

Acknowledgements

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“Get a bicycle. You will not regret it, if you live.”

Mark Twain

The Role of TASK Two-Pore-Domain Potassium Channels in General Anaesthesia

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Declaration of originality

I, Daniel Pang, declare that this thesis is my own work. Information derived from the work of others has been acknowledged in the text and a list of references is given in the bibliography.

Abstract

TASK channels, members of the two-pore-domain potassium channel family, contribute towards the resting membrane potential and have been implicated in the mechanism of general anaesthesia. Previous work from our group with a TASK-3 channel knockout (T3KO) mouse showed a reduction in halothane sensitivity using a loss of righting reflex (LORR) assay, and absence of the theta brain oscillation induced in wild type (WT) mice by halothane anaesthesia.

Two further strains of knockout mice, the TASK-1 knockout (T1KO) and the double knockout (DKO: TASK-1 and -3 channels), were compared with WT using the LORR assay, cortical electroencephalogram recording in response to halothane and during sleep. The mechanistic basis for the diminished theta oscillation in T3KO mice was investigated by recording in CA1 pyramidal cells of the hippocampus.

The LORR assay revealed a decrease in halothane sensitivity in T1KO but not DKO compared with WT. The T1KO strain had a theta oscillation induced by exposure to halothane similar to that of WT mice, whereas that observed in the DKO was intermediate between WT and T3KO. T1KO differed from other strains in that the distribution of sleep and wake periods was uniform across the diurnal cycle. The resting membrane potential did not differ between strains during control or halothane exposure. During control there was no strain difference in action potential (AP). Halothane altered AP shape in WT but not the T3KO strain. WT had a greater ability to sustain AP firing than T3KO during halothane.

These data show that T1KO mice have decreased anaesthetic sensitivity and altered sleep structure compared with WT, indicating a role for this channel in anaesthetic sensitivity and sleep. The similar resting membrane potential and lack of response to halothane in the T3KO makes pyramidal cells an unlikely source of the theta ablation observed.

Table of Contents

Acknowledgements	1
The Role of TASK Two-Pore-Domain Potassium Channels in General Anaesthesia	2
Abstract	4
Table of Contents	5
List of Figures	8
List of Tables	12
1.0 Introduction	13
1.1 Two-pore-domain potassium channels (K2P)	16
1.1.1 The potassium leak.....	16
1.1.2 K2P structure and electrophysiological properties	17
1.1.4 Nomenclature	18
1.2 TWIK-related acid-sensitive K ⁺ channel (TASK) channels	20
1.2.1 TASK channels and general anaesthesia	22
1.3 The electroencephalogram (EEG) and cortical EEG	25
1.3.1 Oscillations and the EEG	26
1.4 The Theta oscillation.....	28
1.4.1 History	28
1.4.2 Generation.....	29
1.4.3 Theta oscillation differentiation	34
1.4.4 Interneurons and the theta oscillation	36
1.4.5 Halothane and type 2 theta oscillation.....	37
1.4.6 TASK channels and the theta oscillation	38
1.4.7 TASK channel expression and sleep	38
1.5 Aims and hypothesis	39
2.0 Materials and methods	40
2.0.1 Creation of knockout mouse strains and genotyping.....	40

2.1 In vivo experiments	41
2.1.1 Animal Instrumentation	41
2.1.2 Cortical EEG recording device: Neurologger 2	45
2.1.3 Loss of righting reflex (LORR) assay	47
2.1.4 Halothane exposure assay	49
2.1.5 Halothane and atropine sulphate assay	51
2.1.6 Intraperitoneal eserine (physostigmine) assay	52
2.1.7 Sleep assay	53
2.1.7.1 Analysis of sleep data	53
2.2 In vitro experiments	58
2.2.1 Preparation of acute brain slices	58
2.2.2 Whole cell patch clamping	61
2.2.3 Solutions	69
2.3 Statistics	71
2.4 Gentoyping	71
3.0 Results	75
3.1 Behavioural phenotypes I - the loss of righting reflex (LORR) and sleep	75
3.1.1 LORR assay	75
3.1.1.1 Halothane	76
3.1.1.2 Sex	77
3.1.1.3 Cyclopropane	79
3.1.2 Sleep	81
3.2 Behavioural phenotypes II - the theta oscillation and the response to halothane, atropine sulphate and eserine	93
3.2.1 Halothane	93
3.2.2 Atropine sulphate	107
3.2.3 Eserine	110
3.3 Mechanistic phenotypes	112
3.3.1 Resting membrane potential	113
3.3.2 Action potential	116

3.3.3 Sustained AP firing	122
3.3.4 sIPSC	125
4.0 Discussion	130
4.1 Loss of righting reflex (LORR)	130
4.1.2 Loss of consciousness (LOC) versus LORR	130
4.1.3 LORR and TASK channel knockout mice	132
4.1.4 LORR and cyclopropane	135
4.1.5 LORR and sex	136
4.1.6 LORR and inter-observer variability	136
4.2 TASK and sleep	137
4.2.1 Sleep and handling stress	137
4.2.2 Sleep in the WT	138
4.2.3 Sleep in the knockout strains	138
4.3 Type 2 theta oscillation is variably altered in KO animals	142
4.3.1 Abolishment of the theta oscillation by atropine sulphate	144
4.3.2 Eserine administration fails to induce type 2 theta oscillation in the T3KO strain	146
4.3.3 Differential brain expression of TASK1 and TASK3	146
4.4 Halothane acts through both GABAA receptors and TASK3 channels in CA1 pyramidal cells	148
4.4.1 Action potential (AP) characteristics	148
4.4.2 Whole cell parameters and halothane	150
4.4.3 Halothane and spontaneous inhibitory post synaptic currents (sIPSCs)	151
4.4.4 Anaesthetic targets	154
4.5 Conclusions	154
4.5.1 Future directions	156
5.0 References	157
6.0 Appendix	171

List of Figures

Figure 1. Blue plaque (left) commemorating the administration of the first general anaesthetic in England	14
Figure 2. A clinically relevant concentration of halothane	15
Figure 3. Membrane structure (top) and current-voltage curves (bottom) for inwardly-rectifying (Kir, left) and two-pore domain (K2P, right) potassium channels.	18
Figure 4. Phylogenetic tree for the 15 identified members of the human K2P gene family. ..	19
Figure 5. A reduced sensitivity to halothane is displayed by TASK-1 knockout (T1KO) animals (-/-)	23
Figure 8. The author having his EEG recorded as part of a neurophysiology experiment. ...	28
Figure 9. Lidocaine injection into the medial septum of a wild type mouse	31
Figure 10. Overview of structures and pathways involved in generation of the theta oscillation in the brain.	33
Figure 11. Overview of excitatory and inhibitory connections.....	34
Figure 12. Cartoon depicting the interaction between inhibitory GABAergic interneurons and a CA1 pyramidal cell of the hippocampus.	37
Figure 13. Cartoon of dorsal surface of mouse skull	43
Figure 14. Photos showing skull before (left) and after (right) placement of skull electrodes. .	44
Figure 15. The Neurologger with batteries fitted.	46
Figure 16. Mice before (left) and after (right) Neurologger fitting.	46
Figure 17. Cartoon of equipment used to assess loss of righting reflex (LORR).	49
Figure 18. Identifiable peaks in the power spectra within the theta frequency band	50
Figure 19. Cartoon depicting the parameters derived from the Lorentzian curve fit	51
Figure 20. Logic rules applied for initial detection of sleep-wake states.	54
Figure 21. Example logic rules for identification of REM sleep.	55
Figure 22. Examples of cortical EEG and EMG data	57
Figure 23. The intact brain of the mouse as it appears following removal from the skull.	59
Figure 24. View of a brain hemisphere with medial surface placed on a petri dish, positioned for the “magic cut”.	60
Figure 25. Cartoon of slice incubation chamber.	61

Figure 26. Cartoon of slice in microscope bath	64
Figure 27. Phase plane plot.	67
Figure 28. Results from genotyping tissue (ear) samples from T3KO and WT animals.	72
Figure 29. Results from genotyping tissue (ear) samples from T1KO and WT animals.	73
Figure 30. Results from genotyping tissue (ear) samples from DKO and WT animals.	74
Figure 31. Dose-response curve of loss of righting reflex data with halothane	76
Figure 32. A comparison between sexes for WT, T1KO, T3KO and DKO strains.	77
Figure 33. Comparison between experimenters for loss of righting reflex (LORR) data.	79
Figure 34. Dose-response curve of loss of righting reflex assay with cyclopropane	80
Figure 35. Percentage of time spent awake per hour during a sleep recording over 29 hours	83
Figure 36. Percentage of time spent in NREM sleep per hour during a sleep recording over 29 hours	85
Figure 37. Percentage of time spent in REM sleep per hour during a sleep recording over 29 hours	87
Figure 38. Time spent as a percentage in wake, NREM sleep and REM sleep (top to bottom, respectively) during the quiet (light) period	88
Figure 39. Time spent as a percentage in wake, NREM sleep and REM sleep (top to bottom, respectively) during the active (dark) period	89
Figure 40. Comparison of quiet (clear bars) and active (black bars) periods for each strain ..	91
Figure 41. Results of exposure to an increasing concentration of halothane	94
Figure 42. Type 2 theta oscillation peak frequency decreases in WT and T3KO	95
Figure 43. Type 2 theta oscillation peak frequency decreases in WT and T1KO	96
Figure 44. Type 2 theta oscillation peak frequency decreases in WT and DKO	97
Figure 45. Type 2 theta oscillation peak frequency decreases	98
Figure 46. Full width at half-magnitude (FWHM) decreases in WT and T3KO strains	99
Figure 47. Full width at half-magnitude (FWHM) decreases in WT and T1KO strains	100
Figure 48. Full width at half-magnitude (FWHM) decreases in WT and DKO strains	101
Figure 49. Full width at half-magnitude (FWHM) decreases in each strain	102
Figure 50. The Q value for WT and T3KO.	103
Figure 51. The Q value for WT and T1KO.	104
Figure 52. The Q value for WT and DKO.	105

Figure 53. The Q value for all strains.....	106
Figure 54. The effect of atropine sulphate	108
Figure 55. Power spectra with normalised power for WT and T3KO strains (n=3 for each strain) with and without eserine	111
Figure 56. Brightfield low power image of a brain slice	112
Figure 57. Scatter plots showing the resting membrane potential	113
Figure 58. Membrane time constant for the sub-threshold response to a brief current injection.	114
Figure 59. Current required to achieve threshold was significantly greater in WT	115
Figure 60. Examples of phase plane plots from T3KO and WT animals	116
Figure 61. The effect of exposure to 1% halothane (approximately 0.40 mM) on the maximum rate of rise, maximum rate of decay and action potential peak height in acute brain slices from WT animals.....	118
Figure 62. The effect of exposure to 1% halothane (approximately 0.40 mM) on the maximum rate of rise, maximum rate of decay and action potential peak height in acute brain slices from T3KO animals	120
Figure 63. A comparison of maximum rate of rise, maximum rate of decay and action potential peak between WT (black bars) and T3KO (red bars)	121
Figure 64. Current - frequency (I-f) curves constructed for WT and T3KO pyramidal cells at rest, during control conditions and exposure to 1% halothane.	123
Figure 65. Current - frequency (I-f) curves constructed for WT and T3KO pyramidal cells held at -60 mV (row B), during control conditions and exposure to 1% halothane.	124
Figure 66. Typical examples of sIPSCs from WT and T3KO	126
Figure 67. Bar graph of sIPSC decay time constant for WT and T3KO.	126
Figure 68. Bar graph of sIPSC area under the curve for WT and T3KO.....	127
Figure 69. Bar graph of sIPSC frequency for WT and T3KO.	128
Figure 70. Bar graph of sIPSC peak height for WT and T3KO.	129
Figure 71. The relationship in EC50 between loss of consciousness (LOC) in humans (defined as failure to respond to a verbal command) and loss of righting reflex (LORR)	131
Figure 72. Halothane sensitivity measured with a loss of righting reflex assay (LORR)	133
Figure 73. Circadian activity in T3KO	139

Figure 74. A network functioning	142
Figure 75. Power spectra from a rat during exposure to 1% halothane	145
Figure 76. TASK3 mRNA expression in a coronal section of the hippocampus from in-situ hybridisation.	147
Figure 77. The percentage increase in IPSC decay half life is a function of volatile agent and concentration	152

List of Tables

Table 1. Commonly used bands for defining EEG frequency ranges.	27
Table 2. Composition of slicing solution.	70
Table 3. Composition of external recording solution.	70
Table 4. Composition of internal recording solution.	70
Table 5. Changes in peak power following atropine administration with and without halothane.	109
Table 6. EC50 values (mean $\pm$ SEM) for halothane (% atm.) reported by Lazarenko et al.	134

1.0 Introduction

Since its introduction as an aid to surgery, the practice of anaesthesia has grown into a specialty in its own right. The first public demonstration of ether anaesthesia was at the Massachusetts General Hospital on the 16th October, 1846 by the dentist William Morton and the news was transmitted by a variety of transportation to Great Britain (Ellis 1976). The first employment of ether in Great Britain is controversial. A blue plaque in Gower Street, London, commemorates the first recorded use of ether in England by another dentist, James Robinson, on 19th December, 1846. However, there is evidence that ether was administered by William Scott for a limb amputation on the same day at the Dumfries Royal Infirmary, Scotland (Baillie 1965). From humble beginnings, as a Faculty within the Royal College of Surgeons of England, the growth of anaesthesia resulted in the formation of the College of Anaesthetists in 1988, which received its royal charter in 1992. Two pioneers of anaesthesia in the UK, John Snow and Joseph Thomas Clover, are depicted on the College's coat of arms (Figure 1). John snow is renowned for administering chloroform to Queen Victoria during the birth of two of her children, thereby increasing acceptance of the contentious issue of pain relief during labour, and ordering the removal of a water pump handle in Soho resulting in the end of a cholera epidemic (www.johnsnowsociety.org). Thomas Clover was a medical student at the time of the introduction of ether into the UK, and he also gained substantial experience in delivering chloroform, developing an apparatus its the safe administration.



Figure 1. Blue plaque (left) commemorating the administration of the first general anaesthetic in England by the dentist James Robinson. The plaque is situated at 52 Gower Street, London, the former home of Francis Boott, friend of James Robinson. The armorial bearings (right) of the Royal College of Anaesthetists depicting John Snow (left) and Joseph Thomas Clover (right). Plaque photo taken by the author. Armorial bearings from The Royal College of Anaesthetists (www.rcoa.ac.uk). Accessed November, 2010.

Several million general anaesthetics are administered each year in the United Kingdom and volatile anaesthetic agents form part of the anaesthetic protocol in 90% of these cases (Campling et al. 1992). Despite widespread use the mechanism of action of the volatile agents remains elusive. Over eighty years after Meyer and Overton identified the relationship between oil solubility and potency (later known as the lipid theory of anaesthetic action), Franks and Lieb shifted focus to a protein theory with their demonstration that clinically relevant concentrations of inhalational anaesthetic agents do not perturb the lipid bilayer (Franks & Lieb 1978, 1982). A strong correlation between potency to inhibit the soluble enzyme luciferase and anesthetic potency was soon reported and followed by the demonstration that volatile anaesthetics (halothane, isoflurane, chloroform and ether) activated a potassium current ($I_{K(An)}$) leading to the inhibition of steady-state firing activity of a particular anaesthetic-sensitive neurone in the snail *Lymnaea stagnalis* (Figure 2) (Franks & Lieb 1984, 1988). Furthermore, $I_{K(An)}$ was shown to demonstrate open rectification and voltage independence, providing an early indicator for the role of two-pore-domain

potassium channels (K_{2P}) in anaesthetic sensitivity. Confirmation that $I_{K(An)}$ was a member of the K_{2P} family was provided in 2007 (Andres-Enguix et al. 2007). An intense period of research over the last 30 years has identified a range of potential anaesthetic targets from the K_{2P} family, with variable effects on anaesthetic sensitivity *in vivo* (Linden et al. 2006, 2007; Heurteaux et al. 2004).

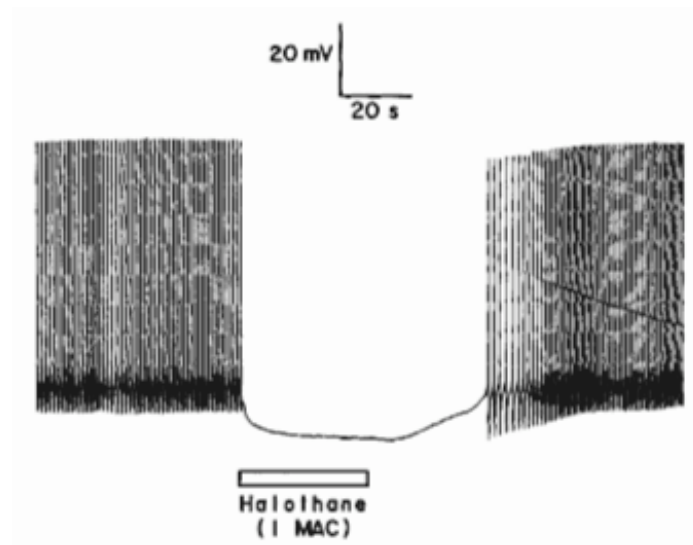


Figure 2. A clinically relevant concentration of halothane (0.0080 atm., period of exposure indicated by open bar) reversibly blocks the activity of an identified neurone in *Lymnaea stagnalis*. MAC is minimal alveolar concentration, the concentration of anaesthetic agent required to prevent gross purposeful movement in response to a nociceptive stimulus. The human value for MAC was used as a guide in selecting a clinically relevant halothane concentration. Adapted from Franks and Lieb (Franks & Lieb 1988).

In the assessment of any potential target as a site of action for general anaesthetics, three criteria have been suggested by Franks (2006) (Franks 2006). These are:

- Plausibility - is the target expressed at a relevant site in the brain and are the known actions of the target likely to promote a state of unconsciousness?
- Sensitivity - the target should be sensitive to clinically relevant concentrations of anaesthetic agents.

- Effect on *in vivo* models - if a transgenic animal is created with the putative target altered in some way and shown to have an altered sensitivity to an anaesthetic agent, this provides strong evidence for the importance of the target.

1.1 Two-pore-domain potassium channels (K_{2P})

The presence of a background (leak) current in neurones was first proposed by Hodgkin and Huxley as a basis for the resting membrane potential (Hodgkin & Huxley 1952). The primary role of a potassium current in setting the resting membrane potential was then suggested (Hille 1973), and around this time it was shown that various anaesthetics, including volatile agents, hyperpolarised neurones (Nicoll & Madison 1982) and that halothane exposure produced an “anaesthetic-induced” potassium current in *Lymnaea stagnalis* (Franks & Lieb 1988). The molecular basis of the potassium leak current was first described almost a decade later with the discovery of K_{2P} channels in *Saccharomyces cerevisiae* and *Caenorhabditis elegans* (Ketchum et al. 1995), followed shortly in *Drosophila melanogaster* (Goldstein et al. 1996) and mammalian systems (Fink et al. 1996; Lesage et al. 1996).

1.1.1 The potassium leak

The presence of a “leak” current emerged from the seminal work of Hodgkin, Huxley, Goldman and Katz and was used to explain the resting membrane potential (Goldman 1943; Hodgkin & Huxley 1952; Hodgkin & Katz 1949). The electrochemical gradient created by active transporters drives the flow of potassium (K^+) ions from the inside of the cell (high potassium concentration) outwards. As K^+ ions leave the cell, the intracellular charge becomes more negative thus opposing further K^+ efflux. Eventually an equilibrium is attained which can be described by the Nernst potential, the membrane voltage at which equilibrium occurs for a given ion at a given transmembrane gradient. This value is approximately -97 mV for K^+ . The final resting membrane potential is determined by the sum of the effects of all ions crossing the plasma membrane. As K_{2P} channels are open at rest, potassium efflux pulls the

resting membrane potential towards the Nernst potential for potassium and any increases in efflux, such as K_{2P} channel activation, will act to hyperpolarise a cell.

1.1.2 K_{2P} structure and electrophysiological properties

Two-pore-domain potassium channels vary in structure from other potassium channels with each subunit having two pore forming domains, with four transmembrane segments (2P/4TM) (Figure 3). In contrast, voltage-gated potassium channels (K_V) have one pore domain and six transmembrane segments (1P/6TM) and inwardly rectifying potassium channels have one pore domain with two transmembrane segments (1P/2TM). K_{2P} channels form dimers and display the following electrophysiological characteristics: (1) weak voltage dependence; (2) activation over a physiological range of membrane potential; (3) exhibit open rectification as predicted by the Goldman-Hodgkin-Katz equation (Goldstein et al. 2001). Rectification is the preferential movement of current in a single direction, and open rectification is the flow of ions down their electrochemical gradient resulting from unequal (physiologic) transmembrane potassium concentrations. In a symmetrical potassium gradient ion flow is linear as described by Ohm's law. In contrast, inwardly rectifying potassium channels have a voltage-dependent block resulting in current flow only when the membrane potential is more hyperpolarised than the Nernst potential (Figure 3). As many K_{2P} channels are open at rest there is minimal delay to current flow in response to a change in voltage. Channel regulation allows control over the resting membrane potential and hence cell excitability. Activation of K_{2P} channels results in hyperpolarisation of the cell membrane and a reduced probability of action potential firing. This regulation is brought about through G protein coupled receptors (GPCRs; see below) and physiological factors.

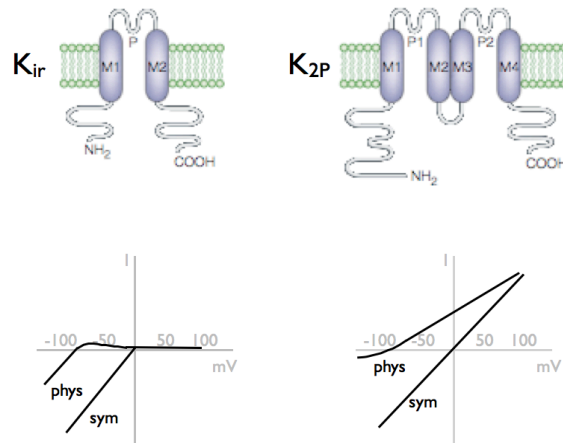


Figure 3. Membrane structure (top) and current-voltage curves (bottom) for inwardly-rectifying (K_{ir} , left) and two-pore domain (K_{2P} , right) potassium channels. K_{ir} channels have one pore (P) domain and two transmembrane (M) segments whereas K_{2P} have two pore domains and four transmembrane segments. Under both symmetrical (sym) and physiologic (phys) potassium concentrations K_{ir} channels pass currents only when the pore is unblocked, a voltage-dependent event. In contrast, K_{2P} channels exhibit open rectification with an outward current over a physiologic range of membrane potentials. Modified from Goldstein et al. (2001) and Hibino et al. (2010).

1.1.4 Nomenclature

The classification of K_{2P} channels is an area of confusion. Initially based on physicochemical features, more recent nomenclature systems have been proposed by the Human Genome Organisation and the International Union of Pharmacology (Figure 4)(Goldstein et al. 2005). For simplicity, the original physicochemical classification will be used here. The acronym TASK refers to “TWIK (“tandem of P domains in a weak inwardly rectifying K^+ channel”)-related acid-sensitive K^+ channel”).

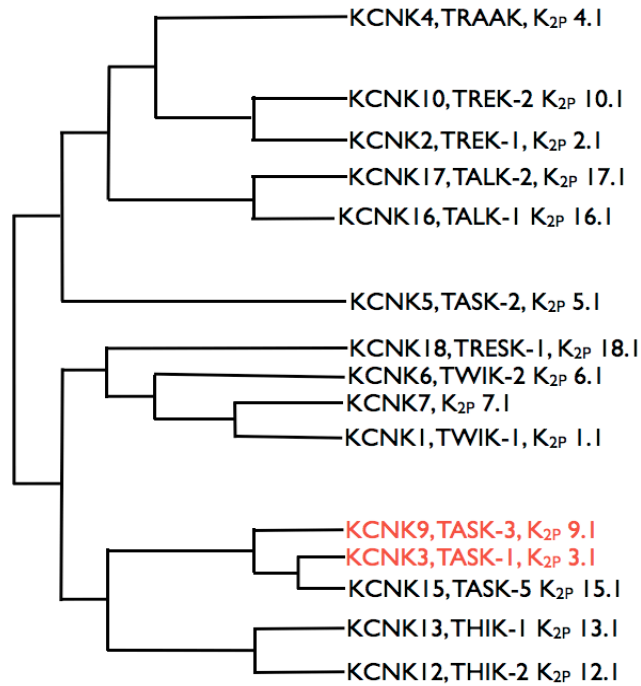


Figure 4. Cladogram for the 15 identified members of the human K_{2P} gene family. TASK-1 and -3 are in red. Naming system is based on the Human Genome Organisation (HUGO) assignments (KCNK X, with “X” based on order of discovery), International Union of Pharmacology (K_{2P} X.X) and physicochemical attributes (e.g. TWIK, “Tandem of P domains in a weak inwardly rectifying K⁺ channel”). Other names based on physicochemical attributes are: TASK, “TWIK-related acid-sensitive K⁺ channel”; TRAAK, TWIK-related arachidonic acid-stimulated K⁺ channel ; TREK, TWIK-related K⁺ channel; TALK, TWIK-related alkaline pH activated K⁺ channel ; TRESK, TWIK-related spinal cord K⁺ channel; THIK, tandem pore domain halothane-inhibited K⁺ channel . Functional channels have not been identified for KCNK 7, 12 and 15. Re-drawn and adapted from Goldstein et al. (Goldstein et al. 2005).

1.2 TWIK-related acid-sensitive K⁺ channel (TASK) channels

The electrophysiological characteristics of the TASK channels TASK-1 (KCNK 3) and -3 (KCNK 9) are well characterised by the general properties ascribed to the K_{2P} family, showing weak rectification, activity across the physiologic range of membrane voltages, and little time dependence. As their name implies, both channels are inhibited by reductions in pH, a property associated with a histidine residue in the first pore domain (Lopes et al. 2001). Unfortunately there are currently no known selective blockers of these channels. As a result, identification of native currents relies on examining electrophysiological and pharmacologic properties. Differentiating TASK-1 and -3 channels is based on differences in pH sensitivity, with the TASK-3 channel less sensitive to acidity having a pK value of 6.7 while the TASK-1 channel has a pK value of approximately 7.3 (Kim et al. 2000; Duprat et al. 1997). Both homo- and heterodimer formation has been identified in heterologous expression systems (Lopes et al. 2001; Berg et al. 2004). Heterodimer formation with both channels leads to an intermediate pH sensitivity, with increasing evidence for their existence in motoneurons (Berg et al. 2004; Lazarenko et al. 2010), and the presence of co-expression (Talley et al. 2001). Additionally, TASK-3 channels have a greater sensitivity to ruthenium red (Czirjak & Enyedi 2003).

TASK-1 and -3 channel regulation has been most studied in cerebellar granule neurones (CGNs) (Aller et al. 2005; Brickley et al. 2001b). Channel inhibition, induced by muscarine or acetylcholine application, results in cell membrane depolarisation and increased probability of action potential firing (Watkins & Mathie 1996). And this inhibition has been shown to be mediated through Gα_q (Czirjak et al. 2001; Talley & Bayliss 2002; Besana et al. 2004; Lopes et al. 2005). The steps following Gα_q activation are the activation of phospholipase C (PLC), which hydrolyses the phospholipid PIP₂, generating diacylglycerol (DAG) and inositol triphosphate (IP₃). In turn, IP₃ and DAG stimulate intracellular calcium release and protein kinase C (PKC) respectively (Fink et al. 1996; Kim et al. 2000; Czirjak et al. 2001; Chemin et al. 2003). Although it is clear that Gα_q activation ultimately results in TASK channel

inhibition, the relative importance of the downstream pathways is unresolved. There are presently roles indicated for PKC ((Lopes et al. 2000), PIP₂ ((Chemin et al. 2003; Lopes et al. 2005)), and G α_q (via direct channel interaction; Chen et al. 2006; Veale et al. 2007).

In vitro anaesthetic sensitivity was demonstrated for TASK-1 and -3 channels with the volatile anaesthetic agent halothane, in addition to other commonly used inhalational anaesthetic agents (Meadows & Randall 2001; Patel et al. 1999; Sirois et al. 2000). The site of this action is in the fourth transmembrane domain, substitution of which abolishes anaesthetic sensitivity (Patel et al. 1999; Sirois et al. 2000; Talley & Bayliss 2002). Activation of TASK-1 by halothane is greater (approximately double) than in TREK-1 at the same concentration (1 mM), and effects are fully reversible (Patel et al. 1999). The same study identified, in the TASK-1 channel, a minimal response to chloroform and inhibition by diethyl ether. A very similar response to 1 mM halothane was also identified in TASK-3 (65% current potentiation at 10 mV; Meadows & Randall 2001).

From *in situ* hybridisation studies, TASK-1 and -3 channel expression is widespread, including the CNS, heart, carotid body and adrenal glands (Aller et al. 2005; Brickley et al. 2007; Talley et al. 2001). TASK-3 channels have a wider distribution in the CNS, with areas of overlap with TASK-1 channels in cerebellar granule cells, motoneurons, locus coeruleus and thalamus. Interestingly, some of these areas play roles in the regulation of sleep and theta oscillation generation (see later). Some of these sites of expression have been validated with the identification of TASK currents in slice preparations (Patel et al. 1999; Sirois et al. 2000; Washburn et al. 2002; Millar et al. 2000; Torborg et al. 2006). These include motoneurons, cerebellar granule cells, locus coeruleus, raphe neurones and hippocampal interneurons.

Other than in general anaesthesia, TASK channels have been implicated in oxygen sensing by carotid body glomus cells (Buckler et al. 2000; Trapp et al. 2008), and secretion of the hormone aldosterone by the adrenal gland (Davies et al. 2008). Carotid body glomus cells, located at the bifurcation of the carotid artery in the neck, function primarily as sensors of oxygen partial pressure and are responsible for hypoxic drive in the face of hypoxia. TASK-1 channels were implicated in this mechanism based on the identification of TASK-like

currents in this cell population and recordings in TASK-1 knockout (T1KO), TASK-3 knockout (T3KO) and TASK-1 and TASK-3 (“double”) knockout (DKO) mouse strains (Buckler et al. 2000; Trapp et al. 2008). The use of TASK-channel knockout strains (male DKO and female T1KO) also enabled identification of a murine model of primary hyperaldosteronism. The steroid hormone aldosterone is produced in the adrenal cortex and contributes to systemic regulation of sodium, potassium and water. Compared to WT, a TASK-like current present in WT zona glomerulosa cells (the site of aldosterone synthesis) was absent in male DKO mice (Davies et al. 2008). The authors proposed that the relative depolarised state of the zona glomerulosa cells promoted increased aldosterone production irrespective of normal regulators.

1.2.1 TASK channels and general anaesthesia

Several papers have been published in the last few years reporting the roles of TASK-1 and -3 channels in anaesthetic sensitivity, more specifically their role in loss of consciousness (LOC), response to a nociceptive stimulus and immobilisation (Lazarenko et al. 2010; Linden et al. 2006, 2007). A summary of the results will be presented here with a more complete discussion in the Discussion chapter.

Using the same strains of TASK knockout mice as employed in our study, Linden et al. reported the results of a range of tests including anaesthetic sensitivities in T1KO and T3KO animals (Linden et al. 2006, 2007). The T1KO strain was found to have minimal differences in basal behaviour compared with WT as assessed by non-interactive observation of activity and subjection to the SHIRPA range of tests, which include the assessment of a range of reflexes and responses to physical handling. Exposure to halothane identified a reduced sensitivity in the knockout using a loss of tail-clamp withdrawal reflex (LOTW) assay, whereby the halothane concentration at which a response (limb or head movement) to clamping of the proximal tail with hemostats was recorded (Figure 5). In contrast, there was no change in the loss of righting reflex (LORR; Figure 5). Assessment of the effects of the gene knockout on mRNA expression failed to show alterations in the expression of other K_{2P} members.

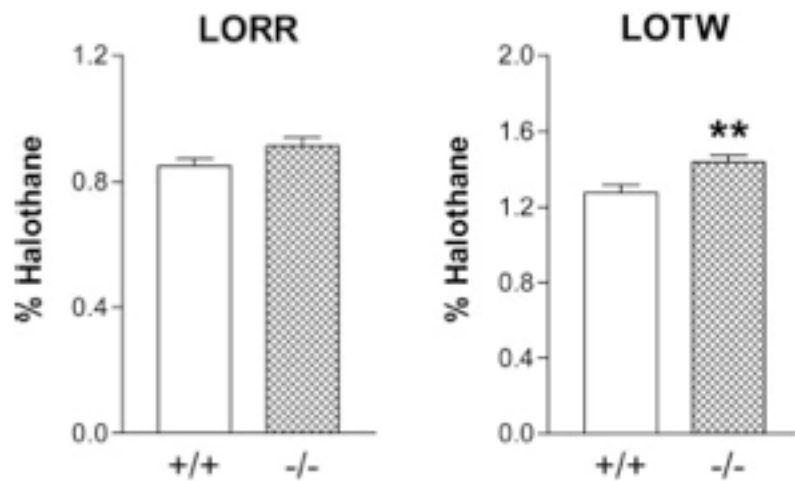


Figure 5. A reduced sensitivity to halothane is displayed by TASK-1 knockout (T1KO) animals (-/-) in a loss of tail-clamp withdrawal (LOTW) assay ($p < 0.01$) compared with wild type (WT; +/+), but no strain difference was identified in a loss of righting reflex (LORR) assay. Comparison performed with a Student's *t* test. Adapted from Linden et al. (Linden et al. 2006).

A second paper by Linden et al. investigated the anaesthetic sensitivity of the T3KO strain (Linden et al. 2007). As with the T1KO strain, basal behaviour and reflexes did not differ greatly from WT. Differences in halothane sensitivity, nocturnal activity and memory were identified. Though LORR was not assessed, the LOTW assay revealed a reduced halothane sensitivity in the knockout (Figure 6) to a similar degree as that identified in T1KO animals. Nocturnal activity (during the lights off period, the more active period in mice) was assessed with the use of infrared beams to denote movement and T3KO animals exhibited significantly greater (38%) activity levels.

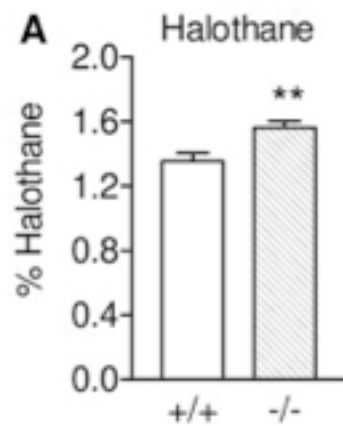


Figure 6. TASK-3 knockout (T3KO) mice (-/-) are less sensitive to wild type (WT; +/+) in a loss of tail-clamp withdrawal (LOTW) assay, requiring a greater halothane concentration to prevent movement in response to the application of a tail clamp, a nociceptive stimulus. $p < 0.01$, Student's *t* test. Adapted from Linden et al. (Linden et al. 2007).

A recent paper from Lazarenko et al. examined the effects of halothane on LORR and LOTW in 3 strains of knockout mice; T1KO, T3KO and DKO. They also studied the effect of the knockout strains on the electrophysiological properties of hypoglossal motoneurons. A reduction in halothane sensitivity was identified in all knockout strains in both the LORR and LOTW assays. There were no differences between knockout strains (Figure 7). The knockout strains had a reduced resting membrane potential (more depolarised) compared with WT, though no significant change in input conductance. The application of halothane also resulted in a reduction in membrane potential in all the knockout strains.

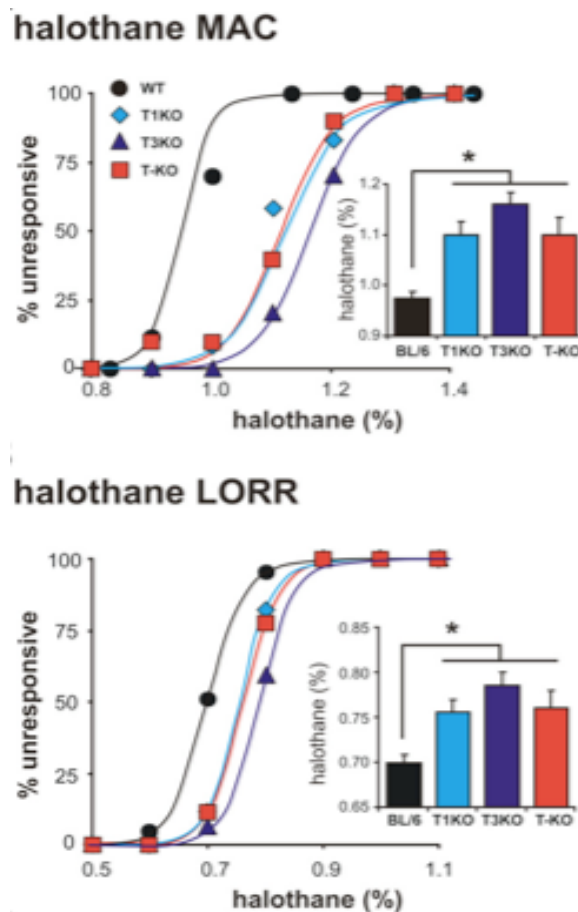


Figure 7. Halothane sensitivity is reduced in TASK-1, TASK-3 and double (both TASK-1 and TASK-3) knockout strains (T1KO, T3KO and T-KO, respectively) compared with wild type (BL/6) with a loss of tail-clamp withdrawal assay (MAC) and loss of righting reflex assay (LORR). Adapted from Lazarenko et al. (Lazarenko et al. 2010).

1.3 The electroencephalogram (EEG) and cortical EEG

The first record in the literature of the EEG was as “electric currents of the brain” by Caton in 1875 (Caton 1875) who reported his findings in rabbits and monkeys, including noting changes in the EEG resulting from behaviour such as mastication. The term “electroencephalogram” was coined by Hans Berger in 1929 (as cited by Haas ;Haas 2003). The monitoring of schizophrenic patients in the 1930s using EEG provided early evidence of the effects of sedative and anaesthetic agents (Lemere 1938). This alteration of the conscious

EEG following drug administration represents a rapidly expanding and lucrative field in the application of EEG-derived parameters for the monitoring of sedated and anaesthetised patients (Anon 2006). There are currently at least six commercially available devices using various proprietary algorithms to generate a simple and user-friendly monitor for clinical use in humans (Jameson & Sloan 2006).

1.3.1 Oscillations and the EEG

The EEG is a measure of macroscopic bioelectric currents some of which are in the form of oscillations (Rampil 1998). At the simplest level an oscillation results from a phasic energy source providing positive feedback within a system cycling between opposing forces (Buzsaki 2006). The generation of current flow resulting from ion flux is a simple example of this. These currents reflect the temporal summation of electrical activity from individual neurones. Excitatory and inhibitory post-synaptic potentials (PSP) are the basis of the EEG and the signal recorded reflects the multitude of synchronised and asynchronised PSP activity. At the level of individual neurones, their contribution to the local field potential stems from changes in transmembrane potential, which in turn depends on current flow across the cell membrane via channel opening. Currents can be viewed as sources or sinks depending on the direction of ion flow *e.g.* the movement of sodium ions into a cell from the extracellular space would be a current sink and *vice versa* for potassium ions. Passive neurones act as capacitive low pass filters, favouring the long-lasting PSPs over action potentials. The longer duration of PSPs (tens of milliseconds versus a few milliseconds for an AP) also favours the likelihood of temporal overlap. The cytoarchitecture of pyramidal cells in particular favours significant PSP formation and temporal summation: the forest of parallel neurones with distinct sites for excitatory and inhibitory inputs generates PSPs which, when combined with shared excitatory/ inhibitory sources, come together to favour temporal summation and generation of measurable electrical signals of sufficient magnitude to be measured at distant sites such as the scalp (Rampil 1998; Buzsaki 2006). The timecourse of PSPs also allows the occurrence of synchrony, that is the overlapping of events in time. This results from the time scale over which PSPs occur, favouring the emergence of one (or more) PSP before the termination of the previous one. Of course there are many factors potentially influencing this likelihood;

neurotransmitter release, receptor activity, states of local channels. And the decay time of PSPs is a variable affected by the leakiness of the cell membrane. Synchrony is favoured by inter-cell connectivity as first proposed in the cell assembly hypothesis of Hebb (Buzsaki 2006).

The source of the measured signal reflects the spatial relationship between the measuring electrode and the neurones in the local area. Fine electrodes inserted into brain tissue may record the activity of a few hundred neurones whilst measurements made at the level of the scalp or dura will reflect that of hundreds of thousands. Furthermore, signal sources may not be the neuronal populations closest to the electrode. Cytoarchitecture (see above), the low resistance of neuronal tissue, capacitive currents of cell membranes and impact of other structures (blood vessels, skull muscles, skin, connective tissue) will all leave an imprint on the final signal. The localisation of EEG sources is a particular challenge in epilepsy and has been compared with earthquake epicentre location in seismology (Buzsaki 2006).

Classification of the EEG into frequency bands was initiated by Berger, who assigned the Greek letter alpha to the first frequency recorded. This nomenclature has been continued, with arbitrary delineations between frequency bands leading to some variation in the frequencies delimiting ranges (Table 1; Rampil 1998).

Band	Range (Hz)
Delta (δ)	0.5 - 3.5
Theta (θ)	3.5 - 7
Alpha (α)	7 - 13
Beta (β)	13 - 30
Beta2 (β_2)/ Gamma (γ)	30 - 50+

Table 1. Commonly used bands for defining EEG frequency ranges. The frequency ranges are not strictly defined and published ranges in the literature vary. Adapted from Rampil, Buzsaki and Steriade et al. (Rampil 1998; Buzsaki 2006; Steriade et al. 1990).

Interestingly, the theta range of frequencies in rodents is described as ranging from 3 - 12 Hz, thus overlapping with alpha in man (Bland et al. 2003; Steriade et al. 1990). This is the range

used for the work presented here.

It should be clarified that the term electroencephalogram is restricted to recordings made using scalp electrodes (Figure 8). Recordings made at a deeper level, such as extradural should be described as cortical EEG or electrocorticogram (ECoG).



