Mapping of Substance P Receptors in Brains of Laboratory Animals by High – Resolution Imaging Systems. *Synapse.* 61:205-215.

Hansson, S.R, Mezey, E. & Hoffman, B.J. (1999). Serotonin transporter messenger RNA in the developing rat brain: early expression in serotonergic neurons and transient expression in non- serotonin neurons. *Neuroscience.* 83 (4) 1185-201.

Hariri, A.R, Weinberger, D.R. (2003). Functional neuroimaging of genetic variation in serotonergic neurotransmission. *Genes, Brain and Behavior.* 2: 341-349.

Hariri, A.R. & Holmes, A. (2006). Genetics of emotional regulation: the role of the serotonin transporter in neural function. *Trends Cogn Sci.* 10 (4) 182-91.

Heils, A, Teufel, A, Petri, S, Stober, G, Riederer, P, Bengel, D. & Lesch, K.P. (1996). Allelic variation of human serotonin transporter gene expression. *J Neurochem.* 66 (6) 2621-4.

Helyes, Z, Elekes, K, Sandor, K, Szitter, I, Kereskai, L, Pinter, E, Kemeny, A, Szolcsanyi, J, McLaughlin, L, Vasiliou, S, Kipar, A, Zimmer, A, Hunt, S.P, Stewart, J.P, Quinn, J.P. (2010). Involvement of prprotachykinin A gene-encoded peptides and the neurokinin 1 receptor in endotoxin – induced murine airway inflammation. *Neuropeptides.* 44, 399-406.

Hoehe, M.R, Wendel, B, Grunewald, I, Chiaroni, P, Levy, N, Morris-Rosendahl, D, Macher, J.P, Sander, T. & Croq, M.A. (1998). Serotonin Transporter (5-HTT) gene polymorphism are not associated with susceptibility to mood disorders. *Am J Med Genet.* 81 (1) 1-3.

Holmes, A, Yang, R.J, Murphy, D.L and Crawley, J.N. (2002). Evaluation of Antidepressant-related Behavioral Responses in Mice Lacking the Serotonin Transporter. *Neuropsychopharmacology 27:914–923.*

Holmes, A, Yang, R.J, Lesch, P.K, Crawley, J.N, Murphy, D.L. (2003). Mice Lacking the Serotonin Transporter Exhibit 5-HT1A Receptor-Mediated Abnormalities in Tests for Anxiety-like behaviour. *Neuropsychopharmacology.* 28, 2077–2088

Hranilovic, D, Stefulj,J, Schwab,S, Borrmann – Hassenbach, M, Albus, M, Jernej, B, Wildenauer, D. (2004). Serotonin Transporter Promoter and Intron 2 Polymorpisms: Relationship Between Allelic Variants and Gene Expression. *Biol Psychiatry.* 55, 1090 – 1094.

Huang, X, Saint – Jeannet, J.P. (2004). Induction of the neural crest and the opportunities of life on the edge. *Dev Biol.* 275 1-11.

Jennings, K.A, Loder, M.K, Sheward, J, Pei,Q, Deacon, R.M.J, Benson,M.A, Olverman, H.J, Hastie, N.D, Harmar, A.J, Shen, S, Sharp, T. (2006). Increased Expression of the 5-HT Transporter Confers a Low Anxiety Phenotype Linked to Decreased 5-HT Transmission. *Neuroscience.*26 : 8955-8964.

Johnson, E,K., J.E. Jones, M. Seidenberg and B.P. Hermann. (2004). The relative impact of anxiety, depression, and clinical seizure features on health-related quality of life in epilepsy. *Epilepsia* 45 (5): 544-550.

Karl T, Duffy L, Herzog H. (2008). Behavioural profile of a new mouse model for NPY deficiency. *Eur J Neurosci.Jul;28(1):173-80.*

Klenova , E, Scott, A.C, Roberts, J, Shamsuddin, S, Lovejoy, E.A, Bergmann, S, Bubb, V.J, Royer, H.D. & Quinn, J.P. (2004). YB-1 and CTCF differentially regulate the 5-HTT polymorphic intron 2 enhancer which predisposes to a variety of neurological disorders. *J. Neurosci.* 24 (26) 5966-73.

