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The Safety, Efficacy and Neuromotor Effects of the Neurosteroid Anaesthetic Alfaxalone in Rats

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Abstract

Rodents are routinely anaesthetised for husbandry and biomedical research purposes. All anaesthetics carry a degree of risk, complications and side effects. An ideal anaesthetic for rodents is safe, predictable, alleviates pain and distress and can be performed without specialist training. A survey was created to identify anaesthetic agents frequently administered to rodents currently utilised by the research community. Findings indicated injectable anaesthetics were very commonly used and complications were often associated with use, highlighting the lack of a safe, predictable laboratory rodent anaesthetic protocol.

Alfaxalone is a neuroactive steroid injectable anaesthetic agent with a high margin of safety, and is commonly used in veterinary anaesthesia as the Alfaxan® formulation. I sought to establish whether alfaxalone could be used as an injectable anaesthetic agent for laboratory rodents. A plasma pharmacokinetics study was performed using IV and IP Alfaxan® in adult female Wistar rats (7-10 weeks). Mean $T_{1/2elim}$ for 2 and 5 $mg.kg^{-1}$ IV was short (~17 minutes), but could not be estimated for IP dosing due to sustained plasma levels for up to 60 minutes after injection. Cl_p for IV injection was calculated at 24.5% and 23% of cardiac output respectively. The observed C_{max} was 2.99 $mg.L^{-1}$ for IP administration, and 2.2 ± 0.9 and 5.2 ± 1.3 $mg.L^{-1}$ for 2 and 5 $mg.kg^{-1}$ IV administration respectively. AUC_{0-60} was 96 $min.mg.L^{-1}$ for IP dosing. The relative bioavailability for IP dosing was 26% and 28%, compared to IV doses of 2 and 5$mg.kg^{-1}$ respectively. Alfaxalone given IP caused longer sleep times than IV dosing, although anaesthesia was not induced in 30% of rats.

Importantly, all rats exhibited muscle twitching after alfaxalone administration. I then sought to test whether combining IP injection of Alfaxalone with common premedication agents could improve anaesthetic induction rates and reduce muscle twitching, to provide a viable anaesthetic regimen for rats. Medetomidine (0.5 $mg.kg^{-1}$), medetomidine-butorphanol (0.35 $mg.kg^{-1}$-0.2 $mg.kg^{-1}$), Acetylpromazine (ACP) -methadone (3 $mg.kg^{-1}$-0.7 $mg.kg^{-1}$) and ACP-xylazine (3 $mg.kg^{-1}$-3 $mg.kg^{-1}$) were administered IP 10 minutes prior to IP alfaxalone (20 $mg.kg^{-1}$). ACP-xylazine, medetomidine and medetomidine-butorphanol all significantly reduced twitching. Medetomidine-butorphanol provided surgical anaesthesia without prolonged recovery and provided an adequate plane of anaesthesia for short painful surgical procedures.

However, none of these premedication agents fully eliminated alfaxalone-induced muscle twitching. I therefore carried out electrophysiological studies of motor neurons (MN), to determine the mechanism causing neuromotor excitation in response to alfaxalone. Previous data has shown that alfaxalone can modulate strychnine-sensitive glycinergic receptors, which may, in part, contribute to the adverse neuromotor excitatory responses manifested as muscle twitching during alfaxalone anaesthesia. The

effects of alfaxalone on inhibitory synaptic transmission to hypoglossal motor neurones (HMNs) were investigated.

Whole cell patch clamp recordings were performed on HMNs in transverse brainstem slices (300 µm thickness) prepared from 10-12 day old Wistar rats anaesthetised with sodium pentobarbitone (100 $mg.kg^{-1}$ IP). Spontaneous and evoked glycinergic inhibitory post-synaptic currents (IPSCs) were recorded at a holding potential of –60mV, using a CsCl-based internal solution, in the presence of the non-NMDA and NMDA glutamate receptor blockers NBQX (10µM) and APV (50µM) and the $GABA_A$ receptor blocker bicuculline (5µM). An alfaxalone dose response study was performed with bath-applied alfaxalone at increasing concentrations of 10nM, 30nM, 100nM, 300nM, 1µM, 3µM and 10µM (n=7). Both spontaneous and paired evoked IPSCs (at 150 ms inter-stimulus interval) were recorded. A reduction in evoked IPSC peak amplitude, rise time and decay time without paired pulse ratio changes was consistent with alfaxalone altering postsynaptic glycine receptors, causing prolongation of both channel opening and closure. A decrease in spontaneous IPSC frequency at higher doses was suggestive of presynaptic modulation by alfaxalone, decreasing glycine release from the presynaptic terminal. At high doses, alfaxalone induced an inward current in HMNs, without significant change in input resistance.

Cannabinoid receptors (CBR) can directly modulate both glycine release and GlyR function in HMNs. I postulated that alfaxalone exerted its effects on glycinergic transmission by increasing postsynaptic endocannabinoid synthesis and activation of cannabinoid 1 receptors (CB_1Rs). The CB_1R inverse agonist-antagonist AM251 (1µM; n= 6) or the CB_1R competitive antagonist NESS0327 (100pM; n=5) both blocked spontaneous IPSC frequency depression, evoked IPSC amplitude reduction, decay time prolongation and baseline inward current produced by alfaxalone at micromolar concentrations. Application of the CB_1R competitive agonist WIN55,212-2 (n=7) alone caused a dose-dependent evoked IPSC peak amplitude depression, starting at nanomolar concentrations. WIN55,212-2 (300nM; n=5) partially blocked IPSC amplitude reduction by co-application of alfaxalone, but did not alter increases in IPSC decay time or induction of an inward current. These findings indicate that alfaxalone induced endocannabinoid signalling to indirectly activate CB_1Rs, which resulted in reduced postsynaptic glycine receptor activity at low alfaxalone doses, and reduced presynaptic release of glycine and induction of an inward current at higher alfaxalone doses. These responses to alfaxalone are likely to make HMNs more excitable, and may therefore underlie neuromotor excitation during alfaxalone anaesthesia. Combination of alfaxalone with CB_1R antagonists or negative modulators of endocannabinoid signalling may therefore provide an optimal anaesthetic regime for laboratory rodents.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

Chapter TWO was submitted and published in the Journal of Veterinary Pharmacology and Therapeutics as a short communication (Appendix Two, publications).

Publications included in this thesis

Publication citation – incorporated as Chapter 2.

Contributor	Statement of contribution
Cora Lau	Designed experiments (80%) Wrote the paper (75%)
Dr Mark Bellingham	Wrote and edited paper (15%)
Dr Ian Shiels	Designed experiments (10%) Edited paper (5%)
Dr Kirby Pasloske	Statistical analysis of data in table 2 Designed experiments (10%) Wrote and edited paper (5%)
Millagahamanda Ranasinghe	Plasma analysis for data in table 2 Wrote and edited paper (5%)
Dr Helen Keates	Edited paper (5%)

Contributions by others to the thesis

Dr Ristan Greer aided in the design of the Rodent anaesthetic survey in Chapter 1.

Dr John Morton supplied technical support for Chapter 2 in helping to design the cross over study.