Figure 8. The author having his EEG recorded as part of a neurophysiology experiment.

1.4 The Theta oscillation

1.4.1 History

First described in 1938 by Jung and Kornmuller (as cited by Bland; Bland 1986), the theta oscillation (sometimes referred to as “rhythmical slow wave”) has remained enigmatic in terms of both formation and function. It is the largest extracellular oscillation of the mammalian brain (Vertes et al. 2004) and has been defined in rodents as a “sinusoidal-like rhythmical slow waveform” occurring within the range of 3-12 Hz (Bland et al. 2003).

Two types of theta oscillation were first described by Kramis et al.(Kramis et al. 1975) and designated type 1 and type 2 theta. This followed from earlier work describing behaviours and

conditions associated with each type of theta oscillation (Vanderwolf 1969; Grastyan et al. 1959). Type 1 theta is associated with voluntary motor movement such as walking, hopping, postural shifts, head movements and grooming (Buzsaki 2005). It is characterised by its location between 7 - 12 Hz.

Type 2 theta is associated with immobility in the presence of sensory stimuli, so-called “alert immobility” (Vanderwolf 1969). It may be elicited by changes in the immediate environment of the animal, which may be perceived as a threat. For example, sudden sounds, hand clapping, hand waving and stroking (Bland 1986). The frequency range at which type 2 theta occurs is 4 – 7 Hz. Two further characteristics help define type 2 theta. Firstly, it is unique in that it can be induced by exposure to the anaesthetic agents ether (Kramis et al. 1975), urethane (Kramis et al. 1975), and halothane (Bland et al. 2003; Robledo 2008), and the anticholinesterase eserine (physostigmine; Leung & Vanderwolf 1980; Tebano et al. 1999). Secondly, the systemic administration of the anticholinergic (antimuscarinic) atropine sulphate ablates this response (Kramis et al. 1975).

Both theta types are also present during REM sleep (Vertes 1977, 1979; Robinson et al. 1977).

In this study, our focus was on the theta oscillation based on previous work from our laboratory showing perturbation of the theta oscillation in a T3KO mouse (Robledo 2008). Disruption of this oscillation was fortuitous as it is the dominant oscillation of the rodent brain, thus simplifying the methods necessary for its measurement (Vertes et al. 2004).

1.4.2 Generation

The exact mechanisms of theta oscillation generation and control remain to be fully elucidated. However, a framework exists describing the current understanding of contributions from the principle brain areas involved. These are: the nucleus reticularis pontis oralis (RPO) of the rostral pontine reticular formation, the supramammillary nucleus (SUM) and posterior hypothalamus, the medial septum and diagonal band of Broca (MS/DBB), and the hippocampus. Briefly, theta oscillation generation involves a pathway from the brainstem (RPO) to the pyramidal cells and interneurons of the hippocampus, via the SUM and MS/

DBB. An initially tonic discharge from the RPO is transformed to phasic one in the SUM, and the net effect of excitatory (cholinergic) and inhibitory (GABAergic) projections from the MS/DBB to the hippocampus results in theta oscillations (Figure 10 and Figure 11).

Early work identified a contribution by the reticular formation of the brainstem, in the form of a tonic discharge, which led to generation of the theta oscillation (Green & Arduini 1954; Petsche et al. 1965, 1962). This was later corroborated by showing that electrical stimulation of the RPO area alone resulted in theta oscillation generation (Vertes 1981; Macadar et al. 1974). Further elucidation of the pathways was performed by tracer studies and autoradiography, which revealed the RPO was connected with the MS/DBB via an intermediary nucleus, the SUM (Vertes 1988; Vertes & Martin 1988). Glutamatergic neurones from SUM were shown to project to cholinergic and GABAergic neurones of the MS/DBB and hippocampus (Vertes 1992; Borhegyi et al. 1998; Haglund et al. 1984; Leranth et al. 1999; Kiss et al. 2000).

Neurones of the SUM exhibit a firing pattern in synchrony with theta which remained if MS/DBB activity was reversibly depressed with local procaine injections (Kirk & McNaughton 1991). Activation of SUM through electrical stimulation or carbachol (a cholinergic agonist) injection resulted in formation of a theta oscillation (Bland et al. 1990; Colom et al. 1991) whereas suppression of SUM with local procaine injection disrupts theta (Bland et al. 1994). Interestingly, the importance of SUM in theta oscillation generation appears to vary with the animal model used: in urethane anaesthetised rats, procaine injection into SUM abolishes theta (Bland et al. 1994), whereas in awake rats, procaine injection into SUM reduces the frequency of theta without eliminating it (Kirk & McNaughton 1993). Similarly, depression of the MS/DBB in urethane-anaesthetised rats abolished theta oscillations (Yoder & Pang 2005; Steffensen 1995; Bland et al. 1996; Petsche et al. 1962), but only resulted in a reduction in theta oscillation amplitude in conscious rats (Kirk & McNaughton 1993). Data from our laboratory has shown a similar effect during halothane exposure in mice (Figure 9). Hence there is some debate in the literature as to the relative importance of inputs from SUM versus a sole pacemaker role for the MS/DBB.

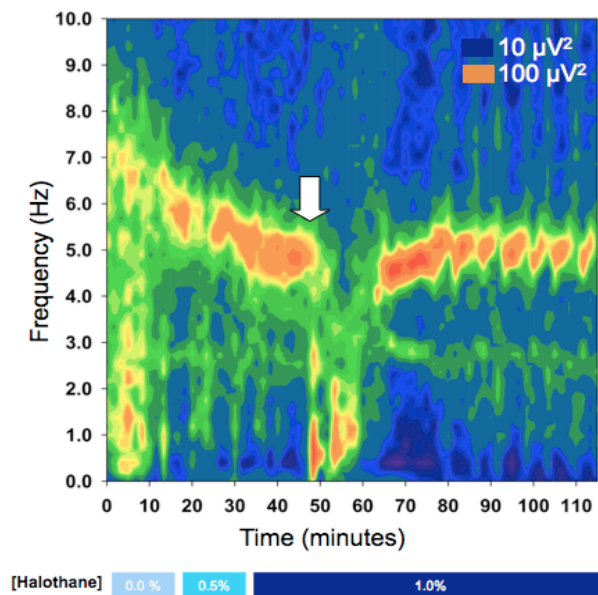


Figure 9. Lidocaine injection into the medial septum of a wild type mouse (solid white arrow) abolishes the theta oscillation induced by halothane. Increasing warmth of colour represents an increase in oscillation power. Adapted from a figure provided by David Carr.

From the MS/DBB cholinergic neurones project on to and excite pyramidal cells and interneurons of the hippocampus (Dutar et al. 1995). GABAergic neurones of the MS/DBB exclusively project on to and inhibit GABAergic interneurons of the hippocampus (Freund & Antal 1988).

Within the hippocampus, recent evidence has shown that multiple dipoles (excitatory and inhibitory) from many layers of the hippocampus summate to produce the local field potential which is recorded at the level of the cortical EEG (Montgomery et al. 2009). The CA1 pyramidal cell has often been used as the classic example to illustrate theta oscillation generation at a single level. They also provide an important contribution to the amplitude of the theta oscillation recorded as a local field potential. A large (approximately 40%) reduction in theta oscillation amplitude results from irreversible damage to CA1 pyramidal cells (Buzsaki et al. 1989). The major contribution of CA1 pyramidal cells to theta oscillations

results from various unique features. Their large dendritic trees (relative to CA3; Pyapali et al. 1998), which receive excitatory input from the EC and CA3 (inhibitory inputs are confined to perisomatic regions and the axon initial segment), may help establish a larger current sink-source gradient, they form the final link in a chain of inhibition-excitation reflecting the activity of downstream connections (MS/ DBB) and their sheer mass of numbers ensures that the final output (local field potential) reflects synchronous activity. At the pyramidal cell, the balance of inhibitory and excitatory inputs result in theta oscillation generation. Inhibitory inputs arise from the MS/DBB via inhibitory interneurons. GABAergic neurons of the MS/DBB project on to inhibitory (GABAergic) interneurons resulting in disinhibition of the pyramidal cell in the perisomatic region. Excitatory cholinergic (and glutamatergic) input arises from the MS/DBB and entorhinal cortex (EC). These dipoles modulate the incoming pacemaker rhythm, altering the amplitude of the theta oscillation. Several lines of evidence for this interplay of dipoles in CA1 pyramidal cells exists, including current source density analysis (Buzsaki et al. 1986; Brankack et al. 1993) and intracellular recordings (Pyapali et al. 1998; Kamondi et al. 1998).

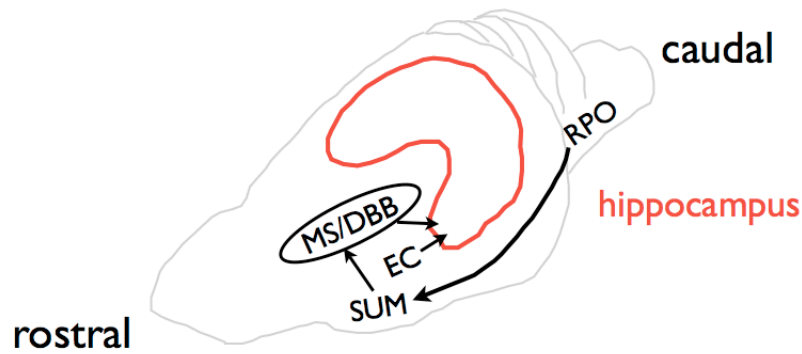


Figure 10. Overview of structures and pathways involved in generation of the theta oscillation in the brain. Tonic discharge from the nucleus reticularis pontis oralis (RPO) of the rostral pontine reticular formation is converted to a rhythmic discharge by the supramammillary nucleus (SUM) which in turn projects on to the medial septum/ diagonal band of Broca (MS/DBB). Cholinergic projections from the MS/DBB provide excitatory inputs to the pyramidal cells and interneurons of the hippocampus. GABAergic projections provide inhibitory inputs to interneurons. Excitatory inputs also arrive from the entorhinal cortex (EC). See text for further details.

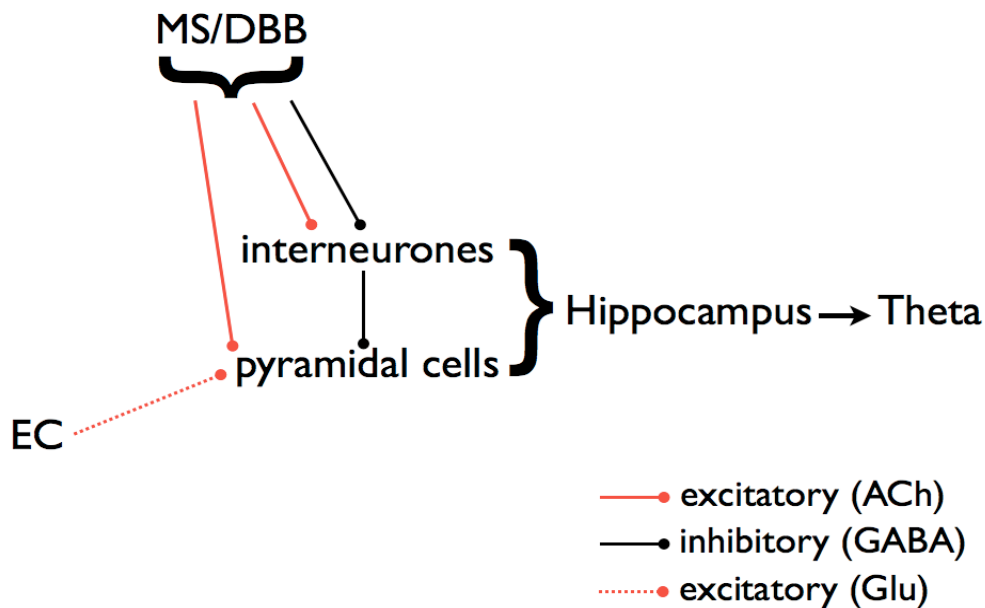


Figure 11. Overview of excitatory and inhibitory connections between the medial septum and diagonal band of Broca (MS/DBB), entorhinal cortex (EC) and hippocampus. Excitatory cholinergic (ACh; solid red lines) projections from the MS/DBB project on to pyramidal cells and inhibitory interneurons of the hippocampus. Pyramidal cells also receive excitatory (glutamatergic; interrupted red lines) input from the EC. Inhibitory GABAergic (GABA; solid black lines) inputs from the MS/DBB terminate on interneurons, and from interneurons on to pyramidal cells. The end result is the generation of a theta oscillation. See text for further details.

1.4.3 Theta oscillation differentiation

As described above, the two types of theta oscillation are classified according to associated behaviours and response to pharmacological agents. The type 1 theta oscillation (7 - 12 Hz) is associated with voluntary motor movement such as walking and grooming and is not disrupted by atropine sulphate administration (Buzsaki 2005). In contrast, the type 2 theta oscillation (4 – 7 Hz) is associated with alert immobility

(Vanderwolf 1969). Furthermore, it is induced by the anaesthetic agent halothane (Bland et al. 2003; Robledo 2008), the anticholinesterase eserine (physostigmine) (Leung & Vanderwolf 1980; Tebano et al. 1999), and is abolished by atropine sulphate administration (Kramis et al. 1975).

Type 1 theta oscillation

The mechanisms underlying the generation of this theta oscillation remain to be fully elucidated. Kamondi et al (Kamondi et al. 1998) showed that surgical removal of the EC in rats resulted in the transformation of a type 1 theta oscillation to one resembling type 2 in its amplitude and frequency profile, and in its sensitivity to atropine. These results indicated that EC inputs are necessary for type 1 theta oscillation generation and Buzsaki (Buzsaki 2002) postulated that the pathway generating type 1 theta oscillation was sensitive to perturbations by urethane. Work by LERANTH et al. (LERANTH et al. 1999) and MORONI et al. (MORONI et al. 1981) showing that the EC contains glutamatergic neurones and that urethane interferes with presynaptic glutamate release, draw attention to a potential role for this neurotransmitter.

Type 2 theta oscillation

The mechanism(s) through which atropine sulphate ablates type 2 theta oscillations is unclear. Although muscarinic cholinergic receptors are widespread in the hippocampus and MS/DBB (Hasselmo & Fehlau 2001; Buzsaki 2002), there is no evidence that direct action of acetylcholine receptors on pyramidal cells within the hippocampus is responsible for the ablation of type 2 theta oscillation. The time course of onset and decay effects on synaptic field potentials was judged to be too slow to contribute to theta oscillation generation, with time constants at least double those in response to GABA application (Hasselmo & Fehlau 2001). Furthermore, following surgical removal of the EC and creation of an atropine sensitive theta oscillation current source density analysis revealed that the dipoles responsible for this oscillation were not associated anatomically with known sites of cholinergic receptors (Kamondi et al. 1998; Buzsaki 2002). Instead, atropine is likely to exert its effects through inhibitory interneurons of the hippocampus (Buzsaki et al. 1983; Stewart & Fox 1990). This effect may be mediated through TASK channels, as channel inhibition resulted from muscarine administration (Watkins & Mathie 1996).

1.4.4 Interneurons and the theta oscillation

The GABAergic interneurons of the hippocampus perform the role of regulating pyramidal cells through inhibition. The large number of interneurons identified and their myriad connections, to each other in addition to pyramidal cells, provides a massive potential for encoding information through a temporal mechanism (O'Keefe & Recce 1993; Buzsaki 2002; Klausberger & Somogyi 2008).

Increasingly, a central role for inhibitory interneurons has been identified in the control of the theta oscillation (Buzsaki et al. 1983; Klausberger & Somogyi 2008; Freund & Antal 1988). Projections from the GABAergic neurons of the MS/DBB target hippocampal interneurons exclusively (Freund & Antal 1988). These anatomical connections place them in a powerful position in terms of affecting theta oscillation generation and modulation (Figure 12). The precise role of these interneurons is a rapidly expanding field, with at least 21 classes of interneurons identified in the hippocampus (Klausberger 2009). Evidence of the contribution of these interneurons to theta oscillations is that they discharge out of phase with the pyramidal cell they innervate, reflecting the rhythm at the heart of theta oscillations (Fox et al. 1986). Unlike pyramidal cells, their study is hindered by difficulties in identifying separate classes in the normal brain based on morphology. Their classification is based on their innervation patterns of pyramidal cell components and molecular expression. Two particular classes of interneurons, parvalbumin (PV)-expressing and cholecystokinin (CCK)-expressing, have been identified of interest based on their interactions with CA1 pyramidal cells and phasic firing during the theta oscillation (Klausberger et al. 2004, 2003, 2005). The specific interaction between the timing of firing and the location of inputs on pyramidal cells allows interneurons to modulate pyramidal cell firing, and in turn, theta oscillations. These attributes have been described as a “spatiotemporal interaction” (Klausberger & Somogyi 2008). For example, the firing of PV-expressing basket cells during the descending phase of the theta cycle, combined with their perisomatic input on pyramidal cells contributes to the inhibition of the pyramidal cell innervated (Klausberger et al. 2003). Inhibition (GABAergic) through hyperpolarisation of the pyramidal cells alters local theta formation with potential effects on the local field potential depending on the relative contribution of the inhibited

pyramidal cell.

Another line of evidence for the role of inhibitory interneurons is the finding that simultaneous blocking of excitatory and inhibitory inputs of the hippocampus resulted in increased firing rates, suggesting that primary hippocampal modulation is through inhibition (Cohen & Miles 2000).

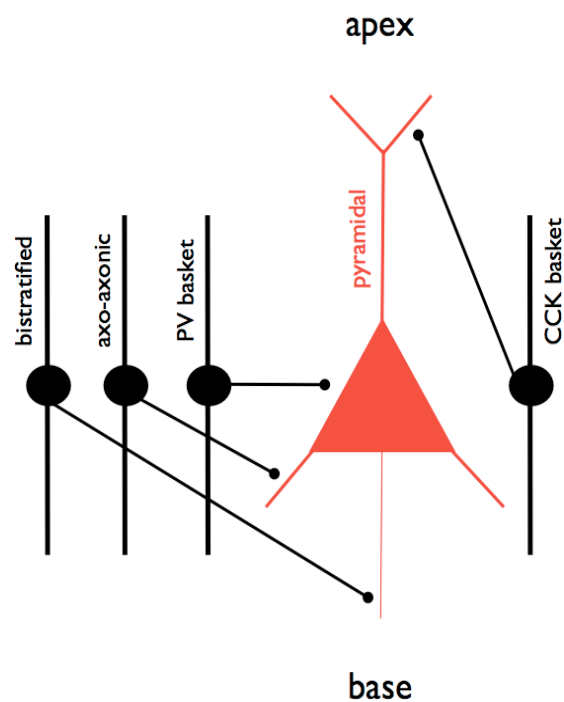


Figure 12. Cartoon depicting the interaction between inhibitory GABAergic interneurons and a CA1 pyramidal cell of the hippocampus. The variation in projection sites of the interneurons promotes modulation of pyramidal cell activity. PV is parvalbumin expressing, CCK is cholecystokinin expressing.

1.4.5 Halothane and type 2 theta oscillation

Bland et al. (Bland et al. 2003) showed that sub-anaesthetic doses of halothane induced the formation of a type 2 theta oscillation in rats. This effect of halothane has since been

observed in wild type mice in our laboratory (Robledo 2008). Halothane involvement in the generation of a theta oscillation involves actions on both hippocampal interneurons and pyramidal cells of CA1. Nishikawa and MacIver (Nishikawa & MacIver 2000, 2001) showed that the application of clinically relevant concentrations of halothane resulted in enhancement of GABAergic synaptic inhibition via depression of evoked EPSCs. This action of halothane is a potential mechanism for the generation of type 2 theta in response to halothane exposure. An alternative mechanism for the action of halothane could be through the activation of TASK channels. The observation that the expected induction of a type 2 theta oscillation was absent in T3KO mice points to a central role of TASK-3 channels in a halothane-induced type 2 theta oscillation (Robledo 2008).

1.4.6 TASK channels and the theta oscillation

The contribution of TASK channels to the resting membrane potential places them in an important position to alter membrane conductance and potentially affect the theta oscillation. The results of *in situ* hybridisation studies have identified the presence of TASK-3 mRNA in the brain areas implicated in the generation of theta oscillations (hippocampus pyramidal cell layer, MS/DBB, RPO, SUM, hippocampal interneurons). In contrast, TASK-1 mRNA expression is more restricted, with lower levels of expression in the hippocampus CA1 pyramidal cell layer and interneurons (Brickley et al. 2007; Talley et al. 2001). Recently, TASK-like currents have been identified in interneurons populating the stratum oriens layer of the hippocampal CA1 region (Torborg et al. 2006) and pyramidal cells (Taverna et al. 2005). The location of these cell populations at the end of the theta oscillation generation pathway could explain some of the observed *in vivo* differences between T3KO and WT mice during halothane exposure.

1.4.7 TASK channel expression and sleep

The uneven expression of TASK mRNA in the structures associated with theta oscillation generation (see above) is continued in those structures associated with the onset of sleep.

These include the pre-optic area of the hypothalamus, reticular and thalamo-cortical relay neurones of the thalamus, and components of the ascending reticular activating system (tuberomammillary nucleus, lateral hypothalamus, locus coeruleus, dorsal raphe nucleus) (McCarley 2007). TASK-3 mRNA expression was found to be more pronounced in all of these areas, except for the lateral hypothalamus (similar expression levels) and pre-optic area (greater TASK-1 expression) (Talley et al. 2001). This differential expression may underlie previous findings in our laboratory that T3KO animals had a slower transition between wake and sleep periods (Robledo 2008) and warrants further investigation in the T1KO and DKO strains.

A detailed discussion of the regulation of sleep in the context of the results is in the Discussion chapter.

1.5 Aims and hypothesis

As identified at the beginning of this chapter, three criteria have been identified for the assessment of putative anaesthetic targets: plausibility, sensitivity and the effect in *in vivo* models (Franks 2006). Previous studies have shown that TASK channels are a plausible anaesthetic target based on relevant expression within the brain and that they have *in vitro* sensitivity to halothane. The purpose of this investigation is to explore the effect of anaesthesia in 3 transgenic mouse strains: TASK-1 knockout (T1KO), TASK-3 knockout (T3KO) and double knockout (DKO; TASK-1 and TASK-3 channels deleted).

The aims of this study are two fold. Firstly to test the hypothesis that T1KO, T3KO and DKO animals have altered behavioural phenotypes compared with wild type animals. These phenotypes include differences in the response to halothane, as measured using a LORR assay and the cortical EEG, and differences in the regulation of sleep. Secondly, significant differences will be further investigated by patch clamp recording in brain slices under control conditions and during exposure to halothane.

2.0 Materials and methods

All experiments were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and were approved by the Ethical Review Process Committee of Imperial College. Procedures were performed under Home Office licence (PPL 70/6811). wild type mice were C57/Bl6 and provided by Harlan (UK). Unless stated otherwise, all animals were male and between 8 - 20 weeks old. All knockout animals were originally provided by Professor William Wisden (Cell Biology and Functional Genomics Section, Imperial College, London, UK). Animals were housed in standard plastic cages (33 x 14.5 x 12 cm) in a humidity and temperature controlled room, under a 12:12 hr light-dark cycle with lights off at 0800. Water and food were provided *ad libitum*. Cages were inspected twice daily and cleaned once weekly.

2.0.1 Creation of knockout mouse strains and genotyping

All knockout strains (T3KO, T1KO and DKO) were created by homologous and insertional recombination (Aller et al. 2005; Brickley et al. 2007) Data for DKO is unpublished. Confirmation of targeted gene deletion was by *in situ* hybridisation and real-time PCR. Gene deletion did not affect the expression of other K_{2P} channels.

Animals were genotyped upon completion of each set of experiments.

To confirm the genetic identity of experimental animals, template DNA was amplified by specific primers using the polymerase chain reaction (PCR):

T1KO

Forward: 5'-GACCCGGGAGGGACGATGAAG-3'

Reverse: 5'-GGTGATGACGGTGATGGCGAAGTA-3'

pIRES

Forward: 5'-TAAGGCCGGTGTGCGTTTGTCTAT-3'

Reverse: 5'-TGGGGTACCTTCTGGGCATCCTTC-3'

T3KO

Forward: 5'-TCTGTCCCGGCTACCGATCCTGC-3'

Reverse: 5'-TTCCGTGGGCGCAGCGGGTTCCGC-3'

(Sigma, UK)

DKO

The 3 sets of above primers used.

Samples of ear (approximately 2 mm²) were digested with 10 µL of 5x buffer PCR, 1.25 µL proteinase K and made up to 50 µL with 38.75 mL miliQ water. Digestion was for 40 minutes at 55 °C, then 10 minutes at 95 °C.

The PCR reaction was carried out using the “Go Taq Green Master Mix” kit (Promega, UK). 3 - 5 µL of DNA was combined with 2.5 µL primer (10 µM), 12.5 µL Mastermix, 3.75 µL miliQ water and 1.25 µL DMSO.

The following PCR steps were used:

5 minutes at 95 °C, followed by 35 cycles of 1 minute at 95 °C, 1 minute at 60 °C and 1 minute at 72 °C, then 5 minutes at 72 °C and held at 4 °C.

Each sample was then run on a 1.3 % agarose gel using 1 x TAE running buffer and compared against a 1500 bp DNA ladder (Promega, UK). Gels were run for 1h at 90 V and visualised following 20 minutes ethidium bromide application, followed by a wash step with water. Controls of miliQ water and the PCR ingredients without DNA were used to check for contamination.

2.1 *In vivo* experiments

2.1.1 Animal Instrumentation

A combination of sterile (autoclaved) and disposable instruments were used for all surgeries. Each animal was weighed before surgery to allow accurate drug dosage. General anaesthesia was induced by placing the animal in a Bell jar with the fresh gas outlet tube from a precision vaporiser (ForTec, Cyprane, UK) through which halothane (Sigma-Aldrich, MO, USA) was carried in oxygen (BOC, , UK). Gas flow rate was controlled by a flowmeter set to 1-2 L

min⁻¹. This was reduced to 0.5 L min⁻¹ once the animal was induced. Once the animal had lost its righting reflex, periodically tested during induction, the animal was removed from the Bell jar and placed on the work surface with its nose in front of the fresh gas outlet tube. Following clipping of the fur overlying the skull, disinfection of the skin with iodine solution, subcutaneous administration of buprenorphine (0.03 mg kg⁻¹, Vetergesic, Alstoe, UK) and 0.9% normal saline (0.5 mL), the animal was transferred to the stereotaxic frame (Harvard Apparatus, UK). Topical lubricant (Lacrilube, Aller-Gan, UK) was applied to eyes. Within the frame, the animal's body was supported by a battery-powered heat pad (Right Spot Far Infra-Red, Small Pad, Equine Right Spot, UK) and the head securely held in place using a tooth bar and nose clamp (Harvard Apparatus, UK). Depth of anaesthesia was assessed by pinching a hind foot prior to the initial incision and regularly assessed during the surgery. If there was no withdrawal reflex, surgery commenced with a skin incision. The skin incision extended along the midline from just rostral to the lateral canthi of the eyes to the caudal border of the ears.

Skin was held clear of the surgical field by placing stay sutures at each corner of the incision. A scalpel blade was used to scrape away the periosteum of the skull and the skull was prepared for later cementing by cleaning with 70% ethanol, applied using a cotton bud. Proposed sites for cortical electroencephalograph (ECoG) electrode placement were measured and marked with a marker pen prior to drilling.

The following sites were identified (Figure 13):

- 1.5 mm Bregma, +1.5 mm midline (recording electrode)
- +1.5 mm Bregma, -1.5 mm midline (recording electrode)
- 2 mm Lambda, 0 mm midline (reference electrode)
- 1.5 mm Bregma, -2.0 mm midline (anchoring screw)

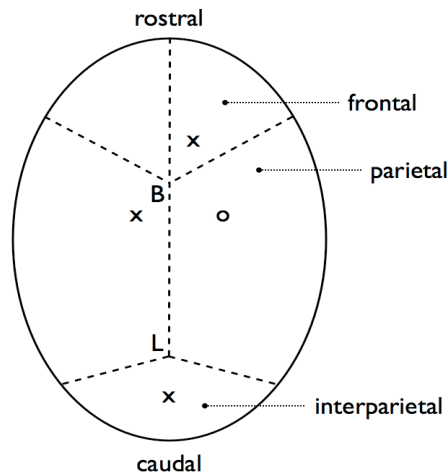


Figure 13. Cartoon of dorsal surface of mouse skull showing recording electrode (x) and anchoring screw (o) sites. Recording and reference electrodes were placed using coordinates relative to Bregma (B; point of convergence of skull suture lines) and Lambda (L; point of convergence of skull suture lines). Recording electrodes: -1.5 mm Bregma, +1.5 mm midline and +1.5 mm Bregma, -1.5 mm midline. Anchoring screw: -1.5 mm Bregma, -2.0 mm midline Reference electrode: +2 mm Lambda, 0 mm midline. Dashed lines are suture lines.

Holes were drilled using a hand-held battery-powered drill (Dremel Model 800, Dremel Europe, NL) with a 1.0 mm drill bit (Figure 14). The recording electrodes were gold plated (Decolletage AG, Switzerland; 2 mm diameter and 2.6 mm length) and the anchoring screw stainless steel. The recording electrodes were soldered to insulated multi-strand stainless steel wire prior to surgery to minimise surgical duration and avoid soldering the electrodes once they were inserted in the skull. Three further electrodes were placed in the nuchal muscles to enable electromyographic (EMG) recording. These electrodes were fashioned from stainless steel insulated wire, with the plastic insulation removed from the last 4-5 mm of each end and coated with solder. One end of each EMG electrode was buried within three distinct sites in the neck musculature by forming the tip into a hook. The opposite ends were soldered to the Neurologger prior to surgery to reduce the surgical duration. The connecting wires were pre-cut to 4-5 cm lengths prior to surgery.

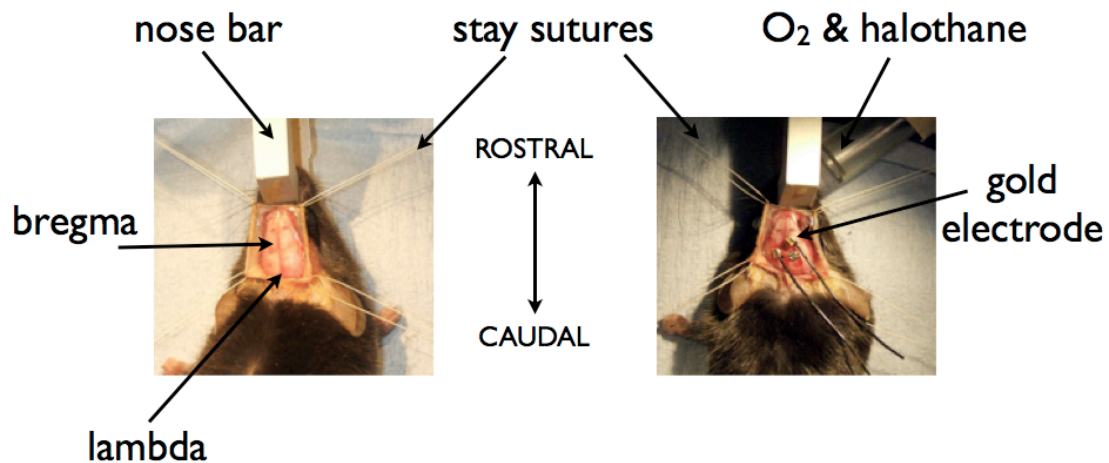


Figure 14. Photos showing skull before (left) and after (right) placement of skull electrodes.

Prior to insertion of the skull electrodes the periosteum overlying the bone was removed and the surface cleaned. The surgical field was maintained with stay sutures and the animal's position fixed with a nose bar. Electrodes (3 gold-plated, recording; 1 stainless steel, anchoring) were positioned relative to bregma and lambda (see text for coordinates). General anaesthesia was maintained with halothane carried in oxygen.

Once all the skull electrodes had been placed, they were covered with an initial layer of dental cement (Orthoresin, DugDent, Dentsply, Germany). As this dried (approximately 10 minutes), the remaining free ends (3 x ECoG electrodes) were soldered on to the connector board. After connection of the electrodes to the connector board, the board was positioned at a slight upward angle ($\approx 10^\circ$) to the plane of the dorsal surface of the skull and held in place with the aid of artery forceps whilst a second, final layer of dental cement was laid. This second layer encased the wires and fixed the connector board in position. Care was taken to ensure that

the angle of the connector board (and Neurologger once attached) would not impede the animal's movement in its cage. Any gaps at either extremity of the skin incision were sutured close and the animal allowed to recover from anaesthesia. Recovery took place in a standard mouse cage under a heat lamp. The animal was observed until it started to move, at which point food and water were placed in the cage. To allow for adequate post-operative observation, surgeries were performed early in the day. Haemorrhage was negligible during instrumentation surgeries. Post-operative analgesia and antibiotics consisted of carprofen (Rimadyl, Pfizer, UK) and enrofloxacin (Baytril, Bayer, UK) respectively. Both were administered in drinking water at a concentration of 100 ppm, for 3 days in the case of carprofen and 5 days in the case of enrofloxacin. During the first 2-3 days after surgery, food pellets and water were placed on the floor of the cage to minimise the effort required to feed.

2.1.2 Cortical EEG recording device: Neurologger 2

The Neurologger is a wireless electronic recording device developed by Dr Alexei Vyssotski (Institute of Neuroinformatics, University of Zurich, Switzerland). The memory capacity of the device we used was 256 Mb, though devices have recently been developed with larger capacities. Its dimensions are 22 x 15 x 5 mm (l x w x h) and total weight (including batteries; 2 x 10ZA hearing-aid batteries) of approximately 2 g (Figure 15). The greatest advantage conferred by the wireless design was the potential to record potentially normal behaviour, previously limited by the use of tethered recording systems (Figure 16). The duration of recordings was limited by sampling frequency and battery capacity. The 400 Hz sampling frequency used allowed recordings of up to 30 hours, enough to record a complete light-dark cycle. Data recording was activated by completing the electrical circuit between the batteries and the Neurologger board with male and female gold-plated connectors. Following completion of an experiment the recording was stopped by disconnecting the batteries. Data was downloaded by connecting the Neurologger to a PC via a USB interface and using proprietary software (Downloader, A Vyssotski). This file could then be read by the software used for EEG analysis, Spike2 (v 5.14 Cambridge Electronic Design, UK). On-line filtering performed by the Neurologger was a bandpass filter between 1 and 70 Hz. Off-line filtering

performed with Spike2 consisted of a bandpass filter for the EMG data of 5-45 Hz (-3 dB), and a low pass filter of 1.0 Hz (-3 dB) for EEG data.

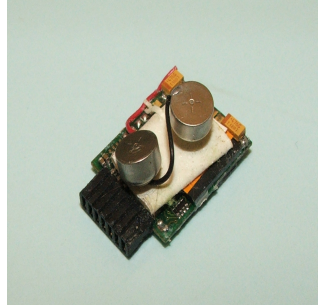


Figure 15. The Neurologger with batteries fitted.

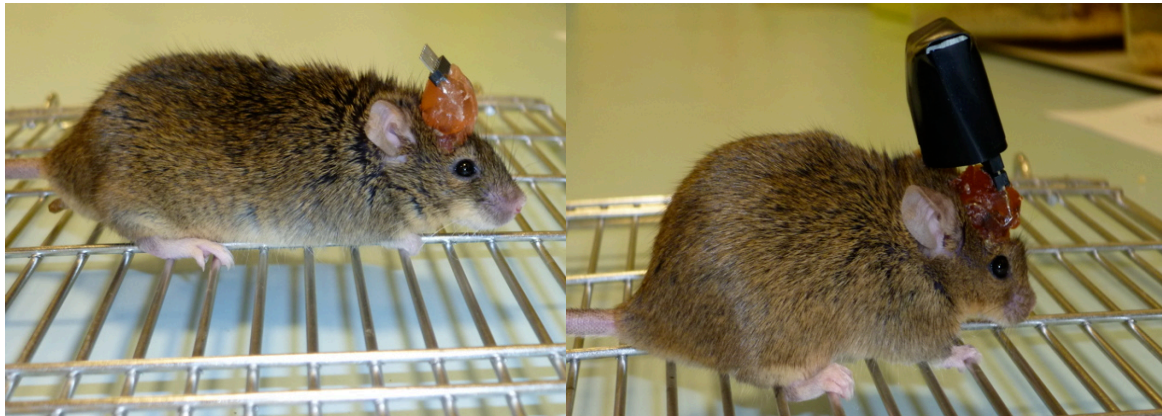


Figure 16. Mice before (left) and after (right) Neurologger fitting. The photograph on the left shows the connector cemented in place with connecting pins exposed ready to accept the Neurologger (right). The Neurologger is covered with masking tape to protect it from water damage.

2.1.3 Loss of righting reflex (LORR) assay

The aim of this experiment was to assess any changes in anaesthetic sensitivity visible as a phenotypic alteration in response to exposure to halothane.

The animal was placed in a glass chamber with a volume of 900 mL (Figure 17). The lid of the chamber had 3 separate ports: one for delivery of gas, one for evacuation of gas and one for gas sampling. The gas analyser (Capnomac II, Datex, Division of Instrumentarium Corporation, Finland) was connected to a PC (Sony Vaio, PCG-141C, Sony Corp., Japan) running monitoring software (Monitor v4.08, Department of Anaesthesia and Intensive Care, Chinese University of Hong Kong). Following placement in the chamber, 100% oxygen was delivered from a cylinder and through a flowmeter and calibrated anaesthetic vaporiser (ForTec, Cyprane Ltd, UK) at a flow rate of 4 L min⁻¹. After a baseline period of 10 minutes, administration of halothane began. A step-wise increase in agent concentration was delivered, from low to high. Each concentration was maintained for 10 minutes (based on time constant calculation to ensure a change in the gas contents of the chamber) and the concentration recorded from the gas analyser and saved as a .xls file by Monitor software. Immediately prior to an increase in concentration, the loss of righting reflex was assessed. This was done by manually rotating the glass jar in an attempt to roll the animal onto its dorsum. A positive response *i.e.* loss of righting reflex was when all four feet were off the ground for a minimum period of 30 seconds. Each animal was tested once at each concentration. Once LORR was achieved the agent concentration was increased by a single step and LORR re-assessed. Normothermia was maintained throughout with a heat lamp. Waste gas was actively scavenged using a Fluovac system (Harvard Instruments) or evacuated through a window. All experiments were performed during the dark (wake) period of the animals' day. The experimenter was blinded to the strain of the animal and animals identified by ear notch identification.

The results of the LORR assay were subjected to a Waud analysis (Zivin & Waud 1992) and the resultant values for EC₅₀ and slope used to fit a sigmoidal dose response curve. Data were plotted with graphical software (GraphPad Prism v4.0c, GraphPad, CA, USA).

Strains studied using LORR assay (and anaesthetic agents used):

Wild type (halothane and cyclopropane)

DKO (halothane and cyclopropane)

T1KO (halothane and cyclopropane)

T3KO (halothane and cyclopropane)

A potential effect of gender was assessed by performing separate experiments with female animals.

For halothane, the following concentrations (% atm.) were delivered: 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1. For cyclopropane, 8, 12, 16, 20 and 25% concentrations were used. As cyclopropane is a gas at room temperature it was mixed with oxygen via an agent specific flowmeter. The following step-wise increments of cyclopropane were added to 500 mL min⁻¹ of oxygen until one step beyond LORR: 40, 60, 80, 100, 125 mL min⁻¹. These flow rates resulted in the following percentage concentrations of cyclopropane when mixed with 0.5 L min⁻¹ O₂: 8, 12, 16, 20, 25% respectively.

Data were compared using a one-way ANOVA and Bonferroni's *post hoc* test and $P < 0.05$ was considered significant. The comparison between female animals was performed with an independent t-test.

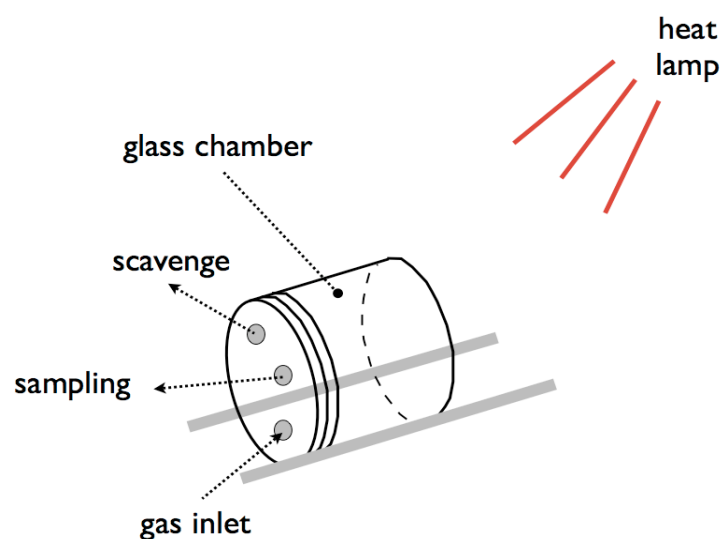


Figure 17. Cartoon of equipment used to assess loss of righting reflex (LORR). The rotating chamber in which the mouse was placed sat on two metal rods (grey), allowing smooth rotation of the chamber. The lid of the chamber had ports for gas entry (oxygen + anaesthetic agent), gas sampling (to the gas analyser) and scavenging. A heat lamp was positioned at a fixed distance for all experiments to maintain normothermia. LORR was assessed once at baseline and at each concentration of anaesthetic. A positive response (loss of LORR) was recorded as the animal was rotated on to its dorsum with four feet off the ground for 30 seconds. Data was analysed with Waud analysis (Zivin & Waud 1992) and results used to fit a sigmoidal dose-response curve.

2.1.4 Halothane exposure assay

The aim of this experiment was to record the variation in ECoG during exposure to increasing levels of halothane on DKO, T1KO and T3KO mice compared with WT.

Each animal was placed in a clear perspex chamber (28 x 28 x 20 cm) which had a tight-fitting lid and ports for oxygen and anaesthetic gas entry, gas sampling and scavenging.