Kurtz MM, Wang R, Clements MK, Cascieri MA, Austin CP, Cunningham BR, Chicchi GG, Liu Q. (2002). Identification, localization and receptor characterization of novel mammalian substance P-like peptides. *Gene. 2002 Aug 21;296(1-2):205-12.*

Kramer, M. S., Cutler, N., Feighner, J., Shrivastava, R., Carman, J., Sramek, J. J., Reines, S. A., Liu, G., Snavely, D., Wyatt-Knowles, E., Hale, J. J., Mills, S. G., MacCoss, M., Swain, C. J., Harrison, T., Hill, R. G., Hefti, F., Scolnick, E. M., Cascieri, M. A., Chicchi, G. G., Sadowski, S., Williams, A. R., Hewson, L., Smith, D., Rupniak, N. M., & . (1998). Distinct mechanism for antidepressant activity by blockade of central substance P receptors. *Science* 281, 1640-1645.

Kraner, S. D., Chong, J. A., Tsay, H. J., & Mandel, G. (1992). Silencing the type II sodium channel gene: a model for neural-specific gene regulation. *Neuron* **9**, 37-44.

Lacoste B, Riad M, Descarries L. (2006). Immunocytochemical evidence for the existence of substance P receptor (NK1) in serotonin neurons of rat and mouse dorsal raphe nucleus. *Eur J Neurosci. Jun;23(11):2947-58.*

Laird JM, Olivar T, Roza C, De Felipe C, Hunt SP, Cervero F. (2000). Deficits in visceral pain and hyperalgesia of mice with a disruption of the tachykinin NK1 receptor gene. *Neuroscience. 2000;98(2):345-52.*

Lauder, J. M. (1993). Neurotransmitters as growth regulatory signals: role of receptors and second messengers. *Tr. Neurosci.* 16, 233-240.

Lauder, J. M. (1988). Neurotransmitters as morphogens. *Prog. Brain Res.* 73, 365-387.

Lauder, J. M., Tamir, H. and Sadler, T. W. (1988). Serotonin and morphogenesis: I. Sites of serotonin uptake and -binding protein immunoreactivity in the midgestation mouse embryo. *Development* 102, 709-720.

Lauder, J. M. and Zimmerman, E. F. (1988). Sites of serotonin uptake in epithelia of the developing mouse palate, oral cavity, and face: possible role in morphogenesis. *J. Craniofac. Genet. Dev. Biol.* 8, 265-276.

Lee, E.C, Yu, D, Velasco, M, Tessarollo, L, Swing, D.A, Court, L.C, Jenkins, N.A, Copeland, N.G. (2000). A Highly Efficient coli-Based Chromosome Engineering System Adapted for Recombinogenic Targeting and Subcloning of BAC DNA. *Genomics.* 73, 56 -65

Lebrand, C. et al. (2006). Transitory uptake of serotonin in the developing sensory pathways of the common marmoset. *J. Comp. Neurol.* 499, 677–689

Lemons D, McGinnis W (2006). Genomic evolution of Hox gene clusters. *Science* 313 (5795): 1918–22.

Lesch, K.P, Mössner. R. (2006). Inactivation of 5HT transport in mice: modeling altered 5HT homeostasis implicated in emotional dysfunction, affective disorders, and somatic syndromes. *Handb Exp Pharmacol.* (175):417-56.

Lesch, K. P. (2005). Serotonergic gene inactivation in mice: Models for anxiety and aggression. *Novartis symposium,* 268:111-40, discussion, 140-6, 167-70.

Lesch, K.P. (2005). Pharmacogenetics of the serotonin transporter. Progressin *Neuro-Psychopharmacology and Biological Psychiatry.* 29, 1062-1073.

Lesch, K.P, Bengel, D, Heils, A, Sabol, S.Z, Greenberg, B.D, Petri, S, Benjamin, J, Muller, C.R, Hamer, D.H. & Murphy, D.L. (1996). Association of anxiety –related traits with a polymorphism in the serotonin transporter gene regulatory region. *Science,*274 (5292) 1527-31.

Li, En., Bestor, T., Jaenish, R. (1992). Targeted mutation of the DNA methyltransferase Gene Results in Embryonic Lethality. *Cell.* Vol 69, pg915-926.

Li, T, Lu, Z. & Lu, L. (2004). Regulation of eye development by transcription control of CCCTC binding factor (CTCF). *J Biol Chem* 279 (26) 27575 – 83.

Liu, H., Mantyh, P. W., & Basbaum, A. I. (1997). NMDA-receptor regulation of substance P release from primary afferent nociceptors. *Nature* 386, 721-724.