Statement of parts of the thesis submitted to qualify for the award of another degree

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Keywords

Neurosteroid, alfaxalone, anaesthesia, rat, pharmacokinetics, glycine, motor neuron, electrophysiology, cannabinoid 1 receptor, TRP channel

Australian and New Zealand Standard Research Classifications (ANZSRC)

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LIST OF ABBREVIATIONS

A	Amplitude
ACP	Acetylpromazine
aCSF	Artificial cerebrospinal fluid
AD_{50}	Anaesthetic dose for 50% of patients
ANZLAA	Australia & New Zealand Laboratory Animal Association
APV	DL-2-amino-5-phosphonopentanoic acid
AUC	Area under the curve
AUC_{extrap}%	Percentage area under the curve extrapolated from last sample time
$AUC_{0\text{-}inf}$	Area under the curve from administration time to infinity
$AUC_{0\text{-}last}$	Area under the curve from time of administration to last blood sample
Ca^{2+}	Calcium
CO_2	Carbon dioxide
CAD	Cath.a differentiated
CBC	Cannabichromene
CBR	Cannabinoid receptor
CB1R	Cannabinoid 1receptor
Cl_p	Plasma clearance
C_{max}	Peak plasma concentration
CNS	Central nervous system
DMSO	Dimethyl sulfoxide
EC_{50}	Half maximal effective concentration
$GABA_A$	Gamma aminobutyric acid type A
$GABA_AR$	Gamma aminobutyric acid type A receptor
$GABA_BR$	Gamma aminobutyric acid type A receptor
GLC	Gas liquid chromatography
GlyR	Glycine receptor
HMN	Hypoglossal motor neuron
HPCD	2-hydroxypropyl-β-cyclodextrin
HPLC	High-performance liquid chromatography
IH	Hyperpolarisation-activated cation current
I_{hold}	Baseline holding current
IM	Intramuscular
IP	Intraperitoneal
IPSC	Inhibitory postsynaptic current
IV	Intravenous
K^+	Potassium
LD_{50}	Lethal dose in 50% of animals
LLOQ	Lower limit of quantification
LOD	Limit of detection
MEC	Minimum effective concentration
MN	Motor neurone
nXII	Hypoglossal motor nucleus
N	Total number
Na^+	Sodium
NMDA	N-methyl-D-aspartate
NSAID	Non-steroidal anti-inflammatory drug
nXII	Hypoglossal motor nucleus
O_2	Oxygen
PPD	Paired pulse depression
PPF	Paired pulse facilitation

LIST OF ABBREVIATIONS (Cont.)

PPR	Paired pulse ratio
R	Receptor
R_n	Input resistance
RSD	Residual standard deviation
SC	Subcutaneous
SD	Standard deviation
SpO_2	Saturation of peripheral oxygen
τ	Decay time constant
T	Time
$T_{1/2elim}$	Elimination half-life
TRP	Transient receptor potential
TRPA1	Transient receptor potential ankyrin 1-type
V_D	Volume of distribution
λ_z	Terminal slope of the curve

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INTRODUCTION

Anaesthesia is frequently used in laboratory animals so that routine husbandry and experimental procedures can be performed. There is a strong need for anaesthetic agents that are easy to administer, provide immobilisation, analgesia and muscle relaxation with an adequate margin of safety. Laboratory animal anaesthesia is often performed by researchers that do not have adequate anaesthetic training or expertise. In many cases, lack of equipment to deliver inhalation anaesthesia and lack of experience in the use of these agents, dictate the use of injectable agents to both induce and maintain unconsciousness. Anaesthesia is an integral part of biomedical research and the extent of surgical intervention dictates the anaesthetic plane and time frame necessary. Procedures range from brief immobilisation for relatively non-painful procedures to prolonged surgical anaesthesia for complex surgery. With rapid advancements in biomedical sciences there have still been no major improvements in rodent anaesthetic protocols. Anaesthetic combinations such as ketamine-xylazine were reportedly used 30 years ago and remain a widely used anaesthetic combination today (Green et al., 1981, Stickrod, 1979, Saha et al., 2007).

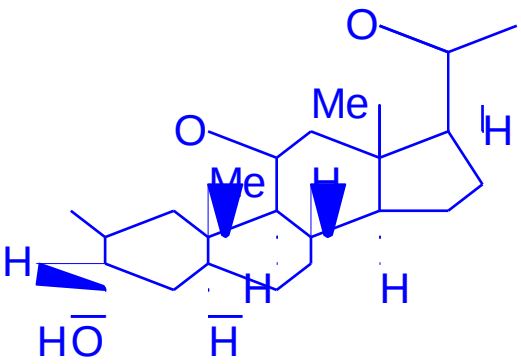
Intravenous (IV) access can be easily obtained in larger animals due to visible superficial vessels. However, this route of access is not as straight forward in small rodents. It is technically difficult to perform IV injections in rodents and is often replaced by a simpler injection technique (Flecknell, 1993a). The intraperitoneal (IP) route of anaesthetic delivery is more commonly used in laboratory rats and mice. IP injections are simple and only require a needle, syringe and minimal expertise (Flecknell, 1993b). In addition, IP injections cause less tissue damage than the intramuscular (IM) route (Smiler et al., 1990) and a larger injection volume can be delivered in a particular site causing minimal discomfort. But no matter how simple it is to deliver an anaesthetic, there is always a degree of risk for developing anaesthetic-associated complications and side effects. Anaesthetics that are delivered in a single bolus injection could lead to variable anaesthetic depth and do not allow for immediate adjustment of dose. This large variation in anaesthetic response can mean that a dose of drug that would produce anaesthesia in one animal could be ineffective in another at worst lethal (Flecknell, 1993b). This highlights the need for an injectable anaesthetic agent that is consistent in effect and has a high margin of safety.

Alfaxalone is a neurosteroid with anaesthetic properties that is commercially available as Alfaxan® CD-RTU (Jurox Pty Ltd, Rutherford). Alfaxalone reportedly has a high margin of safety in a range of species including dogs and horses (Ferre et al., 2006, Muir et al., 2008, Goodwin et al., 2012). This anaesthetic quality would be highly desirable for rodents. In this thesis we aim to determine whether

alfaxalone is a safe and effective anaesthetic agent for use in laboratory rats as a commonly used species in biomedical research.

History of alfaxalone

Hans Selye first documented the existence of steroids that possessed anaesthetic properties in 1941 (Sutton, 1972). It was at this early stage that steroid anaesthetics showed several advantages over barbiturate anaesthetics. An increased therapeutic ratio and progressive liver metabolism with no redistribution into the body tissues meant an increased anaesthetic safety margin (Sutton, 1972). It was also determined at this time that the steroid required an oxygen atom at either end of the steroid molecule to produce anaesthetic effects. In contrast, an increased number of double bonds in A and B rings decreased the steroid-induced anaesthetic activity (Sutton, 1972). In 1965, an anaesthetic steroid 3α-hydroxy-5α-pregnane-11, 20-dione (alfaxalone) was developed and shown to be the most potent steroid molecule tested in an early steroid screening program, when injected intravenously in mice (Davis and Pearce, 1972). Low doses of 1-27mg.kg^{-1} IV were sufficient to cause a loss of righting reflex in the mouse within a few seconds of the injection, and doses as high as 507mg.kg^{-1} IV were administered before death occurred (Davis and Pearce, 1972), demonstrating a wide margin of safety. The IV anaesthetic dose (AD_{50}) in 50% of mice was 1.87mg.kg^{-1} with a lethal dose (LD_{50}) in 50% of mice at 54.7mg.kg^{-1} indicating a therapeutic index of 30.4 (Davis and Pearce, 1972).