Following connection to the Neurologger and initiation of the gas monitoring, a baseline period of at least 5 minutes in room air occurred. After this, halothane carried in 8 L min⁻¹ of oxygen was delivered through two ports in the lid of the gas tight chamber. A step-wise 0.1% increase in halothane concentration was delivered, with an increase in concentration every 10 minutes, from 0.4 – 1.0%. Two final concentrations of 1.2 and 1.5% were also administered. Halothane concentrations were monitored and recorded as described above for the LORR assay. Waste gas was collected by a gas scavenging system (Fluovac, Harvard Apparatus, UK). The last 5 minutes of each concentration step, when halothane concentration had stabilised, was used for data analyses. Power spectra were drawn from 5 minute epochs of ECoG data, following Fast Fourier Transformation (FFT). (Cooley & Tukey 1965) To allow quantitative analyses and comparisons of power spectra between strains, a Lorentzian curve was fitted to peaks identified within the theta frequency band (4 – 12 Hz) using graphing software

(Microcal™ Origin™ v 5.0, MA, USA; Figure 18). Where more than one peak was identified within the theta frequency band, the Lorentzian curve was fitted to the highest identifiable peak. In all cases, there was an easily-identifiable, dominant peak. From fitting of the Lorentzian curve the following parameters were generated or calculated: peak frequency, full width at half magnitude (FWHM) and Q value (peak frequency/ full width at half magnitude; Figure 19).

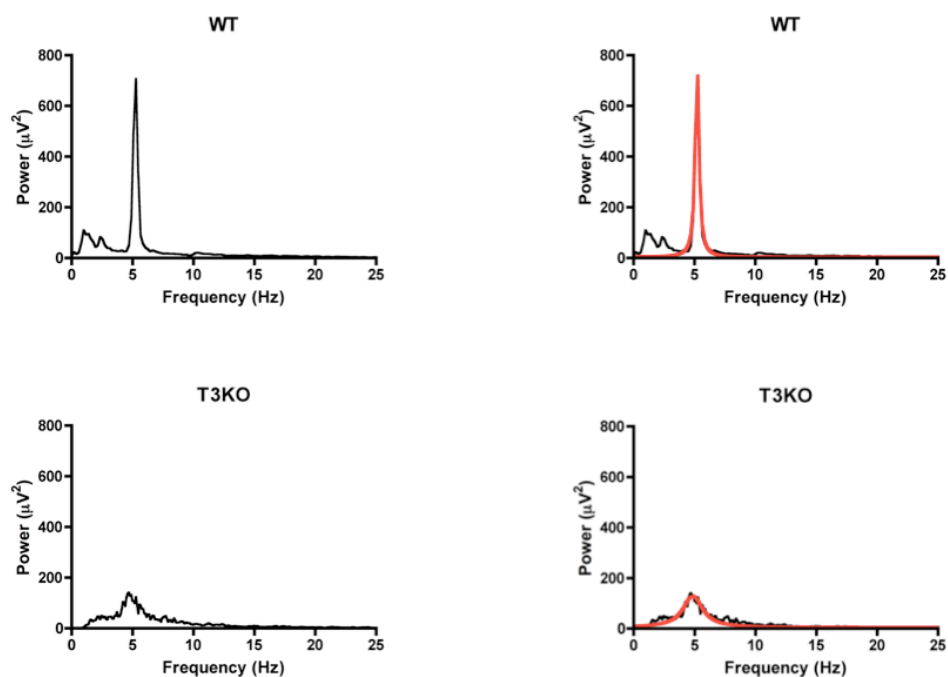


Figure 18. Identifiable peaks in the power spectra within the theta frequency band (4 - 12 Hz) were fitted with a Lorentzian curve. Two examples of power spectra from wild type (WT) and TASK 3 knockout (T3KO) animals during exposure to halothane, before (left column) and after (right column) fitting of the Lorentzian curves (red).

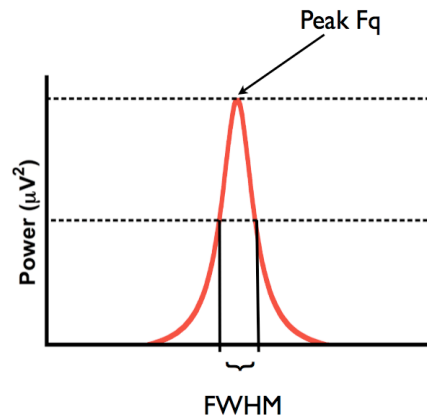


Figure 19. Cartoon depicting the parameters derived from the Lorentzian curve fit: peak frequency (Peak Fq), full width at half-magnitude (FWHM, Hz; the frequency range is depicted by two vertical black bars.) and Q value (= peak fq / FWHM).

Comparisons of Lorentzian curve fit data between strains for peak frequency, full width at half-magnitude and Q value were tested with two-way ANOVA for repeated measures with Bonferroni *post hoc* tests.

2.1.5 Halothane and atropine sulphate assay

The peak induced by halothane has been classified as a type 2 theta oscillation (Bland et al. 2003). A defining feature of this oscillation is that it is ablated by the systemic administration of atropine sulphate (Kramis et al. 1975). Following observations by Robledo that the halothane induced theta oscillation in T3KO animals was sensitive to atropine (Robledo 2008), this sensitivity was tested in T1KO animals, compared to WT. Additionally, the comparison between T3KO and WT was repeated.

The assay was performed with the animal in the same 900 mL glass chamber as that used for the LORR assay. The Neurologger was fitted immediately prior to experimentation. Each

animal was exposed to 4 levels of halothane: 0%, 0.8%, 1.0% and 1.2%. Halothane was carried in oxygen and each step was maintained for 10 minutes before progression to the higher concentration. Each animal acted as its own control in a cross-over design. A saline control of equal volume to the required atropine dose was used. Drugs were administered intraperitoneally (IP). The order of drug administration was randomised but the experimenter was not blind to the drug administered. A minimum of one week was left between the control and the atropine sulphate (Sigma-Aldrich, UK) injection experiment. The atropine dose was 50 mg kg⁻¹. Drugs were administered at the end of the baseline (0% halothane) period.

Data from individual animals was used to draw power spectra (using epochs from the last 5 minutes at each concentration). The peak power for an identifiable peak within the type 2 theta oscillation range of frequencies was normalised to control and the percentage change (relative to control) in the type 2 theta oscillation between control and atropine conditions calculated for each concentration of halothane. The numbers of animals used from each strain, were as follows: T1KO, n = 6; WT, n = 3; T3KO, n = 3. An identical experiment was performed with isoflurane in place of halothane to assess the impact of atropine on type 2 theta oscillation (T1KO, n = 6; WT, n = 4; T3KO, n = 4).

2.1.6 Intraperitoneal eserine (physostigmine) assay

The aim of this experiment was to investigate if the type 2 theta oscillation induced by halothane could also be induced by a second drug, eserine.

Eserine (also known as physostigmine) is an anticholinesterase which has been shown to induce Type 2 theta oscillations when administered in a rats and mice (Leung & Vanderwolf 1980; Tebano et al. 1999). Its administration prevents the enzymatic breakdown of acetylcholine in the brain. A dose of 0.2 mg kg⁻¹ of eserine or the equivalent volume of saline control was administered IP to mice (T3KO, n=3; WT, n=3) instrumented for the Neurologger. Following a baseline period of 10 minutes, the drug/ control was administered

and the cortical EEG recorded for 40 minutes. Data was downloaded from the Neurologger and a five minute epoch was analysed (as described above) and used to draw power spectra.

2.1.7 Sleep assay

Sleep recordings were performed with the animals in an isolated box which was fitted with foam padding to minimise the effects of external noise. The light: dark cycle was maintained as usual. The external dimensions of the box were 80 x 60 x 50 cm. In order to accustom animals to the weight and dimensions of the Neurologger, each animal was fitted with a mock Neurologger for four days preceding the experiment. The mock Neurologger consisted of a piece of circuit board identical in size to the actual Neurologger and weighted with tape and dental cement to match the weight of the actual Neurologger. At the same time, the animal's home cage was modified to allow for the presence of the Neurologger: the wire frame forming the lid, food hopper and water bottle support was turned upside down and taped in place, and a hole was drilled in the end wall of the cage to receive the spout of a drinking bottle. Food was distributed on the floor of the cage rather than in the lid. This modification markedly reduced the risk of the Neurologger catching on the wire of the lid. Climbing behaviour was still observed.

On the day of the experiment, the mock Neurologger was replaced with the real one 2 - 3 hours before the start of the light period. Access to the experimental room was closed to all personnel until termination of the experiment. Each recording period lasted 30 hours.

2.1.7.1 Analysis of sleep data

Following the downloading and filtering of data (as previously described) the sleep data was scored using a script with the Spike2 software. This script was created for us by Geoff Horseman at Cambridge Electronic Design (CED) and based on the sleep score of Costa-Miserachs et al. (Costa-Miserachs et al. 2003). Using the recorded EMG and EEG data, the

score identified three states: awake (W), NREM sleep (N), and REM sleep (R). A fourth state, doubt (D), was produced if data could not be defined as one of the other three states.

The general basis for classification of behaviour according to EEG and EMG data were:
 awake = high EMG and intermediate theta: delta ratio; NREM sleep = low EMG, low theta: delta ratio, high delta; REM sleep = low EMG, high theta: delta ratio.

The first step in generating the sleep score was the extraction of delta and theta oscillations from the filtered EEG signal with bandpass filters and the creation of new data channels within the Spike2 window for delta and the theta: delta ratio. Along with the EMG channel, this information was used as a basis for an initial score performed on consecutive 5 second epochs of data (Figure 20), applied to the entire recording. Epochs not categorised as wake, NREM sleep or REM sleep were scored as doubt.

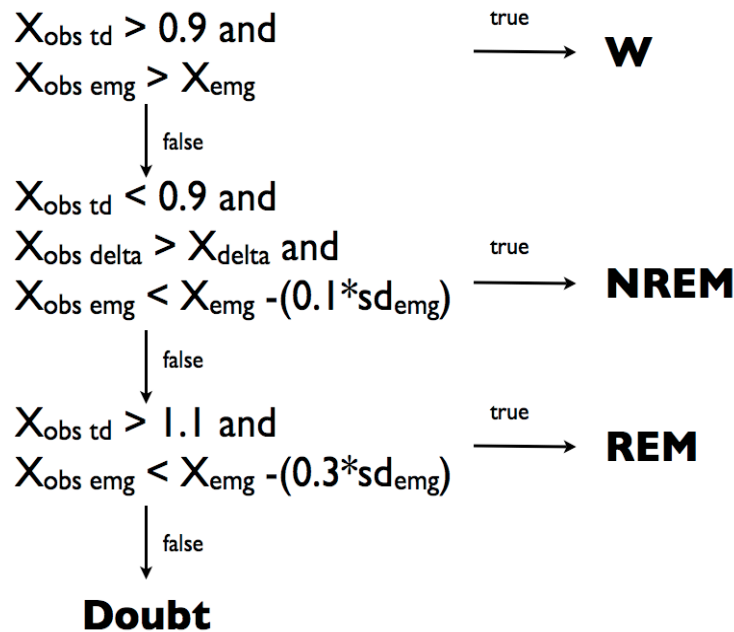


Figure 20. Logic rules applied for initial detection of sleep-wake states. $X_{obs\ td}$, $X_{obs\ emg}$ and $X_{obs\ delta}$ are the mean observed values from 5 sec epochs for the theta:delta ratio, EMG and delta oscillation respectively. X_{delta} , X_{emg} and sd_{emg} are mean delta and EMG and standard deviation of EMG for the entire recording. W, NREM and REM are wake, NREM sleep

and REM sleep respectively. Adapted from Costa-Miserachs et al. (Costa-Miserachs et al. 2003)

The identified wake, NREM sleep and REM sleep periods resulting from the initial scoring were used as a template to score remaining epochs initially scored as doubt. Mean and SD values generated from the initial identification of wake, NREM sleep and REM sleep periods were compared with observed mean values from each 5 sec epoch (Figure 21 below). Where there was close agreement, the observed epochs were scored accordingly. Additionally, the degree of agreement could be modified to optimise the scoring by the changing coefficients controlling the size of the SD.

$$\begin{array}{c} X_{\text{obs td}} > X_{\text{REM td}} - (\alpha * \text{sd}_{\text{REM td}}) \text{ and } \\ X_{\text{obs emg}} < X_{\text{REM emg}} + (\beta * \text{sd}_{\text{REM emg}}) \end{array} \xrightarrow{\text{true}} \mathbf{REM}$$

↓ false

Figure 21. Example logic rules for identification of REM sleep. $X_{\text{obs td}}$ and $X_{\text{obs emg}}$ are the mean observed values from a 5 second epoch. $X_{\text{REM td}}$, $\text{sd}_{\text{REM td}}$, $X_{\text{REM emg}}$, and $\text{sd}_{\text{REM emg}}$ are the mean and standard deviation of theta: delta ratio and EMG from scored REM sleep periods. α and β are coefficients which can be modified to optimise scoring; this was done manually by dragging cursors within the scoring window of Spike2 to alter the level of sensitivity when detecting the theta: delta ratio and EMG (for REM detection). The aim was to maximise detecting REM periods whilst minimising the impact of any artefacts, striking a

balance between REM (or other) period identification and generating doubt scores. Coefficients for EEG, delta and EMG sensitivity could be altered during NREM detection. Adapted from Costa-Miserachs et al. (Costa-Miserachs et al. 2003).

The final step was the grouping of four consecutive 5 sec epochs to generate a scored 20 sec epoch from the predominant state (the order of the states was not considered). This “averaging” of scores removed the fluctuation seen within periods of consistent 5 second epochs e.g. when a muscle twitch during a period of stable NREM sleep resulted in a score of awake being assigned for a 5 second epoch, even though such an epoch is surrounded by epochs scored as NREM sleep. This final step also reduced the number of epochs scored as doubt.

The final percentage of doubt scores was 10% or less for every animal scored. The reliability of the scoring provided a useful tool in training others to use the sleep score script, as poorly scored data resulted in doubt percentages greater than 10%. The final score was exported as a .txt file and plotted with graphing software (GraphPad Prism 4.0c, GraphPad, CA, USA). Figure 22 shows examples of EEG, EMG and theta: delta ratio data resulting in the assignation of wake NREM and REM sleep scores.

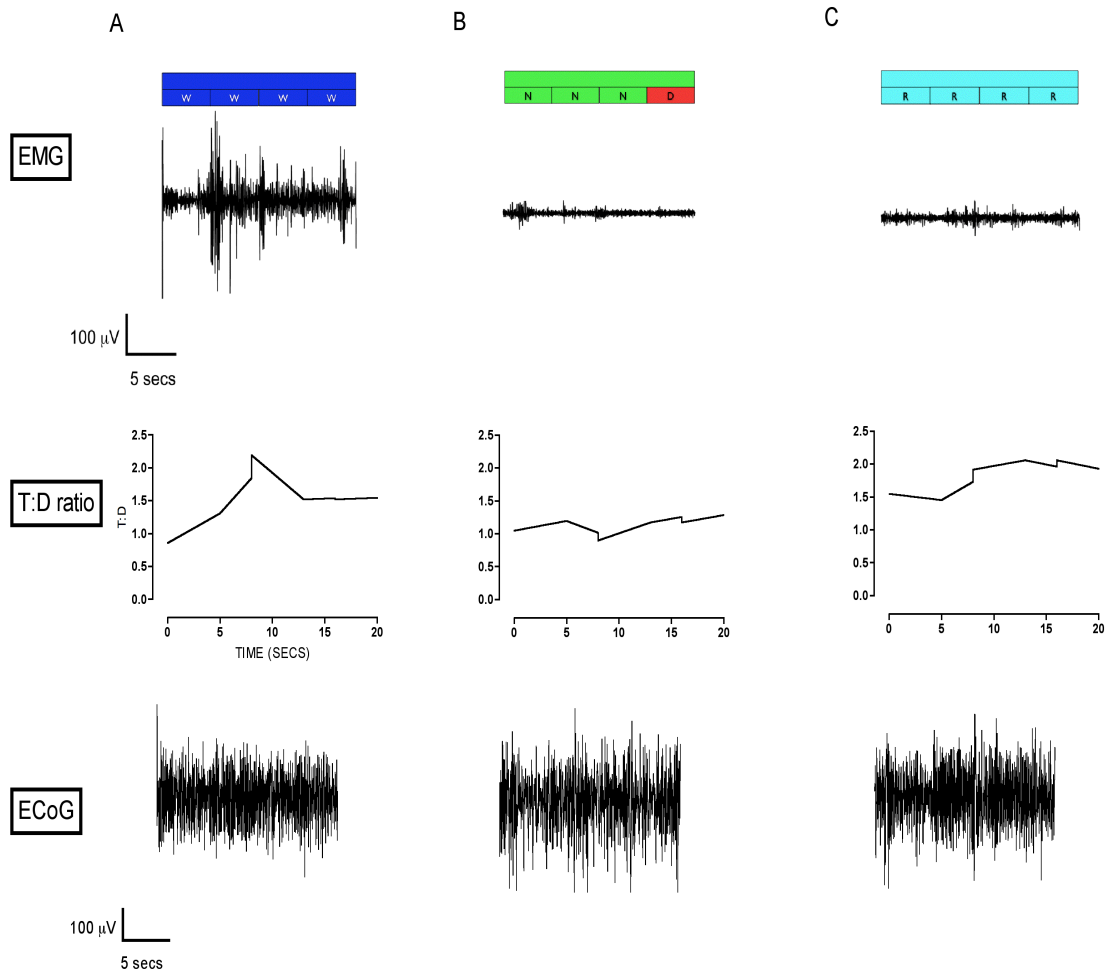


Figure 22. Examples of cortical EEG and EMG data scored as wake (W, column A), NREM sleep (N, column B) and REM sleep (R, column C). Uppermost line shows filtered EMG with results of sleep score, depicted as 5 second and final 20 second epochs. Middle line shows theta: delta (T:D) ratio, extracted from cortical EEG data and lowermost line shows filtered cortical EEG data. A wake score was assigned on the basis of the high EMG activity and high T:D. A NREM sleep score was assigned partially on the basis of the low T:D and low EMG activity. A REM score was assigned on the basis of the low EMG activity and high T:D.

2.2 *In vitro* experiments

This section describes the materials and methods used for the *in vitro* part of this thesis, the preparation of, and recording from brain slices.

2.2.1 Preparation of acute brain slices

In vitro experiments were performed using two strains of mice, T3KO and WT. All animals were male and between 8 and 16 weeks old at the time of sacrifice.

Each animal was anaesthetised with halothane in a Bell jar and loss of righting reflex assured prior to decapitation. The skull was exposed by making an incision through the skin with a scalpel blade along midline extending caudally from between the eyes, followed by an initial immersion of the head in cold slicing solution (Table 2) for approximately 10 seconds before the top of the skull was removed. This was done by inserting one blade of a pair of fine scissors into the foramen magnum and cutting the skull in a line running from the foramen magnum to the lateral canthus of the eye, then medially to the midline. A pair of forceps was used to peel away the cap of skull, exposing the brain. The brain was removed by carefully lifting it off the base of the skull using a small spatula, severing rostral attachments (Figure 23).

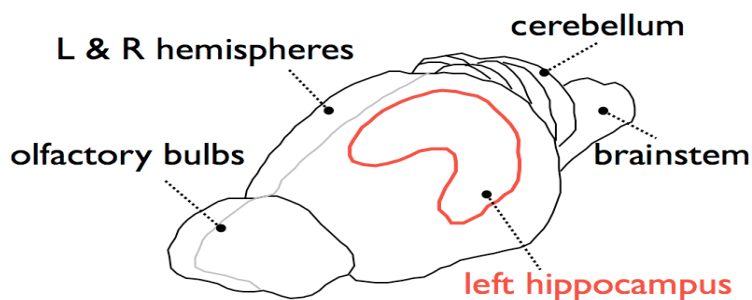


Figure 23. The intact brain of the mouse as it appears following removal from the skull. The positioned of the left hippocampus is delineated in red. Re-drawn from O'Mara et al. (O'Mara et al. 2001).

It was immediately immersed in cold slicing solution which was bubbled with carbogen (95% CO₂, 5% O₂; BOC, UK). Removal of the brain was performed as quickly as possible to maximise tissue viability and the time from decapitation to brain removal took less than a minute. To record from the CA1 region of the hippocampus, the following tissue block was prepared. Keeping the excised brain immersed in the slicing solution, the cerebellum was removed with a transverse cut, the brain bisected along its midline, and placed on the (medial) cut surface in a sylgard coated petri dish. A “magic cut” (Bischofberger et al. 2006) of approximately 10 degrees from a line perpendicular to the petri dish was made (Figure 24). This created a flat surface which could then be glued on to the slicing stage of the slicing machine. This tissue orientation allowed horizontal slices to be made through the hippocampus starting at the ventral surface of the brain (now uppermost on the slicing stage). This method to produce the tissue block optimises preservation of CA1 pyramidal cells (Bischofberger et al. 2006). The tissue block was glued onto the slicing stage of the slicing machine using superglue (Loctite Superglue, UK). The slicing machine (Model 7000smz, Campden Instruments, UK) had been prepared earlier by filling the chamber surrounding the metal stage with a mix of ice and water. This maintained the low temperature necessary

to optimise tissue viability. A few seconds after placing the tissue on the superglue, with the aid of a large spatula, the slicing stage was filled with cold slicing solution immersing the tissue. Carbogen was bubbled continuously through the slicing solution via an inlet in the back of the stage.

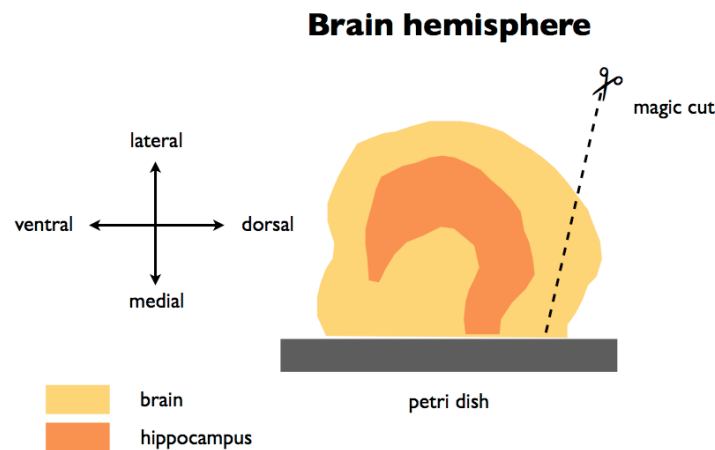


Figure 24. View of a brain hemisphere with medial surface placed on a petri dish, positioned for the “magic cut”. The cut surface forms the base of the tissue block and is glued to the slicing stage of the slicing machine. Horizontal slices are taken starting from the ventral surface, moving dorsally. This orientation optimises preservation of CA1 pyramidal cells. Adapted from (Bischofberger et al. 2006).

The slicing blade was made of stainless steel and was replaced with a new blade every 4-6 experiments. The z deflection of the blade was checked daily and corrected to less than 0.6 μm if necessary.

The slicing machine was programmed to cut 350 μm thick slices, at a slicing speed of 1.0 mm sec^{-1} with 2 mm of lateral blade (amplitude) movement at 80 Hz. Slices were often partially attached to the tissue block by strands of meninges. These connections were severed with the point of a 23 G hypodermic needle and slices left in the the slicing chamber until all the slices had been taken. Identification of the correct depth of tissue at which to start keeping slices

was done by visual identification of the hippocampus. Five usable slices were produced from each brain. At completion of slicing, slices were transferred to a custom-made incubating chamber (Figure 25). Here, they were incubated at 34°C in a beaker placed in a heating block.

The cold slicing solution in the beaker was gradually exchanged for external recording solution (Table 3) via a peristaltic exchange pump (Model 77120-62, Cole Parmer, MasterFlex, US). After 45 minutes the slices were allowed to cool to room temperature and the solution exchange continued for a further 40 minutes.

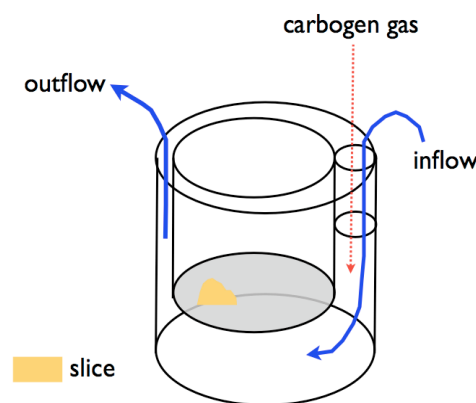


Figure 25. Cartoon of slice incubation chamber. The incubation chamber consisted of a 50 mL glass beaker containing a mesh-bottomed plastic cylinder which housed the slices, a smaller plastic cylinder which braced the larger cylinder within the beaker, and two silicone tubes. One tube supplied fresh external recording solution and the other removed slicing (and recording) solution.

2.2.2 Whole cell patch clamping

The effects of 1% (vaporiser setting) halothane on CA1 pyramidal cells of the hippocampus were investigated using a whole cell patch clamping technique. Wild type and T3KO mouse

strains were studied. Experiments were performed at room temperature (23°C).

Halothane concentration

The concentration of halothane chosen was based on the aqueous phase concentration (C_{aq}) at room temperature (23°C; Franks & Lieb 1993) within the range we found to provide LORR in both T3KO and WT strains, approximately 1%.

Using the following formula:

$$C_{aq} \text{ (mM)} = 121.86 \times \lambda \times P \text{ (%atm)} / (273.15 + T \text{ (°C)})$$

where λ is the Ostwald solubility coefficient, T is temperature and P is the gas phase concentration. A vaporiser setting of 1% at 23°C results in an aqueous phase concentration of 0.5 mM, using a value of 1.20 for the Ostwald solubility coefficient. This calculation is based on an Ostwald solubility coefficient for pure water; a reduction in the solubility coefficient of 10 - 15% occurs in a physiological buffer, with a resultant decrease in calculated aqueous phase concentration (Franks & Lieb 1993).

Equipment

The electrophysiology “rig” was mechanically isolated with the use of an air table (Intracel Isolate System 2000, Intracel, UK) cushioned with nitrogen gas regulated to 65 p.s.i. with a two stage regulator (BOC, UK). Electrical isolation was created by housing the microscope in a Faraday cage and the cage earthed to the amplifier earth.

Slices were placed in a bath and held in place with a “harp” consisting of a U-shaped platinum frame strung with nylon strands, which had been glued on to the frame with superglue (Figure 26). The frame surrounded the slice and the nylon strands pressed down on it, preventing movement. Use of a frame also allowed manipulation of the slice in the bath whilst avoiding direct contact with the slice. The bath was filled with a constantly exchanging external recording solution. The inflow was from a gravity driven system supplying 1-2 mL min⁻¹ of solution, and the outflow was through suction tubing connected to a peristaltic pump (Pump P-1, Pharmacia LKB, Sweden). The external recording solution was at room

temperature and continuously bubbled with carbogen. A silver chloride pellet immersed in the bath solution was connected to the headstage earth.

Slices were visualised with a microscope (Axioskop 2 FS Plus, Zeiss, Germany) with infrared differential interference contrast optics (IR-DIC), and images were viewed with the aid of a digital camera (Model C7500-51, Hamamatsu CCD Camera, Japan) mounted in place of the eye pieces, with images transmitted to a monitor. Visual navigation within the slice was through a low power objective (Zeiss Neofluar 1.5x, Zeiss, Germany) initially, followed by a high power water immersion objective (Zeiss IR-Achroplan 63x, Zeiss, Germany). Further magnification (up to 3x) was provided by the digital camera. Contrast and brightness controls were located on the control box of the digital camera. Once the pyramidal cells of the CA1 region had been identified, the image was optimised by adjusting the amount of light transmitted to the slice and the angle of the condenser. This provided an impression of depth, aiding the approach with the patch pipette.

Patch pipette manufacture and manipulation

Patch pipettes were formed from thin-walled borosilicate glass (OD 1.5 mm, ID 0.86 mm) containing a filament (GC150-F10, Harvard Apparatus, US). Each piece of glass was pulled into two pipettes in a two-stage vertical puller (Model PC-10 Puller, Narishige, Japan). The temperature of the first and second stage pull was adjusted to optimise the shape and length of the pipette tip. As there was minimal shielding around the heating filament of the puller, it was susceptible to slight changes in environmental temperature and air flow, necessitating frequent adjustment. Pipette resistance, once filled with internal solution, ranged from 3 - 8 M Ω . Pipettes were driven through the bath solution and within the slice by a piezo-electric micromanipulator (MP-225, Sutter Instrument Company, UK).

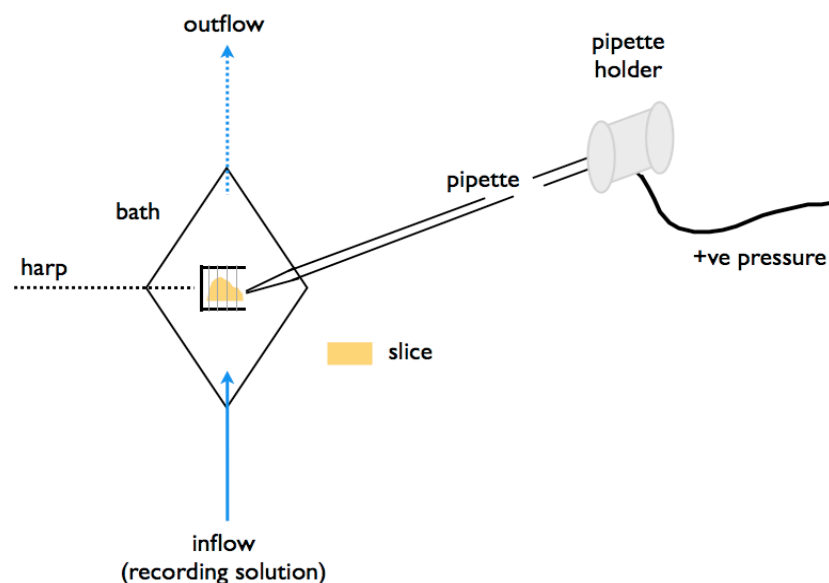


Figure 26. Cartoon of slice in microscope bath, held in place by platinum “harp” with nylon strands (grey). Recording solution inflow ($1 - 2 \text{ mL min}^{-1}$) was gravity driven and outflow was matched by a peristaltic pump. The glass patch pipette was held in a holder driven by a piezo-electric micromanipulator. Positive pressure could be applied to the pipette through tubing connected to a 3-way tap.

Pipettes were secured in place by a pipette holder (DB-S Electrode Holder, G23 Instruments, UCL, UK). Stability and an airtight seal were provided by two rubber O-rings. This airtight seal allowed positive pressure to be delivered through the pipette, preventing obstruction of the fine pipette tip (approximately $2 \mu\text{m}$ diameter) by debris as the pipette was driven down to the neurone of interest. The pipette holder was attached to the headstage amplifier and an electrical connection between the pipette and headstage amplifier provided by a silver-chloride coated stainless steel wire running from the internal solution within the pipette to the headstage amplifier. The headstage amplifier was connected to the patch clamp amplifier (Multiclamp 700B, Axon Instruments, CA, USA), which was located outside the Faraday cage. The amplifier filtered the analogue signal which was then digitised by a National

Instruments digitisation board (PCI-6052E). Stimulation and recording protocols were run using free software: WinWCP (v4.1.6, Strathclyde Institute of Pharmacy and Biomedical Sciences; current clamp recordings) and WinEDR (v3.1.4, Strathclyde Institute of Pharmacy and Biomedical Sciences; voltage clamp recordings).

Whole cell recording

Neurones were recorded using the technique of blind patching. Once an area within the pyramidal cell layer had been selected a pipette was pulled, filled with internal solution and loaded into the pipette holder. A constant positive pressure was applied with nitrogen gas through the pipette from a pico-spritzer (≈ 5 psi; Picospritzer II, General Valve Corporation, US) connected to the pipette holder via plastic tubing as the pipette was submerged in the bath solution. The pipette was manoeuvred until visible in the field of view. It was then lowered in tandem with the objective until it was visible just above the cell layer. Current flow through the pipette was neutralised on the amplifier and pipette resistance calculated by applying a -10 mV square wave. The focus of the field of view was adjusted to the deepest layer that could be visualised, usually 4 - 5 cells below the surface of the slice, and the pipette slowly lowered into focus. From this point the pipette continued to be lowered without direct visualisation. Instead, the -10 mV square wave was observed on the monitor. As a cell was contacted by the pipette tip, the square wave “jumped” suddenly to a more positive value, reflecting an increase in membrane resistance. The subjective evaluation of the speed of this jump gave an indication of the relationship between the pipette and the cell. At this point, positive pressure was removed and a seal allowed to form, encouraged with gentle negative pressure applied by oral suction via a 1 mL syringe. The aim was the formation of a giga-seal (> 1 G Ω). In a healthy cell a giga-seal would form in less than a minute. The capacitive transients were then corrected with the amplifier. Once a stable giga-seal was formed, further negative pressure was applied to the cell membrane to break through and achieve a whole cell configuration. Breaking through resulted in exposure of the internal contents of the cell to the internal solution of the pipette. The slice was exposed to halothane by switching the gravity fed inflow tube from a bottle containing recording solution to one in which halothane carried in carbogen (1 L min^{-1}) was being bubbled. Halothane coming out of solution was

scavenged into a charcoal cannister under the driving force of the gas. Halothane was bubbled into the solution for a minimum of 10 minutes prior to exposing the slice to halothane and once the switch had been made to the halothane containing recording solution, a further 10 minutes were allowed for penetration of the slice before the experimental protocols were started. Nishikawa and MacIver (Nishikawa & MacIver 2000) reported a slow onset (10 minutes) in halothane effects on IPSCs, though they had a larger bath volume than our rig, with a similar fluid rate, resulting in a slower time constant for fluid exchange. Neurones that had stable series resistance and cell capacitance over the recording period were used for analysis.

Current clamp recordings

WinWCP software was used for the current clamp experiments. Under control conditions and in the presence of 1% halothane, the cell was subjected to a brief pulse and a long pulse protocol. Both protocols were run with the cell at its natural resting membrane potential (0 current injected) and at -60 mV (current injected to hold cell at -60 mV). The brief pulse was an incremental increase in voltage, in steps of 1 mV, starting at 3 mV, with a duration of 0.2 ms. The aim of the brief pulse protocol was to elicit an action potential (AP). The long pulse protocol was an incremental increase in voltage steps of 0.2 mV, starting at -0.4 mV, with a duration of 160 ms.

Current clamp data analysis

The data recorded from the current clamp protocols was used to analyse the AP parameters of each cell and generate current-frequency (I-f) input-output curves. Graphing (GraphPad Prism v4.0c) and data handling (Microsoft Excel 2004 v 11.2, WA, USA) software were used.

Action potential (AP) parameters

A time independent method, phase plane plots (PPP; Figure 27), was used to assess and compare AP parameters (Bean 2007). A PPP is a plot of the time derivative of the voltage

(dmV/dt) versus the voltage (mV). The following parameters could be read from each phase plane plot: AP threshold, maximal rate of rise, maximal rate of decay and peak.

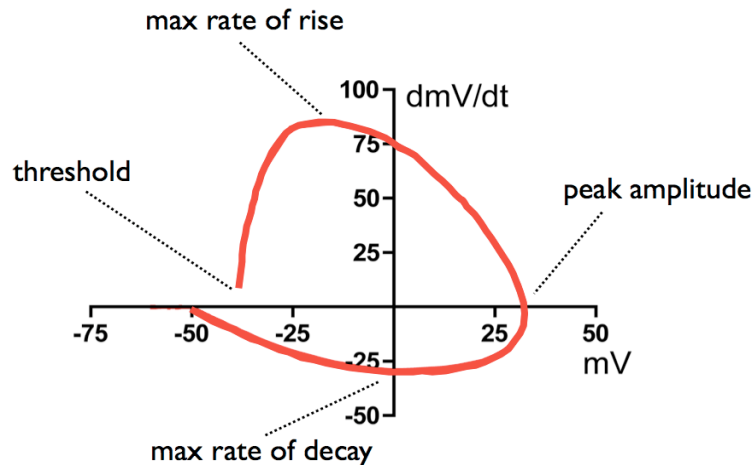


Figure 27. Phase plane plot. By plotting the time derivative of the voltage (dmV/dt) versus the voltage (mV) the following parameters can be easily read from the plot: threshold, maximum rate of rise, peak amplitude, maximum rate of decay.

Current-frequency (I-f) input-output curves

Plots of frequency (Hz) against current (pA) were constructed from the long pulse data by manually counting the number of APs fired in response to each step of voltage injected.

Whole cell parameters

Whole cell parameters were calculated using the current response to a brief pulse immediately prior to the firing of an AP, under control conditions and in the presence of halothane.

The following equations were used:

Input conductance (G ; Siemens) = $1 / R_i$

Where R_i is the input resistance (Ω) = τ / C

with τ (seconds) being the time constant of the decay phase of the cell response to the brief pulse and C is the membrane capacitance (Farads) recorded from the amplifier.

The current injection required to achieve AP threshold was measured by reading off the value displayed on the software (WinWCP; v4.1.6, Strathclyde Institute of Pharmacy and Biomedical Sciences; current clamp recordings) for each recording. The averages under each recording condition were then calculated and compared between strains.

Voltage clamp recordings

In a separate set of experiments, using WinEDR software, cells were held at -60 mV and sIPSCs recorded under control conditions and in the presence of halothane 1%. Recording under each condition lasted 10 minutes with a wash-in period for halothane of 10 minutes. Halothane was applied in the same way as for the current clamp recordings.

Voltage clamp data analysis

Spontaneous IPSCs were detected in WinEDR by setting a threshold of detection for the size of sIPSC peaks. As a result of the presence of APs, and the high frequency of sIPSCs present, the automated detection system was found to be inaccurate, and events were counted manually. A section of data from the last five minutes under each condition was selected and 400 events counted. This data was used to calculate the following parameters: sIPSC frequency, average IPSC decay (from a weighted time constant fitted to the exponential), area under the IPSC curve, and peak height. Average IPSC decay, area, and peak height were calculated from the average of single events which were uncontaminated by synaptic events occurring during the rise or decay phases.

Confocal microscopy

Though the pyramidal cell layer of CA1 is well defined, cell identity and morphology were confirmed with confocal microscopy. A confocal microscope (Zeiss LSM 510, Zeiss, Germany) with a 40x oil immersion lens was used to obtain images in conjunction with ZEN software (ZEN, Zeiss, Germany). The optical source was an argon 458 laser passed through a 475 nm long pass filter. Slice thickness (z interval) was 1 μm . Other settings were optimised for each sample and included pinhole aperture, detector gain, amplifier gain and offset. Slices were first fixed in EDAC (1-Ethyl-3(3-dimethylamino-propyl)carbodiimide) buffer (Sigma E-7750, UK) with 1% N-hydroxysuccinimide (20 mg/10-20 mL; Sigma H-7377, UK) following the protocol of Panula et al (Panula et al. 1984). Both constituents (EDAC, 800 mg; N-hydroxysuccinimide, 20 mg) were diluted in 20 mL of phosphate buffer (0.1 M, pH 7.4). After immersion in the buffer for 24 hours, slices were mounted in Vectashield Mounting Medium with DAPI (4',6-diamidino-2-phenylindole; Vector Lab., Inc., CA, USA).

2.2.3 Solutions

All chemicals were obtained from Sigma-Aldrich UK. Slicing and external recording solution was made up as a 10x stock in 1 and 2 L bottles respectively. Sucrose, glucose, calcium chloride and magnesium chloride were omitted from the concentrated stock solution and added on each experimental day to a 1x solution made up by dilution with MiliQ water. Internal recording solution was made up as a 25 mL batch without Mg-ATP or Na-GTP and stored at -20°C in 1 mL aliquots. Aliquots were stored for 5-6 weeks. Mg-ATP and Na-GTP solutions were freshly made up each week, stored in 30 μL aliquots at -20°C and added to the internal solution each day. All solutions were buffered to a pH of 7.3 - 7.4. Lucifer yellow (1 mg; Sigma, US) was added to each aliquot of internal solution immediately prior to use. Over the course of the experimental day the internal solution was stored on ice to minimise degradation of Mg-ATP and Na-GTP.

Low potassium slicing solution	mM
NaCl	85
KCl	2.5
NaH ₂ PO ₄ ·H ₂ O	1.25
NaHCO ₃	26
Sucrose	75
CaCl ₂	1
MgCl ₂	4
Glucose	25

Table 2. Composition of slicing solution.

Low potassium recording solution	mM
NaCl	125
KCl	2.5
NaH ₂ PO ₄ ·H ₂ O	1.25
NaHCO ₃	26
CaCl ₂	2
MgCl ₂	1
Glucose	25

Table 3. Composition of external recording solution.

Internal recording solution	mM
KCH ₃ SO ₄	120
NaCl	4
HEPES	10
EGTA	5
MgCl ₂	1
CaCl ₂	1
Mg-ATP	3
Na-GTP	0.3
KOH	1M stock to pH solution to 7.3-7.4

Table 4. Composition of internal recording solution.

2.3 Statistics

The statistical tests used to make comparisons between treatment conditions (control and halothane) and between strains (WT and T3KO) for the *in vivo* experiments were ANOVA for repeated measures or 2-way ANOVA with the appropriate *post hoc* test depending on the number of comparisons being made. The results of the *in vitro* experiments were analysed with paired or unpaired t tests depending on the comparison being made. The choice of test for each result is described in the each figure legend. For all comparison, a p-value < 0.05 was considered significant.

2.4 Gentoyping

Animals used were genotyped following termination of the experiments to confirm genetic identity (Figure 28, Figure 29, Figure 30). Ear tissue samples were used for template DNA and amplified using the PCR (see Methods).