Liu, Y, Oakley, E.J, Sun, L. & Jost, J.P. (1998). Multiple domains are involved in the targeting of the mouse DNA methyltransferase to the DNA replication foci. *Nucleic Acids Res.* 26(4) 1038-45.

Liu, H., Cao, Y., Basbaum, A. I., Mazarati, A. M., Sankar, R., & Wasterlain, C. G. (1999a). Resistance to excitotoxin-induced seizures and neuronal death in mice lacking the preprotachykinin A gene. *Proc.Natl.Acad.Sci.U.S.A* 96, 12096-12101.

Liu, H., Mazarati, A. M., Katsumori, H., Sankar, R., & Wasterlain, C. G. (1999b). Substance P is expressed in hippocampal principal neurons during status epilepticus and plays a critical role in the maintenance of status epilepticus. *Proc.Natl.Acad.Sci.U.S.A* 96, 5286-5291.

Liu, H., Sankar, R., Shin, D. H., Mazarati, A. M., & Wasterlain, C. G. (2000). Patterns of status epilepticus-induced substance P expression during development. *Neuroscience* 101, 297-304.

Liu, P, Jenkins, N,A, Copeland, N,G. (2003). A Highly Efficient Recombineering-Based method for Generating Conditional Knockout Mutations. *Genome Research.* 13:476-484.

Livy D. J. and Wahlsten D. (1997). Retarded formation of the hippocampal commissure in embryos from mouse strains lacking a corpus callosum. *Hippocampus* 7, 2-14. \par

Lothe A, Didelot A, Hammers A, Costes N, Saoud M, Gilliam F, Ryvlin P. (2008). Comorbidity between temporal lobe epilepsy and depression: a (18F)MPPF PET study. *Brain* Oct; 131: 2765-82.

Lovejoy, E.A, Scott, A.C, Fikerstrand,C.E, Bubb, V.J, Quinn, J.P. (2002). The serotonin transporter intronic VNTR enhancer correlated with a predisposition to affective disorders has distinct regulatory elements within the domain based on the primary DNA sequence of the repeat unit. *Neuroscience.* 17, 1-4.

Mackenzie, A, Payne, C, Boyle, S, Clarke, A.R, Quinn, J.P. (2000). The Human Preprotachykinin-A Gene Promoter Has Been Highly Conserved and Can Drive Human – Like Marker Gene Expression in the Adult Mouse CNS. *Neuroscience.* 16, 620-630.

Mackenzie, A. and Quinn, J.P. (2004). Post – genomic approaches to exploring neuropeptide gene mis-expression in disease. *Neuropeptides.* 38 (1): 1-15.

Mackenzie, A, Quinn, J. (2002). A Yeast Artificial Chromosome Containing the Human Preprotachykinin-A Gene Expresses Substance P in Mice and Drives Appropriate Marker – Gene Expression during Early Brain Embryogenesis. *Molecular and Cellular Neuroscience.* 19, 72 -87.

Mackenzie, A, Quinn, J. (1999). A serotonin transporter gene intron 2 polymorphic region, correlated with affective disorders, has allele-dependent differential enhancer-like properties in the mouse embryo. *PNAS.* 26,15251-15255.

Magara, F, Muller, U, Li, Z.W, Lipp, H.P, Weissmann, C, Stagljar, M. & Wolfer, D. (1999). Genetic background changes the pattern of forebrain commissure defects in transgenic mice underexpressing the B-amyloid-precursor protein. *Proc. Natl. Acad. Sci* 96 4656-4661.

Mathis C, Paul SM, Crawley JN. (1994). The neurosteroid pregnenolone sulfate blocks NMDA antagonist-induced deficits in a passive avoidance memory task. *Psychopharmacology (Berl). 1994 Oct;116(2):201-6.*

Maubach, K.A, Cody, C, Jones, R.S.G. (1998). Tachykinins may modify spontaneous Epileptiform activity in the rat entorhinal cortex *in vitro* by activating gabaergic Inhibition. *Neuroscience* Vol. 83, No. 4, pp. 1047 1062.

Mayer, E.A. (2000). The neurobiology of stress and gastrointestinal disease. *Gut bmjjounals.* 47:861 – 869.

Mellerup, E, Bennike, B, Bolwig, T, Dam, H, Hasholt, L, Jorgensen, M.B, Plenge, P. & Sorensen, S.A. (2001). Platelet serotonin transporters and the transporter gene in control subjects, unipolar patients and bipolar patients. *Acta Psychiatr Scand.* 103 (3) 229-33.