3α-hydroxy-5α-pregnane-11, 20-dione (Alfaxalone) chemical structure

Due to the lipophilic nature of alfaxalone, it was initially solubilised in a non-ionic detergent polyoxyethylenated castor oil, Cremaphor EL (Brewster et al., 1989). This product was commercially available as Althesin®, or Saffan® (the veterinary version), and contained alphadolone acetate to increase the solubility of alfaxalone in Cremaphor EL (Davis and Pearce, 1972). Althesin® showed greater antinociceptive potency than chloralose-urethane, urethane and pentobarbital in Sprague Dawley rats, as well as producing minimal to no suppression of core body temperature and mean arterial blood pressure (Ching, 1984). This alfaxalone-alfadalone formulation had promising

anaesthetic potential, but the availability and use of this preparation was discontinued due to the allergic side effects associated with the Cremaphor EL excipient (Brewster et al., 1989, Lorenz et al., 1977). However, in the time of its availability as Althesin®, a number of studies were performed using this formulation of alfaxalone/alfadalone in rats and mice. Tissue distribution studies of Althesin® and metabolism and excretion studies in rats were performed along with AD_{50} and LD_{50} of mice (Davis and Pearce, 1972, Card et al., 1972, Child et al., 1972). Studies were also conducted on the effect of Althesin on reproduction in mice and rats (Child et al., 1972)

Cyclodextrins have an amphiphatic property that can form soluble and reversible inclusion complexes with water-insoluble compounds (Brewster et al., 1989). This has led to the formulation of alfaxalone that is available today. Using 2-hydroxypropyl-β-cyclodextrin (HPCD) has improved the solubility of alfaxalone by 375 times, therefore removing the need for the addition of alfadalone (Estes et al., 1990, Muir et al., 2008). An HPCD-alfaxalone formulation, Alfaxan®-CD RTU was developed by Jurox Pty Ltd, and is commercially available in Australia for dogs and cats.

Alfaxalone administration, distribution, metabolism and elimination (ADME)

Plasma pharmacokinetics of the HPCD preparation of alfaxalone has been performed in cats, dogs and horses. The pharmacokinetics of alfaxalone show non-linear pharmacokinetic parameters after IV administration of Alfaxan® in cats (Whittem et al., 2008), dogs (Ferre et al., 2006), horses (Goodwin et al., 2011). A rebound effect of plasma levels was observed in some cats and dogs and majority of horses, where an intermittent elevation in plasma level was evident and followed by a return of plasma levels to the presumed slope of the pharmacokinetic time profile (Whittem et al., 2008, Ferre et al., 2006, Goodwin et al., 2011). Alfaxalone clearance (Cl_p) at clinically relevant doses was approximately 5-10% of cardiac output for cats compared with 50-60% in the beagle dog and 46% in horses (Ferre et al., 2006, Whittem et al., 2008, Goodwin et al., 2011). These results show considerably similar pharmacokinetic profiles between species administered Alfaxan®.

Alfaxalone pharmacokinetic studies in rats have been performed using various solvents, including Cremaphor EL, dimethyl sulfoxide (DMSO), acetonitrile and HPCD, as it is well known that formulation variations could affect the pharmacokinetic parameters of anaesthetic drugs (Whittem et al., 2008). Althesin® alfaxalone plasma half-life was approximately 7 minutes in rats using gas liquid chromatography (GLC) extraction methods (Child et al., 1972). The distribution half-life of 0.8 ± 0.1 min and elimination half-life of 14 ± 0.7 minutes was reported in male Wistar rats using high-performance liquid chromatography (HPLC) extraction method, where it was also shown that HPCD did not alter alfaxalone pharmacokinetic parameters when compared to DMSO vehicle (Visser et al.,

2002). The Alfaxalone clearance rate reportedly almost equalled the rat liver blood flow, which indicated that body weight was important for alfaxalone clearance (Visser et al., 2002). Radio-active labelled ^{14}C-alfaxalone showed that alfaxalone became widely distributed throughout body tissues following a single intravenous injection in Sprague Dawley rats and became localised in the central nervous system (CNS) within one minute (Card et al., 1972). At three minutes post injection, the highest level of ^{14}C-alfaxalone was found in the liver and by ten minutes, alfaxalone isotopes had reached the renal medulla (Card et al., 1972). Alfaxalone was almost completely cleared from body tissues within an hour following IV administration (Card et al., 1972). Furthermore, the majority of radio-labelled alfaxalone was retrieved from faeces and urine within the first 48 hours with 97.9% recovered within a period of five days post intravenous injection (Child et al., 1972). However, to date, there has been no pharmacokinetic study of alfaxalone administered in the commercially available formulation to rats.

Intraperitoneal administration of alfaxalone

It is technically difficult to acquire intravenous access in a rat or mouse, and therefore the intraperitoneal (IP) route of administration of substances to rodents is most frequently used. Adult female Wistar rats displayed periods of electroencephalographic silence from 6-8 minutes after 0.5mL per 100g body weight IP Althesin® injection (Fink et al., 1982). Alfaxalone appears to show an age dependent increase in dosage required to induce anaesthesia. An elevated dose requirement of IP Althesin® was necessary to maintain surgical anaesthesia for 30 day old compared with 17 day old female Wistar rats (Fink et al., 1982). 17 day old male and female rats of comparable weight required a similar dose, but at 90 days of age, male rats required four times the dose of female rats to maintain surgical anaesthesia (Fink et al., 1982). No clinically significant pharmacokinetic differences between sexes have been reported (Ferre et al., 2006).

Neurosteroids and their neuronal actions

Neurosteroids are a class of steroids that do not interact with hormone steroid receptors (Visser et al., 2000). They are synthesised in response to several physiological states, including stress and pregnancy (Hosie et al., 2006). Endogenous neurosteroids are synthesised in the central and peripheral nervous systems from cholesterol, with one of the earliest observations being their anaesthetic properties (Sutton, 1972, Shiraishi et al., 2003, Compagnone and Mellon, 2000, Belelli and Lambert, 2005). It is the function that neurosteroids have on producing anaesthesia that we are most interested.

The anaesthetic potency of many anaesthetic compounds follow the Meyer-Overton rule which states that the action of general anaesthetics is proportional to their lipid membrane partition coefficient

(Heimburg and Jackson, 2007, Thompson and Wafford, 2001). But also, he anaesthetic potency correlates well with their activity at the γ-aminobutyric acid A receptor ($GABA_AR$) (Thompson and Wafford, 2001). The $GABA_AR$ complex is a multimeric structure made up of five subunits and it is well known that the majority of the fast inhibitory synaptic transmission in the mammalian brain is mediated by the neurotransmitter γ-aminobutyric acid (GABA), which is the endogenous ligand for the $GABA_AR$ (Lambert et al., 1995, Paul and Purdy, 1992). Activation of the, $GABA_AR$ by GABA or other ligands, rapidly decreases CNS excitability and it is thought that neurosteroids induce anaesthesia by this mechanism (Cottrell et al., 1987, Lambert et al., 1995, Paul and Purdy, 1992, Hill-Venning et al., 1996). Both endogenous and synthetic neurosteroids are well established positive allosteric modulators of the $GABA_AR$ (Lambert et al., 1995, Harrison and Simmonds, 1984, Compagnone and Mellon, 2000, Weir et al., 2004, Hill-Venning et al., 1996, Belelli and Lambert, 2005). More specifically, endogenous neurosteroids act as potent modulators of all major $GABA_AR$ isoforms and regulates $GABA_AR$ through two distinct binding sites in the transmembrane domains by potentiation or direct activation of $GABA_AR$ (Hosie et al., 2006, Belelli and Lambert, 2005). The presence of a β subunit has been shown to play an important role in the allosteric modulatory effects of IV anaesthetics on the $GABA_A R$ (Carlson et al., 2000). More specifically, alfaxalone also directly activates $GABA_A Rs$ containing αγ subunits, therefore demonstrating the importance of the γ2 subunit for allosteric $GABA_AR$ modulation by alfaxalone (Carlson et al., 2000). A dual mechanism of action was reported for alfaxalone in isolated bovine chromaffin cells under voltage clamp conditions (Cottrell et al., 1987). Low alfaxalone concentrations (>30nM) potentiated GABA-induced ion current amplitude and increased mean open time of $GABA_AR$ chloride channels (Cottrell et al., 1987, Nolan et al., 1997). At higher concentrations (>1μM), alfaxalone also directly activated $GABA_ARs$, causing an increase in chloride channel conductance (Cottrell et al., 1987, Nolan et al., 1997, Visser et al., 2002). The $GABA_AR$ effects of neurosteroids are well established and the resultant effects of $GABA_AR$ activation by alfaxalone is the induction of anaesthesia.