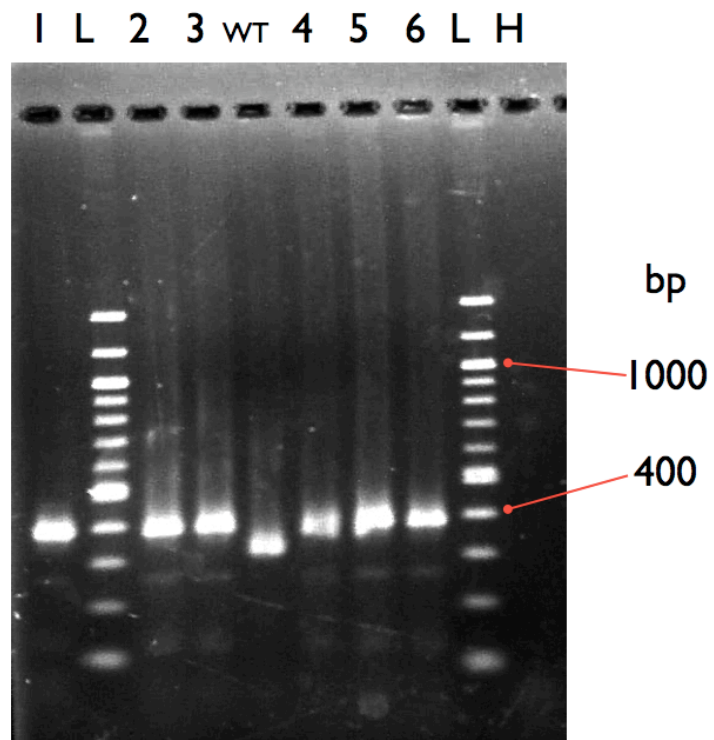


Figure 28. Results from genotyping tissue (ear) samples from T3KO and WT animals. The analysis was performed blind. T3KO animals (samples 1-6) show a band at 400 base pairs (bp), and WT at 350 bp. L is ladder and H is the DNA-free water sample.

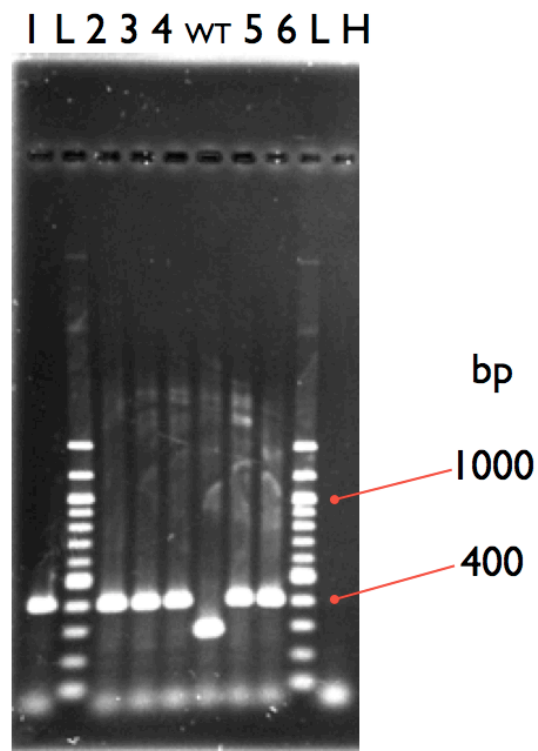


Figure 29. Results from genotyping tissue (ear) samples from T1KO and WT animals. The analysis was performed blind. T1KO animals (samples 1-6) show a band at 400 base pairs (bp), and WT at 300 bp. A multiplex PCR was performed with primers for both T1KO and pIRES. L is ladder and H is the DNA-free water sample.

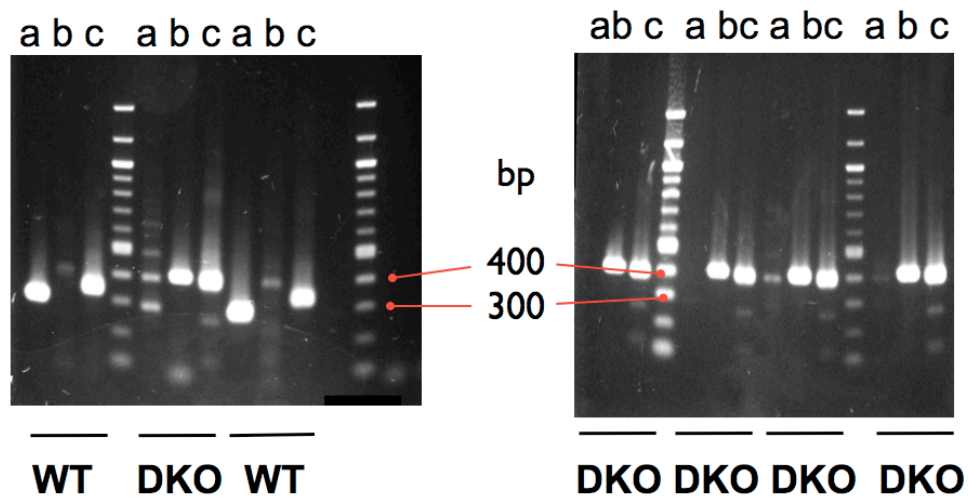


Figure 30. Results from genotyping tissue (ear) samples from DKO and WT animals. Primers for TASK-1 and TASK-3 genes were used in to identify DKO animals. The analysis was performed blind. DKO animals show a band at 400 base pairs (bp) for both TASK-1 (b) and TASK-3 (c) primers. WT show a band at 300 bp (a) and 350 bp (c). L is ladder.

3.0 Results

Some of the *in vivo* data presented in this, and the following chapter have been published (Appendix; Pang et al. 2009).

3.1 Behavioural phenotypes I - the loss of righting reflex (LORR) and sleep

3.1.1 LORR assay: is there a difference in anaesthetic sensitivity between T3KO, T1KO, DKO and WT strains of mice?

The origins of one of our earliest investigations, the LORR, were the findings of Dr Alex Caley (a previous PhD student in our laboratory) that the T3KO animal was markedly less sensitive to halothane than WT. This initial work was consolidated and further comparisons made, with the anaesthetic agent cyclopropane, and between the T3KO, T1KO, DKO and WT strains. Other groups have made similar comparisons with variable results (Lazarenko et al. 2010; Linden et al. 2006, 2007). Potential reasons for these differences will be described in the discussion.

Full details of the methodology are described in Chapter 2. Briefly, each animal was exposed to a step-wise increase in anaesthetic concentration following a baseline period with 100% oxygen. Experiments were performed in a rotatable glass chamber maintained a fixed distance from a heat lamp. The anaesthetics were delivered from a precision, temperature calibrated vaporiser (halothane), or by mixing directly with oxygen (cyclopropane).

3.1.1.2 Halothane

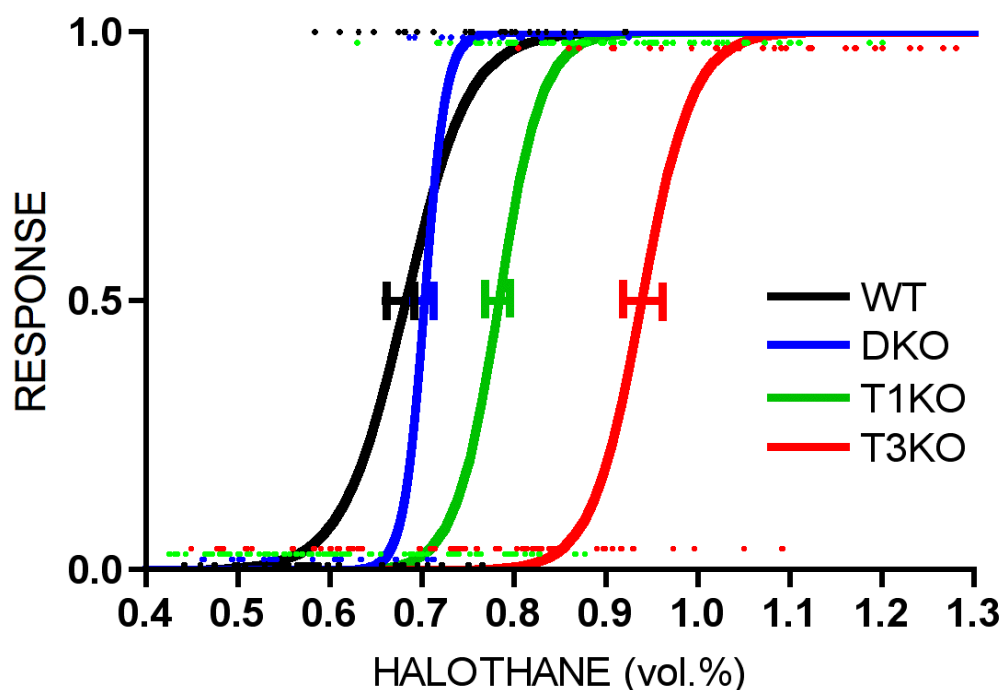


Figure 31. Dose-response curve of loss of righting reflex data with halothane following Waud analysis for WT, DKO, T1KO and T3KO strains of mice. The y-axis represents the response to halothane where 0 is consciousness and 1 is loss of righting reflex and the x-axis shows the range of halothane concentrations used. Solid circles represent raw data for individual animals, plotted staggered for clarity. The $EC_{50} \pm SEM$ values, slopes $\pm SEM$ and group sizes for the strains tested are: WT, 0.68 ± 0.02 , 12.8 ± 3.0 , $n=19$; DKO, 0.70 ± 0.01 , 37.9 ± 18.0 , $n=8$; T1KO, 0.78 ± 0.01 , 18.6 ± 3.2 , $n=20$; T3KO, 0.94 ± 0.02 , 15.9 ± 3.4 , $n=12$. There was a significant difference between T1KO and WT ($p<0.001$), T3KO and WT ($p<0.001$), T1KO and T3KO ($p<0.001$), DKO and T1KO ($p<0.05$), DKO and T3KO ($p<0.001$). There was no significant difference between WT and DKO. WT data were pooled between Daniel Pang ($n=11$) and Alex Caley ($n=8$). Statistical differences were tested with one-way ANOVA and a Bonferroni post hoc test.

Compared with WT and DKO strains, which did not have differing EC_{50} values (0.68 ± 0.02 and 0.70 ± 0.01 , respectively), the T3KO strain was markedly less sensitive to halothane ($EC_{50} = 0.94 \pm 0.02$) with a 38% decrease in sensitivity (Figure 31, above). Halothane sensitivity of the T1KO strain ($EC_{50} = 0.78 \pm 0.01$) was intermediate between WT (and DKO) and T3KO.

3.1.1.3 Sex

An early question that arose during the LORR experiments was whether a sex difference existed in the response to anaesthetics. To test this, female DKO, T3KO and WT animals were compared to males in the presence of halothane (Figure 32).

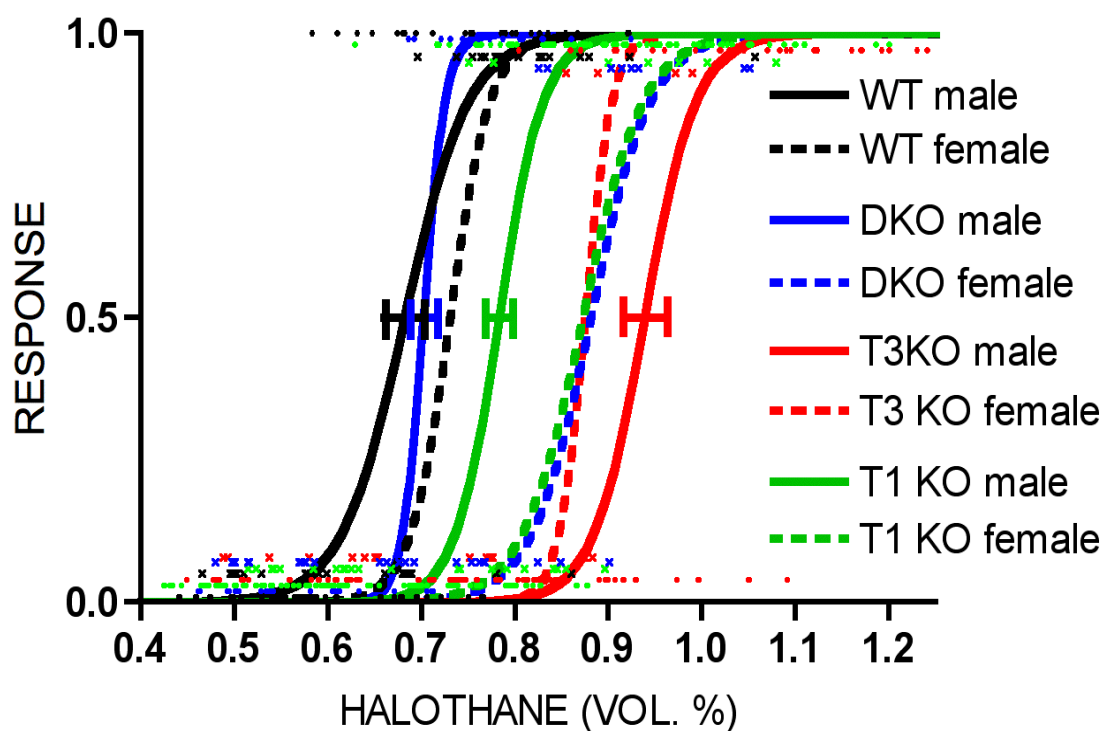


Figure 32. A comparison between sexes for WT, T1KO, T3KO and DKO strains. Dose-response curve of loss of righting reflex data with halothane following Waud analysis for WT, DKO, T1KO and T3KO strains of mice. The y-axis represents the response to halothane where 0 is consciousness and 1 is loss of righting reflex and the x-axis shows the

range of halothane concentrations used. Error (SEM) bars have been omitted for clarity from the lines representing the female data. Solid circles represent raw data for individual male animals and crosses represent corresponding female data, plotted staggered for clarity. A rightward shift in halothane sensitivity was evident for the T1KO and DKO strains. The converse occurs with the T3KO strain. There was a significant difference between sexes for the DKO strain ($p < 0.001$) and T1KO ($p < 0.01$), but not for WT or T3KO. The $EC_{50} \pm SEM$, slope $\pm SEM$, and group sizes were as follows: WT male, 0.68 ± 0.02 , 12.8 ± 3.0 , $n=19$; WT female, 0.73 ± 0.02 , 20.2 ± 6.9 , $n=8$; DKO male, 0.70 ± 0.01 , 37.9 ± 18.0 , $n=8$; DKO female, 0.88 ± 0.04 , 13.6 ± 5.2 , $n=5$; T1KO male, 0.78 ± 0.01 , 18.6 ± 3.2 , $n=20$; T1KO female, 0.87 ± 0.04 , 13.4 ± 5.7 , $n=8$; T3KO male, 0.94 ± 0.02 , 15.9 ± 3.4 , $n=12$; T3KO female, 0.87 ± 0.04 , 31.2 ± 12.1 , $n=9$. Statistical differences were tested with unpaired t-tests.

This experiment was performed to investigate a potential bias from using mixed sex groups. The inconsistent nature of the results, the significant differences between the sexes for DKO and T1KO strains but not WT or T3KO, serves to highlight the potential variability. Performing experiments without assessing the potential impact of sex may serve to hide differences within a single sex group. Alternatively, large sample sizes may be required before a significant difference is achieved.

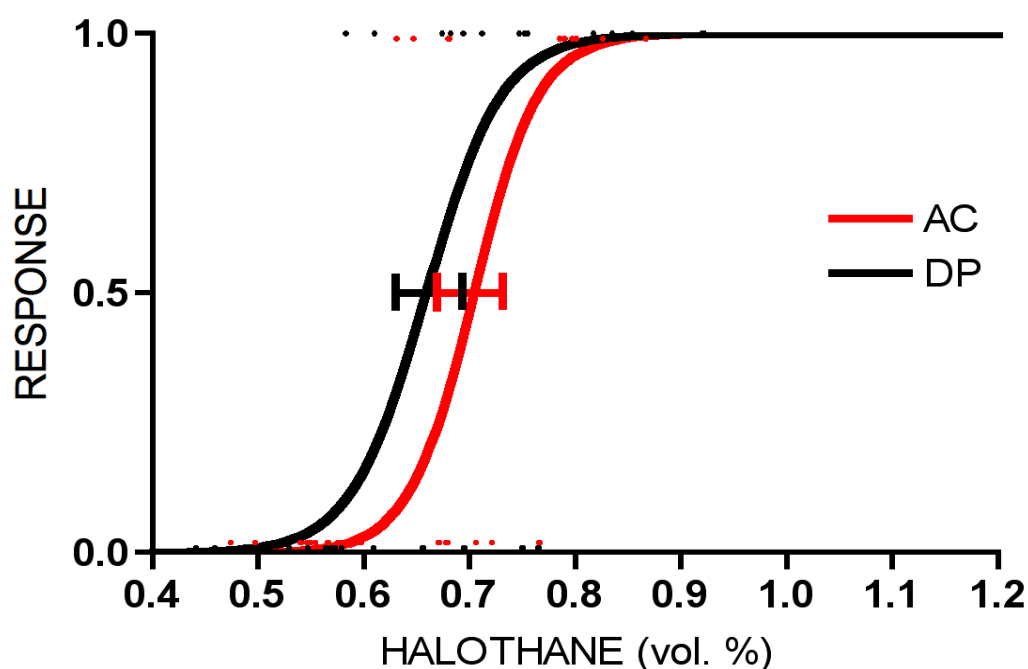


Figure 33. Comparison between experimenters for loss of righting reflex (LORR) data. Dose-response curve of LORR data with halothane following Waud analysis for WT mice, where AC is data collected by Dr Alex Caley ($EC_{50}=0.70 \pm 0.03$, slope = 14.3 ± 5.0 , $n=8$), and DP is data collected by Daniel Pang ($EC_{50}=0.66 \pm 0.03$, slope = 12.3 ± 3.9 , $n=11$). The y-axis represents the response to halothane where 0 is consciousness and 1 is loss of righting reflex and the x-axis shows the range of halothane concentrations used. Solid circles represent raw data for individual animals, plotted staggered for clarity. There is a no significant difference between the EC_{50} values obtained. Error bars represent SEM.

These data show the good inter-observer agreement for the method of measuring LORR we used (Figure 33). This is important to demonstrate given the manual rotation technique employed and risk of variability introduced by the effect of different technique between experimenters. Additionally, our rigorous adherence to our definition of LORR, that all four paws of the animal must be off the ground for at least 30 seconds in order to be classed as a LORR.

3.1.1.4 Cyclopropane

Next, the effect of exposure to cyclopropane was tested as it is an anaesthetic agent that does not affect TASK-3 channels in vitro (Gruss et al. 2004). As cyclopropane is a gas at room temperature, it was delivered by mixture with a known flow rate of oxygen through an agent specific flowmeter.

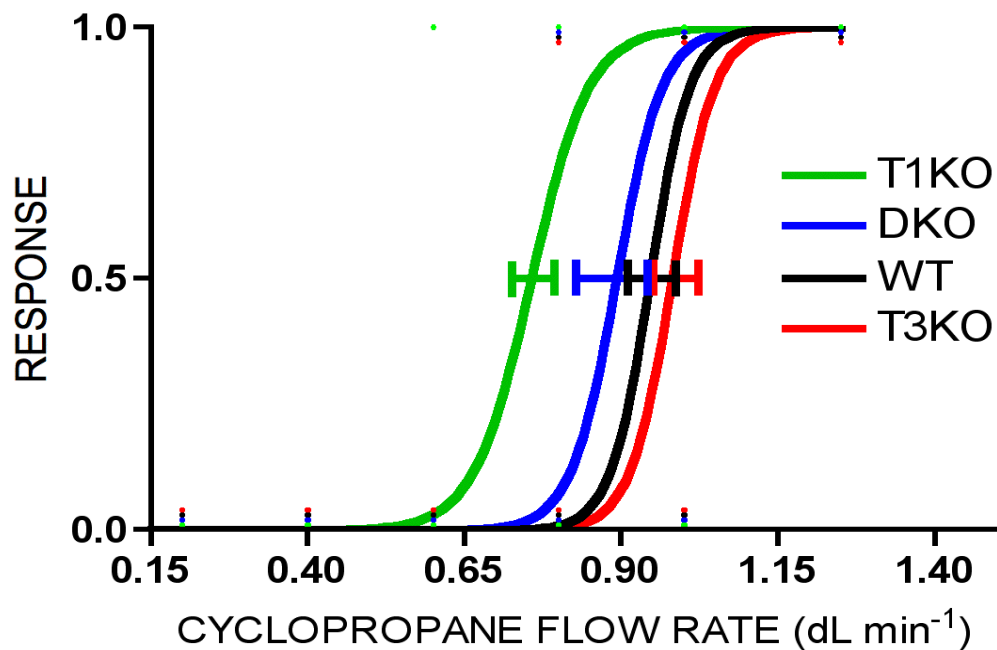


Figure 34. Dose-response curve of loss of righting reflex assay with cyclopropane following Waud analysis for WT, DKO, T1KO and T3KO strains of mice. The y-axis represents the response to cyclopropane where 0 is consciousness and 1 is loss of righting reflex and the x-axis shows the range of cyclopropane flow rates used. Solid circles represent raw data for individual animals, plotted staggered for clarity. Raw data overlap as cyclopropane concentration was determined by the flowmeter. A gas analyser specific to cyclopropane was not available (unlike for the halothane LORR assay). Cyclopropane was mixed with 500 mL min⁻¹ oxygen and the following concentrations tested: 8, 12, 16, 20 and 25%. The EC₅₀ ± SEM values (equivalent % cyclopropane concentrations), slopes ± SEM and group sizes for the strains tested are: WT, 0.95 ± 0.04 (19.0% ± 0.8), 13.8 ± 5.2, n=10; DKO, 0.89 ± 0.06 (17.8% ± 1.2), 12.0 ± 5.8, n=4; T1KO, 0.76 ± 0.03 (15.2% ± 0.6), 9.5 ± 2.2,

n=18; T3KO, 0.98 ± 0.04 ($19.6\% \pm 0.8$), 12.9 ± 4.6 , n=10. There was a significant difference between WT and T1KO ($p < 0.01$) and between T1KO and T3KO ($p < 0.001$). Data were tested for significance with a one-way ANOVA and a Bonferroni post hoc test.

With the exception of the T1KO strain, there was no difference in the response to cyclopropane between the other strains tested (Figure 34, above). T1KO ($EC_{50} = 15.2\% \pm 0.6$) showed greater sensitivity to cyclopropane than WT ($EC_{50} = 19.0\% \pm 0.8$) and T3KO ($EC_{50} = 19.6\% \pm 0.8$). The explanation for this is not apparent, though cyclopropane has effects at other putative anaesthetic targets, as discussed later.

3.1.2 Sleep: does an aspect of normal behaviour differ between strains?

Having a non-tethered system for monitoring the cortical EEG with the Neurologger, it provided an ideal tool to study normal behaviour. Given the occurrence of theta oscillations during REM sleep and the identification in previous work from our laboratory that the T3KO strain had a different sleep architecture compared with WT (Robledo 2008), and the demonstration that the T3KO strain have altered nocturnal activity (Linden et al. 2007), it was important to investigate any alterations in sleep in the T1KO and DKO strains.

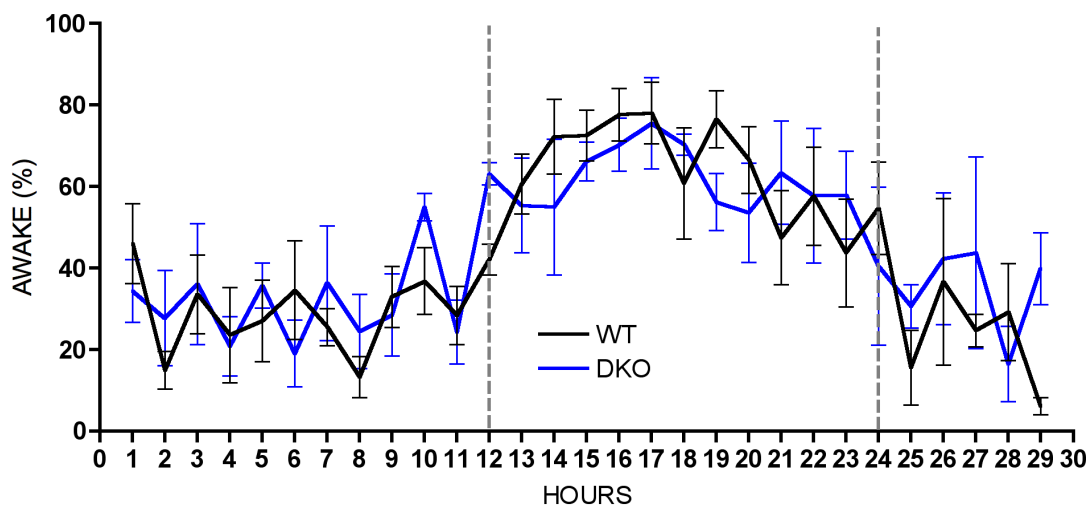
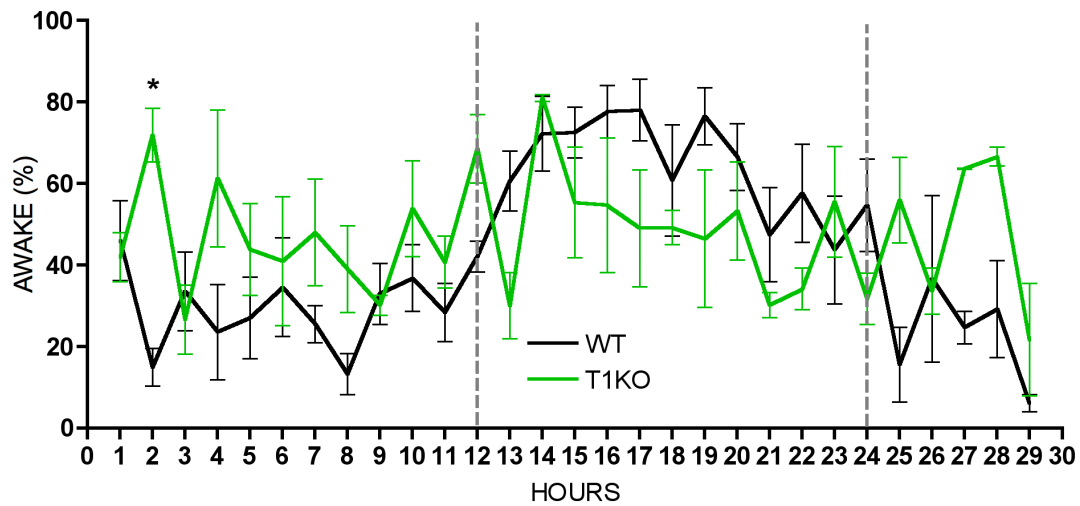
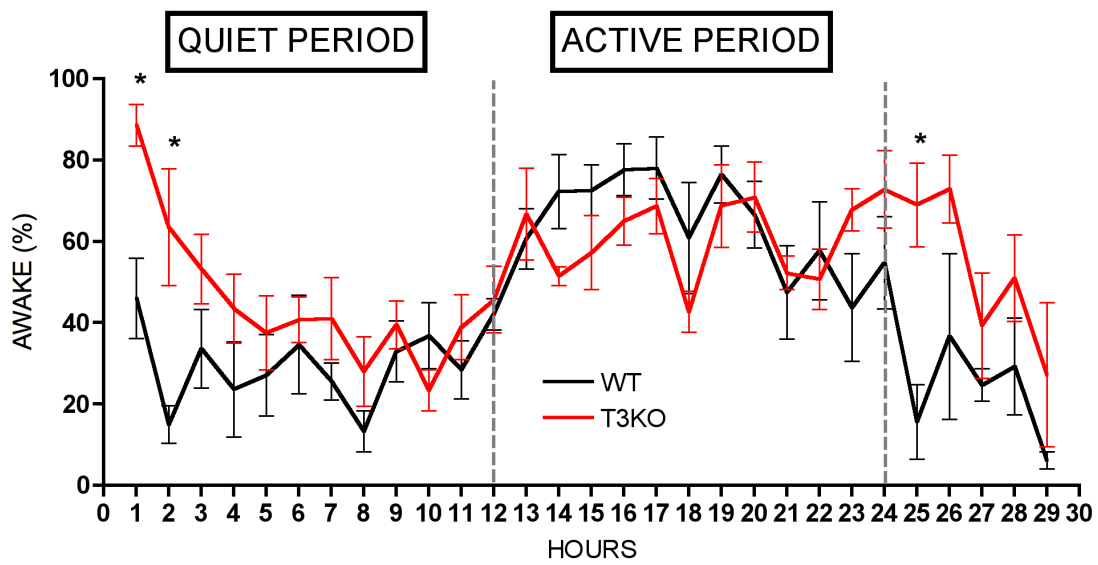
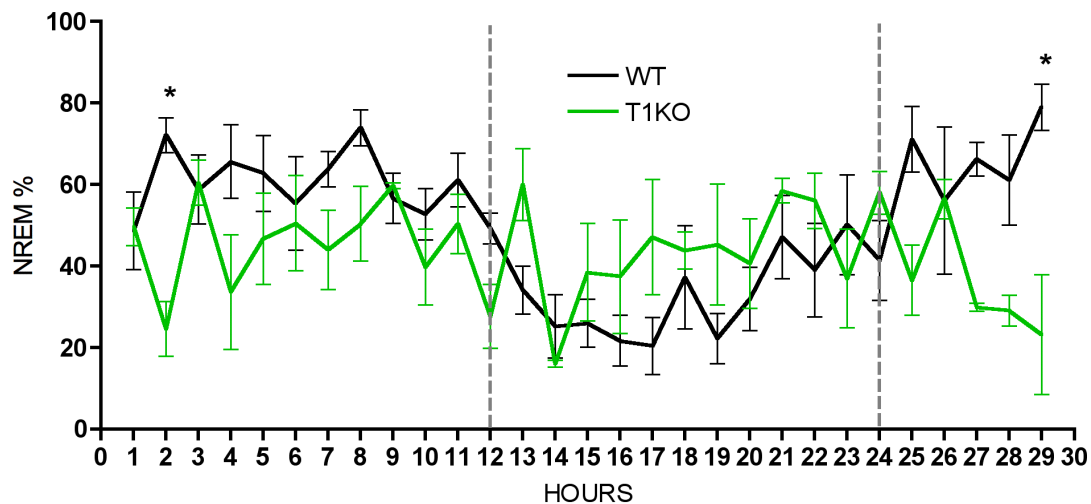
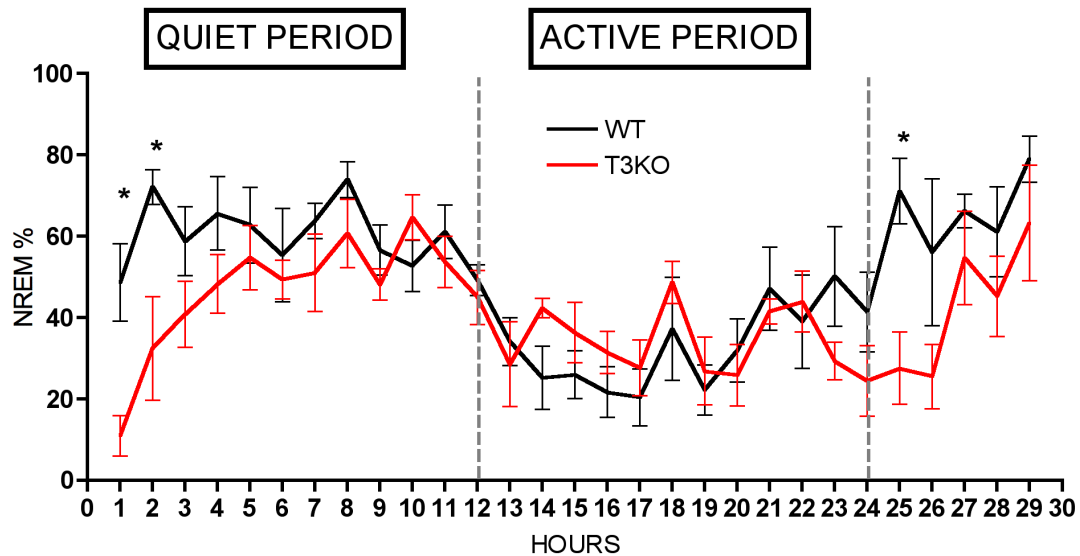


Figure 35. Percentage of time spent awake per hour during a sleep recording over 29 hours with the mouse in its home cage. Red = T3KO (n=5), green = T1KO (n=3), blue = DKO (n=4), black = WT (n=5). Data for T3KO is from CR, data for WT is pooled from DP and CR. During the first hour there was a greater percentage of time spent awake in the T3KO strain ($88.6 \pm 5.1\%$) than the DKO ($p < 0.01$, $34.4 \pm 7.7\%$), WT ($p < 0.05$, $46.1 \pm 9.9\%$) and T1KO ($p < 0.05$, $42.0 \pm 6.0\%$) strains. During the second hour there was a greater percentage of time spent awake in the T3KO ($63.5 \pm 14.4\%$) than the WT ($p < 0.01$, $15.0 \pm 4.6\%$), and in the T1KO ($72.0 \pm 6.5\%$) than the WT ($p < 0.01$) strains. During the 25th hour there was a greater percentage of the time spent awake in the T3KO ($69.0 \pm 10.3\%$) than the WT ($p < 0.05$, $15.6 \pm 9.2\%$) strains. Error bars represent SEM. These data were analysed with 2-way ANOVA for repeated measures with Bonferroni *post hoc* tests. Asterisk indicates significant ($p < 0.05$) difference from WT.

Sleep data has been presented as “quiet” and “active” periods rather than “light” and “dark” periods to avoid confusion with the nocturnal behaviour of the mice. Similarly, the terms “wake” and “sleep” have been avoided as rodents do not have the well defined division of these behaviours exhibited in humans. Our data for wild type show that even during the quiet (or “sleep”) period, 30% of each hour is spent awake. This doubles during the active (or “wake”) period (64%).

The percentage of time spent awake during the active period is similar for WT, T3KO and DKO strains (WT $64.1 \pm 3.4\%$, DKO $60.2 \pm 2.7\%$, T3KO $61.3 \pm 2.8\%$; Figure 35). During the quiet period there is a clear difference between T3KO and the other strains, with a substantially greater time spent awake in the first hour ($88.6 \pm 5.1\%$) than the other strains (DKO $34.4 \pm 7.7\%$, WT $46.1 \pm 9.9\%$, T1KO $42.0 \pm 6.0\%$). A similar situation persists in the second hour (with the exception of T1KO, which does not differ from T3KO) before the time spent awake by T3KO falls into line with that of the other strains. The general distribution of a lower percentage of time spent awake in the quiet period and vice versa for the active period is displayed by all strains except T1KO. This strain has a near even distribution of time spent awake during both periods (quiet $47.3 \pm 4.1\%$, active $47.6 \pm 4.2\%$). The data beyond the 24 hour period (hours 25-29) were included to verify that any stress induced by handling prior to the experiment (when the mock Neurologger was replaced with the real

Neurologger) did not affect the results. This was borne out by the similar relationship between strains observed in hour 25 as in hour 1 *i.e.* the T3KO strain spent a significantly greater period of time awake than the WT strain. This would indicate that T3KO has a slower transition between the active and quiet periods compared with other strains. The time spent awake by the DKO strain was striking in its similarity to wild type, with no significant differences at any time point.



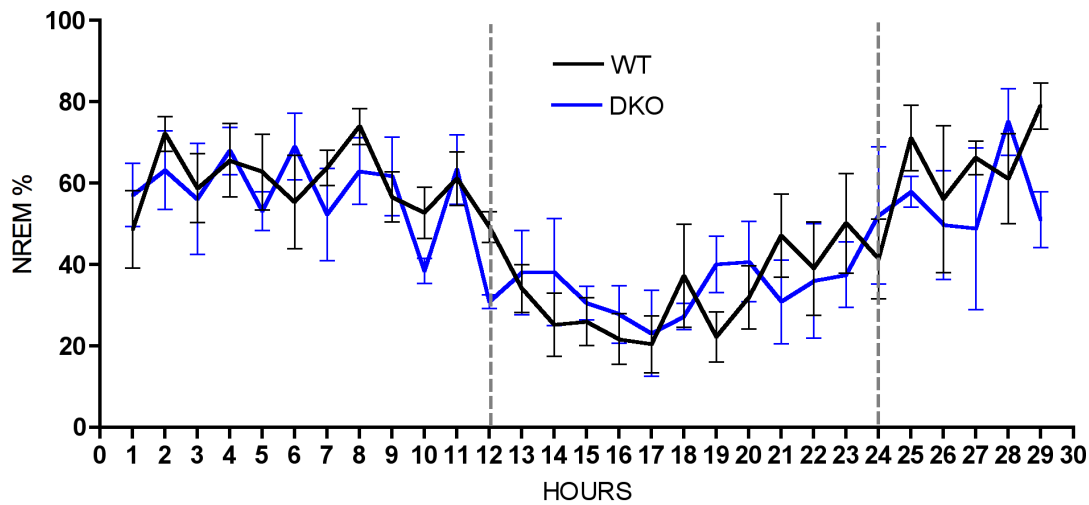
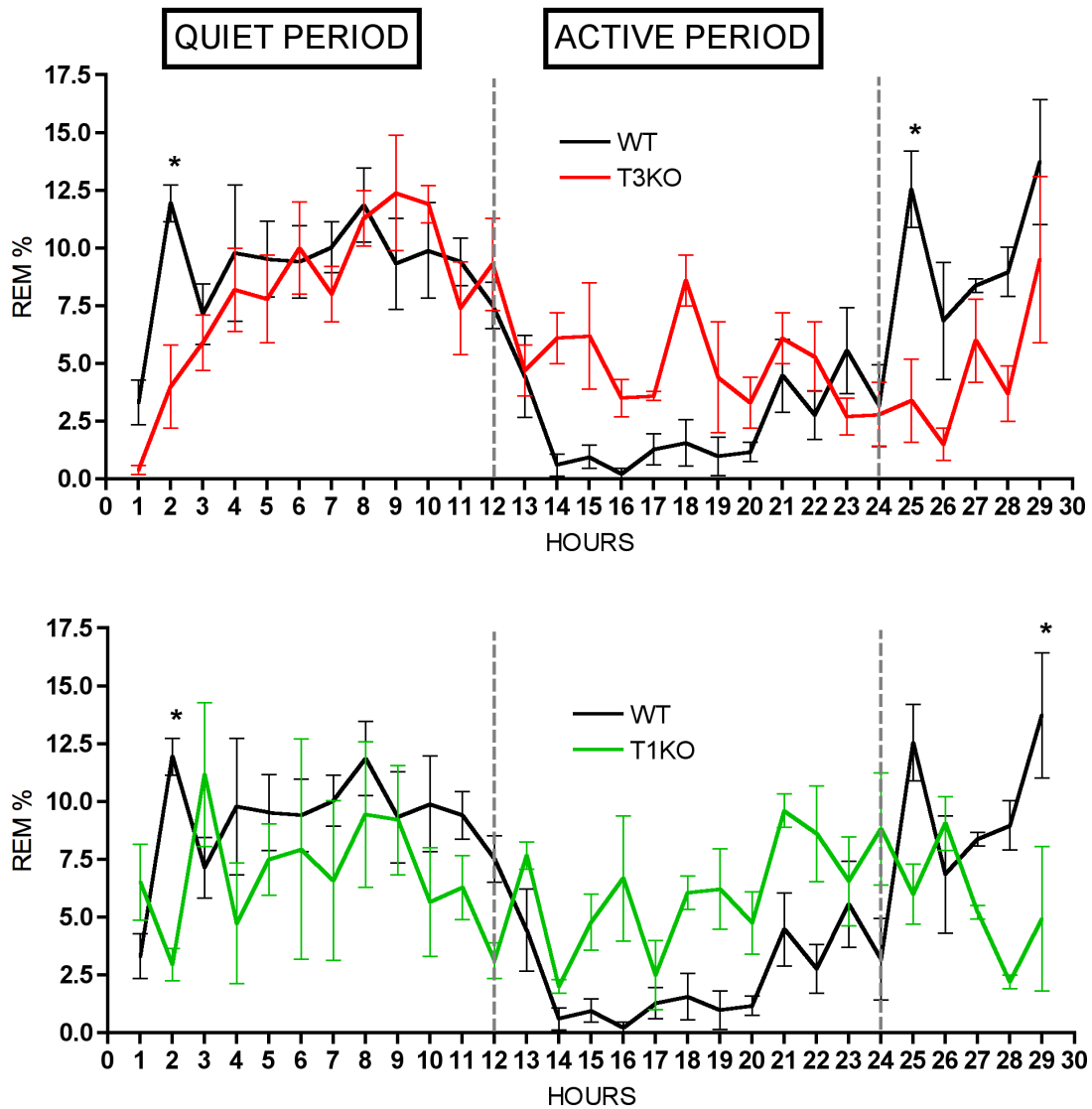


Figure 36. Percentage of time spent in NREM sleep per hour during a sleep recording over 29 hours with the mouse in its home cage. Red = T3KO (n=5), green = T1KO (n=3), blue = DKO (n=4), black = WT (n=5). Data for T3KO is from CR, data for WT is pooled from DP and CR. During the first hour there was a greater percentage of time spent in NREM sleep in the DKO ($57.1 \pm 7.8\%$) than the T3KO ($p < 0.01$, $11.0 \pm 5.0\%$), and the WT ($48.7 \pm 9.5\%$) than the T3KO ($p < 0.05$) strains. In the second hour there was a greater percentage of time spent in NREM sleep in the WT ($72.2 \pm 4.3\%$) than the T3KO ($p < 0.05$, $32.5 \pm 11.7\%$) and the WT than the T1KO ($p < 0.05$, $24.7 \pm 6.7\%$) strains. The WT strain ($71.1 \pm 8.0\%$) spent more time in NREM sleep compared with T3KO ($p < 0.01$, $27.6 \pm 8.9\%$) during the 25th hour, and compared with T1KO ($23.2 \pm 14.7\%$; WT $79.1 \pm 5.7\%$) during the 29th hour ($p < 0.001$). Error bars represent SEM. These data were analysed with 2-way ANOVA for repeated measures with Bonferroni *post hoc* tests. Asterisk indicates significant ($p < 0.05$) difference from WT.

Like the data for percentage time spent awake, a similar pattern appears for NREM sleep. Again most strains behave similarly during the active period, spending around a third of the time in NREM sleep (Figure 36). The exception, T1KO, spends a similar percentage of time in NREM sleep during both periods (quiet period $44.8 \pm 3.3\%$, active period $44.9 \pm 3.6\%$). A notable strain difference becomes apparent during the first hour (quiet period), when T3KO ($11.0 \pm 5.0\%$) spends substantially less time in NREM sleep than DKO ($57.1 \pm 7.8\%$) and

WT ($48.7 \pm 9.5\%$) strains. This pattern continues into the second hour after which T3KO behaviour joins that of the other strains. Again, T3KO displays a slower transition from active to quiet periods, lagging behind the other strains. Once more, the DKO strain shows a striking similarity to WT.