Metwali, A, Blum, A.M, Elliott, D.E, Setiawan, T, Weinstock, J.V. (2004). Cutting Edge: Hemokinin has substance P-Like Function and Expression in Inflammation. *J. Immunology.* 172: 6528-6532.

Mitchell PJ, Tjian R (1989). "Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins". *Science* 245 (4916): 371–8.

Mori, N., Schoenherr, C., Vandenbergh, D. J., & Anderson, D. J. (1992). A common silencer element in the SCG10 and type II Na+ channel genes binds a factor present in nonneuronal cells but not in neuronal cells. *Neuron* **9**, 45-54.

Morrison, R. S., Wenzel, H. J., Kinoshita, Y., Robbins, C. A., Donehower, L. A., & Schwartzkroin, P. A. (1996). Loss of the p53 tumor suppressor gene protects neurons from kainate-induced cell death. *J.Neurosci.* **16**, 1337-1345.

Muller, U. (1999). Ten years of gene targeting: targeted mouse mutants, from vector design to phenotype analysis. *Mechanisms of Development.* 82, 3-21.

Muyers,JPP, Zhang, Y, Stewart, AF. (2001). Techniques: Recombinogenic engineering – new options for cloning and manipulating DNA. *Biomedical Sciences.* 26.5.

Nagy, A. (2000). Cre Recombinase: The universal Reagent for Genome Tailoring. *Genesis.* 29:99-109.

Nakamura, M. Ueno, S. Sano, A. & Tanabe, H. (2000). The human serotonin transporter gene linked polymorphism (5-HTTLPR) shows ten novel allelic variants. *Mol Psychiatry.* 5 (1) 32-8.

Nielsen, L.B, McCormick, S.P, Pierotti, V, Tam, C, Gunn, M.D, Shizuya, H and Young, S.G. (1997). Human apolipoprotein B transgenic mice generated with 207- and 145-kilobase pair bacterial artificial chromosomes. Evidence that a distant 5-element confers appropriate transgene expression in the intestine. *J Biol Chem.* 272 (47): 29752-29758.

Nyman, M.J, Eskola, O, Kajander, J, Vahlberg, T, Sanabria, S, Burns, D, Hargreaves, R, Solin, O, Hietala, J. (2006). Gender and age affect NK1 receptors in the human brain – a positron emission tomography study with [18F] SPA-RQ. *International Journal of Neuropsychopharmacology.* 1 – 11.

O'Halloran DJ, Jones PM, et al. (1990). The regulation of neuropeptide expression in rat anterior pituitary following chronic manipulation of estrogen status:a comparison between substance P, neuropeptide Y, neurotensin, and vasoactive intestinal peptide. *Endocrinol* 1990;127:1463–1469.

Olivier Jr, A.A, Jiang, M, Lam, T, Smith, K.L, Swann, J.W. (2000). Novel Hippocampal Interneuronal Subtypes Identified Using Transgenic Mice That Express Green Fluorescent Protein in GABAergic Interneurons. *Neuroscience.* 20: 3354 – 3368.

Ooi L, Wood IC (July 2007). "Chromatin crosstalk in development and disease: lessons from REST". *Nat. Rev. Genet.* **8** (7): 544–54.

Osborne CK, Schiff R, Fuqua SA, Shou J (2001). Estrogen receptor: current understanding of its activation and modulation. *Clin. Cancer Res.* 7 (12 Suppl): 4338s–4342s; discussion 4411s–4412s.

Ozaki HS, Wahlsten D. (1993). Cortical axon trajectories and growth cone morphologies in fetuses of acallosal mouse strains. *J Comp Neurol. Oct 22;336(4):595-604.*

Ozsarac, N, Santha, E, Hoffman, B.J. (2002). Alternative non-coding exons support seroyonin transporter mRNA expression in the brain and gut. *Neurochemistry.* 82, 336-344.

Palm, K., Belluardo, N., Metsis, M., & Timmusk, T. (1998). Neuronal expression of zinc finger transcription factor REST/NRSF/XBR gene. *J.Neurosci.* **18**, 1280-1296.

Palm, K., Metsis, M., & Timmusk, T. (1999). Neuron-specific splicing of zinc finger transcription factor REST/NRSF/XBR is frequent in neuroblastomas and conserved in human, mouse and rat. *Brain Res.Mol.Brain Res.* **72**, 30-39.