It has been suggested that alfaxalone may possess antinociceptive effects and potential antinociceptive properties of neurosteroids have been investigated at several receptor sites (Shiraishi et al., 2003, Horishita et al., 2002, Nadeson and Goodchild, 2000, Nadeson and Goodchild, 2001, Winter et al., 2003, Britton et al., 1991). However there is conflicting evidence of antinociceptive activity for alfaxalone. Spinal muscarinic acetylcholine receptors are involved in antinociception, and inhibition of muscarinic receptor function has been demonstrated for IV anaesthetics and analgesic agents (Shiraishi et al., 2003). Alfaxalone at clinically relevant doses was shown to inhibit M1 and M3 muscarinic receptors expressed in *Xenopus laevis* oocytes (Shiraishi et al., 2003). An alternative antinociceptive pathway has been suggested, in which noradrenergic neurotransmission is enhanced

by noradrenaline transporter inhibition (Horishita et al., 2002). Alfaxalone competitively suppressed noradrenaline uptake by interfering with desipramine binding and noradrenaline recognition in cultured bovine adrenal medullary cells (Horishita et al., 2002). Antinociceptive actions were also reported for neurosteroids, in which alfadalone-alfaxalone combination reportedly produced antinociceptive effects by positive modulation of spinal cord $GABA_AR$s (Nadeson and Goodchild, 2000). However, the antinociceptive properties were attributed to the neurosteroid alfadalone and not to alfaxalone (Nadeson and Goodchild, 2001). Similar reports support the lack of antinociceptive effects of alfaxalone. Clinical studies involving the tail flick latency test for antinociception reported pain suppressive properties for alfadalone, but not alfaxalone, at a non-sedative dose, $10mg.kg^{-1}$ alfaxalone IP (Winter et al., 2003). Similarly, no antinociceptive benefits were apparent in adult Wistar rats administered $8mg.kg^{-1}$of alfaxalone in which no significant change in tail-flick latency was observed compared with control (Britton et al., 1991). This data suggests that an ideal anaesthetic regimen based on alfaxalone would incorporate an antinociceptive agent.

Like $GABA_AR$, glycine receptors (GlyR) also play a role in mediating fast inhibitory neurotransmission in the CNS, particularly in brainstem and spinal cord circuits. GlyRs are ligand-gated chloride ion channels activated by the synaptically release amino acid glycine, composed of pentamers of α and β subunits. Glycinergic neurotransmission is involved in the coordination of reflex responses, sensory signal processing and nociception in the adult CNS (Mukhtarov et al., 2005). Strychnine-sensitive GlyRs are involved in the modulation of spinal sensory input, nociceptive signals and pain processing, and enhancement of inhibitory GlyR activity could lead to suppression of nociceptive signals (Ahrens et al., 2008, Laube et al., 2002, Betz and Laube, 2006).
Along with $GABA_AR$ modulation, alfaxalone has also been shown to potentiate the actions of glycine on the GlyR. Alfaxalone and the neurosteroids ORG20599 and minaxolone did not elicit a detectable current response on α1 GlyRs expressed in *Xenopus laevis* oocytes when applied in the absence of glycine, but produced a dose-dependent increase in inward current responses elicited by glycine (Weir et al., 2004). The potentiation of α1 GlyRs was much less pronounced for alfaxalone than that of ORG20599 and minaxolone, suggesting that GlyR modulation does not play a critical role for the anaesthetic action of neurosteroids (Weir et al., 2004). Similarly, the concentration of alfaxalone necessary to potentiate the α1 GlyR subunits, transiently transfected into transformed human embryonic kidney (HEK 293) cells, was much higher than that required for $GABA_AR$ modulation, suggesting that GlyR potentiation by neurosteroids would fall outside a clinically relevant range (Ahrens et al., 2008).

Clinical use of alfaxalone

Alfaxan® has been widely used for anaesthetic induction in cats and dogs since its introduction into the veterinary anaesthetic market (Ferre et al., 2006). The removal of the Cremaphor EL solvent abolished many of the side effects associated with the Althesin® preparation. Several studies have been carried out to determine the efficacy of this new formulation, and all results were promising, with alfaxalone showing a large margin of safety. IV alfaxalone produced a smooth and uneventful anaesthetic induction in premedicated horses and was rapidly cleared from plasma (Goodwin et al., 2011). Additionally, alfaxalone use in neonatal foals showed good quality of induction and recovery from anaesthesia (Goodwin et al., 2012). Alfaxalone provided a smooth and rapid induction of anaesthesia in beagle dogs at clinical ($2mg.kg^{-1}$) and supra-clinical ($10mgkg^{-1}$) IV doses, demonstrating its safety at five times the effective dose (Ferre et al., 2006). Similarly, mixed-breed dogs showed rapid induction, good muscle relaxation and uneventful anaesthetic recovery with $6mg.kg^{-1}$ and $20mg.kg^{-1}$ IV doses (Muir et al., 2008). A fast and stable anaesthetic induction was obtained with alfaxalone anaesthesia in cats at $5mg.kg^{-1}$ IV (Whittem et al., 2008, Muir et al., 2009). Young healthy cats aged between 3 to 12 months showed smooth and rapid anaesthetic induction followed by rapid and unremarkable recovery after a titrated dose of IV alfaxalone (Zaki et al., 2009).

However, one common observation with alfaxalone anaesthesia was the presence of neuro-excitation during anaesthetic induction and recovery. This was manifested as muscle twitching and limb paddling, and was observed in 7 of 47 and 10 of 47 cats given IV alfaxalone anaesthetic respectively (Mathis et al., 2012). Twitching was also reported in pigs; 20 of 60 pigs anaesthetised with alfaxalone displayed muscle and jaw twitching after anaesthetic induction, and 6 pigs shook during the anaesthetic recovery period (Keates, 2003). Dogs showed less pronounced neuro-excitatory signs, such as agitation and noise hypersensitivity when stimulated, during alfaxalone anaesthetic recovery (Ferre et al., 2006). This neuro-excitatory effect of alfaxalone has also been reported with other neurosteroids, where pro-convulsant properties have been described in at least twenty neurosteroid compounds causing seizures following IV administration in mice (Paul and Purdy, 1992).