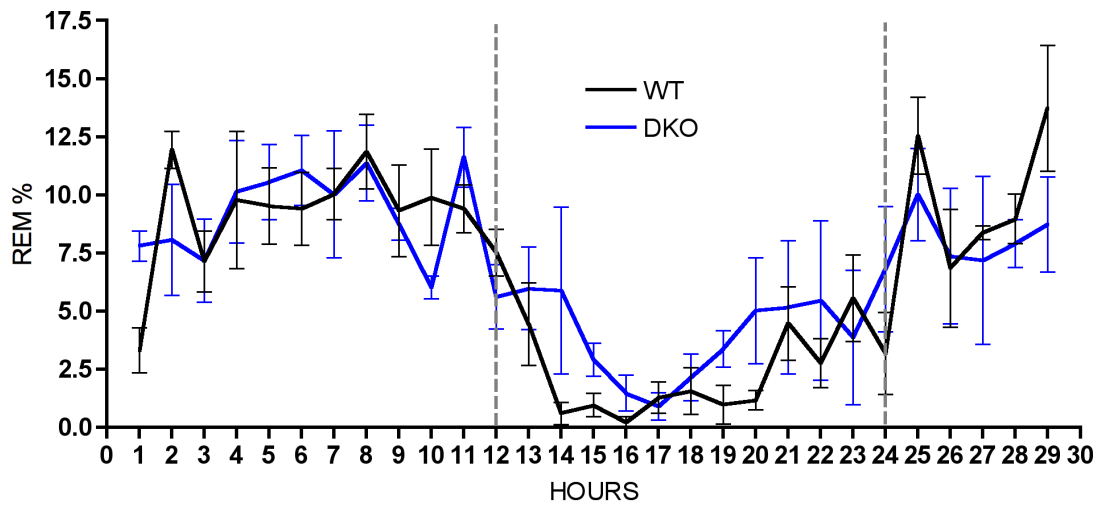


Figure 37. Percentage of time spent in REM sleep per hour during a sleep recording over 29 hours with the mouse in its home cage. Red = T3KO (n=5), green = T1KO (n=3), blue = DKO (n=4), black = WT (n=5). Data for T3KO is from CR, data for WT is pooled from DP and CR. During the second hour WT ($11.9 \pm 0.8\%$) spent more time in REM sleep than the T3KO ($p < 0.05$, $4.0 \pm 1.8\%$) and T1KO ($p < 0.05$, $2.9 \pm 0.7\%$) strains. Similarly WT (hour 25, $12.5 \pm 1.6\%$; hour 29, $13.7 \pm 2.7\%$) spent more time in REM sleep than the T3KO ($p < 0.01$, $3.4 \pm 1.8\%$) and T1KO ($p < 0.05$, $4.9 \pm 3.1\%$) strains during the 25th and 29th hours respectively. Error bars represent SEM. These data were analysed with 2-way ANOVA for repeated measures with Bonferroni *post hoc* tests. Asterisk indicates significant ($p < 0.05$) difference from WT. Note the change in y-axis scale compared with Figure 35 and Figure 36.

During the quiet period the general pattern observed during NREM sleep is maintained; the T3KO strain sleeps less than WT at the beginning of the period before following the same pattern for the rest of the period. During the active period there is no difference between strains in the time spent in REM sleep. The distribution of REM sleep between quiet and active periods is very similar for the T1KO strain (quiet $6.8 \pm 0.7\%$, active $6.2 \pm 0.7\%$), failing to show the cyclical pattern observed in the other strains. DKO and WT show a very similar distribution of REM sleep throughout (Figure 37).

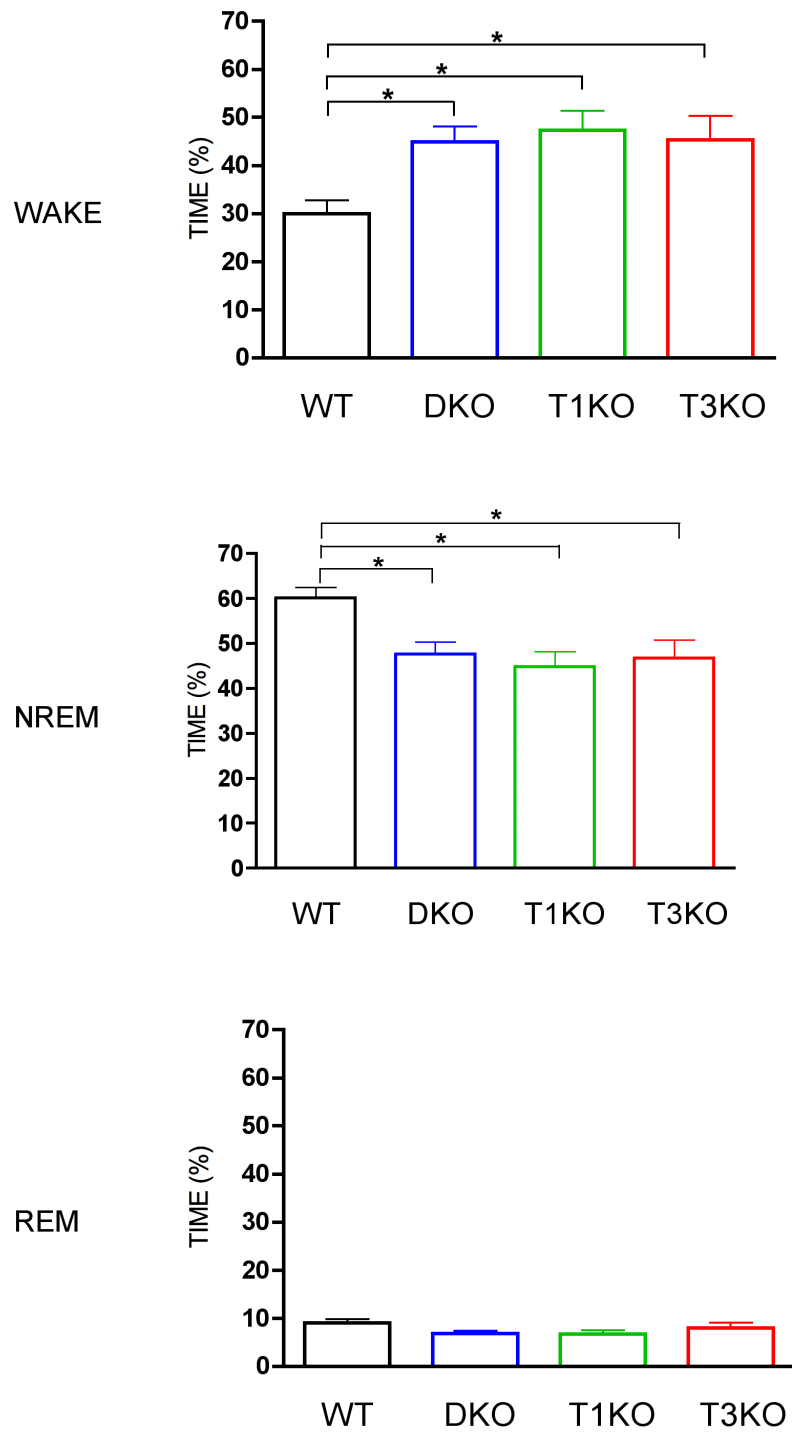


Figure 38. Time spent as a percentage in wake, NREM sleep and REM sleep (top to bottom, respectively) during the quiet (light) period (12 hours) of the circadian cycle. Less time was spent awake in WT ($29.9 \pm 2.8\%$) than each of the other strains ($p < 0.05$; DKO, $44.9 \pm 3.2\%$; T1KO, $47.3 \pm 4.1\%$; T3KO $45.3 \pm 4.9\%$), whereas more time is spent by WT (60.1

$\pm 2.3\%$) in NREM sleep than each of the other strains ($p < 0.05$; DKO, $47.7 \pm 2.7\%$; T1KO, $44.8 \pm 3.3\%$; T3KO $46.8 \pm 4.1\%$). During REM sleep, there was no difference between strains WT, 9.1 ± 0.7 ; DKO, $6.8 \pm 0.6\%$; T1KO, $6.8 \pm 0.7\%$; T3KO $8.0 \pm 1.0\%$). Data were subjected to unpaired t-tests. Error bars represent SEM.

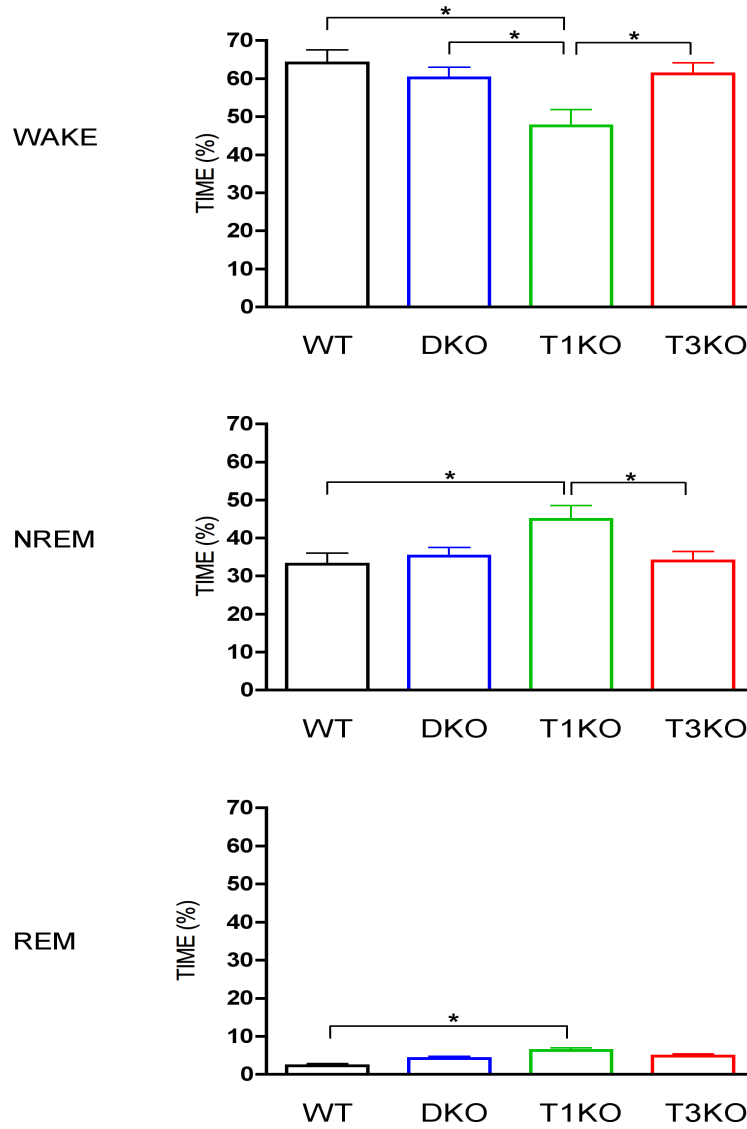


Figure 39. Time spent as a percentage in wake, NREM sleep and REM sleep (top to bottom, respectively) during the active (dark) period (12 hours) of the circadian cycle. The T1KO strain spent less time ($47.6 \pm 4.2\%$) awake than all other strains ($p < 0.05$; WT, $64.1 \pm 3.4\%$; DKO, 60.2 ± 2.7 ; T3KO, $61.3 \pm 2.8\%$), and more time in NREM sleep (T1KO, $44.9 \pm 3.6\%$) than WT ($p < 0.05$, $33.1 \pm 2.9\%$) or T3KO strains ($p < 0.05$, $34.0 \pm 2.4\%$). There was no difference compared with DKO ($35.2 \pm 2.2\%$). Compared to WT ($2.3 \pm$

0.5%), T1KO spent more time in REM sleep ($p < 0.05$, $6.2 \pm 0.7\%$). There was no difference between T1KO and DKO ($4.1 \pm 0.6\%$) or T3KO ($4.8 \pm 0.5\%$) strains. Data were subjected to unpaired t-tests. Error bars represent SEM.

Analysis of the data split into 12 hour periods (quiet and active) shows some interesting differences (Figure 38 and Figure 39). During the quiet period (Figure 38) WT spends substantially less time awake compared to all other strains, and the situation is reversed during NREM sleep. A different picture is apparent during the active period (Figure 39) when time spent awake is similar across strains with the exception of T1KO, in which it is lower. Again, the situation is reversed during NREM sleep. T1KO also spends more time in REM sleep than WT. These results reflect the importance of analysing the data in different ways. The hourly analyses (Figure 35, Figure 36, Figure 37) revealed the differences in transitional behaviour between T3KO and the other strains. Whereas, pooling the hourly data into 12 hour periods showed that the WT strain spent less time awake during the quiet period than the knockout strains, with a concomitant increase in NREM sleep. A difference that was inapparent by displaying and analysing data hour by hour.

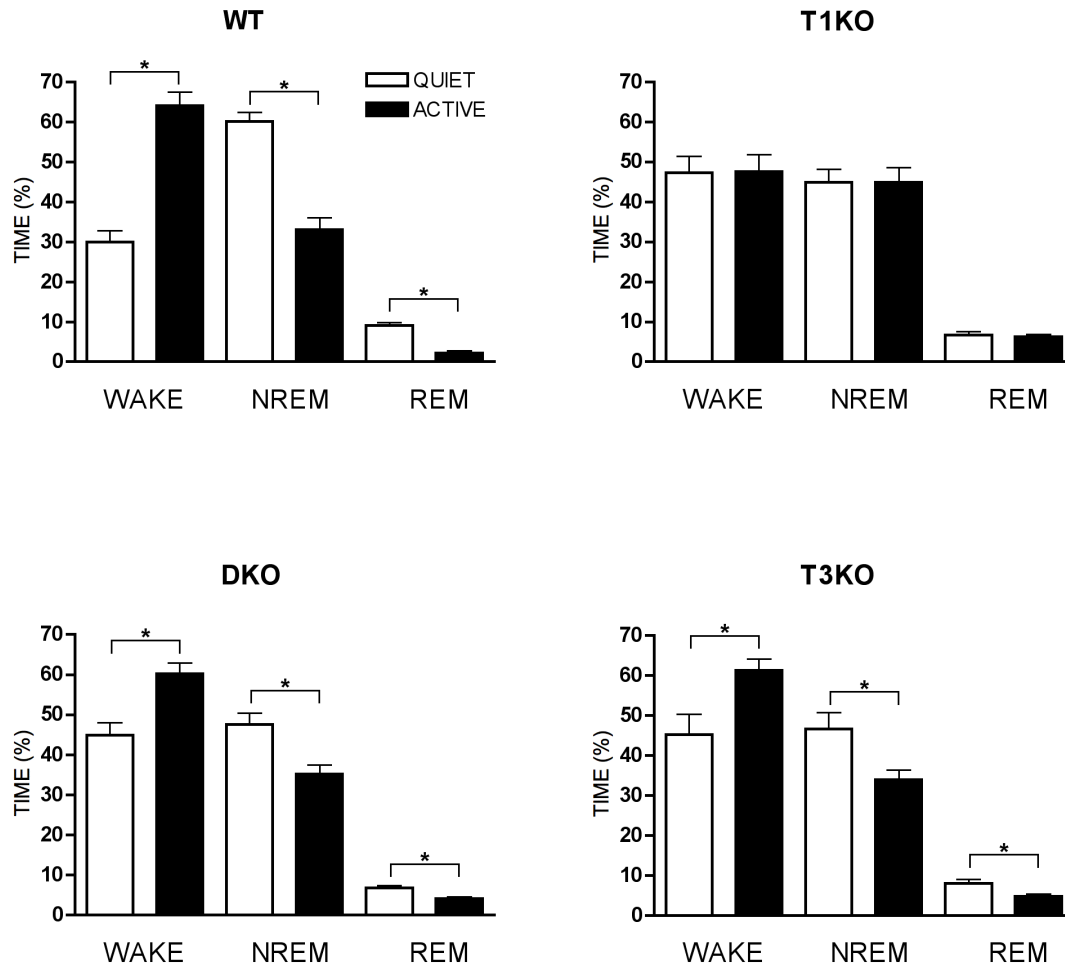


Figure 40. Comparison of quiet (clear bars) and active (black bars) periods for each strain during wake, NREM sleep and REM sleep states, expressed as percentage time. The WT (quiet $29.9 \pm 2.8\%$; active 64.1 ± 3.4), T3KO (quiet $45.3 \pm 4.9\%$; active 61.3 ± 2.8) and DKO (quiet $44.9 \pm 3.2\%$; active 60.2 ± 2.7) strains showed the same patterns of behaviour with less time spent awake during the quiet period (WT, $p < 0.0001$; T3KO, $p < 0.05$; DKO, $p < 0.05$) and more time spent in NREM (WT, $p < 0.0001$, [quiet 60.1 ± 2.3 ; active 33.1 ± 2.9]; T3KO, $p < 0.05$, [quiet 46.7 ± 4.1 ; active 34.0 ± 2.4]; DKO, $p < 0.05$, [quiet 47.7 ± 2.7 ; active 33.2 ± 2.2]) and REM (WT, $p < 0.0001$, [quiet 9.1 ± 0.7 ; active 2.3 ± 0.5]; T3KO, $p < 0.05$, [quiet 8.0 ± 1.0 ; active 4.8 ± 0.5]; DKO, $p < 0.05$, [quiet 6.8 ± 0.6 ; active 4.1 ± 0.6]) sleep. The T1KO strain showed a very different pattern, with no difference in time spent in each state during both active and quiet periods (awake [quiet 47.3 ± 4.1 ;

active 47.6 ± 4.2], NREM [quiet 44.8 ± 3.3 ; active 44.9 ± 3.6], REM [quiet 6.8 ± 0.7 ; active 6.2 ± 1.2].

Figure 40 highlights the difference between T1KO and the other strains. The cyclical pattern of wake versus sleep varying during the quiet and active periods, as shown by all other strains, is broken by the behaviour of the T1KO strain, which shows a very stable distribution of wake and sleep behaviours throughout the circadian cycle.

3.2 Behavioural phenotypes II - the theta oscillation and the response to halothane, atropine sulphate and eserine

3.2.1 Halothane

The TASK1 and TASK3 channels are activated by the volatile anaesthetic agents halothane and isoflurane (Berg et al. 2004; Talley & Bayliss 2002; Meadows & Randall 2001; Sirois et al. 2000; Patel et al. 1999). The degree of activation by halothane is greater for the TASK3 than TASK1 channel and the effects of halothane are greater than isoflurane in either channel. Previous work by our group showed the induction of a type 2 theta oscillation in T3KO mice exposed to halothane (Robledo 2008). This series of experiments was performed to verify these findings and investigate the effects of halothane in the T1KO and DKO strains. As expression of TASK1 mRNA is absent in the hippocampus, one area involved in generation of the theta oscillation, it was predicted that behaviour might be similar to WT (Aller et al. 2005). The response of each animal was measured individually by exposure to a step-wise increase in halothane concentration as the cortical EEG was recorded with the Neurologger (see Chapter 2 for detailed methods).

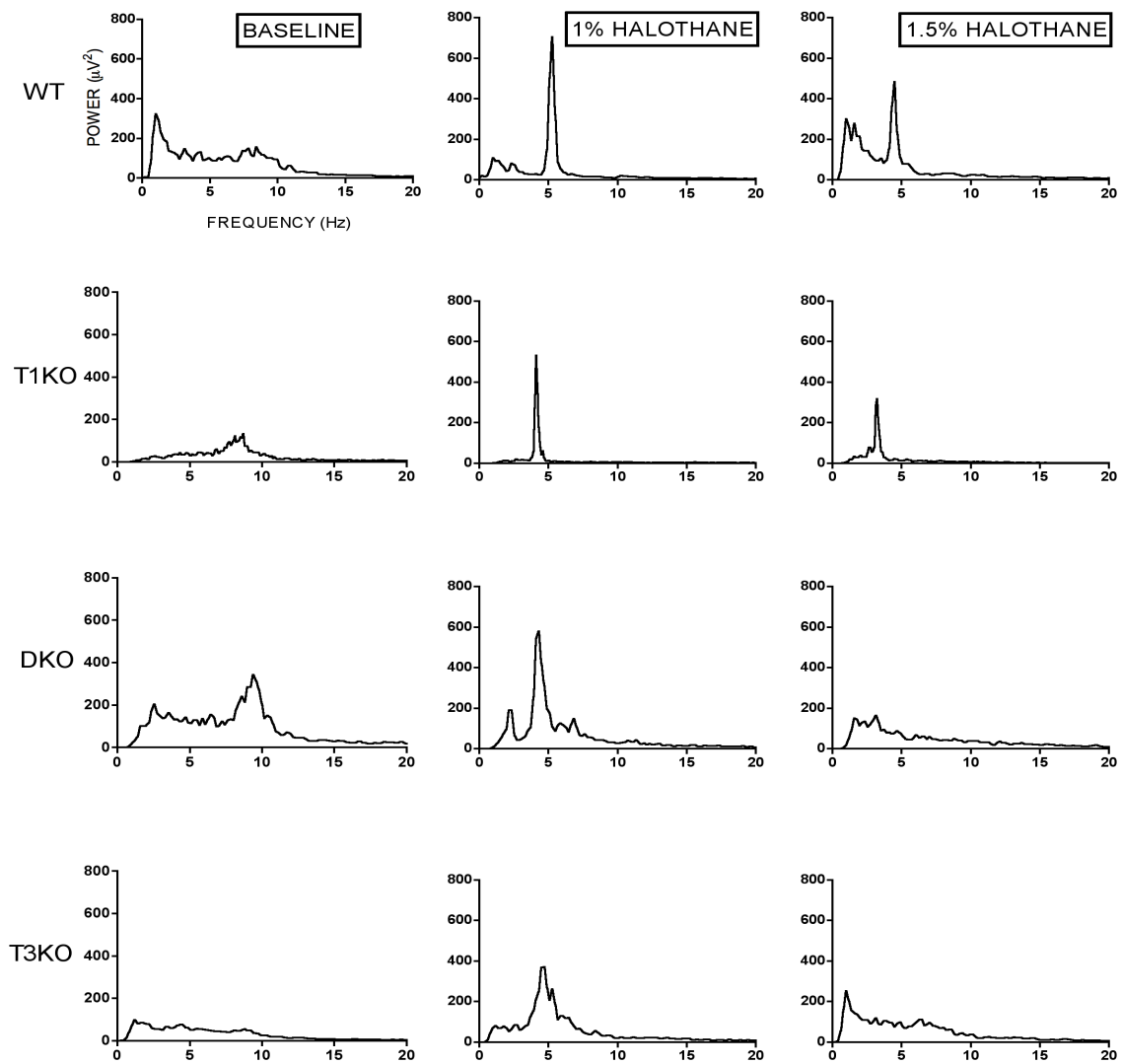


Figure 41. Results of exposure to an increasing concentration of halothane in 4 strains of mice instrumented with a Neurologger. Each graph is a representative example of one animal. The WT strain shows the induction of a type 2 theta oscillation by exposure to a clinically relevant concentration of halothane. The T1KO strain shows a similar response. The DKO and T3KO strains differ from WT and T1KO in the shape of peak produced as a result of halothane exposure. Quantification of this response was performed by fitting a Lorentzian curve to identified peaks (see below).

Exposure to halothane in each strain induces a change in the power spectrum derived from the cortical EEG. The induction of a peak centred at approximately 5 Hz (within the theta

oscillation range) is visible for each strain studied, but the quality of this peak varies. It is narrower and better defined (more “tuned”) in the WT and T1KO strains, becoming slightly broader in the DKO strain, and even more so in the T3KO strain (Figure 41). As the concentration of halothane is increased to a supraclinical level the peak is reduced in size and frequency (WT and T1KO) or disappears entirely (DKO and T3KO). In order to quantify these observed responses to halothane, identifiable peaks in the power spectra were fitted with a Lorentzian curve.

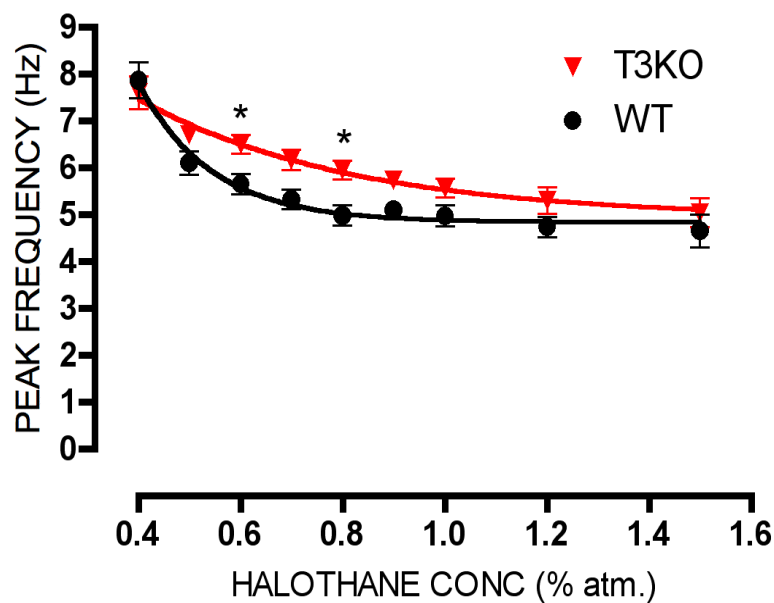


Figure 42. Type 2 theta oscillation peak frequency decreases in WT and T3KO as halothane concentration increases, with the highest frequency occurring at the lowest concentration of halothane administered. Significant differences in peak frequency occurred at 0.6% and 0.8% ($p < 0.05$). Sample sizes were: WT, $n=8$; T3KO, $n=4$. Error bars represent SEM. A single exponential curve was fitted to discrete data points. Data were analysed with 2-way ANOVA with repeated measures followed by Bonferroni *post hoc* tests.

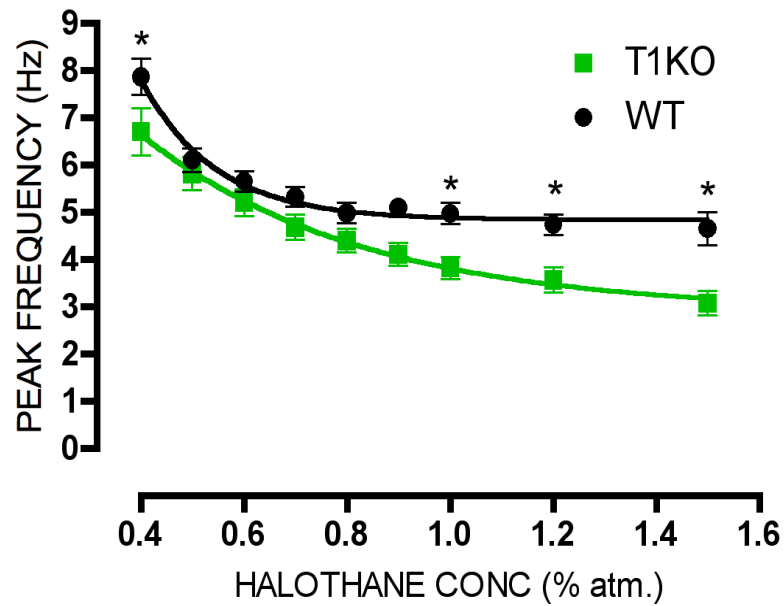


Figure 43. Type 2 theta oscillation peak frequency decreases in WT and T1KO as halothane concentration increases, with the highest frequency occurring at the lowest concentration of halothane administered. Significant differences in peak frequency occurred at 0.4%, 1%, 1.2% ($p < 0.05$) and 1.5% ($p < 0.001$). Sample sizes were: WT, $n=8$; T1KO, $n=6$. Error bars represent SEM. A single exponential curve was fitted to discrete data points. Data were analysed with 2-way ANOVA with repeated measures followed by Bonferroni *post hoc* tests.

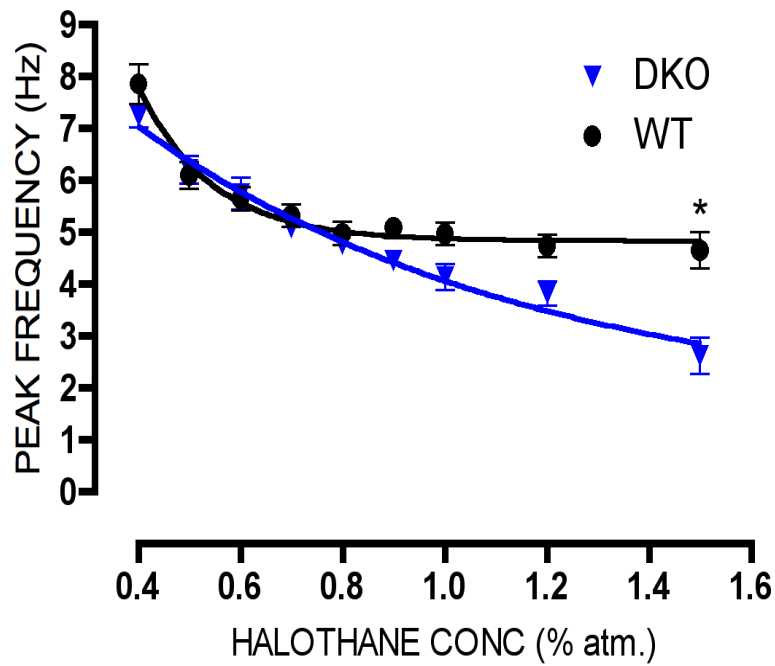


Figure 44. Type 2 theta oscillation peak frequency decreases in WT and DKO as halothane concentration increases, with the highest frequency occurring at the lowest concentration of halothane administered. Significant differences in peak frequency occurred at 1.5% ($p < 0.001$). Sample sizes were: WT, $n=8$; DKO, $n=3$. Error bars represent SEM. A single exponential curve was fitted to discrete data points. Data were analysed with 2-way ANOVA with repeated measures followed by Bonferroni *post hoc* tests.

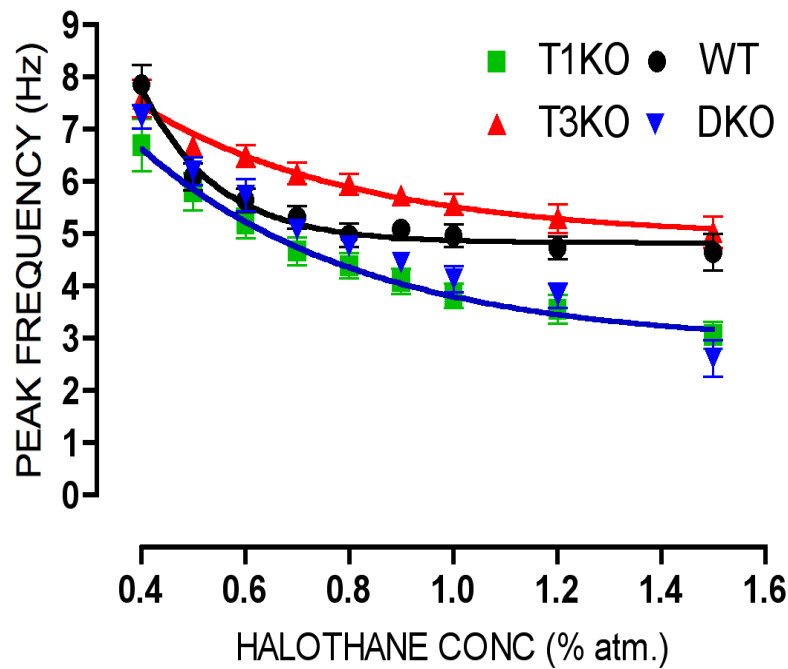


Figure 45. Type 2 theta oscillation peak frequency decreases in WT, T3KO, T1KO and DKO as halothane concentration increases, with the highest frequency occurring at the lowest concentration of halothane administered. Significant differences in peak frequency occurred between the following strains and concentrations (in addition to the differences listed in the previous 3 figures): T3KO vs. T1KO, all concentrations between 0.6 - 1.5% inclusive ($p < 0.001$). T3KO vs. DKO, 0.9% and 1.0% ($p < 0.05$), 1.2% ($p < 0.01$) and 1.5% ($p < 0.001$). Sample sizes were: WT, $n=8$; T1KO, $n=6$; DKO, $n=3$; T3KO, $n=4$. Error bars represent SEM. A single exponential curve was fitted to discrete data points. Data were analysed with 2-way ANOVA with repeated measures followed by Bonferroni *post hoc* tests.

The peak frequency data (Figures 42-45) show a progression in peak position from a sub-anaesthetic (sub-LORR) concentration to a concentration greater than that required for LORR. The initial theta oscillation peak appeared at approximately 7 Hz in all strains, then differed in the rate of decrease for each strain until exposure to the final, highest halothane concentration. The response in the T1KO and DKO strains were very similar, exhibiting the lowest peak frequency at the highest halothane concentration (3.1 ± 0.3 Hz and 2.6 ± 0.3 Hz,

respectively). WT and T3KO strains had the smallest changes in peak frequencies, both culminating in peak frequencies of approximately 5 Hz.

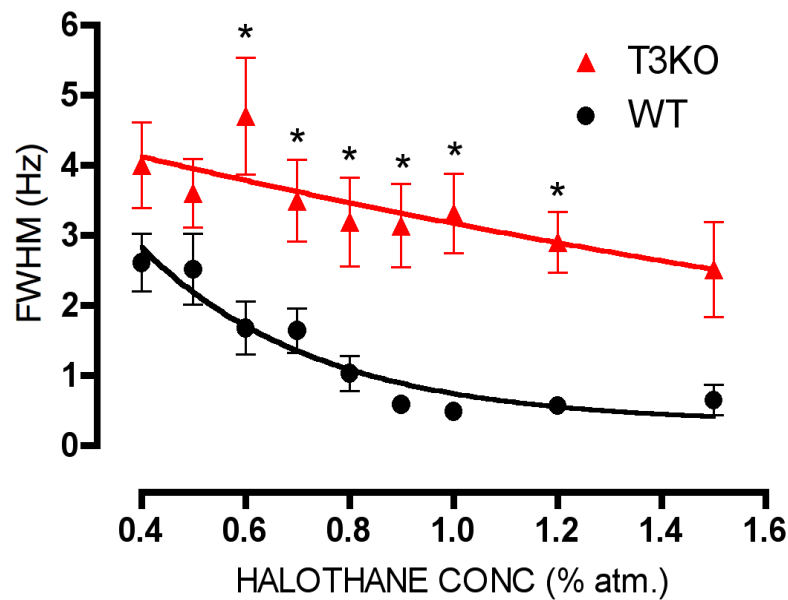


Figure 46. Full width at half-magnitude (FWHM) decreases in WT and T3KO strains as halothane concentration increases, reflecting the narrowing of the theta oscillation generated. Significant differences in FWHM occurred at 0.6% ($p < 0.001$), 0.7% ($p < 0.05$), 0.8% ($p < 0.01$), 0.9% and 1.0% ($p < 0.001$), 1.2% ($p < 0.01$). Sample sizes were: WT, $n=8$; T3KO, $n=4$. Error bars represent SEM. A single exponential curve was fitted to discrete data points. Data were analysed with 2-way ANOVA with repeated measures followed by Bonferroni *post hoc* tests.

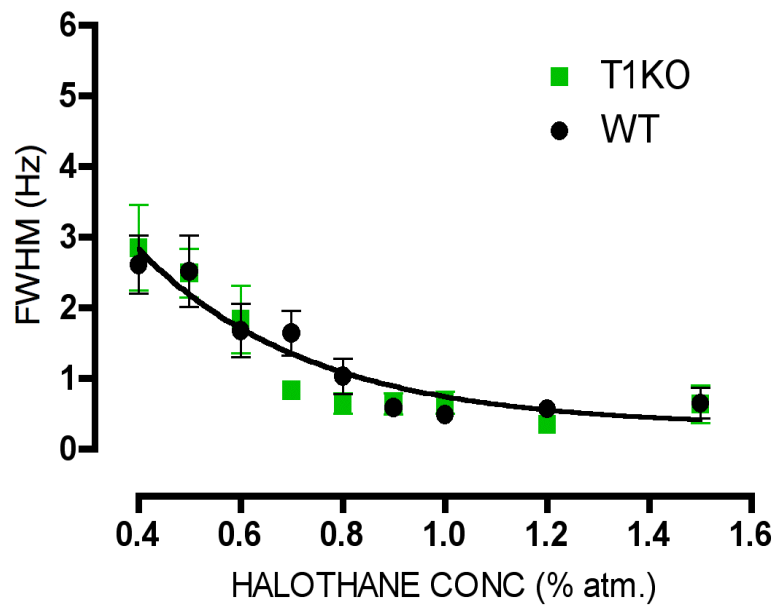


Figure 47. Full width at half-magnitude (FWHM) decreases in WT and T1KO strains as halothane concentration increases, reflecting the narrowing of the theta oscillation generated. There were no significant differences in FWHM between WT and T1KO at any concentration. Sample sizes were: WT, n=8; T1KO, n=6. Error bars represent SEM. A single exponential curve was fitted to discrete data points. Data were analysed with 2-way ANOVA with repeated measures followed by Bonferroni *post hoc* tests.

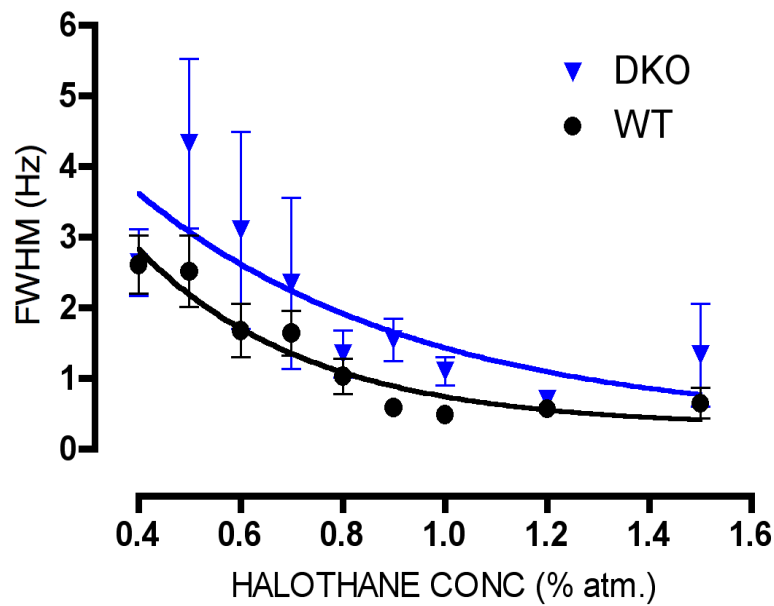


Figure 48. Full width at half-magnitude (FWHM) decreases in WT and DKO strains as halothane concentration increases, reflecting the narrowing of the theta oscillation generated. There were no significant differences in FWHM between WT and T1KO at any concentration. Sample sizes were: WT, $n=8$; DKO, $n=3$. Error bars represent SEM. A single exponential curve was fitted to discrete data points. Data were analysed with 2-way ANOVA with repeated measures followed by Bonferroni *post hoc* tests.

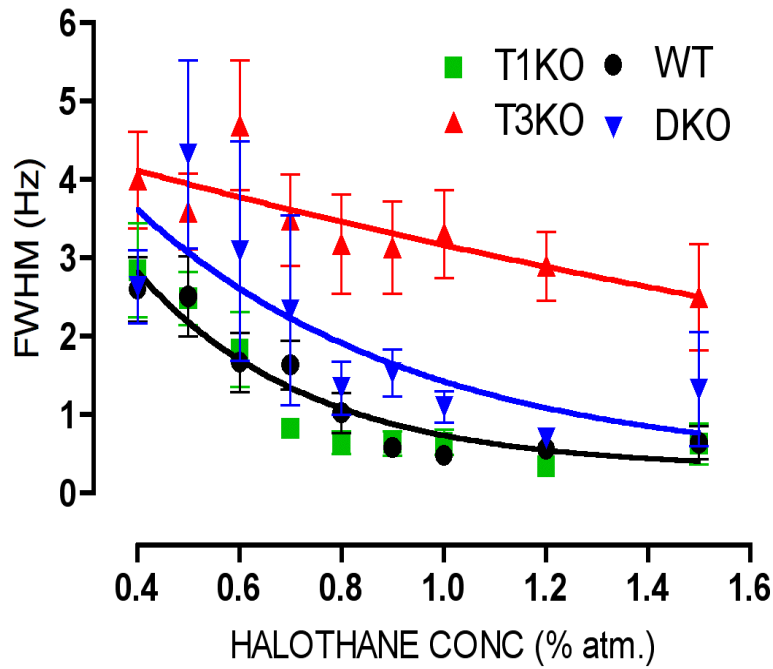


Figure 49. Full width at half-magnitude (FWHM) decreases in each strain as halothane concentration increases, reflecting the narrowing of the theta oscillation generated. The decrease in FWHM across the range of halothane concentrations was least for T3KO (0.4%, 4.0 ± 0.6 Hz ; 1.5%, 2.5 ± 0.7 Hz). The values of FWHM for WT, DKO and T1KO at 0.4% and 1.5% halothane were: WT, 2.6 ± 0.4 and 0.6 ± 0.2 Hz; DKO, 2.6 ± 0.5 and 1.3 ± 0.7 Hz; T1KO, 2.8 ± 0.6 and 0.6 ± 0.3 Hz. Sample sizes were: WT, n=8; T1KO, n=6; DKO, n=3; T3KO, n=4. Error bars represent SEM. A single exponential curve was fitted to discrete data points. Data were analysed with 2-way ANOVA with repeated measures followed by Bonferroni *post hoc* tests.

The basic pattern of a narrowing peak was visible in all strains (Figures 46-49), with the WT and T1KO showing a near identical narrowing as the halothane concentration was increased. The T3KO strain showed the least change in shape of the theta oscillation peak, with a FWHM value of 4 ± 0.6 Hz decreasing to 2.5 ± 0.7 Hz at the highest concentration of halothane. This 40% decrease is small compared to the approximately 80% decrease in T1KO and WT strains. Again, this reflects the pattern seen in the raw data (Figure 41), where

the theta oscillation peak remains broad in the T3KO strain. The DKO strain falls between these extremes, with a 50% decrease in FWHM over the range of concentrations studied.