Parks, C, Robinson, P, Sibille, E, Shenk, T. & Toth, M. (1998). Increased anxiety of mice lacking the serotonin 1A receptor. *Proc. Natl, Acad, Sci, USA.* 95 10734 – 10739.

Patak E, Candenas ML, Pennefather JN, Ziccone S, Lilley A, Martin JD, Flores C, Mantecon AG, Story ME, Pinto FM (2003) Tachykinins and tachykinin receptors in human uterus. *Br J Pharmacol* 139:523-532.

Payne, C. M., Heggie, C. J., Brownstein, D. G., Stewart, J. P., & Quinn, J. P. (2001). Role of tachykinins in the host response to murine gammaherpesvirus infection. *J.Virol.* **75**, 10467-10471.

Pekarik, V and Belmonte J.C. (2008). NFIX- one gene, two knockouts, multiple effects. *J. Biology.* 7:29

Plioplys S, Dunn DW, Caplan R (2007). "10-year research update review: psychiatric problems in children with epilepsy". *J Am Acad Child Adolesc Psychiatry* 46 (11): 1389–402.

Poliseno L, Salmena L, Zhang J, Carver B, Haveman WJ, Pandolfi PP. (2010). A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. *Nature. 2010 Jun 24;465(7301):1033-8.*

Porsolt RD, Bertin A, Jalfre M. (1977). Behavioral despair in mice: a primary screening test for antidepressants. *Arch Int Pharmacodyn Ther. 1977 Oct;229(2):327-36.*

Ptak K, Burnet H, Blanchi B, Sieweke M, De Felipe C, Hunt SP, Monteau R, Hilaire G. (2002). The murine neurokinin NK1 receptor gene contributes to the adult hypoxic facilitation of ventilation. *Eur J Neurosci*, 16(12):2245-52.

Quinn, J,P, Fiskerstrand, Gerrard, L, MacKenzie, A, Payne, C,M. (2000). Molecular models to analyse preprotachykinin-A expression and function. *Neuropeptides.* 34, 292-302.

Quinn, J.P, Kipar, A, Hughes, D.J, Bennett, E, Cox, H, McLaughlin, L, Zimmer, A, Hunt, S.P, Stewart, J.P. (2010). Altered host response to murine gammaherpesvirus 68 infection in mice lacking the tachykinin 1 gene and the receptor for substance P. *Neuropeptides.* 45, 49-53.

Racine, R. J. (1972). Modification of seizure activity by electrical stimulation. II. Motor seizure. *Electroencephalogr Clin Neurophysiol* 32, 281-94.

Ramon and Cajal (1911). *Histologie du Systeme Nerveux de l'Homme et des Vertebretes*, Vols. 1 and 2 ed. A. Maloine. Paris. 1911.

Rance NE, Krajewski SJ, Smith MA, Cholanian M, Dacks PA. (2010). Neurokinin B and the hypothalamic regulation of reproduction. *Brain Res.* 2010 Dec 10;1364:116-28. Epub 2010 Aug 25.

Reif, A, Rosler, M, Freitag, C.M, Schneider, M, Eujen, A, Kissling, C, Wenzler, D, Jacob, C.P, Retz-Junginger, P, Thome, J, Lesch, K.P, Retz, W. (2007). Nature and Nurture Predispose to Violent Behavior: Serotonergic Genes and Adverse Childhood Environment. *Neuropsychopharmacology.* 32: 2375 – 2383.

Ren- Patterson, R.F, Cochran, L.W, Holmes, A, Sherrill, S, Huang, S, J, Tolliver, T, Lesch, K.P, Lu, B, Murphy, D,L. (2004). Loss of Brain – Derived Neurotrophic Factor Gene Allele Exacerbates Brain Monoamine Deficiencies and Increases Sress Abnormalities of Serotonin Transporter Knockout Mice. *Neuroscience.* 79, 756 -771.

Ribeiro-da-Silva A, Hökfelt T. (2000). Neuroanatomical localisation of Substance P in the CNS and sensory neurons. *Neuropeptides. Oct;34(5):256-71. Review.*

Rise, L.M, Frankel, W.N, Coffin, J.M, Seyfried, T,N. (1991). Genes for Epilepsy Mapped in the Mouse. *Science,* vol 253: 669-673.

Ristevski, S. (2005). Making Better Transgenic Models. *Molecular Biotechnology.* 29.

Rodgers, R.J., Gentsch, C., Hoyer, D., Bryant, E., Green, A.J., Kolokotroni, K.Z., Martin, J.L. (2004). The NK1 receptor antagonist NKP608 lacks anxiolytic-like activity in Swiss-Webster mice exposed to the elevated plus-maze. *Behavioural Brain Research.* 154: 183-192.