Alfaxalone for rodent anaesthesia

Alfaxalone anaesthesia is widely used in veterinary medicine for its ease of administration, wide margin of safety, and its titratable nature when given IV. Current studies indicate rapid and smooth anaesthetic induction and recovery in most veterinary species, and it is believed that these favourable characteristics of alfaxalone anaesthesia could be translated for use in laboratory rodents. Initially, this thesis identified currently utilised anaesthetic regimens from a rodent anaesthetic survey aimed at researchers. In this survey, several anaesthetic-related complications were identified with commonly

used injectable anaesthetic combinations, highlighting the need for a safer and more reliable injectable anaesthetic combination for laboratory rodents.

My initial hypothesis provided some evidence that alfaxalone could fill these criteria and potentially improve on current rodent anaesthetic protocols. In rats, the IV route is not possible without invasive intervention and is technically difficult to perform. In this case, the IP route of drug administration is common place for laboratory rodents. A pharmacokinetic investigation in rats was performed to determine whether alfaxalone was suitable for IP administration. Results were promising, as sustained plasma levels were seen up to 60 minutes post IP administration of 20mg.kg^{-1}alfaxalone. This was a major benefit, as it provided a simple route of anaesthetic delivery as well as longer sleep times than IV dosing. Following this, an alfaxalone clinical trial was performed in adult Wistar rats using IP, IM and SC administration of alfaxalone. Pilot studies revealed that neuro-excitation was prominent via all routes of alfaxalone delivery. So a plan was devised to incorporate various premedication agents into an alfaxalone anaesthetic regimen to see if neuroexcitation could be eliminated. Findings showed, however, that neuro-excitation was reduced by some premedication agents, but not abolished. As the neuro-excitatory effects appeared to be one of the major limitations in providing an effective alfaxalone anaesthetic protocol for rats, it was prudent to find the mechanism for alfaxalone neuro-excitation using electrophysiological studies.

The effects of alfaxalone on inhibitory synaptic transmission to motor neurons (MN) was investigated using whole cell patch clamp technique in hypoglossal motor neurons (HMNs) in brainstem slices from Wistar rats. A reduction in evoked inhibitory postsynaptic current (IPSC) peak amplitude, rise time and decay time without paired pulse ratio changes, was consistent with alfaxalone altering the activity of postsynaptic glycine receptors, causing prolongation of both channel opening and closure. A decrease in spontaneous IPSC frequency at higher doses of alfaxalone was suggestive of presynaptic modulation by alfaxalone, decreasing glycine release from the presynaptic terminal. At high doses, alfaxalone also induced an inward current in HMNs, without significant change in input resistance. We then postulated that alfaxalone exerted its effects on glycinergic transmission by increasing postsynaptic endocannabinoid synthesis and activation of cannabinoid 1 receptors (CB_1Rs) through transient receptor potential (TRP) channel activation. Our results were supportive of these mechanisms possibly underlying increased HMN excitability and neuromotor excitation. Ultimately, addressing the neuro-excitation will not just improve anaesthesia for laboratory rodents, but improve anaesthesia for all veterinary species.

CHAPTER ONE

Rodent Anaesthetic Survey

Abstract

It is commonly accepted that pain and distress should be minimised in animals undergoing any surgical procedure. This view has superseded previous ideas where anaesthesia was available merely to facilitate the researcher, and the animals' welfare was overlooked. Fortunately, this outdated attitude is no longer the norm. An ideal anaesthetic protocol for research animals would be safe, predictable, alleviates pain and distress and could be performed by scientists untrained in anaesthetic practice. A survey of rodent anaesthetic techniques was conducted to identify commonly used anaesthetic protocols currently being utilised by the research community in Australasia. 30 survey responses were received from various research Institutions around Australia and New Zealand detailing their anaesthetic and analgesic protocols in rats and mice. Results identified ketamine-xylazine anaesthetic combination and isoflurane as the most common injectable and inhalation anaesthetic respectively. The predominant anaesthetic delivery method was by injection. 61.5% respondents used injectable anaesthetic agents for rats as compared with 38.5% gaseous anaesthesia. Buprenorphine was the predominant analgesic chosen for both rats and mice and non-steroidal anti-inflammatory medications were not favoured for use in mice. Anaesthetic associated complications most frequently encountered were inadequate depth of anaesthesia, hypothermia, long recovery time, apnoea and unexpected wake up from anaesthesia. Anaesthetic related rodent mortality was not uncommon ranging markedly between researchers up to 100% deaths. The rodent anaesthetic survey identified several commonly chosen anaesthetic combinations and analgesic agents and with them their associated anaesthetic complications. Results highlighted a need for increased anaesthetic training for research scientists and technicians. Regardless of the time consuming nature of training, ultimately this would be more cost and time effective given the high complication rate faced by researchers. At present, only a limited number of anaesthetic agents are utilised and they are still accompanied by a number of complications. Therefore it is prudent to find a simple and safe anaesthetic protocol for rats and mice that provides a wide margin of safety to ultimately reduce anaesthetic related complications and death. Alfaxalone could potentially provide a safer and easy alternative laboratory rodent anaesthetic to currently available anaesthetic agents.

Introduction

Laboratory rats and mice are frequently anaesthetised for husbandry and biomedical research purposes. Historically anaesthesia was merely required to provide immobilisation and no emphasis was placed on the physiological and metabolic effects caused by the anaesthetic agents on the animals themselves (Wixson et al., 1987c). A good anaesthetic protocol needs to be safe, predictable and ensure that the mouse or rat cannot experience any pain or distress during the procedure (Flecknell, 1993b). Unfortunately, all anaesthetic agents carry with them a degree of risk for complications and side effects. Importantly, poor peri-operative care could result in complications such as prolonged recovery, unnecessary stress on the animal and potential mortality (Flecknell, 1993b).

Regardless of the anaesthetic regimen chosen, anaesthetic maintenance is difficult in rodents due to their small size and requirement for specialised rodent-specific monitoring equipment (Flecknell, 1993a). This is compounded by the lack of veterinary anaesthetic expertise where anaesthetic monitoring is performed by researchers and not by trained veterinary anaesthetists. As an additional complicating factor, researchers must take into account the possible differences in anaesthetic tolerance between species, and also the possible differences between the growing number of genetically modified rodent strains (Avsaroglu et al., 2007).

Over the last few decades, some investigators have tried to identify the most commonly administered anaesthetic and analgesic agents used in biomedical research for laboratory rodents (Richardson and Flecknell, 2005, Stokes et al., 2009). These studies involved the use of a structured literature review from published peer reviewed articles. The results from these literature searches gave us an insight into drug progression for rodent anaesthesia.

Between 1990 and 2000, there was a marked reduction in the use of ether. This was a convenient anaesthetic agent, however it was not only irritant to rodents, but also posed an occupational risk as it was potentially explosive when combined with oxygen and air (Richardson and Flecknell, 2005). As a potential replacement for ether, many other gaseous agents including isoflurane and halothane were employed but appropriate delivery apparatus is necessary along with equipment for the removal of waste gases (Richardson and Flecknell, 2005). As a consequence of the considerably expensive gaseous anaesthetic delivery systems, the use of injectable agents became a common alternative anaesthetic route. A shift towards using combination anaesthetic agents was seen within the early 2000s as an attempt to minimise adverse effects attributed to using sole agents (Stokes et al., 2009).

This was also followed by a noticeable increase in the use of analgesic agents, which were given in addition to an anaesthetic regimen to provide analgesia (Stokes et al., 2009).