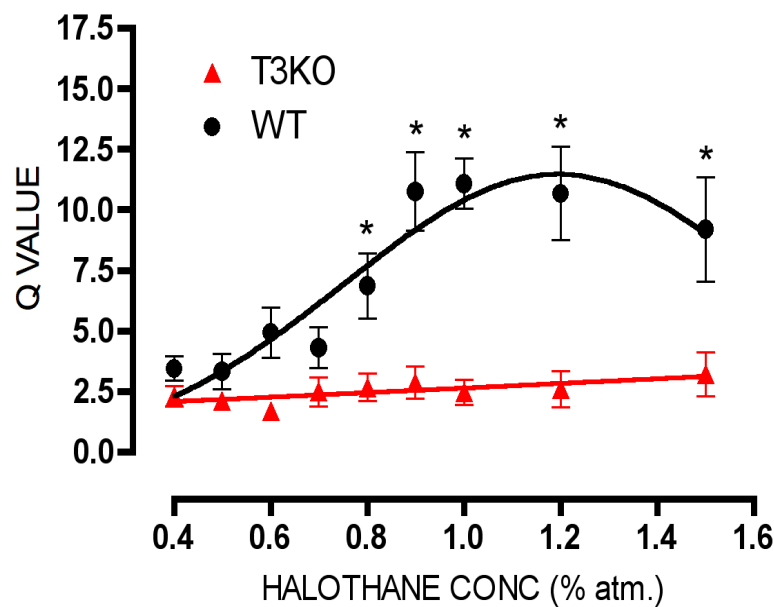


Figure 50. The Q value for WT and T3KO. It is calculated from peak frequency/ full width at half-magnitude, and reflects the “sharpness” of the theta oscillation. Significant differences were identified between WT and T3KO strains at 0.8% ($p < 0.05$), 0.9%, 1.0%, 1.2% ($p < 0.001$) and 1.5% ($p < 0.01$). Sample sizes were: WT, $n=8$; T3KO, $n=4$. Error bars represent SEM. A Gaussian curve was fitted to discrete data points. Data were analysed with 2-way ANOVA with repeated measures followed by Bonferroni *post hoc* tests.

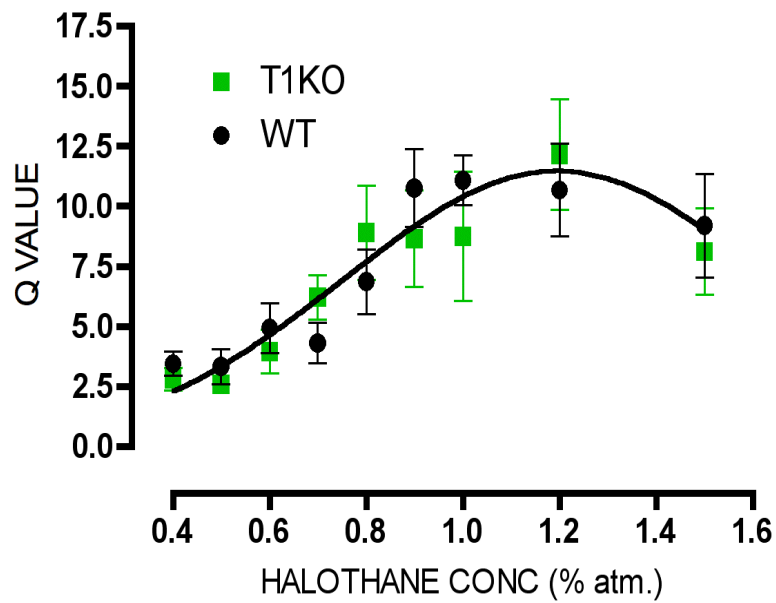


Figure 51. The Q value for WT and T1KO. It is calculated from peak frequency/ full width at half-magnitude, and reflects the “sharpness” of the theta oscillation. No significant differences were identified at any concentration between WT and T1KO strains. Sample sizes were: WT, n=8; T1KO, n=6. Error bars represent SEM. A Gaussian curve was fitted to discrete data points. Data were analysed with 2-way ANOVA with repeated measures followed by Bonferroni *post hoc* tests.

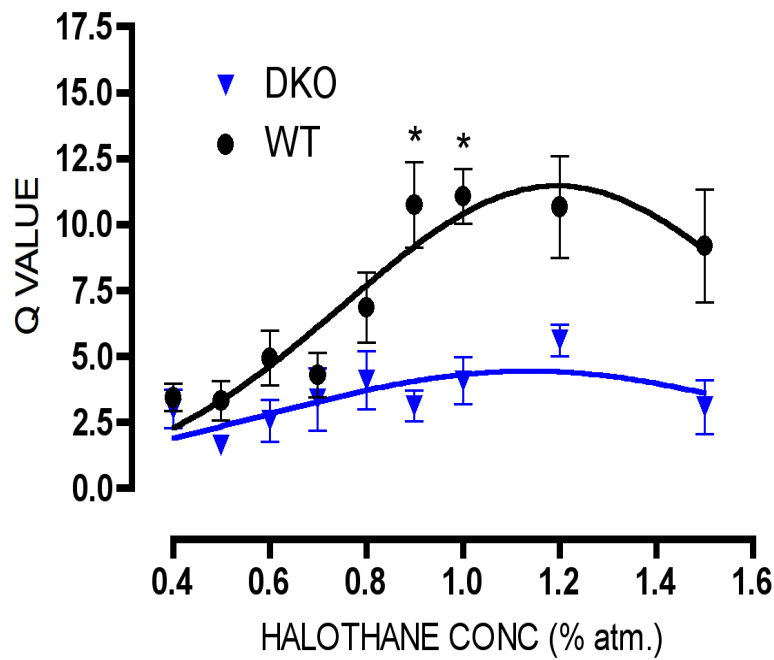


Figure 52. The Q value for WT and DKO. It is calculated from peak frequency/ full width at half-magnitude, and reflects the “sharpness” of the theta oscillation. Significant differences were identified between WT and DKO strains at 0.9% and 1.0 ($p < 0.01$). Sample sizes were: WT, $n=8$; DKO, $n=3$. Error bars represent SEM. A Gaussian curve was fitted to discrete data points. Data were analysed with 2-way ANOVA with repeated measures followed by Bonferroni *post hoc* tests.

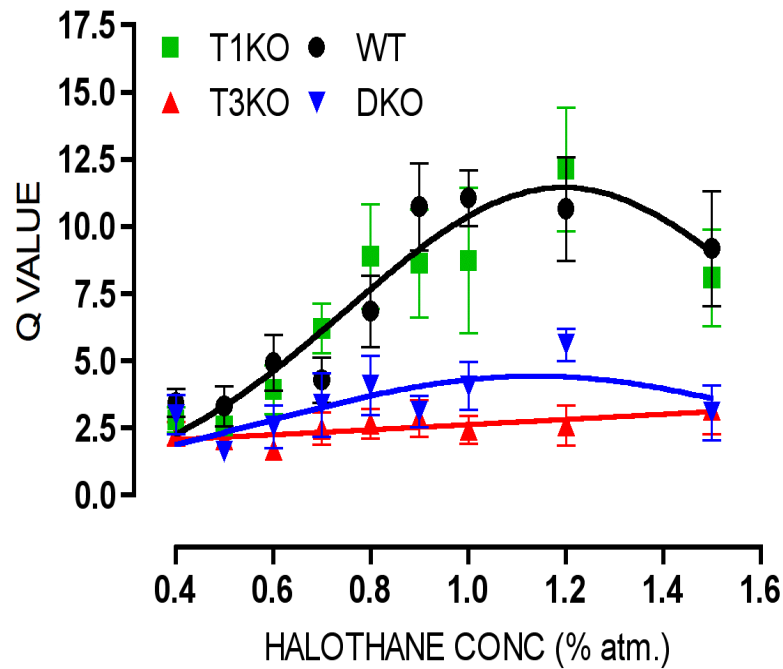


Figure 53. The Q value for all strains. The strains studied can be divided into two groups, those showing a gradual increase in Q value up to 1.2% halothane (WT and T1KO) and those showing a little change over the range of halothane concentrations (DKO and T3KO). WT and T1KO strains had a three to four fold increase in Q value between 0.4 - 1.2% (WT 0.4 - 1.2%, $3.5 \pm 0.5 - 10.7 \pm 2.0$; T1KO 0.4 - 1.2%, $2.8 \pm 0.5 - 12.1 \pm 2.3$). There was no significant change over the same range for DKO and T3KO strains. Significant differences were identified between the following strains and concentrations (in addition to the differences listed in the previous 3 figures): T3KO vs. T1KO 0.8%, 0.9%, 1.0%, 1.2% ($p < 0.001$), 1.5% ($p < 0.01$). T1KO v DKO: 1.2% ($p < 0.05$). There were no significant differences between T3KO and DKO strains. Sample sizes were: WT, $n=8$; T1KO, $n=6$; DKO, $n=3$; T3KO, $n=4$. Error bars represent SEM. A Gaussian curve was fitted to discrete data points. Data were analysed with 2-way ANOVA with repeated measures followed by Bonferroni *post hoc* tests.

The Q value result highlights the difference between strains, splitting them into two groups (Figures 50-53). The smallest change in Q value occurred with the T3KO and DKO strains,

and the greatest change with the WT and T1KO strains. This confirms the picture presented in the power spectra data (Figure 41), with WT and T1KO showing near identical changes over the range of halothane concentrations, whereas T3KO, and to a lesser extent DKO, did not develop such a finely tuned theta oscillation peak. For these data, the Q value is particularly influenced by the marked difference in FWHM between T3KO and the other strains. The fitting of a Lorentzian curve to the power spectra enabled an objective comparison of the response to halothane exposure between strains. These data showed the near-identical type 2 theta oscillation induced by halothane in T1KO and WT strains, the marked absence of a sharply tuned theta peak in the T3KO strain and an intermediate response in the DKO strain. Furthermore, the results of the T3KO response repeat previous findings in our laboratory (Robledo 2008).

3.2.2 Atropine sulphate

The anticholinergic (muscarinic antagonist) agent atropine sulphate has been shown to block the generation of type 2 theta oscillations and this effect was originally described as a defining feature of the type 2 theta oscillation, “atropine-sensitive theta” (Kramis et al. 1975). This sensitivity to atropine sulphate distinguished type 2 from the atropine-resistant type 1 theta oscillation (Kramis et al. 1975). Furthermore, both cholinergic input from the MS/ DBB is an essential component in the generation of the theta oscillation (Yoder & Pang 2005). Bland et al. have shown the abolishment of a halothane-induced theta oscillation in rats (Bland et al. 2003). The aim of our experiment was to investigate the effect of systemic atropine sulphate administration on the halothane-induced theta oscillation in WT and T1KO strains, which produce a theta oscillation upon exposure to halothane, and the effect on the cortical EEG in the T3KO strain. Abolishment of the theta oscillation in WT and T1KO would provide further evidence that halothane-induced theta is a type 2 theta oscillation. In this experiment, a 10 minute baseline period was followed by IP administration of 50 mg kg⁻¹ atropine sulphate and exposure to an increasing concentration of halothane. The peak power for an identifiable peak within the type 2 theta range of frequencies was normalised to control (Figure 54) and the percentage change in the type 2 theta oscillation between control and atropine conditions calculated for each concentration of halothane.

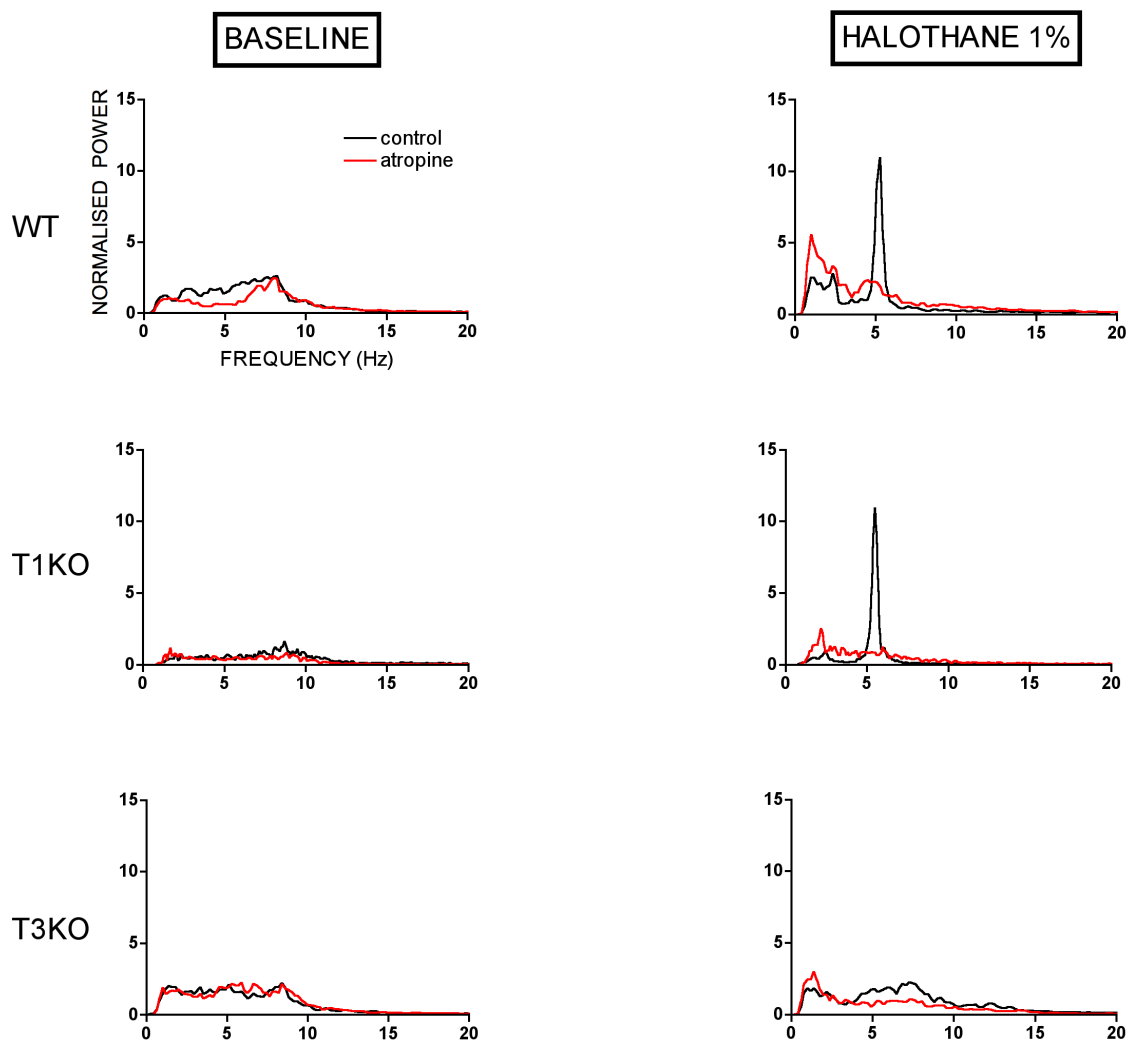


Figure 54. The effect of atropine sulphate (50 mg kg⁻¹, IP) on WT, T1KO and T3KO strains with and without 1% halothane; representative data. The data were normalised to the control condition in the absence of atropine. The characteristic halothane-induced peak, centred around 5 Hz, is visible in the WT and T1KO strains and absent in the T3KO strain. During the baseline period (no halothane), there was no theta oscillation and no

effect of atropine administration on the cortical EEG. In contrast, the presence of atropine sulphate prevented induction of the theta oscillation in the WT and T1KO strains. $n=4$ for each strain.

The type 2 theta oscillation is clearly visible as a peak centred around 5 Hz in the power spectrum of WT and T1KO strains. In these strains it was abolished by the administration of 50 mg kg⁻¹ atropine. In T3KO, there is no clear Type 2 theta oscillation in the presence of halothane and the administration of atropine does not significantly change the appearance of the power spectrum. These findings are similar to those of Bland et al. in the rat (Bland et al. 2003).

To quantify the effects of atropine sulphate administration in the presence and absence of anesthetic agent for each strain studied, the peak power following atropine sulphate administration was compared with the corresponding control period (Table 5).

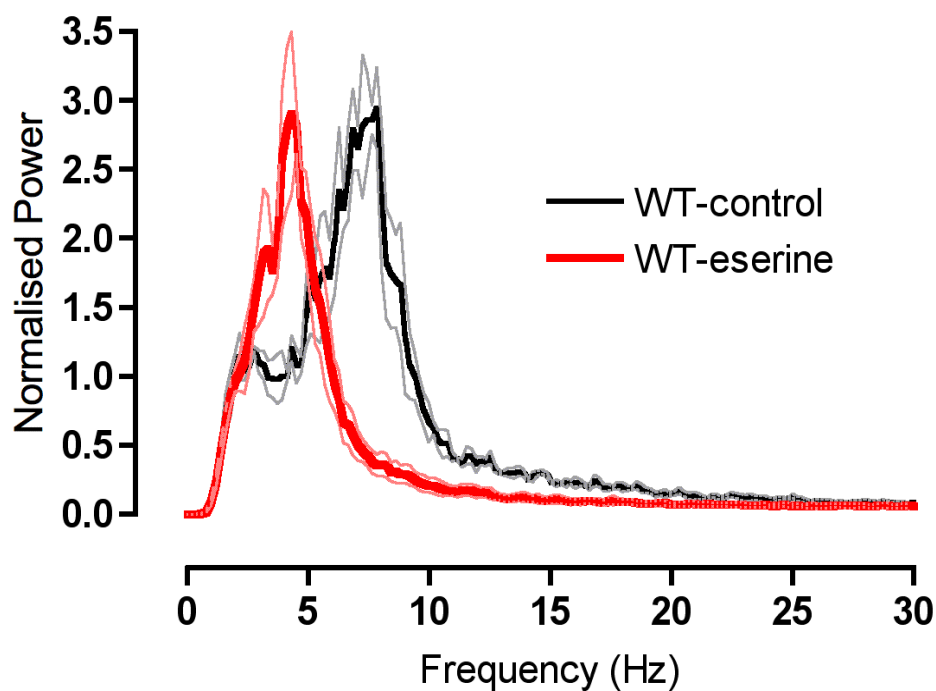
	WT	T1KO	T3KO
Baseline + atropine	84.89	70.74	83.94
halothane 1% + atropine	16.79	14.17	82.43

Table 5. Changes in peak power following atropine administration with and without halothane. Values are the mean percentage change ($n=4$ for each strain) relative to control condition (for each strain with and without halothane), following atropine administration. During exposure to 1% halothane, there is a large decrease of approximately 60% in the peak power following atropine sulphate administration compared with control conditions for WT and T1KO strains. There is no change associated with atropine administration in the T3KO strain.

These data illustrate the changes observed in the power spectra (Figure 54), quantifying the large reduction in theta oscillation in the WT and T1KO strains, and absence of effect on the T3KO power spectrum.

3.2.3 Eserine

The anticholinesterase agent eserine, also known as physostigmine, has been shown to induce type 2 theta oscillations in the rat and mouse (Leung & Vanderwolf 1980; Tebano et al. 1999). Its mechanism of action, prevention of the breakdown of ACh, provides an alternative means of investigating the role of cholinergic input in generation of the type 2 theta oscillation. Although it has been reported that eserine does not effect the generation of the type 1 theta oscillation, its administration is associated with immobility, reducing the likelihood of observing type 1 theta in the spontaneously moving animal (Leung & Vanderwolf 1980). Eserine (0.2 mg kg^{-1} , IP) or an equal volume of saline was administered systemically to mice (WT and T3KO) instrumented with the Neurologger (Figure 55).



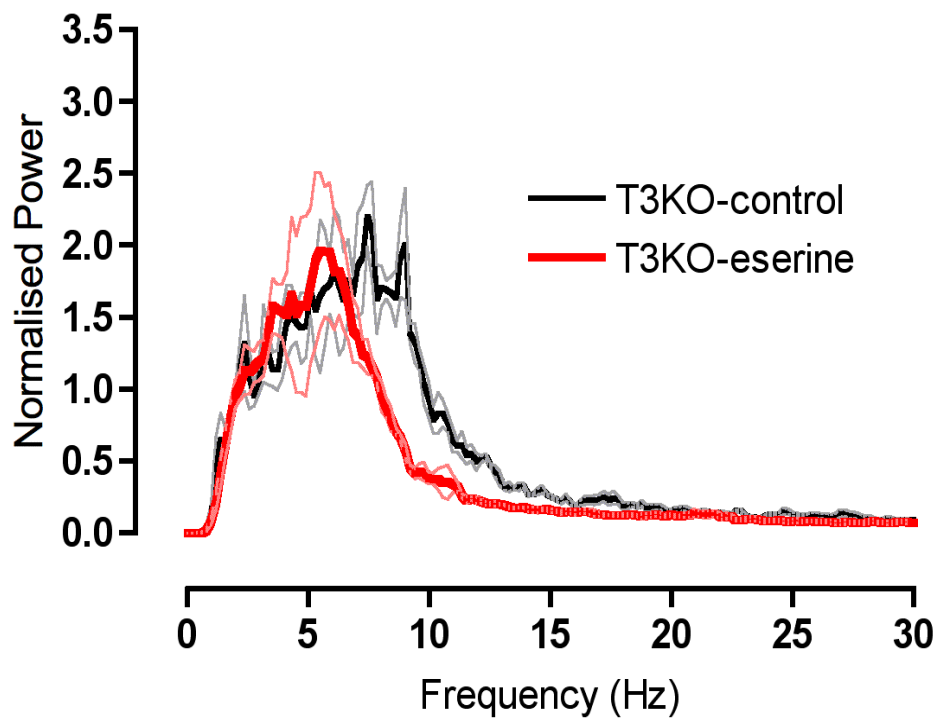


Figure 55. Power spectra with normalised power for WT and T3KO strains ($n=3$ for each strain) with and without eserine (0.2 mgkg^{-1} , IP). The traces in bold represent the mean and the fainter traces, the standard error for each strain. The administration of eserine to WT animals induced a theta oscillation peak centred around 4.5Hz. In this strain, eserine administration resulted in a $270 \pm 60\%$ increase in theta power ($p<0.05$). In contrast, in the T3KO strain there was no definable theta oscillation peak induced compared to the control condition.

These data provide further evidence of the role of cholinergic inputs in the generation of the type 2 theta oscillation, and that this pathway is non-functional in the T3KO strain.

3.3 Mechanistic phenotypes - characterisation of the TASK3 knockout (T3KO) action potential in hippocampal CA1 pyramidal cells

Following the results of the *in vivo* work, showing the difference in cortical EEG between mouse strains in response to exposure to halothane, it was logical to begin the pursuit of the underlying mechanisms of this difference. The decision to compare T3KO and WT strains was based on several factors: the greatest difference in type 2 theta oscillation was between the T3KO strain and WT, TASK3 mRNA is normally expressed in the CA1 area of the hippocampus in WT animals and absent in the T3KO strain (Brickley et al. 2007), there is a large body of evidence that the septohippocampal formation is crucial for theta oscillation generation and that CA1 pyramidal cells form the major contribution to the recorded cortical EEG (Buzsaki 2002; Vertes et al. 2004).

The distinctive anatomy of the hippocampus and clearly defined stratum pyramidale containing the soma of pyramidal neurones made identification of the appropriate region relatively straightforward (Figure 56).

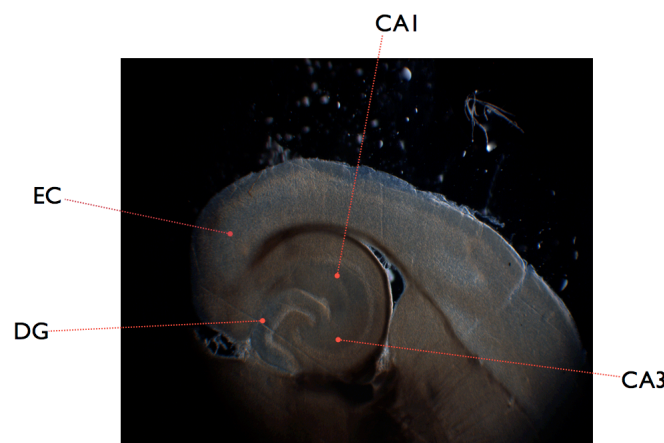


Figure 56. Brightfield low power image of a brain slice with pertinent structures labelled: EC, entorhinal cortex; DG, dentate gyrus; CA 1 and 3, cornu ammonis areas 1 and 3 respectively.

3.3.1 Resting membrane potential

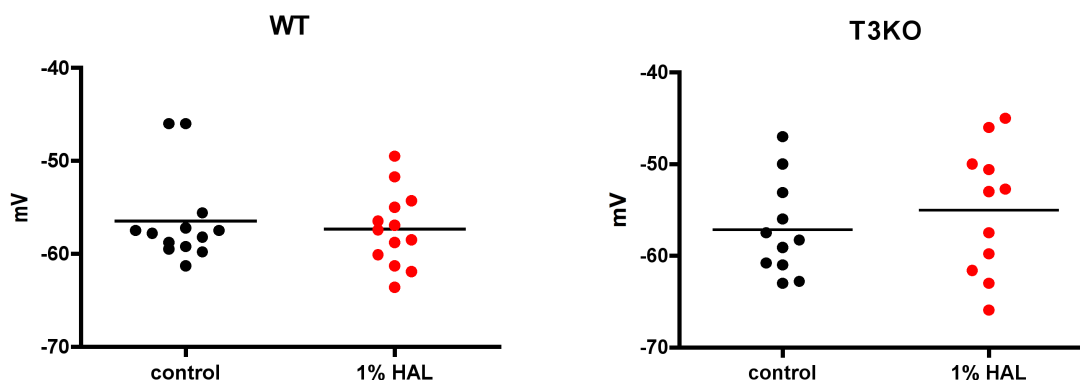


Figure 57. Scatter plots showing the resting membrane potential (RMP; the horizontal black bar is the mean, and filled circles represent individual data points) for WT (n=13) and T3KO (n=11) strains under control conditions and during exposure to 1% halothane (HAL). No current is being injected into the cell. There was no significant difference in resting membrane potential between strains or conditions. Values of RMP were: WT control -56.5 ± 1.3 mV; WT halothane -57.4 ± 1.1 mV; T3KO control -57.2 ± 1.6 mV; T3KO halothane -55.0 ± 2.1 mV.

The lack of change in resting membrane potential (RMP) during halothane exposure, compared with control conditions (Figure 57) was similar to the findings of Nishikawa and MacIver (Nishikawa & MacIver 2000). They did not record a significant change in rat CA1 pyramidal cells upon exposure to 0.35 mM halothane. The lack of difference in the T3KO strain was surprising given the potential contribution of the TASK3 channel to setting the RMP, and in contrast to the findings of Lazarenko et al. (Lazarenko et al. 2010) who found T3KO cells to be approximately 4% more depolarised, albeit in motoneurons.

Whole cell parameters

Capacitance

There was no difference between strains under control conditions. The mean $\pm$ SEM capacitance for WT was 132.9 ± 17.1 pF, and for T3KO it was 128.0 ± 17.2 pF.

Membrane time constant

There was no difference in the sub-threshold membrane time constant (Figure 58) between strains or within a strain under control conditions or during halothane application.

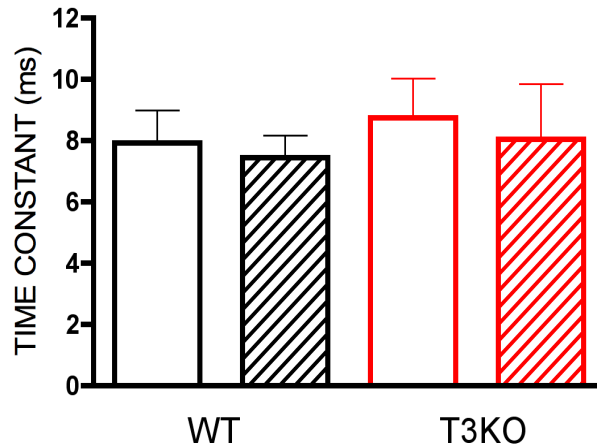


Figure 58. Membrane time constant for the sub-threshold response to a brief current injection. There was no difference between strains or conditions. Shaded bars are results of halothane application. Control conditions: WT 7.9 ± 1.1 ms (n=14), T3KO 8.8 ± 1.3 ms (n=9). Halothane: WT 7.4 ± 0.7 ms (n=12), T3KO 8.0 ± 1.8 ms (n=5). Data were subjected to an unpaired t test. Error bars represent SEM.

Current required to attain AP threshold

The current required to achieve AP threshold increased with exposure to halothane in the WT (Figure 59). There was no change between control or halothane conditions in the T3KO strain. Interestingly, Brickley et al., examining the electrophysiological characteristics in cerebellar granule cells using the same knockout strain, reported that the T3KO strain fired APs at a significantly lower current injection than WT, a reflection of the more depolarised resting membrane potential in the T3KO strain (Brickley et al. 2007). The lack of difference in the current injection required to achieve AP threshold between strains in this study probably reflects the similar resting membrane potential. We did identify an increase in the current required to achieve AP threshold during halothane exposure in WT but not in T3KO which may reflect an increased input conductance associated with halothane activation of TASK-3 channels in the WT.

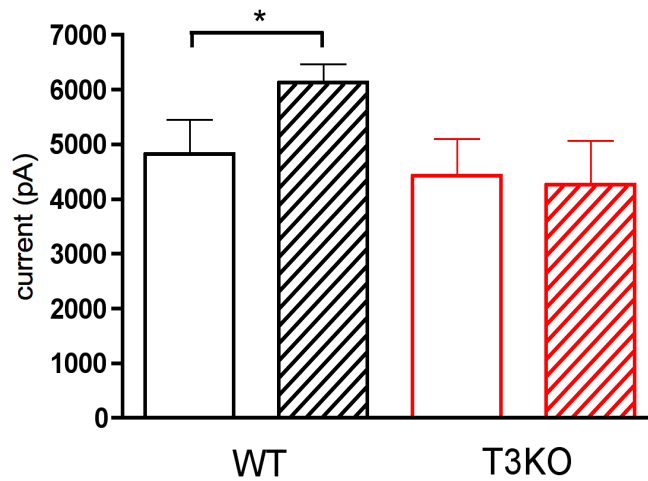


Figure 59. Current required to achieve threshold was significantly greater in WT upon exposure to 1% halothane ($p = 0.014$). The addition of halothane did not affect the current required to achieve threshold in the T3KO strain ($p = 0.35$). There was no difference between strains under control conditions ($p = 0.33$). Data were compared with paired (pre- and post-halothane) and unpaired (WT versus T3KO control) t tests. Sample sizes were $n = 11$ for WT and $n = 8$ for T3KO. Error bars represent SEM.

3.3.2 Action potential

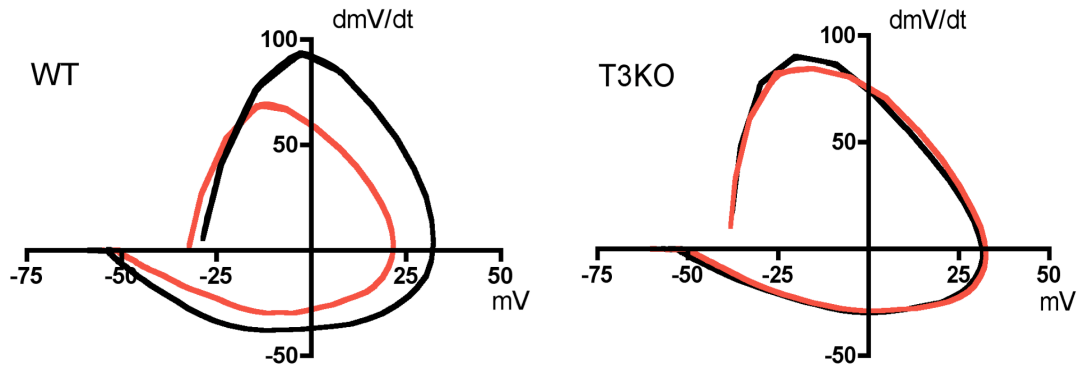


Figure 60. Examples of phase plane plots from T3KO and WT animals constructed from action potentials during current clamp. In each case, the black curve represents control and the red curve exposure to 1% halothane. Action potentials were elicited with a brief pulse. The brief pulse was a depolarising incremental increase in voltage, in steps of 1 mV, starting at 3 mV, with a duration of 0.2 ms.

The phase plane plots (Figure 60) show at a glance that there is no difference in the AP stimulated under control or halothane conditions for the T3KO strain, whereas there is a clear difference for WT.

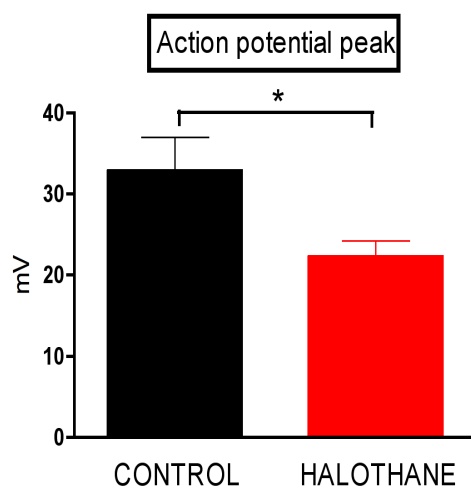
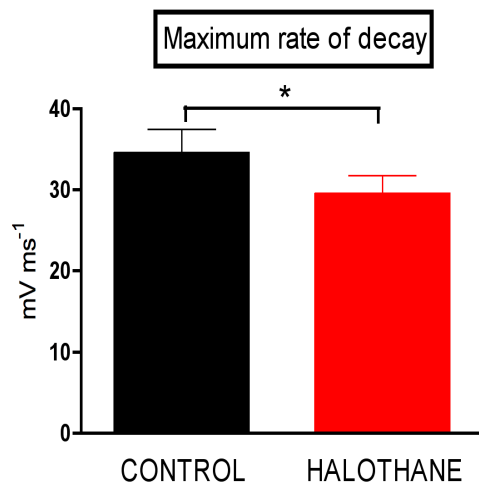
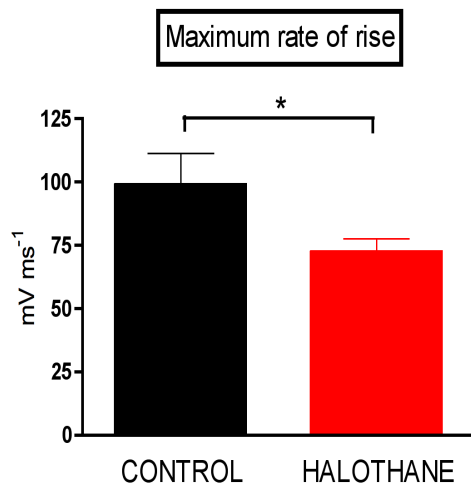


Figure 61. The effect of exposure to 1% halothane (approximately 0.40 mM) on the maximum rate of rise, maximum rate of decay and action potential peak height in acute brain slices from WT animals (n=8). There are significant decreases in each parameter following exposure to halothane. The maximum rate of rise decreased from 98.9 ± 12.7 to 72.5 ± 5.2 mV ms⁻¹ (p<0.05), the maximum rate of decay decreased from 34.5 ± 3.0 to 29.5 ± 2.3 mV ms⁻¹ (p<0.05), and the action potential peak decreased from 32.9 ± 4.2 to 22.3 ± 2.0 mV (p<0.05). Data were compared with a paired t test.

The application of halothane resulted in a reduction in the maximal rate of rise, rate of decay and peak action potential height in the WT strain (Figure 61). The decrease in these parameters was 27%, 15% and 32% respectively. Halothane application did not result in a change in these parameters in the T3KO strain (Figure 62).

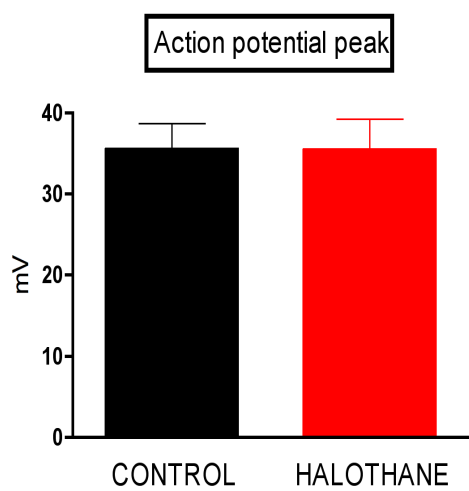
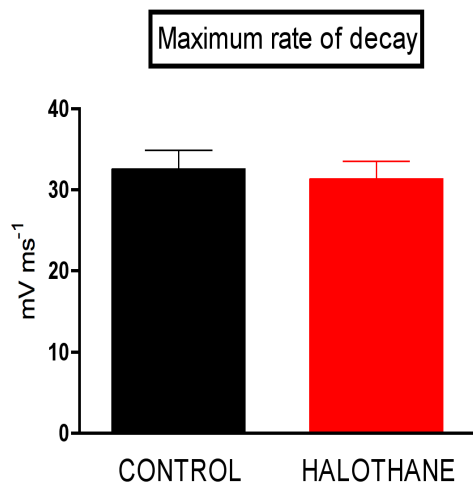
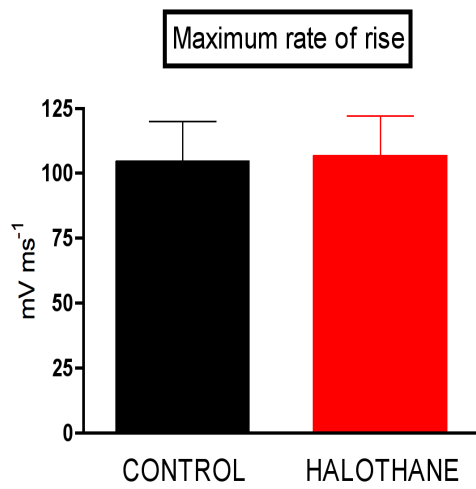


Figure 62. The effect of exposure to 1% halothane (approximately 0.40 mM) on the maximum rate of rise, maximum rate of decay and action potential peak height in acute brain slices from T3KO animals (n=7, control; n=6, halothane). There are no significant decreases in each parameter following exposure to halothane. The maximum rate of rise was 104.5 ± 15.6 and 106.5 ± 15.6 mV ms⁻¹ during control and halothane conditions, respectively. The maximum rate of decay was 32.5 ± 2.4 and 31.3 ± 2.3 mV ms⁻¹ during control and halothane conditions, respectively. The action potential peak was 35.5 ± 3.2 and 35.4 ± 3.8 mV, respectively.

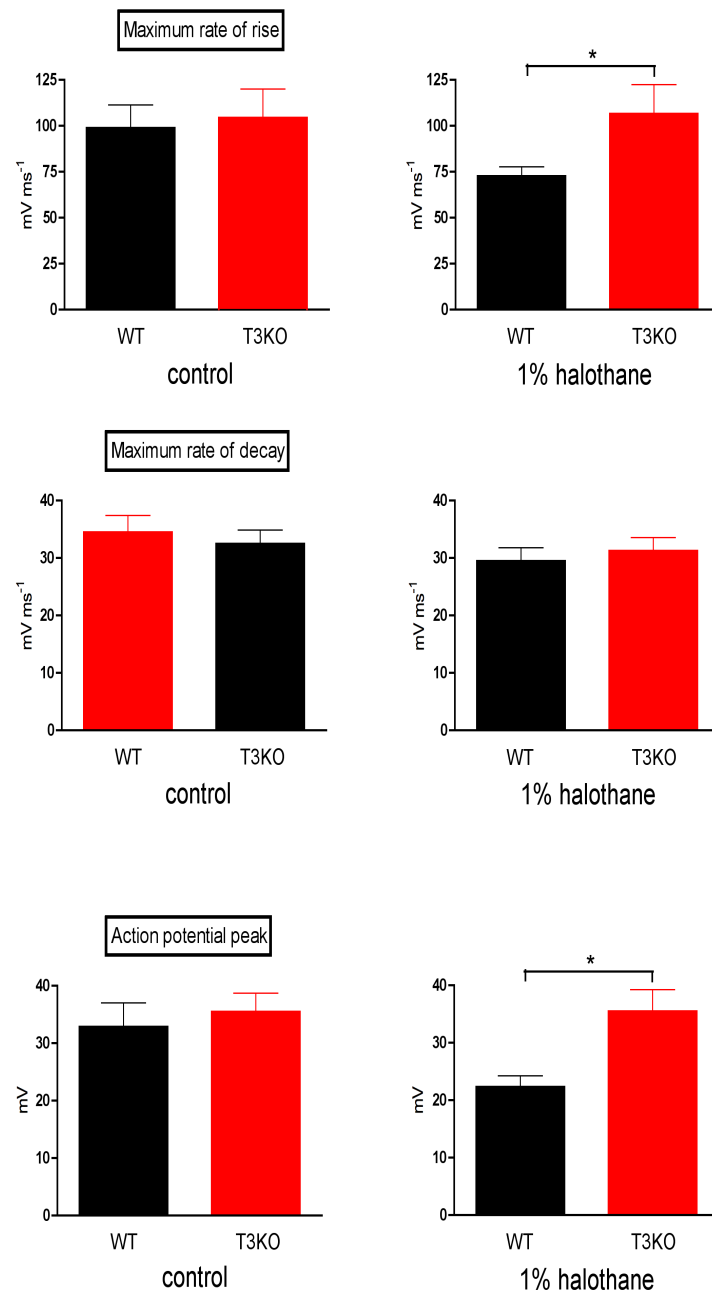


Figure 63. A comparison of maximum rate of rise, maximum rate of decay and action potential peak between WT (black bars) and T3KO (red bars) under control conditions (left column) and exposure to 1% halothane (right column). The mean $\pm$ SEM values are as reported in the previous two figures. During exposure to halothane, WT slices exhibited a significantly slower maximum rate of rise ($p < 0.05$) and action potential peak ($p < 0.01$) compared with T3KO slices.