Romijn, H.J. et al. (1991). At what age is the developing cerebral cortex of the rat comparable to that of the full-term newborn human baby? *Early Hum. Dev.* 26, 61–67

Roopra, A., Huang, Y., & Dingledine, R. (2001). Neurological disease: listening to gene silencers. *Mol.Interv.* **1**, 219-228.

Said, S. I. And V. Mutt (1970). Polypeptide with broad biological activity, isolation from small intestine. *Science* 169 (951): 1217-1218.

Santarelli, L., Gobbi, G., Debs, P. C., Sibille, E. T., Blier, P., Hen, R., & Heath, M. J. (2001). Genetic and pharmacological disruption of neurokinin 1 receptor function decreases anxiety-related behaviors and increases serotonergic function. *Proc.Natl.Acad.Sci.U.S.A* 98, 1912-1917.

Santarelli, L., Saxe, M., Gross, C., Surget, A., Battaglia, F., Dulawa, S., Weisstaub, N., Lee, J., Duman, R., Arancio, O., Belzung, C., & Hen, R. (2003). Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science* 301, 805-809.

Satake H, Ogasawara M, Kawada T, Masuda K, Aoyama M, Minakata H, Chiba T, Metoki H, Satou Y, Satoh N. (2004). Tachykinin and tachykinin receptor of an ascidian, Ciona intestinalis: evolutionary origin of the vertebrate tachykinin family. *J Biol Chem.Dec 17;279(51):53798-805.*

Sarajari S, Oblinger MM. (2010). Estrogen effects on pain sensitivity and neuropeptide expression in rat sensory neurons. *Exp Neurol.* 2010 Jul;224(1):163-9. Epub 2010 Mar 19.

Sarnyai, Z, Sibille, E.L, Pavlides, C, Fenster, R.J, McEwen, B.S, Toth, M. (2000). Impaired Hippocampal- dependent learning and functional abnormalitoes in the hippocampus in mice lacking serotonin (1A) receptors. *Pro, Natl, Acad, Sci.* 97 14376-14731.

Sergeyev V, Hökfelt T, Hurd Y. (1999). Serotonin and substance P co-exist in dorsal raphe neurons of the human brain. *Neuroreport. 1999 Dec 16;10(18):3967-70.*

Schenkel, L,C, Bragatti, J,A, Torres C,M, Martin K,C, Gus-Manfro,G, Leistner –Segal, S, Bianchin, M,M. (2011). Serotonin transporter gene (5HTT) polymorphisms and temporal lobe epilepsy. *Epilepsy Res.* 10.1016.

Scmitt, A, Mossner, R, Gossmann, A, Fischer, I.G, Gorboulev, V, Murphy, D.L, Koepsell, H, Lesch, K.P. (2003). Organic Cation Transporter Capable of Transporting Serotonin Is Up-Regulated in Serotonin Transporter- Deficient Mice. *Journal of Neuroscience Research.* 71: 701 – 709.

Severini, C., Improta, G., Falconieri-Erspamer, G., Salvadori, S., & Erspamer, V. (2002). The tachykinin peptide family. *Pharmacol.Rev.* 54, 285-322.

Sever P, Leys K, Schachter M. (1987). Autoradiographic localisation of NPY receptors in rabbit kidney: comparison with rat, guinea-pig and human. *Eur J Pharmacol. 1987 Feb 10;134(2):233-7.*

Shen, X, Xiao, H, Ranallo, R, Wu, W.H. & Wu, C. (2003). Modulation of ATP- dependent chromatin-remodeling complexes by inositol polyphosphates. *Science,* 299 (5603), 112 – 4.

Shimojo, M., Paquette, A. J., Anderson, D. J., & Hersh, L. B. (1999). Protein kinase A regulates cholinergic gene expression in PC12 cells: REST4 silences the silencing activity of neuron-restrictive silencer factor/REST. *Mol.Cell Biol.* **19**, 6788-6795.

Shuey, D. L., Sadler, T. W., Tamir, H. and Lauder, J. M. (1993). Serotonin and morphogenesis- Transient expression of serotonin uptake and binding protein during craniofacial morphogenesis in the mouse. *Anat. Embryol.* 187, 75-85.