These surveys focused only on results from peer reviewed journals and the anaesthetic protocols used for that particular study. We believe that many rodent anaesthetics are performed not only by researchers, but also animal technicians that routinely perform rodent anaesthesia for husbandry procedures. Therefore we designed a rodent anaesthetic survey to help us identify the most commonly used anaesthetic regimens for researchers and animal technicians. This survey focused directly on typical anaesthetic practices used within laboratories and animal facilities. Survey data was collected between 2009 and 2012, approximately five years after the last survey report. The results of this survey would help determine which anaesthetic regimen are currently most utilised and also whether there is a market for a new anaesthetic agent such as alfaxalone to enter the laboratory rodent industry.

Materials and Methods

The rodent anaesthetic survey was limited to three A4 pages to encourage participation (Appendix 1). Wording of the survey was chosen carefully so that respondents felt comfortable answering sensitive questions pertaining to mortality rates and complications encountered. A list of anaesthetic related complications was supplied to help prompt a response from survey participants.

Key information requested from this survey was sorted into sections. Section 1 of the survey ascertained the animals' signalment, number of animals and anaesthetic regimen used for that particular protocol. Section 2 asked specific questions associated with anaesthetic experience and participants' thoughts on the quality of that anaesthetic regimen (Appendix 1).

Anaesthetic survey distribution

The anaesthetic survey was emailed to a variety of rodent users in the research industry ranging from rodent breeding and housing facilities to biomedical research Institutes around Australia and New Zealand. The rodent anaesthetic survey was uploaded to the Australia and New Zealand Laboratory Animal Association (ANZLAA) member's website. ANZLAA members are largely employed within the rodent breeding and housing industry in Australasia. Surveys were also circulated around more local research facilities including the Pharmacy Australia Centre of Excellence, Mater Medical Research Institute and Queensland Institute of Medical Research. Within The University of Queensland, surveys were distributed to The University of Queensland Veterinary Teaching Hospital, The University of Queensland Biological Resources and The University of Queensland School of Biomedical Sciences. Research Institutes within the University of Queensland campus, Australian Institute for Bioengineering and NanoTechnology, Institute for Molecular Biosciences and Queensland Brain Institute members were also invited to take part in this rodent anaesthetic survey. Printed copies of the anaesthetic survey with a postage paid envelope, were made available to participants preferring a hard copy of the document. During an annual ANZLAA meeting held in Sydney in 2010, surveys were promoted and distributed via self addressed and postage paid envelopes.

Anaesthetic survey data compilation

Survey replies were received from all around Australia and New Zealand. In the interest of confidentiality, survey participants and facility names were not disclosed and instead a survey number, from 1 to 30, was allocated. All data collected were collated and tabulated identifying anaesthetic regimen, procedural impact, analgesic use, anaesthetic complications and mortality rate for rats and mice. Anaesthetic agent combinations were categorised as injectable or inhalation anaesthesia. The procedural impact on the rodents was categorised into non-invasive, moderate and

invasive interventions. Non-invasive intervention was defined as a procedure that required minimal physical contact where the anaesthesia was merely performed to provide immobilisation. Moderate intervention was defined as any procedures requiring administration or removal of substances such as injections or blood collection. Invasive intervention was defined as any procedures involving surgical intervention requiring surgical site incision and wound closure. Analgesic agents were defined as analgesic agents used in addition to that used for anaesthesia, therefore not inclusive of anaesthetic agents that possessed analgesic properties. All data were presented in a table with data presented as total numbers, mean, range and percentages.

Results

Approximately 300 survey forms were distributed around Australia between July of 2009 and 2012 and a total of thirty anaesthetic survey replies were collected. Investigators were not contacted for additional information due to the sensitive nature of some of the survey questions and the confidential manner in which the survey information was treated. Contact details were not requested and therefore survey responses received by mail were anonymous. In total, thirty survey responses were received over the allocated period resulting in an approximate response rate of 10%.

The majority of anaesthetics were delivered by injection

The predominant anaesthetic delivery method described in the anaesthetic survey was by injection. 61.5% of respondents used injectable anaesthetic agents for rats (Table 1.1). This bias towards the use of injectable agents was more so in mice than for rats. 75% of mouse anaesthetics were administered by injection. Ketamine-xylazine combination was the most popular injectable anaesthetic agent for both rats and mice. Tiletamine-zolazepam (Zoletil®) - xylazine combination anaesthesia was the next most popular injectable anaesthetic combination for rats and mice. Almost all anaesthetics here were delivered by intraperitoneal injection. Only one survey respondent administered anaesthesia via the intramuscular injection.

Inhalation agents were more common for rats

38.5% of respondents used inhalation agents in rats compared with 25% for mice. Isoflurane was the only inhalation agent administered to rats. Both isoflurane and methoxyflurane anaesthesia was utilised for mouse anaesthesia.

ANAESTHETIC AGENTS	Rats N (%)	Mouse N (%)
Total survey responses	26 (100)	12 (100)
Injectable Anaesthetic Agents		
Ketamine & Xylazine	9 (34.6)	6 (50)
Tiletamine-Zolazepam & Xylazine	6 (23.1)	2 (16.7)
Pentobarbital	1 (3.8)	
Tribromoethanol		1 (8.3)
Inhalation Anaesthetic Agents		
Isoflurane	10 (38.5)	1 (8.3)
Methoxyflurane		2 (16.7)

Table 1.1. Anaesthetic agent combinations used in rats and mice.

Lists survey response data for injectable and inhalation anaesthetic agent combinations as described by rodent anaesthetic survey respondents. The responses were separated into rat and mouse anaesthesia and displayed as the number of responses in total number (N) and as a percentage (%).

Analgesic use in laboratory rodents

Of the 30 surveys collected, 28 investigators responded to the analgesic question. From these responses, additional analgesics were administered to 47.8% of rats and 40% of mice. Additional analgesia was not administered to all rodents where the procedures were categorised as moderately invasive and invasive (Table 1.2). The most commonly administered analgesic agent for both rats and mice was the opioid drug buprenorphine. Non-steroidal anti-inflammatory agents, meloxicam, carprofen and paracetamol were reported. One local anaesthetic agent, bupivacaine was reportedly used for rat analgesia (Table 1.2).

Drug restrictions did not limit anaesthetic and analgesic choice

Drug restrictions did not limit the use of ketamine or opioid analgesic agents. Only 3 of 30 respondents chose tiletamine-zolazepam over ketamine based on the difficulties with handling a schedule 8 drug restricted agent. Rather, 14 respondents stated their choice of anaesthetic was not affected by drug restrictions, but based on its familiarity, convenience and anaesthetic qualities.

a)

	Procedural impact	Analgesia	No Analgesia	No Response
Rat	Non-invasive	1	3	0
	Moderate	2	5	0
	Invasive	8	2	2
	Total (%)	**11 (47.8)**	**10 (43.5)**	**2 (8.7)**
Mouse	Non-invasive	0	1	0
	Moderate	1	4	0
	Invasive	3	1	0
	Total (%)	**4 (40)**	**6 (60)**	**0 (0)**

b)

Analgesia	Rat	Mouse
Buprenorphine	5	4
Meloxicam	3	0
Carprofen	2	0
Paracetamol	1	1
Bupivicaine	1	0
Total	**12**	**5**

Table 1.2. Analgesic agent use in correlation with procedural impact for rats and mice.

a) Analgesic usage for non-invasive, moderate and invasive procedures in both rats and mice

b) Analgesic agents most commonly reported for use in rats and mice

Reported anaesthetic related complications

Investigators reported a range of anaesthetic related complications but two survey respondents mentioned no complications were observed in their rat anaesthetics and three survey respondents noted no complications with mouse anaesthesia. Additionally, no complications were observed with inhalation anaesthesia in mice.