Under control conditions there was no difference between strains in maximum rate of rise, maximum rate of decay or the action potential peak (Figure 63, left column). In contrast, exposure to 1% halothane (Figure 63, right column) resulted in a lower maximum rate of rise (WT $72.5 \pm 5.2 \text{ mV ms}^{-1}$, T3KO $106.5 \pm 15.6 \text{ mV ms}^{-1}$) and action potential peak (WT $22.3 \pm 2.0 \text{ mV}$, T3KO $35.4 \pm 3.8 \text{ mV}$) in WT compared with T3KO. There was no difference between strains in the maximum rate of decay achieved (WT $29.5 \pm 2.3 \text{ mV ms}^{-1}$, T3KO $31.3 \pm 2.3 \text{ mV ms}^{-1}$).

3.3.3 Sustained AP firing

Current-frequency curves (Figure 64 and Figure 65) were drawn for each strain under control and halothane conditions with the cells allowed to sit at their resting membrane potential and whilst held at -60 mV. A similar pattern was visible for each condition; in each case the WT strain was able to sustain AP firing at a greater current injection than the T3KO strain. This difference was more pronounced during exposure to halothane, when the T3KO strain exhibited a deterioration in APs beyond approximately 400 pA of current, whilst WT was able to sustain AP firing until over 550 pA of current injection.

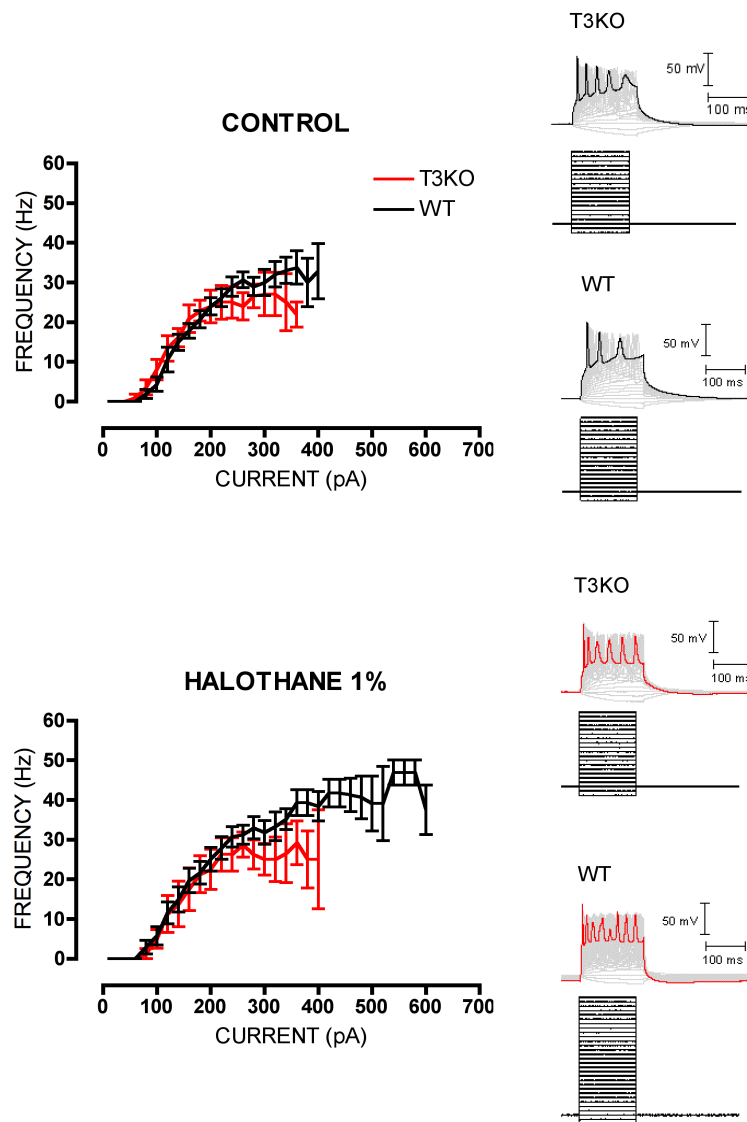


Figure 64. Current - frequency (I-f) curves constructed for WT and T3KO pyramidal cells at rest, during control conditions and exposure to 1% halothane. The frequency count included action potentials up to the point at which action potential height deteriorated to less than 75% of the height of the first action potential in a train. During exposure to 1% halothane, WT pyramidal cells were able to sustain AP firing at a higher level of current injection. The rate of firing did not differ between strains at the equal levels of current injection and there was no strain difference at the point of initial AP firing. WT, n=11. T3KO, n=8. Inset graphs show example data for control (black) and halothane (red) conditions. The ability of the WT to sustain APs is noticeable from the greater number of

current steps tolerated. The long pulse protocol was an incremental increase in current steps of 20 pA, starting at -40 pA, with a duration of 160 ms.

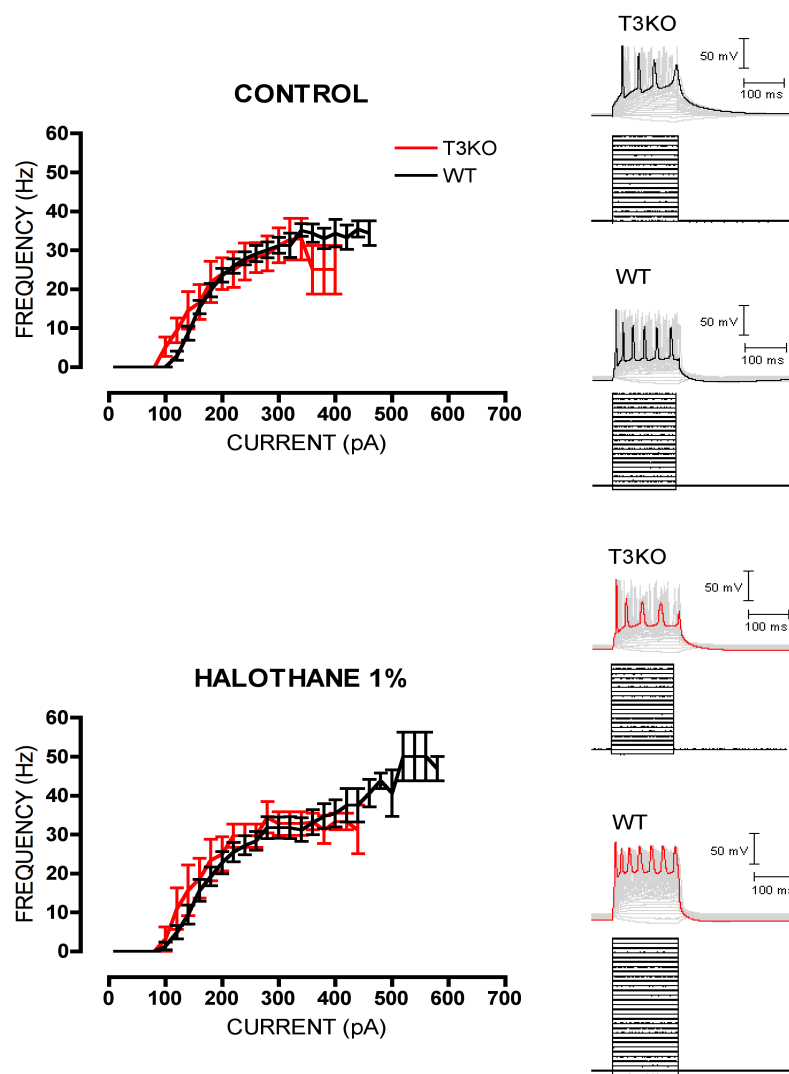


Figure 65. Current - frequency (I-f) curves constructed for WT and T3KO pyramidal cells held at -60 mV (row B), during control conditions and exposure to 1% halothane. The frequency count included action potentials up to the point at which action potential height deteriorated to less than 75% of the height of the first action potential in a train. During exposure to 1% halothane, WT pyramidal cells were able to sustain AP firing at a higher level of current injection. The rate of firing did not differ between strains at the equal

levels of current injection and there was no strain difference at the point of initial AP firing. WT, n=11. T3KO, n=8. Inset graphs show example data for control (black) and halothane (red) conditions. The ability of the WT to sustain APs is noticeable from the greater number of current steps tolerated. The long pulse protocol was an incremental increase in current steps of 20 pA, starting at -40 pA, with a duration of 160 ms.

These data are similar to findings by Brickley et al. who reported a decreased ability to fire sustained APs by cerebellar granule cells in a T3KO strain compared with WT (Brickley et al. 2007). They postulated that the reduced input conductance associated with the TASK-3 channel knockout resulted in greater inactivation of voltage gated sodium channels, limiting the ability to sustain AP firing. Nishikawa and MacIver did not find an increase in AP firing upon exposure to halothane, though their protocol used a single current injection step unlike the depolarising steps we employed (Nishikawa & MacIver 2000).

3.3.4 sIPSC decay time and area are increased, and frequency decreased by halothane



Figure 66. Typical examples of sIPSCs from WT and T3KO strains under control conditions and during halothane application.

The following data result from the average of 400 sIPSCs manually identified for each experiment. Typical individual examples are shown in Figure 66. The application of halothane significantly slowed the IPSC decay for each strain (Figure 67). The increase in time constant was 15% for WT and 32% for T3KO. There was no difference between strains during control or halothane conditions. Similarly, with application of a similar halothane concentration in rat CA1 pyramidal cells, Nishikawa and MacIver (2000) reported that the recorded stimulus evoked (at the Schaffer collateral pathway) mIPSCs showed an increase in the decay time constant (Nishikawa & MacIver 2000).

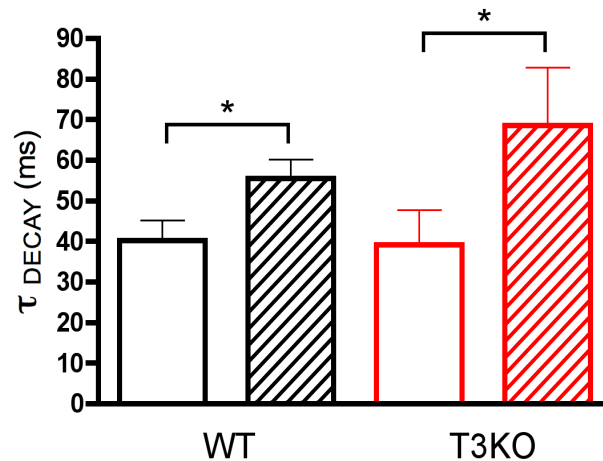


Figure 67. Bar graph of sIPSC decay time constant for WT and T3KO. Shaded bars are results for 1% halothane application. There was a significantly slower time constant under control conditions for both strains ($p < 0.05$). Control conditions: WT 40.3 ± 4.9 ms,

T3KO 32.2 ± 7.0 ms. Halothane: WT 55.5 ± 4.7 ms, T3KO 42.7 ± 13.0 ms. Data were subjected to a paired t test. Error bars represent SEM.

The area under the sIPSC curve did not differ between strains (Figure 68). The application of halothane resulted in a 40% increased area under the curve in the WT strain. A similar change in the mean values (58% increase) occurred in the T3KO strain though this did not achieve significance.

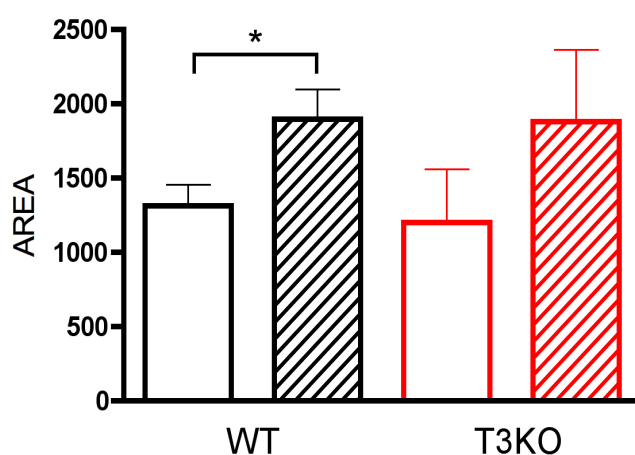


Figure 68. Bar graph of sIPSC area under the curve for WT and T3KO. Shaded bars are results for 1% halothane application. There was a significantly smaller area under the curve under control conditions for WT ($p < 0.05$). Control conditions: WT 1317.0 ± 138.0 , T3KO 1469.0 ± 340.0 . Halothane: WT 1897.0 ± 202.0 , T3KO 2333.0 ± 251.1 . Data were subjected to a paired t test. Error bars represent SEM.

The frequency of sIPSCs did not differ between strains under control or halothane conditions (Figure 69). However there was an effect of halothane in each strain. In the WT halothane application resulted in a 40% decrease in IPSC frequency, and in the T3KO strain a similar (30%) change in mean values occurred though this did not achieve significance. In contrast, Nishikawa and MacIver (2000) reported an increase in mIPSC frequency with the application of halothane (Nishikawa & MacIver 2000). That study included AMPA and NMDA receptor

antagonists and a sodium channel blocker in the internal solution which may have contributed to the difference in findings.

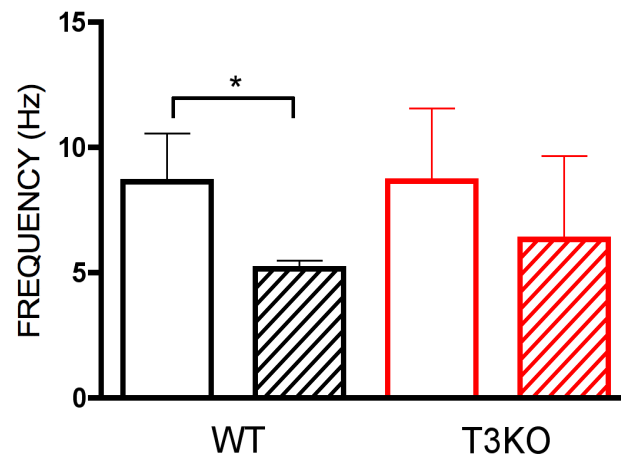


Figure 69. Bar graph of sIPSC frequency for WT and T3KO. Shaded bars are results for 1% halothane application. There was a significantly lower frequency during halothane application for WT ($p < 0.05$). Control conditions: WT 8.6 ± 2.0 Hz, T3KO 8.7 ± 3.0 Hz. Halothane: WT 5.2 ± 0.3 Hz, T3KO 6.3 ± 3.3 Hz. Data were subjected to a paired t test. Error bars represent SEM.

There was no difference in sIPSC peak height (Figure 70) between strains or within a strain under control conditions or during halothane application. An equipotent halothane concentration applied to CA1 pyramidal cells of the rat did not alter IPSC amplitude (Lukatch & MacIver 1997).

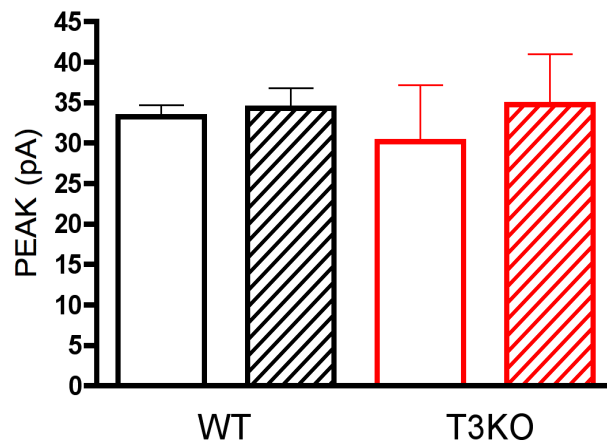


Figure 70. Bar graph of sIPSC peak height for WT and T3KO. Shaded bars are results for 1% halothane application. There is no difference between strains or treatment conditions. WT n=6, T3KO n=4. Control conditions: WT 33.3 ± 1.5 pA, T3KO 30.2 ± 7.0 pA. Halothane: WT 34.3 ± 2.5 ms, T3KO 34.8 ± 6.2 ms. Data were subjected to a paired t test. Error bars represent SEM.

4.0 Discussion

This study was broadly divided into two sections. The first, covering the *in vivo* work, investigated the anaesthetic sensitivity, cortical EEG response and sleep behaviours of three strains of transgenic mice. We have shown that the TASK1 and 3 channels are targets for the volatile anaesthetic halothane, the cortical EEG response to halothane exposure is markedly altered in the T3KO strain, and the structure of sleep is changed in terms of transitions between the active and quiet periods (T3KO) and distribution of activity between these periods (T1KO). Additionally, our results with the DKO strain highlight the possibility of compensation occurring when global knockout animals are created.

The second section, examining the underlying mechanism of the theta oscillation through acute brain slice preparations, showed there was no difference in the RMP between WT or T3KO strains, confirmed the effect of halothane in WT hippocampal CA1 pyramidal cells and the contribution of the TASK3 channel to input conductance, and identified a role for TASK3 channels in sustaining action potential firing.

4.1 Loss of righting reflex (LORR)

4.1.2 Loss of consciousness (LOC) versus LORR

General anaesthesia has been traditionally defined as a triad of loss of consciousness, analgesia and muscle relaxation. This definition originated from a sentence at the end of a paper examining the properties of methyl-N-propyl ether in the 1950s (Rees & Gray 1950) and was rapidly accepted (Gray & Rees 1952). It simply described the desirable outcomes of a general anaesthetic when delivered to facilitate surgery. Though this definition suits its intended purpose, it brings a degree of confusion to the investigation of general anaesthesia as anaesthetic agents have variable efficacy in each component of the triad. For example, ketamine (in humans) given alone can induce loss of consciousness and analgesia but provides poor muscle relaxation. From the standpoint of research into mechanisms of action, a simpler

definition has been recently proposed, the “reversible, drug-induced loss of consciousness”(Franks 2006). This clear endpoint eliminates confusion, provided loss of consciousness can be clearly identified. Comparing the effects of increasing concentrations of anaesthetics would involve measurable end-points. Loss of consciousness in humans is typically identified by the failure to respond to a verbal command such as “open your eyes”(Bonhomme et al. 2000). Loss of righting reflex in rodents is used as a surrogate measure of loss of consciousness and a positive correlation has been shown between these measures (Figure 71; Franks 2008).

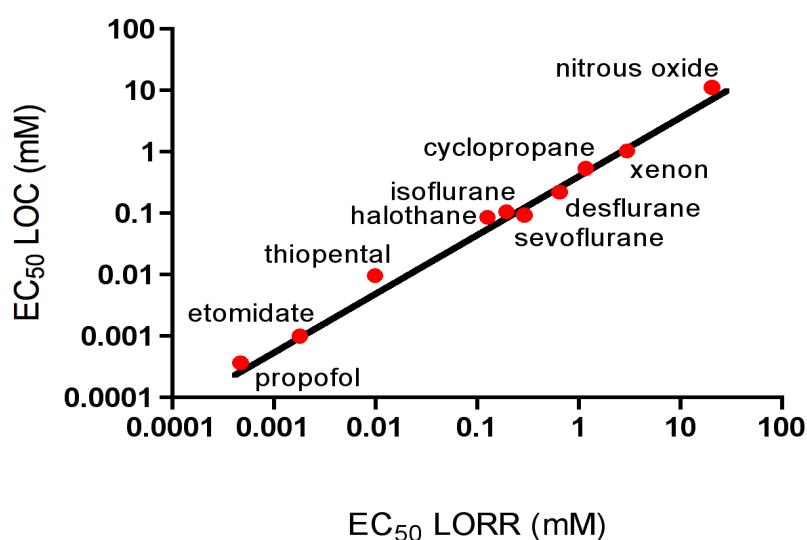


Figure 71. The relationship in EC₅₀ between loss of consciousness (LOC) in humans (defined as failure to respond to a verbal command) and loss of righting reflex (LORR) in rats and mice. There is a clear correlation between LOC in humans and LORR in rodents for both injectable and inhalational anaesthetic agents. Concentrations are for aqueous solution at 37°C. Graph re-drawn from data provided in Franks (2008) (Franks 2008).

4.1.3 LORR and TASK channel knockout mice

Linden et al (Linden et al. 2006) tested LORR in a T1KO mouse with halothane (Figure 72). Contrary to our findings, they reported no significant difference between the T1KO (mean approximately 0.85% atm.) and wild type (mean approximately 0.9% atm. Figure 5).

Comparisons of LORR data should be done carefully as there is no standardised methodology or definition of LORR. The definition used in our laboratory is that all 4 feet are off the ground for a period of at least 30 seconds. Some of the methodological differences may explain the differing results in LORR in T1KO reported by Linden et al. (2006).

Compared to our methodology for testing LORR, Linden et al. (2006) tested the righting reflex every 2 minutes (versus every 10 minutes) with each test consisting of placing the mouse in a supine position on 3 consecutive occasions. If the animal was unable to right itself during each trial it was scored as a loss of righting reflex and the anaesthetic concentration recorded. It is not stated if the manipulation of the animal was performed by rotating the chamber (as in our studies) or picking up the mouse directly. It is also unclear what duration the animal had to remain in a supine position for the result to be scored as a LORR, or if all four feet had to be off the ground. Such differences in methodology could explain the observed differences in anaesthetic sensitivity between studies.

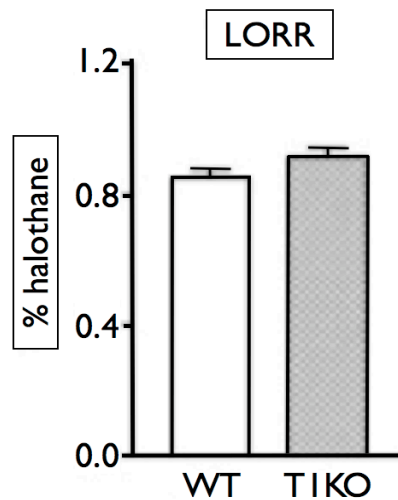


Figure 72. Halothane sensitivity measured with a loss of righting reflex assay (LORR) in T1KO (n=14-20) and wild type (n=14-20) mice. There was no significant difference between strains. These data differ from ours in that we found a significantly higher concentration of halothane is required to achieve LORR in T1KO mice (Figure 31). Re-drawn from Linden et al. (Linden et al. 2006).

A separate paper by Linden et al. (2007) investigated the anaesthetic sensitivity of the T3KO strain (Linden et al. 2007). A LORR assay was not performed but results for a loss of tail withdrawal (LOTW) assay were reported. A significantly higher concentration of halothane was required ($p < 0.01$) for LOTW in knockout (approximately 1.5% atm.) compared with wild type (approximately 1.3% atm.) mice. It is difficult to infer effects on LORR from these data as LOTW requires a nociceptive response, and it is not therefore possible to separate potential alterations in LORR from the response to a nociceptive stimulus.

Another group, investigating the sensitivity of T1KO, T3KO and DKO to halothane (and isoflurane) described slightly different results from our findings (Lazarenko et al. 2010; Table 6).

	Our study	Lazarenko et al. (2010)
Wild type	0.68 ± 0.02	0.70 ± 0.01
T1KO	0.78 ± 0.01*	0.84 ± 0.01*
T3KO	0.94 ± 0.02*	0.83 ± 0.01*
DKO	0.70 ± 0.01	0.81 ± 0.02*

Table 6. EC₅₀ values (mean ± SEM) for halothane (% atm.) reported by Lazarenko et al. (Lazarenko et al. 2010) compared with those of our study using a loss of righting reflex assay. *Represents a significant difference compared to wild type (p<0.05). Lazarenko et al. (2010) found a decreased sensitivity to halothane in all knockout strains. This is in contrast to our findings, of a decrease in halothane sensitivity in T1KO and T3KO compared with WT, but no difference for DKO. Furthermore, we found a significant difference between T1KO and T3KO, with T3KO requiring a higher concentration of halothane to achieve LORR. n>6 was used for each strain by Lazarenko et al. (2010).

They reported that all strains of knockout animals were less sensitive to halothane than wild type, but without differences between strains. The most striking differences compared with our results was the difference in halothane sensitivity identified in T3KO and DKO. In T3KO, whilst we found an EC₅₀ of 0.94% atm., they reported 0.83% atm., a value similar to T1KO. Their findings in the DKO strain (EC₅₀= 0.81% atm.) did not differ from the other knockout strains, whereas we found an EC₅₀ of 0.70% atm., no different from wild type. There are two key differences in the study of Lazarenko et al. (2010) compared with ours. Firstly, the methodology employed by Lazarenko et al. (2010) was based on that of Quinlan et al. (Quinlan et al. 1998). This methodology differed from ours in that mice were exposed to a single concentration of anaesthetic agent and allowed to recover before being subjected to the next concentration. Information is not provided regarding the nature of loss of righting reflex assessment, particularly the number of tests at each concentration, the definition of loss of righting reflex and whether the test chamber was rotated manually or motor-driven and whether this was performed intermittently or continuously. Despite these methodological differences, the similar halothane sensitivity reported for the wild type strains used would suggest that the explanation for differing results lies elsewhere. Secondly, the generation of the knockout animals differs. In creating the mouse employed by Lazarenko et al., exon 2 of the

TASK3 gene was removed (by flanking with LoxP), leaving behind exon 1 (Mulkey et al. 2007; Lazarenko et al. 2010). Though both exons are required to code a fully functioning channel, there is recent evidence that partial transcription of a two-pore domain potassium channel (TREK1 was used) can interact with and affect intact subunits (Veale et al. 2010). Such a truncated protein reduced the native wild type channel activity and expression, an example of a dominant negative. In TASK1 and TASK3 channels, exon 1 codes for the first transmembrane domain and part of the P1 loop and exon 2 codes the remaining channel components (Vega-Saenz de Miera et al. 2001; Kim et al. 2000). Therefore, it is possible that the remaining exon 1 in the mouse of Lazarenko et al. (2010) successfully coded a truncated protein which may have affected the function of the remaining non-targeted TASK channel (TASK1 or 3 depending on the knockout). The creation of truncated proteins also raises the intriguing possibility that such proteins formed dimers with remaining K_{2P} channels in the DKO strain, negatively affecting their function.

In contrast, our animals (and those of Linden et al. (Linden et al. 2006, 2007)) were created with multiple inverted insertions of exon 1 at the TASK3 locus in the T3KO strain, and disruption of exon 1 at the TASK1 locus in the T1KO strain. This approach entirely prevents channel transcription. These differences in the creation of the knockout strains offer an explanation for the differing results between our studies.

4.1.4 LORR and cyclopropane

Following the results of the halothane LORR assay, it was logical to assess the T3KO strain against an anaesthetic agent known to not have an effect on the TASK3 channel. On this basis, cyclopropane was chosen (Gruss et al. 2004). Intriguingly, the T1KO strain was more sensitive to cyclopropane than the other strains tested (T3KO, DKO, WT; Figure 34).

These results indicate an effect of cyclopropane on the TASK1 channel. This mechanism is unlikely to be mediated through GABA_A receptors, which are upregulated in the T1KO (Linden et al. 2008), as cyclopropane does not act at this site either via potentiation of GABA or directly at the receptor (Raines et al. 2003). As predicted, there was no increase in alteration in sensitivity compared with WT in the T3KO strain.

4.1.5 LORR and sex

Our data clearly showed a significant effect of sex for the DKO and T1KO strains (Figure 32) with female mice (DKO 0.88 ± 0.04 % atm, T1KO 0.87 ± 0.04 % atm) showing a lesser sensitivity to halothane than male mice (DKO 0.70 ± 0.01 % atm, T1KO 0.78 ± 0.01 % atm). Linden et al. (Linden et al. 2006) did not find a significant sex effect in the T1KO strain with a LORR assay. As the genetic background of our strains is identical, this difference is likely due to methodological differences. Sex differences in anaesthetic sensitivity have been reported for injectable agents such as the neurosteroid alphaxalone (Fink et al. 1982) and these differences have been ascribed to a combination of pharmacokinetic and pharmacodynamic differences. The evidence for sex differences in sensitivity to volatile agents is less clear, largely as a result of underpowered clinical studies (Pleym et al. 2003). One mechanism through which sex could have an effect on volatile agent sensitivity is through cyclic regulation of GABA_A receptor populations during the oestrus cycle (Maguire et al. 2005). In order to explore this source of potential bias further, the oestrus cycle of mice would have to be monitored and data analysed according to the stage of oestrus.

4.1.6 LORR and inter-observer variability

Finally, given the variation in methodology used to assess LORR, and that our method involved manual rotation of the container containing the animal, it was important to assess inter-observer variability. Using data previously collected by AC in wild type animals, a comparison with data collected by DP showed near identical results (Figure 33). This demonstrates the repeatability of our technique, contributing to the consistency of data.

An alternative measure of LOC

A recent, alternative measure in rodents, the forced walking test has been proposed, with the purported advantage of identifying the moment of loss of consciousness as reflected in the

surrogate marker of loss of motion (Hwang et al. 2010). This loss of motion is measured using a triaxial accelerometer attached to a headstage. For the anaesthetic agents studied, propofol and a xylazine/ ketamine combination, the point of LORR did not differ statistically from the loss of motion.

4.2 TASK and sleep

Several automated sleep scoring systems are now available for use in mice (Brankack et al. 2010; Pack et al. 2007; Veasey et al. 2000). Most recently, a system also based on data from the Neurologger has been published (Brankack et al. 2010). This system used a supervised classifier technique (versus the threshold algorithms used in our study) and reported good agreement with manually scored cortical EEG and EMG data. An alternative approach is an interesting system scoring activity as sleep or wake based on the breaking of infra-red beams (Pack et al. 2007). The advantages of such a system are that there is no requirement for surgery and animal instrumentation and that it provides a quick method to screen animals for gross differences. The disadvantage is that it is non-specific and unable to identify different sleep states.

4.2.1 Sleep and handling stress

Great effort was made to ensure that animals were not disturbed during the sleep experiments as there is evidence that disruption to light: dark cycles effects the distribution of NREM and REM sleep (Deboer et al. 2007). Additionally, the recording period we used exceeded the 12:12h light: dark cycle in order to assess the possibility of handling stress affecting the initial period of recording. This became particularly important with the differences we observed in the transition between active and quiet periods between T3KO and the other strains. The effect of brief (5 minutes) periods of manual restraint on the ensuing light: dark cycle in rats showed an increase in total sleep over both periods, comprising of increased NREM sleep during both periods and increased REM sleep during the active (dark) period (Tang et al. 2007). Though the greatest difference appeared in the first hour after handling, the

differences were maintained over the remaining time. A study performed with C57BL/6 mice showed that 1 hour of manual restraint (in the middle of the quiet period) resulted in increased NREM and REM sleep during the following active period (Meerlo et al. 2001).

In our study, the transition into the second circadian cycle approximated that of the first most closely in the WT, followed by the DKO and T3KO strains. The behaviour of the TIKO animals differed in hours 25 - 29, compared with hours 1 - 5 (corresponding phases of the circadian cycle). It is not clear if the the greatest contributing factor is the effect of handling stress on the initial recording period, or the small number of animals studied.

The similar pattern of behaviour in the WT, DKO and T3KO animals may have resulted from our effort to perform the Neurologger switch several hours before the onset of the quiet period, that the restraint was brief (less than one minute), and recordings were always performed in each animal's home environment.

4.2.2 Sleep in the WT

The percentage time spent awake or in each sleep state during the active or quiet periods in WT mice were very similar to the findings of Brankack et al. (Brankack et al. 2010), who used an automated system based on Neurologger recordings, and Huber et al. (Huber et al. 2000), using manual scoring with a tethered system. Our data for WT during wake, NREM and REM sleep in the active and quiet periods were: active period; $64.1 \pm 3.4\%$ (wake), $33.1 \pm 2.9\%$ (NREM), $2.3 \pm 0.5\%$ (REM). Quiet period; $29.9 \pm 2.8\%$ (wake), $60.1 \pm 2.3\%$ (NREM), $9.1 \pm 0.7\%$ (REM). Those of Huber et al. (Huber et al. 2000) were: active period; $66.9 \pm 1.9\%$ (wake), $29.4 \pm 1.6\%$ (NREM), $3.6 \pm 0.4\%$ (REM). Quiet period; $35.9 \pm 1.0\%$ (wake), $54.4 \pm 0.7\%$ (NREM), $9.7 \pm 0.5\%$ (REM).

4.2.3 Sleep in the knockout strains

Other than T1KO, there was no difference for sleep or wake between strains during the active (dark) period (Figure 39). In contrast, the knockout strains spent more time awake and less time in NREM sleep than WT during the quiet (light) period (Figure 38).

Linden et al. studied the circadian rhythm of T3KO mice (Linden et al. 2007). Their findings differ significantly from ours in that T3KO mice exhibited a greater degree of activity during the active period compared with wild type animals, and there was no identifiable difference between groups (T3KO vs. WT) during the quiet period. It is difficult to directly compare findings between their study and ours as different methods were used to identify activity/wake. Whilst we used sleep scoring based on EMG and cortical EEG data, Linden et al. (Linden et al. 2007) used a photobeam system, with breaks of the photobeams indicating movement. Thus, as they describe it, this is a measure of locomotor activity rather than sleep. The other major difference was that the start of the recording period was at the beginning of the active period, opposite to our recording timing, which started towards the end of the active period. Though not commented on by the authors, similar to our findings, there was a greater mean activity in the T3KO strain, albeit non significant, at the transition between active and quiet periods (Figure 73).

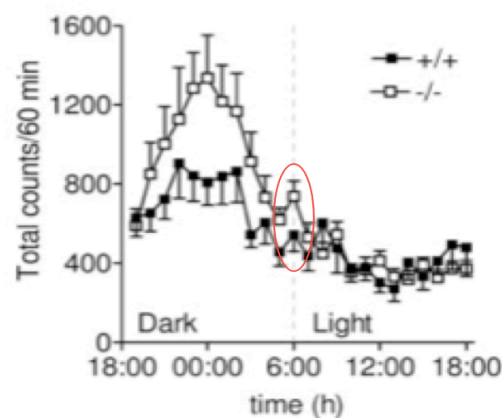


Figure 73. Circadian activity in T3KO (open squares, -/-) and wild type (closed squares, +/+) mice over a 24 hour period, beginning in the active (dark) period. Activity was classified using a photobeam system, generating counts as the photobeams were broken by animal movement. The red oval encircles the transition from active to quiet period. Similar to our

study, these data show the T3KO strain taking longer to reduce its activity levels (a surrogate marker for wakefulness) compared with WT. Modified from Linden et al. (Linden et al. 2007).

The near equal distribution of wakefulness and sleep states across the active and quiet periods in the T1KO strain was an interesting finding (Figure 40). The balance between wake and sleep is seated in the hypothalamus, as first postulated by von Economo (von Economo 1930). Current thinking regarding the initiation of sleep is that it is a combination of passive and active processes. A passive entry into sleep results from the gradual accumulation of activity-dependent metabolites such as adenosine, (Liu & Gao 2007; Porkka-Heiskanen et al. 1997), the synthesis of which is associated with the increased metabolic rate of wakefulness, with accumulation dampening the activity of wake-promoting neurones of arousal centres (see below). These neurones project on to and inhibit thalamic reticular neurones. The resultant disinhibition frees thalamic reticular neurones to inhibit thalamo-cortical relay neurones, severing communication of sensory information between the thalamus and cortex (cortical deafferentation) and resulting in sleep. This view of the onset of sleep as a passive process, following the obstruction of wakefulness, was proposed in the first half of the 19th century (Moruzzi 1964). Evidence of an active component to entry into sleep came from work showing activity of pre-optic area (POA) neurones (“sleep-active”) of the rostral hypothalamus during sleep (Kaitin 1984; Szymusiak & McGinty 1989), with a central role played by GABA (Gaus et al. 2002; Gong et al. 2004). These neurones provide inhibitory projections on arousal areas of the posterior hypothalamus and brainstem (including the ascending reticular activating system (Moruzzi & Magoun 1949): comprising the tuberomammillary nucleus [histaminergic], lateral hypothalamus [hypocretinergic], locus coeruleus [noradrenergic], dorsal raphe nucleus [serotonergic], pedunculopontine tegmentum [cholinergic] and laterodorsal tegmentum [cholinergic]). (Sherin et al. 1996, 1998; Steininger et al. 2001; Yoshida et al. 2006; Uschakov et al. 2007; Zardetto-Smith & Johnson 1995). In turn, these arousal centres promote wakefulness via inhibitory projections on to orexin neurones of the lateral and posterior hypothalamus (Sakurai et al. 2005; Muraki et al. 2004). The complexity of sleep-wake homeostasis makes it difficult to pinpoint a cause for the sleep behaviour identified in the T1KO strain. The data presented (particularly Figure 40) show the

striking loss of a circadian rhythm in this strain, with no difference between time spent in any stage of sleep or wakefulness, between the quiet and active periods. Given the small number of animals studied ($n = 3$), more work is required to confirm this finding. It is possible that the compensation described by Linden et al. (2008) by upregulation of GABA receptors is enough to alter the balance of the normal sleep-wake cycle (Linden et al. 2008). The alteration in TASK channel expression levels in the brain could have a profound effect on the control of sleep and wake periods. Variable brain expression of these channels may provide clues to the pathways underlying the observed changes (section 1.2). The data on the T3KO strain, provided by Dr Christian Robledo has been discussed in more detail elsewhere (Robledo 2008; Pang et al. 2009; see Appendix).

The striking similarity between the DKO strain and wild type was unexpected, though perhaps not surprising. The occurrence of developmental compensation in lines of knockout mice has been appreciated since the early days of the introduction of the technology (Picciotto & Wickman 1998; Routtenberg 1995) and the mechanisms underlying compensation are increasingly appreciated (Greenspan 2001; Brickley et al. 2001b; Swensen & Bean 2005; Marder & Goaillard 2006). The maintenance of a phenotype despite network alterations such as gene knockout is “degeneracy”, the ability of a network to produce the same result through different strategies (Greenspan 2001; Figure 74). Compensatory functional upregulation of certain GABA_A receptor subtypes has been shown in the T1KO strain, exhibited as increased sensitivity to the injectable anaesthetic agents propofol and pentobarbital (Linden et al. 2008). It could be argued that compensation is more likely to occur when channels of particular importance have been removed. Therefore the difficulty lies in assessing the true importance of the knocked out gene in the face of compensation.

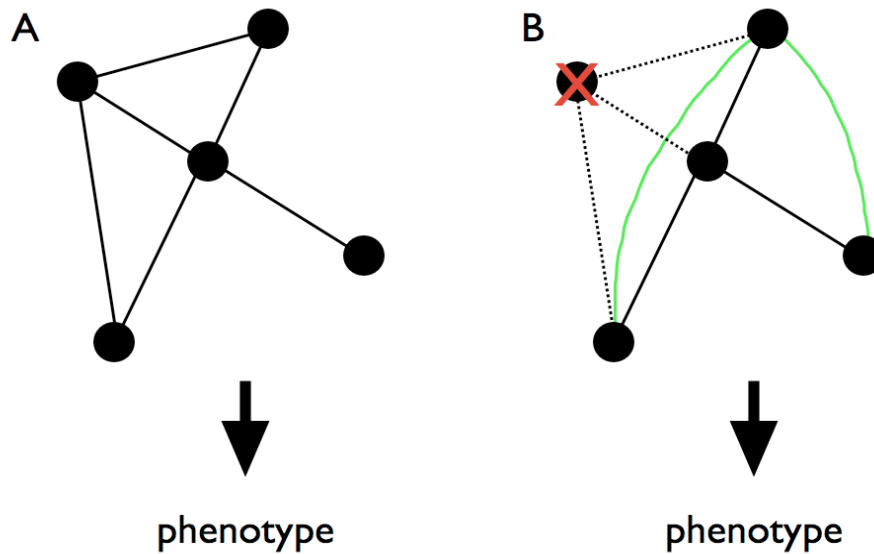


Figure 74. A network functioning under normal circumstances (A) results in an identifiable phenotype. Following perturbation (B), such as knockout of a gene (red cross), compensatory connections (green lines) may result. The overall effect is to produce a phenotype, which may appear identical to that of the normal situation (“degeneracy”). Identifying differences between phenotypes will depend upon the sensitivity of the investigation performed. Closed circles are elements of a network (such as genes). Continuous black lines are intact connections. Dashed black lines are interrupted connections. Adapted from Greenspan (Greenspan 2001).

4.3 Type 2 theta oscillation is variably altered in KO animals

The technique of EEG recording employed in this study provided unique advantages over traditional tethered systems, namely the opportunity to express normal behaviour. The placement of the skull electrodes overlying the dura mater resulted in the recording of a cortical EEG. The advantage of this technique was the ease and speed of placement of

electrodes and low failure rate. The primary disadvantage is that the information recorded reflects local field potentials and is not specific to a single neurone or group of neurones. However, the dominance of the theta oscillation in the mammalian brain and its well-characterised localisation to the hippocampus ensured that this recording technique was not a barrier to generating meaningful data.

The most striking finding of the series of experiments exposing each strain of mouse to halothane was the difference in response in the T3KO compared with the other strains. This difference was first observed by Dr Christian Robledo (Robledo 2008). The T3KO strain failed to generate the classic finely tuned peak within the theta range of frequencies that is elicited in WT animals in response to halothane exposure. Characterisation of this difference by fitting a Lorentzian curve to the theta oscillation provided a quantitative measure of the peak characteristics. Compared to WT, the peak generated in T3KO was considerably broader, with a FWHM value of approximately 3.5 Hz, versus 1.5 Hz in WT at a halothane concentration corresponding with the population LORR. This difference was reflected in the resultant Q value (peak frequency/ FWHM), where T3KO failed to demonstrate the increased sharpness of the theta oscillation peak observed in WT as the halothane concentration increased. Instead, the Q values of the two strains diverged, with the T3KO strain maintaining a non-changing Q value over the range of halothane concentrations studied. These objective measures reflect the power spectra observed (Figure 41).

Of the remaining strains, T1KO and DKO, their cortical EEG response to halothane is split between resembling the WT and the T3KO. The T1KO closely resembled the WT response across the range of halothane concentrations used. Functional compensation, with the upregulation of GABA_A receptors in the T1KO strain has recently been reported (Linden et al. 2008). As GABA_A is a target of halothane (section 4.4.4), and GABAergic projections are essential for the generation of the theta oscillation (section 1.4.2), this compensation is a possible explanation for the ability of the T1KO strain to generate a type 2 theta oscillation. The alteration in type 2 theta oscillation observed in the DKO (similar in Q value to T3KO) may reflect a degree of compensation occurring to enable type 2 theta generation, but not sufficient to return the oscillation to a shape equivalent to that recorded in the WT. Despite the DKO strain showing an identical sleep pattern to WT, its cortical EEG response to halothane differs from WT. In contrast, the T1KO strain differed markedly from WT (and

the other knockout strains) in sleep structure, but showed a near identical cortical EEG response to halothane as WT. These opposing effects indicate that the pathways involved in type 2 theta oscillation generation differ from those involved in sleep.