Shulz. L.D, Ishikawa, F.& Greiner, D.L. (2007). Humanized mice in translational biomedical research. *Nature.7.*

Soeby, K, Larsen, S.A, Olsen, L, Rasmussen, H.B. & Werge, T. (2005). Serotonin transporter: evolution and impact of polymorphic transcriptional regulation. *Am J Med Genet B Neuropsychiatr Genet.* 136(1): 53-7.

Specht and Schorpfer. (2001). Deletion of the alpha synuclein locus in a subpopulation of C5BC/6J inbred mice. *BMC neuroscience 2:11.*

Spencer, E,M, Chandler, K,E, Haddley, K, Howard, M,R, Hughes, D, Belyaev, N,D, Coulson, J,M, Stewart, J,P, Buckley, N,J, Kipar,A, Walker, M,C, Quinn, J,P. (2006). Regulation and role of REST and REST4 variants in modulation of gene expression in *in vivo* and *in vitro* in epilepsy models. *Neurobiology of Disease.* 24(1): 41-52.

Stefulj, J, Bordukalo-Niksic, T, Hecimovic H, Demarin, V, Jernej, B. (2010). Epilepsy and serotonin (5HT): Variations of 5HT – related genes in temporal lobe epilepsy. *Neuroscience Letters.*10,1016.

Szapacs, M.E, Mathews, T.A, Tessarollo, L, Lyons, W.E, Marmounas, L.A, Andrews, A.M. (2004). Exploring the relationship between serotonin and brain-derived neurotrophic factor: analysis of BDNF protein and extraneuronal 5-HT in mice with reduced serotonin transporter or BDNF expression. *Neuroscience.* 140, 81-92.

Tabuchi A, Yamada T, Sasagawa S, Naruse Y, Mori N, Tsuda M. (2002). REST4-mediated modulation of REST/NRSF-silencing function during BDNF gene promoter activation. *Biochem Biophys Res Commun. 2002 Jan 11;290(1):415-20.*

Taketo M., Schroeder A. C., Mobraaten L. E., Gunning K. B., Hanten G., Fox R. R., Roderick T. H., Stewart C. L., Lilly F., Hansen C. T., and Overbeek P. A. (1991). FVB/N: An inbred mouse strain preferable for transgenic analyses. *Proc. Natl. Acad. Sci. USA* 88, 2065-2069. \par

Talley N.J. (2001). Drug therapy options for patients with irritable bowel syndrome. *Am J manag care* 7 S261-S267.

Tam OH, Aravin AA, Stein P, Girard A, Murchison EP, Cheloufi S, Hodges E, Anger M, Sachidanandam R, Schultz RM, Hannon GJ. (2008). Pseudogene-derived small interfering RNAs regulate gene expression in mouse oocytes. *Nature. 2008 May 22;453(7194):534-8.*

Tauscher J, Kielbasa W, Iyengar S, Vandenhende F, Peng X, Mozley D, Gehlert DR, Marek G. (2010). Development of the 2nd generation neurokinin-1 receptor antagonist LY686017 for social anxiety disorder. *Eur Neuropsychopharmacol. Feb;20(2):80-7.*

Tecott, L. H., Sun, L. M., Akana, S. F., Strack, A. M., Lowenstein, D. H., Dallman, M. F. and Julius, D. (1995). Eating disorder and epilepsy in mice lacking 5-HT2C serotonin receptors. *Nature* 374, 542-546.

Thippeswamy T, Howard MR, Cosgrave AS, Arora DK, McKay JS, Quinn JP. (2007). Nitric oxide-NGF mediated PPTA/SP, ADNP, and VIP expression in the peripheral nervous system. *J Mol Neurosci.;33(3):268-77. Epub Sep 11.*

Tie, Li, Zhenyu, Lu, Luo, Lu.(2004). Regulation of Eye Development by Transcription Control of CCCTC Binding Factor (CTCF). *Biological Chemistry.* 279, 27575-27583.

Toth, K., Wittner, L., Urban, Z., Doyle, W.K., Buzsaki, G., Shigemoto, R., Freund, T.F., and Magloczky, Z. (2007). Morphology and synaptic input of Substance P receptor-immunoreactive interneurons in control and epileptic Human Hippocampus. *Neuroscience,* 144, 495-508.

Trillat AC, Malagie I, Mathe-Allainmat M, Anmella MC, Jacquot C, Langlois M, et al.(1998). Synergistic neurochemical and behavioral effects of fluoxetine and 5-HT1A receptor antagonists. *Eur J Pharmacol* 357:179–84.