The most common anaesthetic complication reported was inadequate depth of anaesthesia after a single dose of an injectable agent (Table 1.3). 66.6% of survey participants experienced this complication in mice and 56% in rats during injectable anaesthesia. One investigator reported 50% of their mouse ketamine-xylazine anaesthetics were inadequate (Table 1.5).

Unexpected anaesthetic wake-up was observed in rats following both injectable and inhalation anaesthesia where it affected 25% of injectable anaesthesia and 30% of inhalation anaesthesia (Table 1.4). This complication was not observed during mouse anaesthesia.

37.5% of rats appeared cold during injectable anaesthesia in rats and similarly 33.3% for mice (Table 1.4 & Table 1.5). Four survey participants specifically mentioned that the animals appeared cold even when a supplementary heat pad was provided.

Apnoea was observed with both injectable and inhalation anaesthetics. 25% of rats showed periods of apnoea when given an injectable anaesthetic (Table 1.2). One Investigator reported an 83% incidence of apnoea as a consequence of administering ketamine-xylazine. Apnoea was observed by three of twelve investigators performing mouse anaesthesia (Table 1.5).

Prolonged anaesthetic recovery was predominantly seen with injectable anaesthesia with 37.5% of (Table 1.3). One Investigator mentioned prolonged recovery from anaesthesia in 50% of the rats anaesthetised with pentobarbital (Table 1.4). Additionally, another investigator observed a 10% prevalence of prolonged recoveries following ketamine-xylazine anaesthesia in mice (Table 1.5).

Only 50% of survey participants reported their anaesthetic related mortality rates. Mortality rates identified in this rodent anaesthetic survey ranged from no deaths to 100% mortality (Table 1.7).

	rat				mouse			
	injectable		inhalation		injectable		inhalation	
	N	%	N	%	N	%	N	%
No complications	1	6.3	1	10	1	11.1	2	66.7
No response to this question	0	0	3	30	1	11.1	1	33.3
Inadequate depth after initial administration	9	56	1	10	6	66.6	0	0
Unexpected wakeup	4	25.0	3	30	0	0	0	0
Cold	6	37.5	1	10	3	33.3	0	0
Apnoea	4	25.0	2	20	3	33.3	0	0
Long recovery	6	37.5	1	10	3	33.3	0	0
Total Survey Responses	**16**		**10**		**9**		**3**	

Table 1.3. Survey findings for anaesthetic complications associated with injectable and inhalation anaesthetic agents for rats and mice.

Complications as experienced by survey respondents are represented as a percentage of responses compared with total survey responses. Where N = total number of survey respondents experiencing the complication

Survey number	Inadequate Anaesthesia	Cold	Apnoea	Unexpected Wakeup	Prolonged Recovery
1					
2		12.5			
3	10				
4	10	10			50
5	20		1	1	20
6	5		83.3	16.7	
8					% not specified
9		33.3		1	
10	2	% not specified			% not specified
11					
12			% not specified		
13					1
14	0.05			2.5	1
15	1				% not specified
16	0.025				
17	% not specified	% not specified	7.1	7.1	
18			5	30	
20					
21		10			
22				50	
23					
24	10	% not specified			
25					
27					
Range	**0.025 to 20%**	**10 to 33.3%**	**1 to 83.3%**	**1 to 50%**	**1 to 50%**

Table 1.4. Survey findings representing the most commonly experienced anaesthetic-related complications in rats.

The frequency each anaesthetic-related complication was experienced by survey respondents as a percentage of their total anaesthetics performed in rats.

Survey number	Inadequate Anaesthesia	Cold	Apnoea	Prolonged Recovery
4	2		1	
7	1	1		
10			% not specified	1
13				
14	5			
16				1
17	10		% not specified	
19	50			
26	% not specified	% not specified		10
28				
29				
30				
Range	**5 to 50%**	**1%**	**1%**	**1 to 10%**

Table 1.5. Survey findings representing the most commonly experienced anaesthetic-related complications in mice.

The frequency each anaesthetic-related complication was experienced by survey respondents as a percentage of their total anaesthetics performed in mice.

	Rat		Mouse	
Survey number	**Mortality (%)**	**Anaesthetic regimen**	**Mortality (%)**	**Anaesthetic regimen**
1	15.6	Tiletamine-Zolazepam & Xylazine		
2	0.4	Tiletamine-Zolazepam & Xylazine		
3	1	Tiletamine-Zolazepam & Xylazine		
4	No response	Pentobarbitone	2	Tiletamine-Zolazepam & Xylazine
5	10	Ketamine & Xylazine		
6	1	Ketamine & Xylazine		
7			1	Tribromoethanol
8	100	Ketamine & Xylazine		
9	No response	Ketamine & Xylazine		
10	No response	Ketamine & Xylazine	No response	Ketamine & Xylazine
11	No response	Isoflurane		
12	No response	Isoflurane		
13	0.6	Ketamine & Xylazine	No response	Ketamine & Xylazine
14	1	Ketamine & Xylazine	1	Ketamine & Xylazine
15	No response	Tiletamine-Zolazepam & Xylazine		
16	No response	Ketamine & Xylazine	No response	Methoxyflurane
17	0	Ketamine & Xylazine	0	Ketamine & Xylazine
18	No response	Isoflurane		
19			15	Ketamine & Xylazine
20	5	Isoflurane		
21	2.9	Tiletamine-Zolazepam & Xylazine		
22	0	Isoflurane		
23	No response	Isoflurane		
24	No response	Isoflurane		
25	No response	Isoflurane		
26			1.3	Ketamine & Xylazine
27	No response	Isoflurane		
28			No response	Tiletamine-Zolazepam & Xylazine
29			0	Isoflurane
30			No response	Methoxyflurane
Total Responses	**12 of 24 (50%)**		**5 of 12 (41.7%)**	

Table 1.6. Anaesthetic associated mortality rates as experienced by survey respondents

Reported anaesthetic mortality rates defined by each survey respondent for rats and mice.

Anaesthetic regimen	Survey Number	Rat		Mouse	
		Mortality (%)	Average	Mortality (%)	Average
Tiletamine-Zolazepam & Xylazine	1	15.6		2	
	2	0.4			
	3	1			
	15	No response			
	21	2.9	**5.0**		**2**
Ketamine & Xylazine	5	10		1	
	6	1		No response	
	8	100		No response	
	9	No response		0	
	10	No response		15	
	13	0.6			
	14	1			
	16	No response			
	17	0	**18.76**		**5.33**
Isoflurane	11	No response		0	
	12	No response			
	18	No response			
	20	5			
	22	0			
	23	No response			
	24	No response			
	25	No response			
	27	No response	**2.5**		**0**
Methoxyflurane	16	N/a	**N/a**	No response	**N/a**
Tribromoethanol	7	N/a	**N/a**	1	**1**

Table 1.7. Comparison of mortality rates associated with various anaesthetic agent combinations.