Though not tested directly, there is indirect evidence that the type 2 theta oscillation is unrelated to the loss of righting reflex (or loss of consciousness). From Figure 41, it is clear that the type 2 theta oscillation begins to appear at sub-anaesthetic (sub-LORR) concentrations of halothane. This is true for all the strains studied. Whilst it was an initially attractive idea that the two were related, this does not appear to be the case.

4.3.1 Abolishment of the theta oscillation by atropine sulphate confirms the theta oscillation induced by halothane is type 2

The administration of systemic atropine sulphate during type 2 theta oscillation abolishes the oscillation, and this response is a defining characteristic of this oscillation, also known as “atropine-sensitive theta” (Kramis et al. 1975). Previous observations in rats identified that the halothane-induced theta oscillation was type 2 theta based on this response to atropine administration during exposure to halothane (Bland et al. 2003; Figure 75). We were interested in confirming the type of theta oscillation we had observed in the WT and T1KO strains, which generated a well-defined oscillation during halothane exposure, and observing what changes might occur in the T3KO strain, which failed to generate a clear oscillation in response to halothane.

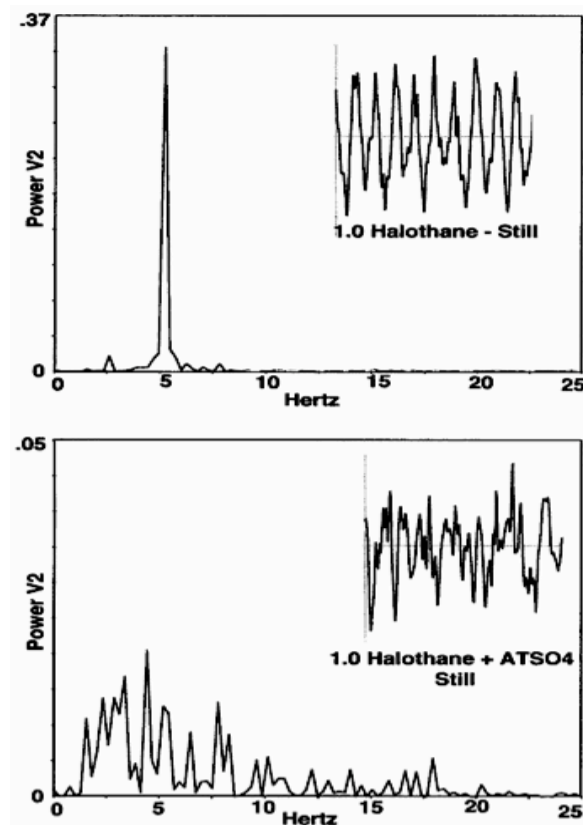


Figure 75. Power spectra from a rat during exposure to 1% halothane alone (top, peak frequency of 5.2 Hz) or after administration of systemic atropine sulphate (ATSO₄, 50 mg kg⁻¹, IP, bottom). The theta oscillation induced by halothane is abolished following atropine sulphate administration. The x axis depicts the frequency (Hz) and the y axis the power (V²). Note change in y axis scale between plots. Adapted from Bland et al. (Bland et al. 2003).

Our findings in the WT and T1KO mirrored that of Bland et al. (Bland et al. 2003). This provided further evidence, in addition to the frequency and induction by halothane, that the oscillation observed was type 2 theta. In the T3KO strain, there was no change in the appearance of the power spectrum, confirming the involvement of acetylcholine in type 2 theta oscillation generation. The effect of atropine sulphate reflects the essential contribution of the cholinergic system in the generation of the type 2 theta oscillation (Yoder & Pang 2005) (section 1.4.2).

4.3.2 Eserine administration fails to induce type 2 theta oscillation in the T3KO strain

The administration of eserine, an anticholinesterase, induces the type 2 theta oscillation (Leung & Vanderwolf 1980; Loizzo et al. 1996). Therefore a further confirmation of the identity of the oscillation (halothane-induced, atropine sulphate ablated) was provided by eserine administration. This resulted in the induction of a type 2 theta oscillation in the WT. As expected, following the results from atropine sulphate administration, eserine administration in the T3KO strain did not induce a type 2 theta oscillation (Figure 55).

4.3.3 Differential brain expression of TASK1 and TASK3 may explain the difference in cortical EEG and sleep observed in T3KO and T1KO strains

As described in the introduction (section 1.2) there are marked differences in brain expression of mRNA between TASK1 and TASK3 channels and some of these sites are central to generation of the theta oscillation and sleep (Brickley et al. 2007; Talley et al. 2001). In terms of type 2 theta oscillation generation apparatus TASK3 mRNA expression was very high in the supramammillary nuclei and dentate gyrus, high in the RPO and moderate in the CA1 and CA3 pyramidal cell layers, and medial septum. TASK1 mRNA expression was moderate in the supramammillary nuclei and dentate gyrus, high in the RPO and low-to-moderate in the CA1 and CA3 pyramidal cell layers, and medial septum (Talley et al. 2001). Similar findings were reported by Brickley et al. who showed a greater difference in TASK3 versus TASK1 mRNA expression in the hippocampus (Brickley et al. 2007).

Perhaps crucially, Talley et al. reported TASK3 expression in regions of the hippocampus distinct from the pyramidal cell layers and postulated the location within the interneurone population (Figure 76; Talley et al. 2001). The role of interneurons is integral to the

generation of the theta oscillation and it may be the absence of expression in the T3KO strain within this population that underlies the lack of type 2 theta oscillation observed.

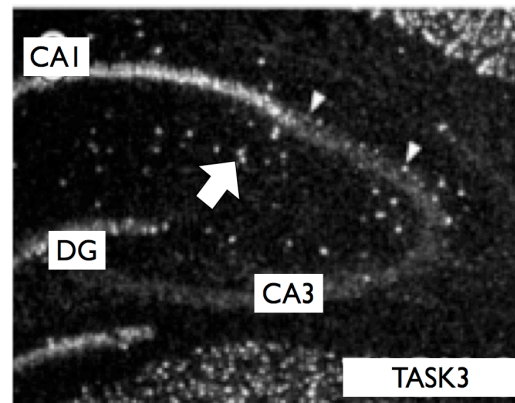


Figure 76. TASK3 mRNA expression in a coronal section of the hippocampus from in-situ hybridisation. The white arrow indicates areas of expression outwith the pyramidal cell layer, possibly indicating expression within interneurons. CA1 and CA3 are the cornu ammonis areas 1 and 3 respectively. DG is dentate gyrus. Modified from Talley et al. (Talley et al. 2001).

These variations in expression between TASK1 and 3 across regions involved in type 2 theta generation provides insight into our results. The greater degree of TASK3 expression, which would be maintained in the T1KO strain, would explain the ability of this strain to generate a type 2 theta oscillation. In contrast, in the T3KO, missing critical channel expression rendered it unable to generate the oscillation.

The anatomic locations implicated in the regulation of sleep (section 4.2.3) also show unequal expression patterns between TASK3 and TASK1 mRNA, with substantially greater TASK3 expression in the brainstem nuclei (dorsal raphe nuclei, locus coeruleus) and thalamic relay nuclei, and greater TASK1 expression in the thalamic reticular nucleus. The change in balance in expression levels within various nuclei provides a potential mechanism which may begin to explain our findings (and those of Linden et al. (2007)). For example, the increase in the time spent awake in the T3KO strain (compared to WT) may be a result of the loss of

TASK3 expression in the pre-optic nuclei (sleep promoting) of the hypothalamus. And the equal division of sleep and wake across quiet and active periods in the T1KO strain may result from loss of TASK1 expression in the sleep promoting thalamic reticular nucleus combined with maintained TASK3 expression in the thalamic relay nuclei. Despite the complex interplay of sleep pathways, this differential expression provides an appealing explanation for the variation in sleep patterns observed between strains.

4.4 Halothane acts through both GABA_A receptors and TASK3 channels in CA1 pyramidal cells

Blind patching

The decision to employ the technique of blind patching was based on optimising neuronal health as recorded cells were situated within a deeper level of the slice thereby minimising dendritic damage from slicing, and providing an unbiased sample population (Castaneda-Castellanos et al. 2006). The density and homogeneity of pyramidal cells within the easily identifiable stratum pyramidale also supported use of this technique. Absolute confirmation of the identity of the cells studied was planned with the use of confocal microscopy to identify (and measure) cells which had been patched. This analysis is currently in progress.

4.4.1 Action potential (AP) characteristics

The AP properties of the WT CA1 pyramidal cells was similar to those reported in the literature for C57BL/6 mice (McColl et al. 2006; Routh et al. 2009; Biscoe & Duchen 1985). It is interesting to note that significant differences in electrophysiological properties of CA1 pyramidal cells have been identified between mouse strains and between mice and rats (Routh et al. 2009). These differences include the resting membrane potential, action potential amplitude, input resistance and cell morphology. Compared with Sprague-Dawley rats, C57BL/6 mice had a more hyperpolarised resting membrane potential, slower action

potential upstroke and smaller amplitude, whereas 129/SvEv mice had a smaller dendritic surface area, greater input resistance, action potential threshold and amplitude. These differences highlight the importance of appropriate comparisons when comparing data between studies.

Halothane and the AP

Under control conditions there was no difference in RMP between WT or T3KO mouse strains. This is at odds with the findings of Lazarenko et al. (2010) in motoneurons, who reported that their T3KO strain had a resting membrane potential 5 mV more depolarised compared with WT (Lazarenko et al. 2010). Our finding is surprising as removal of the T3KO channel would be expected to lead to membrane depolarisation as the contribution to the potassium leak is removed. A possible explanation for the lack of change in RMP between the strains studied may be due to other sources of leak contributing to the RMP. In this case the removal of TASK-3 channels alone may not be sufficient to result in an alteration of RMP. Though this explanation is at odds with the findings of Lazarenko et al. (2010) and Brickley et al. (2007), this may reflect the different cell populations studied; motoneurons and cerebellar granule cells respectively (Brickley et al. 2007; Lazarenko et al. 2010).

The application of 1% atm. halothane to the slices did not result in a change in RMP in either WT or T3KO strains. The lack of effect in WT is the same as reported by Nishikawa and MacIver in rat CA1 pyramidal cells, using a slightly higher concentration of halothane (approximately 1.2% atm.) (Nishikawa & MacIver 2000). However, the lack of difference between strains is surprising as the expected effect of halothane would be to activate TASK channels, thus hyperpolarising the cell membrane, and accentuating a difference between the T3KO and WT strains. A change in RMP (more depolarised) was reported by Lazarenko et al. (Lazarenko et al. 2010) in motoneurons of T3KO mice at a significantly higher concentration of halothane (0.8 mM) than used in this study. And the change in RMP was relatively small (approximately 3 mV). The application of supraclinical concentrations of halothane (3 - 12 x MAC) to rat CA1 pyramidal cells only resulted in a 5 mV hyperpolarisation (Nicoll & Madison 1982). The differences between our findings and those of Lazarenko et al. (2010) may lie in the different cell population studied (motoneurons vs.

CA1 pyramidal cells) or potential compensation by other K_{2P} channels in setting the RMP, or differences in construction of the KO animals used (section 4.1.3).

We found that AP properties were altered by the application of halothane only in the WT, where the maximum rate of rise, rate of decay and AP peak were decreased (Figure 61). This confirmed that halothane was penetrating the slice in the face of the lack of RMP change. There was no effect of halothane in the T3KO strain and the AP did not differ between strains under control conditions. Although we did not observe changes in RMP in the T3KO strain, the lack of AP change in response to halothane application, when an alteration was observed in the WT, indicates a mechanism of action via the TASK3 channel.

4.4.2 Whole cell parameters and halothane

In response to a train of depolarising current steps during current clamp recording we found that halothane application had little effect on the ability of T3KO to produce APs. In contrast, WT were able to continue to produce APs until a substantially greater current injection, a reduction in accommodation. This ability of the WT strain was not identified by Nishikawa and MacIver in rat CA1 pyramidal cells (Nishikawa & MacIver 2000), who reported no change in discharge activity in with halothane application. A possible explanation for this difference is that a single pulse of current was used (120 pA, 300 ms), rather than the series of pulses reported here. We injected over 500 pA of current at the end of our stimulus protocol, at which point a difference in the response to halothane was discernible (Figure 64 and Figure 65). At the lower current injection employed by Nishikawa and MacIver (2000) there was no difference between halothane and control in our data. Our results indicate that the TASK3 channel is necessary for sustained AP firing. Similar findings were reported by Brickley et al. who reported the inability of cerebellar granule neurones to sustain AP firing in T3KO mice (Brickley et al. 2007). They proposed that the difference resulted from an indirect effect of the potassium conductance provided by TASK3 channels to reduce the steady-state inactivation of voltage-gated sodium channels (via membrane hyperpolarisation).

In our study we did not find a change in RMP between the T3KO or WT, a difference identified by Brickley et al. (the T3KO strain had a more depolarised RMP) which was associated with the lower current injection reported to reach AP threshold. In keeping with our findings (no difference in RMP), there was no difference in the current required to reach AP threshold under control conditions between strains. In spite of the similar RMP values during halothane exposure, our data showed that there was an increase in the current required to achieve AP threshold in the WT but not the T3KO strain (Figure 59). The greater current required to achieve AP threshold in the WT strain during halothane exposure, compared with the control period, likely reflects the increased input conductance associated with halothane activation of TASK-3 channels. This is supported by the absence of effect of halothane on the current to reach AP threshold in the T3KO strain.

4.4.3 Halothane and spontaneous inhibitory post synaptic currents (sIPSCs)

Similar to the findings of Nishikawa and MacIver (2000), and Banks and Pearce (1999), we also identified an increase in the sIPSC decay time, indicating a role for halothane in GABA_A mediated synaptic inhibition (Nishikawa & MacIver 2000; Banks & Pearce 1999). The size of this increase in IPSC decay time was less than that reported by Nishikawa and MacIver (2000) and may reflect the lower concentration of halothane used in our study (Nishikawa & MacIver 2000). The percentage change in decay time for an equipotent anaesthetic concentration appears to be related to the individual volatile agent and is also concentration dependent, with enflurane and isoflurane having a greater effect on decay time than halothane (Figure 77; Jones & Harrison 1993; Gage & Robertson 1985; Banks & Pearce 1999).

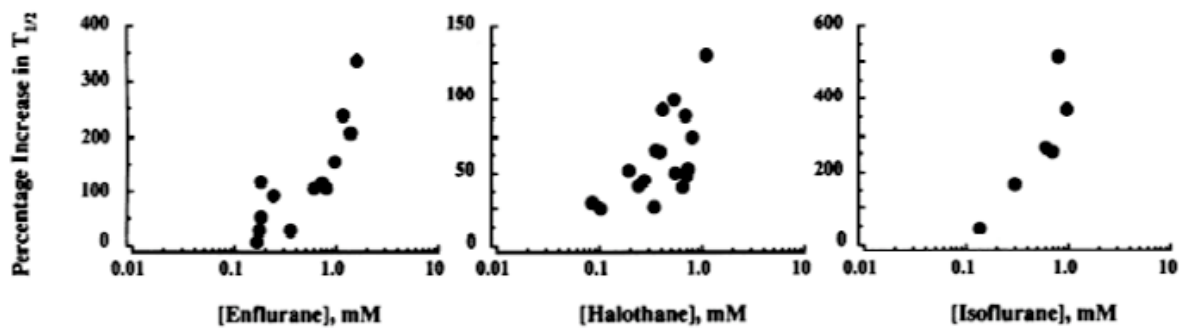


Figure 77. The percentage increase in IPSC decay half life is a function of volatile agent and concentration (compared with control conditions). Enflurane and isoflurane application resulted in a marked prolongation in IPSCs compared to the increase resulting from halothane application. As anaesthetic concentration of each agent increased, a further IPSC prolongation is observed. Note change in y-axis scales. Adapted from Jones and Harrison (Jones & Harrison 1993).

The area under the sIPSC curve (AUC) did not differ between strains under control or halothane exposure conditions. The effect of halothane exposure was a significant increase in the AUC in the WT and a non-significant increase in mean values in the T3KO strain. These changes reflect the IPSC prolongation observed as a result of halothane exposure in both strains, and the absence of strain difference reflects the $GABA_A$ mediated action of halothane.

Changes in IPSC amplitude reflect postsynaptic effects of anaesthetic agents. The lack of effect of halothane on IPSC amplitude is in keeping with the literature (Mody et al. 1991; Gage & Robertson 1985; Banks & Pearce 1999). Although Nishikawa and MacIver (2000) reported a modest change in stimulus-evoked IPSC amplitude, these results differed from an earlier study from the same group when no change in amplitude was identified (Nishikawa & MacIver 2000; Lukatch & MacIver 1997). Banks and Pearce (1999) reported a significantly greater dose dependent decrease in mIPSC amplitude with isoflurane (and enflurane) indicating an inter-agent difference (Banks & Pearce 1999). Interestingly, Banks and Pearce (1999) showed that the effects of isoflurane and enflurane on mIPSC amplitude and decay time constant were not equal for a given concentration between or within agents. This could

explain why we did not observe an effect of halothane on sIPSC amplitude, but did observe an increase on IPSC decay.

Changes in IPSC frequency reflect presynaptic effects of anaesthetic agents by their actions on the release of GABA from presynaptic terminals. Nishikawa and MacIver (Nishikawa & MacIver 2000) reported a significant increase (2-3 fold) in mIPSC frequency following the application of halothane (0.35 mM). In contrast, Banks and Pearce identified a much smaller (less than 1 Hz) increase in mIPSC frequency at 0.6 mM halothane (Banks & Pearce 1999). It is unclear why such different results were observed in these studies despite similar experimental methodology. Our results showed a 30 - 40% decrease in sIPSC frequency in both strains in response to halothane. Two important differences between our study and those of Nishikawa and MacIver (2000) and Banks and Pearce (1999) may explain this difference. Firstly, both cited studies included bath application of 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX, approximately 20 μ M) during IPSC recording. CNQX has been shown to increase IPSC frequency 2 - 4 fold at concentrations four times lower than used in these studies (Brickley et al. 2001a; McBain et al. 1992). Secondly, the animals (rats) used ranged from 2 - 6 weeks old, spanning the time at which GABA_A receptor subtypes undergo dramatic changes, specifically an increase in $\alpha 1$ expression, a subunit involved in halothane's effects on GABA_A (Nishikawa et al. 2002; Fritschy et al. 1994). These differences may account for the different findings of our study.

These data indicate a postsynaptic (increase in decay time constant) mode of action for halothane. Evidence for a presynaptic effect is less clear for the reasons outlined above. These effects were similar in both strains studied reflecting either minimal contribution from the TASK3 channel or a degree of compensation masking potential effects.

4.4.4 Anaesthetic targets

A complicating factor in the investigation of anaesthetic action is that the volatile general anaesthetics have multiple targets. Therefore the results of studies in global knockout models require careful interpretation for this reason and the possibility of compensatory central nervous system changes (Linden et al. 2008; Brickley et al. 2001b).

Halothane has been shown to have effects in a range of receptors at clinically relevant concentrations. Indeed, within the K_{2P} family alone, halothane activates TASK1 (Sirois et al. 2000; Putzke et al. 2007; Patel et al. 1999; Berg et al. 2004; Linden et al. 2006), TASK3 (Meadows & Randall 2001; Lazarenko et al. 2010; Berg et al. 2004; Linden et al. 2007), TREK1 (Heurteaux et al. 2004) and TRESK (Liu et al. 2004) channels. A TREK1 knockout mouse showed a decreased sensitivity to halothane with both LORR and MAC testing, though not to the same degree as reported here with the T3KO strain (Heurteaux et al. 2004). The recently identified K_{2P} TRESK appears particularly sensitive to halothane *in vitro* (Liu et al. 2004).

Our work concentrated on halothane as this has the greatest effect (compared with isoflurane) on TASK1 and TASK3 channels (Patel et al. 1999; Andres-Enguix et al. 2007).

It is this *in vitro* evidence coupled with expression of TASK, and K_{2P} channels as a family, that makes them such attractive targets for the mechanism of action volatile anaesthetic agents.

4.5 Conclusions

Our findings show roles for the TASK1 and 3 channels in anaesthetic sensitivity, generation of the type 2 theta oscillation and the sleep-wake cycle.

The T3KO strain was markedly less sensitive to the volatile anaesthetic than the T1KO strain, whilst there was no significant difference between the DKO and WT. A type 2 theta oscillation, induced by halothane and eserine and blocked by atropine sulphate in the WT, was completely disrupted in the T3KO strain. The shape of the type 2 theta oscillation was

minimally altered in the T1KO strain and the effect on the DKO strain was intermediate between that of the WT and T3KO strains. All knockout strains spent more time awake and less time asleep during the quiet period compared with WT. Additionally, the T3KO strain exhibited slow transition periods between active and quiet periods (Robledo 2008), and the T1KO strain showed similar amounts of sleep and wake throughout the sleep-wake cycle. A comparison of hippocampal CA1 pyramidal cells between WT and T3KO strains reflected the action of halothane through the TASK3 channel and GABA_A receptor. Halothane application slowed the action potential and reduced accommodation in the WT but had no effect in the T3KO strain. This indicates that TASK3 channels in CA1 pyramidal cells may contribute to the difference in type 2 theta oscillation observed between strains. Actions on the GABA_A receptor were reflected by an increase in sIPSC decay times and decrease in frequency upon exposure to halothane in both strains. Much of the *in vivo* data presented in this thesis was recently published (Appendix; Pang et al. 2009).

4.5.1 Future directions

In vivo

Further exploration of the sleep phenotype exhibited by the T1KO strain is warranted. This could involve repetition of the experiment to verify the results and increase the sample size.

As the type 2 theta oscillation is also associated with certain behaviours such as immobility, it would be interesting to verify if the phenotype exhibited by the T3KO is preserved.

The theta oscillation also has roles in navigation and memory consolidation, therefore natural avenues to pursue would be to investigate these functions in the knockout strains.

In vitro

Further investigation of the mechanistic framework underlying the difference in type 2 theta oscillation observed in the T3KO strain could be focused on the role of inhibitory interneurons in the generation of type 2 theta. Parvalbumin positive (parv-positive) interneurons exhibit TASK-like currents (Torborg et al. 2006) and could be a promising target. The difficulty in patching interneurons is that it is not possible to distinguish classes based on anatomic location or morphology and parv-positive interneurons only represent approximately 20% of all interneurons. Therefore to increase the likelihood of patching an interneuron of the desired class, aid identification and reduce animal numbers, generation of a mouse line with a means of identifying the desired class of interneurons (such as with GFP) and selective TASK channel deletion in these cells.

5.0 References

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6.0 Appendix

An unexpected role for TASK-3 potassium channels in network oscillations with implications for sleep mechanisms and anesthetic action

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TASK channels are acid-sensitive and anesthetic-activated members of the family of two-pore-domain potassium channels. We have made the surprising discovery that the genetic ablation of TASK-3 channels eliminates a specific type of theta oscillation in the cortical electroencephalogram (EEG) resembling type II theta (4–9 Hz), which is thought to be important in processing sensory stimuli before initiating motor activity. In contrast, ablation of TASK-1 channels has no effect on theta oscillations. Despite the absence of type II theta oscillations in the TASK-3 knockout (KO) mice, the related type I theta, which has certain neuronal pathways in common and is involved in exploratory behavior, is unaffected. In addition to the absence of type II theta oscillations, the TASK-3 KO animals show marked alterations in both anesthetic sensitivity and natural sleep behavior. Their sensitivity to halothane, a potent activator of TASK channels, is greatly reduced, whereas their sensitivity to cyclopropane, which does not activate TASK-3 channels, is unchanged. The TASK-3 KO animals exhibit a slower progression from their waking to sleeping states and, during their sleeping period, their sleep episodes as well as their REM theta oscillations are more fragmented. These results imply a previously unexpected role for TASK-3 channels in the cellular mechanisms underlying these behaviors and suggest that endogenous modulators of these channels may regulate theta oscillations.

EEG | knockout | REM | theta | wavelet

If a sufficient number of neurons participate in network oscillations, then the local field potentials summate, and measurable voltage oscillations can be recorded in the electroencephalogram (EEG). These oscillations are observed over a wide range of frequencies and reflect the synchronous neuronal activity that occurs during a variety of different behaviors. For example, as animals explore their environments, as they learn and lay down memories, as they process sensory input, and as they sleep, characteristic oscillations occur in the “theta” range of frequencies (4–12 Hz) (1). These theta oscillations are often divided into two types (1–5): Type I, which occurs at slightly higher frequencies (6–12 Hz), and type II (also known as arousal theta), which occurs at the lower end of the range (4–9 Hz). Type I theta is associated with exploratory behavior, walking, running, and rearing, whereas type II theta is associated with immobility during the processing of sensory stimuli relevant to initiating, or intending to initiate, motor activity.

The neuronal networks that generate these theta oscillations involve ascending pathways from the brainstem that project to the hypothalamus and then to the medial septum/diagonal band of Broca and the hippocampus (6–9). Where the true pacemaker is located is unclear, but the basic requirements for a neuron to oscillate are a depolarizing drive (such as a sodium current) together with a restoring drive, such as a repolarizing potassium current. Most computational models (10–12) include several different ionic currents, some of which are well-characterized

and attributed to known ion channels (e.g., HCN channels underlying I_h), whereas others are only defined operationally (e.g., slow potassium currents).

We have been studying the role TASK-3 potassium channels might play in general anesthesia. This channel is a member of a family of 15 “background” or “leak” potassium channels (13) that is directly inhibited by acid and activated by certain inhalational anesthetics (14, 15). During our initial experiments, we monitored the cortical EEG as a function of anesthetic concentration and made a striking observation. In wild-type mice, a highly-tuned anesthetic-induced peak in the theta band of frequencies (4–9 Hz), which appeared at around the concentrations that induced a loss of righting reflex, was absent in the TASK-3 knockout (KO) animals. TASK-1 KO mice, on the other hand, appeared identical to wild-type animals.

In this paper we show that the ablation of TASK-3 potassium channels removes type II theta oscillations, but leaves type I theta oscillations and exploratory behavior unaffected. Moreover, the TASK-3 KO mice show altered anesthetic sensitivity, disrupted sleep behavior, and a fragmentation of both sleep episodes and theta oscillations during REM sleep. These results suggest that TASK-3 channels play key roles in anesthetic sensitivity and the regulation of sleep.

Results

Anesthetic-Induced Loss of Righting Reflex. To assess any differences in anesthetic sensitivity between wild-type, TASK-1 KO, and TASK-3 KO animals, we used the loss of righting reflex (LORR) as an assay. In rodents, LORR is observed at the same concentrations as loss of consciousness in humans (16), with a comparably steep concentration-response curve reflecting a sharp transition between the awake and anesthetized states. We first investigated halothane because of its great efficacy in activating TASK channels (14, 15, 17–19). We found that the TASK-3 KO mice were significantly ($P < 0.001$) less sensitive to halothane, with an EC_{50} of $0.94 \pm 0.02\%$ atm ($n = 12$) compared with $0.68 \pm 0.02\%$ atm ($n = 19$) for wild-type animals (Fig. 1A). In contrast, TASK-1 KO mice showed only a small change in anesthetic sensitivity with an EC_{50} of $0.78 \pm 0.01\%$ atm; $n = 20$. To help assess if the difference between wild-type and TASK-3

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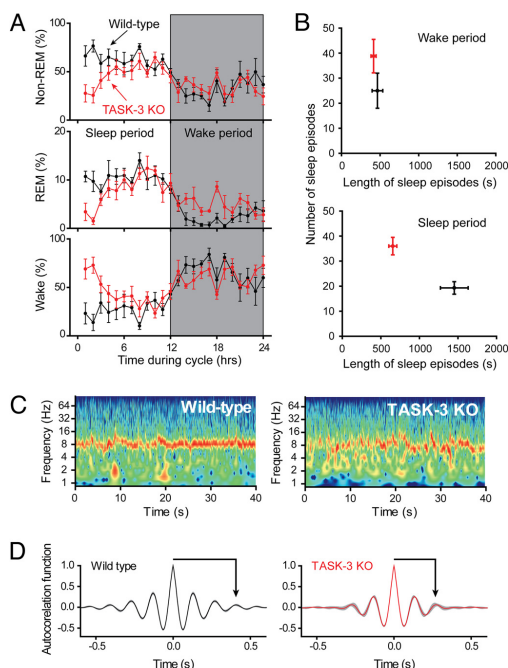


Fig. 4. The ablation of TASK-3 channels disrupts natural sleep and fragments both sleep episodes and theta oscillations. (A) TASK-3 KO mice show a much slower progression into sleep (both into non-REM and REM) at the start of the natural sleep period ("lights on"). During the natural wake period ("lights off") the sleep behavior of the two genotypes is broadly similar. (B) The upper panel shows that there were no significant differences in sleep fragmentation during the natural wake period. During the natural sleep period (lower panel), however, the TASK-3 KO animals show a fragmented sleep pattern with significantly more ($P < 0.001$), but significantly shorter ($P < 0.005$) sleep episodes. (C) REM sleep episodes during the natural sleep period showed a clear difference in the fragmentation of the theta oscillations. The Wavelet power spectra show illustrative examples of 40-s segments of EEG during REM for wild-type and TASK-3 KO mice. The autocorrelations in (D) reflect this quantitatively and show that the wild-type mice ($n = 5$) display more coherent REM theta oscillations ($P < 0.05$) than do TASK-3 KO mice ($n = 5$). The gray shading represents the SEM envelope, and the arrows indicate the time at which a peak in the autocorrelation function is no longer significantly different from zero.

Because the most striking phenotypic difference between the wild-type and TASK-3 KO animals was the loss of the type II theta oscillations (Fig. 1C and D), and because natural sleep had been disrupted, we next investigated whether the theta oscillations that occur during REM sleep were also affected. We calculated the Wavelet power spectrum as a function of time during periods of sustained REM sleep that occurred during the natural sleep period (“lights on”). Increased fragmentation of the theta oscillations was evident in the Wavelet power spectra of the TASK-3 KO mice. Fig. 4C shows representative examples of Wavelet power spectra for the two genotypes, where it can be seen that in wild-type animals, theta oscillations tend to be less interrupted. This is shown quantitatively by the autocorrelation functions shown below (Fig. 4D) calculated on 120-s segments of data from both wild-type (left graph; $n = 5$) and TASK-3 KO animals (right graph; $n = 5$). A test between the peak heights in

the autocorrelation functions show a significant difference ($P < 0.05$) between the wild-type and KO animals.

Discussion

The TASK-3 KO mice were significantly less sensitive to the general anesthetic halothane. Indeed, they showed the greatest genetically-engineered decrease in sensitivity to an inhalational anesthetic yet reported for anesthetic-induced hypnosis (16, 33). This decrease in halothane sensitivity is comparable with that observed with TREK-1 KO mice in response to a painful stimulus (33). The fact that the TASK-3 KO mice displayed an unchanged sensitivity to cyclopropane, an agent that does not measurably activate TASK-3 channels (20), supports the idea that the decrease in halothane sensitivity was a direct consequence of their absence. Thus, involvement of TASK-3 channels in anesthetic-induced LORR seems likely. A possible role for anesthetic-activated potassium channels was suggested many years ago (34). The first such channel that was characterized was discovered in the pond snail *Lymnaea stagnalis* (18), and when recently cloned (17), found to be closely related to mammalian TASK channels that have been shown to be activated by a variety of volatile general anesthetics (14, 15). Nonetheless, other channels, such as GABA_A receptors and other two-pore-domain potassium channels are almost certain to be involved (16, 35, 36), because at only 40% higher concentrations, the TASK-3 KO mice are also anesthetized.

The loss of type II theta oscillations in the TASK-3 KO mice was completely unexpected. This theta oscillation has been widely studied, using a variety of different anesthetics (4, 6, 8, 37). The increased tuning of the oscillation under halothane anesthesia implies that the opening of TASK-3 channels promotes neuronal synchronicity. The oscillation is characterized by its sensitivity to atropine, and it has been postulated to mediate the processing of sensory stimuli before initiating motor activity (2–5). The absence of type II theta was specific to the loss of the TASK-3 channels, because the removal of TASK-1 channels left the oscillations unchanged.

Our finding that type I exploratory theta oscillations are identical in the wild-type and TASK-3 KO animals has important implications for the extent to which the pathways and molecular mechanisms that mediate the two types of theta oscillations overlap. Clearly, TASK-3 potassium channels are a necessary component for type II oscillations but play no evident role in type I oscillations. Although the circuitry responsible for generating these oscillations is not certain, it is widely believed that a pathway from the brainstem, ascending through the hypothalamus to the medial septum and hippocampus is involved (1, 6–9). This is consistent with our observation that the type II theta oscillation can be reversibly blocked by lidocaine injection into the medial septum.

Given that the recombinant TASK-1 and TASK-3 channels have similar biophysical properties with respect to anesthetic sensitivity (15), why are the deficits in oscillatory activity and sleep patterns specific to the TASK-3 KO strain? This is probably because of differences in neuronal expression between the two genes (38); we surmise that the TASK-1 gene is not expressed, or not expressed highly, in the neurons either driving or supporting the oscillations. In the adult mouse forebrain the TASK-3 gene has much stronger expression than TASK-1. In particular, TASK-3 mRNA is abundant in layers 2 to 6 of the neocortex, CA1 hippocampal pyramidal cells, dentate granule cells, and the septum (38, 39); the TASK-3 gene is also expressed in parvalbumin-positive GABAergic interneurons (40) in the hippocampus, some subtypes of which aid the generation of theta oscillations (41, 42). The TASK-1 gene, by contrast, is poorly expressed in the mouse hippocampus (38). One scenario is that, as a consequence of losing TASK-3 channels, a change in the biophysical behavior of a specific type of hippocampal interneu-

ron (e.g., a subtype of parv-positive interneuron) might produce the selective loss of type II theta with minimal impact on type I theta. It has recently been shown, for example, that the loss of TASK-3 channels in cerebellar granule neurons affects their ability to sustain high-frequency firing of action potentials (43). Alternatively, TASK-3 channels might govern the regulation of selective cholinergic input to the hippocampus, so their absence might disrupt theta oscillations. More work is needed to elucidate the cellular mechanism.

Previously, establishing the physiological roles of type II theta oscillations has been problematic because they are only sustained when anesthetic is present, or can only be blocked when atropine is present. Both of these drug treatments will affect neuronal behavior in ways that do not involve theta oscillations per se. TASK-3 KO animals should allow us to test hypotheses about where and when these oscillations might play a role. Whether or not the theta oscillations that are elicited by various drug treatments correspond to the theta oscillations that are observed during specific behavioral states requires further investigation.

Network oscillations and sleep are intimately connected, with sleep states largely characterized by the frequency and amplitude of oscillations in the EEG. Transient spindles and K-complexes appear before sleep onset, and the relative amplitudes of theta and delta oscillations (together with the EMG) allow REM and non-REM sleep states to be distinguished. It is too early to be certain that the absence of type II theta oscillations in the TASK-3 KO animals is directly responsible for the changes we observe in sleep behavior. Nonetheless, the differences we see in both the time to transition into both REM and non-REM sleep, as well as the fragmentation in the sleep episodes are a strong indication that modulation of TASK-3 potassium channels is part of the mechanism used in natural sleep regulation, perhaps via muscarinic acetylcholine receptors (44). Interestingly, atropine treatment, which blocks type II theta oscillations, also shortens REM episodes in both cats (45) and rats (46). The fragmentation of the theta oscillations during REM episodes is further evidence of TASK-3 involvement in sleep mechanisms in general and theta oscillations in particular.

In summary, we have made the surprising discovery that TASK-3 channels are required for atropine-sensitive type II theta oscillations. In contrast, they make no contribution to type I exploratory theta oscillations. Animals lacking these channels display a reduced sensitivity to the general anesthetic halothane, have a significantly slower progression into sleep, and exhibit fragmented sleep behavior. Because of the many ways in which TASK-3 channels can be modulated, it is easy to imagine that this might lead to the regulation of behavior via the promotion or reduction of theta oscillations.

Materials and Methods

Mice. All experiments were in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 and approved by the Ethical Review Committee of Imperial College London. Animals were housed in a humidity- and temperature-controlled room, under a 12:12-h light-dark cycle. Water and food were provided ad libitum. The TASK-1 (39) and TASK-3 KO (43) mice, each with a disruption of the first coding exon, were as described previously.

Anesthetic-Induced Loss of Righting Reflex. An animal was placed in a cylindrical glass chamber (900 mL) and, following a 10-min baseline period with 100% oxygen, the anesthetic was introduced, initially at 0.4% for halothane or 8% for cyclopropane. The anesthetic concentration was then increased stepwise (steps of 0.1% for halothane and 4% for cyclopropane), and after 10 min equilibration, LORR was assessed by manually rotating the glass cylinder and scoring a LORR if the animal had all four feet off the ground for 30 s or more. The observer was blinded to the genotype of the animal. Each animal was tested once at each anesthetic concentration. Normothermia was maintained using a heat lamp placed 45 cm above the glass cylinder. A quantal concentration-response curve was calculated using the method of Waud (47).

EEG Recording. Surgery was carried out under halothane (0.8–1.5% in oxygen) anesthesia. Three gold-plated EEG electrodes (Decolletage AG) were inserted through the skull onto the dura mater, the first in the frontal bone (+1.5 mm to Bregma, −1.5 mm from midline), the second in the parietal bone (−1.5 mm to Bregma, +1.5 mm from midline), and the third in the interparietal bone over the cerebellum (−2.0 mm from Lambda, 0.0 mm from midline) for the reference electrode. Three lengths of Teflon-insulated stainless steel wire (with the distal 3 mm of insulation removed) were inserted in the neck muscle for EMG recording. Once all of the electrodes were in place, they were covered with dental cement (Orthoresin; DeguDent GmbH). The animals were allowed at least 7 days to recover from surgery. The EEG and EMG signals were recorded on a miniature data logging device (48) containing a 256 MB memory chip (<http://www.vyssotski.ch/neurologger2>). This device was sufficiently small (about 2 g including batteries) to be attached directly to the animal's skull. Four data channels (up to 30 h at 10-bit resolution) could be recorded at a sampling rate of 400 Hz and were bandpass-filtered (−3 dB corner frequency) between 1 and 70 Hz followed by high-pass (0.6 Hz, −3 dB) offline digital filtering. The recording device allowed EEG-EMG signals to be recorded in free-moving animals, either in an activity monitor or in their home cages, where data could be recorded during a complete sleep-wake cycle in a natural and familiar environment.

It should be noted that our EEG measurements were from the cortex, so that the sleep state of the mice could be determined. Previous work on theta oscillations has usually been done with rats, often using hippocampal electrodes. However, because of the small distance between the hippocampus and the cortex in mice, it is almost certain that oscillations generated in the hippocampus would be detected by our cortical EEG electrodes.

EEG Analysis. EEG data were analyzed using either conventional FFT power spectra (Fast Fourier transforms of the autocorrelation function) or Morlet Wavelet analysis. FFT power spectra were calculated using the program Spike (Spike 2, v5.14; Cambridge Electronic Design) with the area being normalized to 100. Where a theta peak was observed, a good fit to the data could be obtained using a Lorentzian function:

$$p = p_0 + \left\{ \frac{a_0 \gamma^2}{\gamma^2 + (f - f_0)^2} \right\}$$

where p is the EEG power, p_0 is a baseline, a_0 is the height of the Lorentzian, γ is the half-width at half maximum, f is the frequency and f_0 is the peak frequency. The Q-factor, a measure of the sharpness of the peak, was calculated as $f_0/(2\gamma)$.

EEG data were also analyzed using Wavelet transforms (49), which are appropriate when the EEG signal is nonstationary (which is often the case); the Wavelet power spectrum is the square of the Wavelet transform. This method involves convoluting the EEG signal with a series of "Daughter" wavelets, which are time-scaled variants of a "Mother" wavelet. In general, the wavelet transform is defined as:

$$W(s, \tau) = \frac{1}{\sqrt{s}} \int x(t) \psi\left(\frac{t - \tau}{s}\right) dt$$

where s and τ represent the scale and local center of the wavelet $\psi(s, \tau)$, and $x(t)$ is the EEG signal as a function of time. We used the most commonly used Mother wavelet, the Morlet function, which is a complex sinusoid, windowed by a Gaussian:

$$\psi_0(\eta) = \pi^{-1/4} e^{i\omega_0 \eta} e^{-\eta^2/2}$$

where η is a dimensionless "time" parameter, and ω_0 is the dimensionless wavelet central "frequency" that was set to 6 to satisfy the admissibility criterion (50). The Wavelet power spectra were calculated using Matlab (MathWorks) using a script based on that of Torrence and Compo (49).

Sleep Scoring. For the sleep experiments, mice were placed in a temperature-controlled, sound-proof box illuminated within on a 12:12-h light-dark cycle. Data were recorded both from animals in their home cages as well as from animals exposed to a novel environment (an activity test chamber). In these latter experiments, as well as the EEG-EMG signals, the coordinates of the animal were recorded using two orthogonal sets of 16 infrared beams (Activity test chamber; Med Associates) and analyzed with Activity Monitor software (Med Associates). The activity box was thoroughly cleaned with ethanol between experiments.

The sleep state (rapid-eye-movement sleep, REM; nonrapid-eye-movement sleep, non-REM; or wake, W) was scored automatically using an established

protocol (51). Briefly, the scoring consisted of filtering the EEG into "delta" (0.5–4 Hz) and "theta" (6–10 Hz) frequency bands and scoring 20-s epochs as one or other of the three states based on W being periods of high EMG and intermediate theta/delta ratio, REM being periods with high theta/delta ratio and low EMG and non-REM being periods with high delta waves, low theta/delta ratio, and low EMG.

Statistics. Unless otherwise stated, Student's *t*-test was used to test for significance. Where shown, errors bars represent the SEM.

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