Valentino, R.J., and Commons, K.G. (2005). Peptides that fine-tune the serotonin system. *Neuropeptides*, 39, 1-8.

Vasiliou, A.S, Mackenzie, A, Morris, R, McLaughlin, L, Bubb, V.J, Haddley, K, Quinn, J.P. (2007). Generation of a transgenic model to address regulation and function of the human neurokinin 1 receptor (NK1R). *Neuropeptides.* 41, 194-205.

Vendruscolo, L., Takahashi, R.N., Bruske, G., and Ramos, A. (2003). Evaluation of the anxiolytic-like effect of NKP608, a NK1-receptor antagonist, in two rat strains that differ in anxiety-related behaviours. *Psychopharmacology,* 170:287-293.

Von Euler, U. S. & Gaddum, J. H. (1931). An unidentified derepressor substance in certain tissue extracts. *Journal of Physiology* **72**, 74-87.

Wang WT, Hu SJ, Han D. (2005). Hippocampal intrinsic bursting-firing neurons and temporal lobe epilepsy. *Sheng Li Ke Xue Jin Zhan. 2005 Apr;36(2):137-40.*

Watson, SA, Grabowska, AM, El-Zaatari, M, Takhar, A. (2006). Gastrin – active participant or bystander in gastric carcinogenesis? *Nat Rev Cancer,* 6: 936–946.

Weiss, L.A, Abney, M, Cook Jr, E.H, Ober, C. (2005). Sex Specific genetic Architecture of Whole Blood Serotonin levels. *Am. J. Hum. Genet.* 76:33-41.

Wendland, J.R, Martin, B.J, Kruse, M.R, Lesch, K.P & Murphy, D.L. (2006). Simultaneous genotyping of four functional loci of human SLC6A4, with a reappraisal of 5_HTTLPR and rs25531.*Mol Psychiatry.* 11(3), 224 – 6.

Wasterlain, C. G., Mazarati, A. M., Naylor, D., Niquet, J., Liu, H., Suchomelova, L., Baldwin, R., Katsumori, H., Shirasaka, Y., Shin, D., & Sankar, R. (2002). Short-term plasticity of hippocampal neuropeptides and neuronal circuitry in experimental status epilepticus. *Epilepsia* 43 Suppl 5, 20-29.

Wolfe, S. A., Nekludova, L., & Pabo, C. O. (2000). DNA recognition by Cys2His2 zinc finger proteins. *Annu.Rev.Biophys.Biomol.Struct.* **29**, 183-212.

Wolfer, D.P, Wim, E, Crusio. & Lipp, H.P. (2002). Knockout mice: simple solutions to the problems of genetic background and flanking genes.*Neurosciences.* 25: 7.

Workman, JL, Kingston, RE. (1998). Alteration of nucleosome structure as a mechanism of transcriptional regulation. *Annu Rev Biochem,* 67;545-79.

Yang X, Model, P, Heintz, N. (1997). Homologous recombination based modification in *Escherichia coli* and germline transmission in transgenic mice of a bacterial artificial chromosome. *Nature Biotechnology.* 15, 859 - 865.

Yang X, Schadt EE, Wang S, et al. (2006). Tissue-specific expression and regulation of sexually dimorphic genes in mice. *Genome Res.* 16:995-1004

Young, H.M, Turner, K.N, Bergner, A.J. (2005). The location and phenotype of proliferating neural – crest-derived cells in the developing mouse gut. *Cell Tissue Res.* 320:1-9.

Yu, D, Ellis, H.M, Lee E-C, Jenkins, N.A, Copeland, N.G, Court, D.L. (2000). An efficient recombination system for chromosome engineering in Escherichia coli. *Proc. Natl. Acad. Sci. USA.* 97, 11, 5978 – 5983.

Zhang Y, Paige CJ (2003) T-cell developmental blockage by tachykinin antagonists and the role of hemokinin 1 in T lymphopoiesis. *Blood* 102:2165-2172.

Zhou, F.C, Sari, Y, Zhang, J.K. (2000). Expression of serotonin transporter protein in developing rat brain. *Developmental Brain research.* 119, 33-45.

Zimmer, A., Zimmer, A. M., Baffi, J., Usdin, T., Reynolds, K., Konig, M., Palkovits, M., & Mezey, E. (1998). Hypoalgesia in mice with a targeted deletion of the tachykinin 1 gene. *Proc.Natl.Acad.Sci.U.S.A* 95, 2630-2635.