Reported anaesthetic percentages associated with the anaesthetic regimen for rats and mice

Discussion

A survey response of 30 replies was lower than anticipated from the large number circulated. The low response could be in part due to the busy nature of staff and researchers, unwillingness to disclose confidential information, or that they just did not know the answers to the questions. Because of the reduced number of survey responses, the results may not be representative of the Australasian research community. In spite of this, the rodent anaesthetic survey findings were still invaluable in providing us with first hand information about rodent anaesthesia within the research community.

Ketamine-xylazine is still the most commonly used injectable anaesthetic regimen

Ketamine-xylazine anaesthetic combination was identified as the most commonly used injectable regimen in mice and rats. This result is consistent with previously published survey findings from 2000-2001 and 2005-2006 that showed an increasing trend towards its use and a decrease in use of pentobarbital (Stokes et al., 2009). A veterinary survey established ketamine combinations as the most widely used anaesthetic agent for almost every species including laboratory rodents due to its perceived safety, minimal cardiovascular and respiratory depressive effects (Wagner and Hellyer, 2000, Saha et al., 2007). However, the safety aspect of this combination did not translate from the veterinary to the research field. One investigator mentioned they experienced an inadequate depth of anaesthesia in 50% of the ketamine-xylazine anaesthetics in mice. Another investigator reported a 83% incidence of apnoea following ketamine-xylazine injection in rats. Additionally, significant mortality rates were reported in the survey findings that were consistent with other published literature (Wixson et al., 1987c, Smith, 1993, Wixson et al., 1987a, Arras et al., 2001). Xylazine itself has been implicated in several anaesthetic-related adverse issues. Twenty-five percent of complications in one study involved the use of xylazine in the veterinary anaesthetic protocol (Dyson et al., 1998). And an increased anaesthetic related mortality rate was observed in cats and dogs given xylazine (Clarke and Hall, 1990). But even with these side effects, ketamine-xylazine combination, first reported for used in rats over 30 years ago, is still the most commonly used rodent anaesthetic today (Stickrod, 1979, Green et al., 1981, Saha et al., 2007).

Zolazepam-tiletamine-xylazine is the alternative injectable anaesthetic

The zolazepam-tiletamine combination anaesthetic, marketed as zoletil® (Virbac Australia, Pty Ltd, Milperra NSW, Australia) is a commonly used veterinary anaesthetic (de la Pena et al., 2012). This combination provides rapid induction, immobilisation, good muscle relaxation, and smooth recovery from anaesthesia (Ferrari et al., 2005). And these features could account for zolazepam-tiletamine and xylazine anaesthetic mixture being the second most chosen injectable anaesthetic protocol in this study. There are many similarities between zolazepam-tiletamine-xylazine and ketamine-xylazine

combinations. Both these anaesthetic regimen incorporate a muscle relaxant, xylazine and a dissociative agent, ketamine or tiletamine. The inclusion of zolazepam provides additional relaxant effects. Zolazepam is a benzodiazepine tranquilizer and tiletamine is an arylaminoalkalone, non-narcotic dissociative anaesthetic agent. They work in combination to provide these anaesthetic qualities (Ferrari et al., 2005, Saha et al., 2007). As with any anaesthetic regimen, there are still some associated side effects. Sprague Dawley rats anaesthetised with zolazepam-tiletamine developed dose and time dependent hypertension similar to known cardio-stimulatory effects of other dissociative anaesthetic agents (Wilson et al., 1993). However, cardiovascular depression, consisting of persistent hypotension and bradycardia, was observed when zolazepam-tiletamine was administered in combination with xylazine (Wilson et al., 1993). Zolazepam-tiletamine-xylazine combination in horses demonstrated that respiratory depression was associated with both CNS sedative effects of xylazine and the dose dependent respiratory depression of tiletamine (Wilson et al., 1993, Hubbell et al., 1989).

The use of pentobarbital as an injectable rodent anaesthetic has been superseded

Previously, pentobarbital was the most commonly used laboratory rodent anaesthetic. However its use has dropped from 33% of the total reported anaesthetic use in 2000-2001, to 16% in 2005-2006 with an increase in isoflurane use from 2% to 16% and ketamine-xylazine combination from 15% to 31% in this time (Stokes et al., 2009). This drop in pentobarbital use is consistent with our findings, with only one in thirty survey respondents picking pentobarbital as their anaesthetic of choice. This investigator also noted they experience a 10% of rats were inadequately anaesthetised, 10% were appeared cold, and 50% of rats experienced prolonged recovery (Table 1.4). When used a sole anaesthetic agent, pentobarbital is considered a poor analgesic and is associated with many complications including severe cardiovascular and respiratory depression and a low safety margin (Ferrari et al., 2005, Flecknell, 1993b, Field et al., 1993, Danneman and Mandrell, 1997). With safety concerns such as these, and an abundance of more reliable anaesthetic combinations available, the shift away from pentobarbital use was inevitable.

Tribromoethanol anaesthesia causes gastrointestinal side effects

Tribromoethanol and its combinations can provide rapid and deep anaesthesia followed by rapid recovery in mice and rats (Weiss and Zimmermann, 1999, Cho et al., 2011, Gopalan et al., 2007, Gopalan et al., 2005). One survey respondent used tribromoethanol frequently in mice and from their experience however, observed approximately 1% mortality rate. Tribromoethanol has also been associated with increased post-operative mortality and intestinal complications such as abdominal adhesions, peritonitis and ileus (Tarin and Sturdee, 1972, Arras et al., 2001, Zeller et al., 1998, Cho et

al., 2011). Contrasting reports state that tribromoethanol has a very low mortality rate when used correctly and has few complications (Weiss and Zimmermann, 1999). But in line with increasing animal welfare concerns, there must be good justification in choosing this anaesthetic over currently available anaesthetic combinations that bring with them fewer post-anaesthetic complications. Additionally, the development of anaesthetic-related intestinal complications could compromise the biomedical research data.

A shift towards the use of inhalation anaesthesia

The shift towards the use of inhalation anaesthesia could likely be driven by the moral and ethical considerations associated with the use of animals in research (Flecknell, 1993a). Inhalation agents are delivered in combination with 100% oxygen and this in itself would improve anaesthetic recovery times and would alone increase its safety. Regardless of the increase in inhalation anaesthetic use, there is still an increased anaesthetic risk to rodents due to their small size. It is technically difficult to intubate rodents so therefore the majority of inhalation anaesthetics are delivered via a face mask and relying on the animal breathing spontaneously (Flecknell, 1993a). Along with risks to rodents, handlers could also potentially be exposed to waste gases where isoflurane exposure has been linked to a higher incidence of infertility and spontaneous abortion in women (Mazze, 1985).

Isoflurane is fast becoming an anaesthetic of choice for rats

Isoflurane gaseous anaesthesia was the most frequently used inhalation anaesthetic agent in this study. This high proportion of gaseous anaesthetic use reflects a shift from older perceptions where anaesthetics merely provided immobilisation to animals, to reducing anaesthetic recovery time and ultimately improving animal welfare (Wixson et al., 1987c). Stokes (2009) reported an increase in isoflurane use from 2.6% to 17.4% within five years for rats (Stokes et al., 2009). This progressive move towards inhalation anaesthesia appears to be continuing with 38.5% of survey respondents within this study choosing isoflurane as their anaesthetic of choice for rats. In general, delivery of gaseous anaesthesia is costly due to the expense of the anaesthetic delivery systems in comparison to the use of a simple needle and syringe for injectable anaesthetic delivery (Flecknell, 1993b).

Methoxyflurane is the inhalation anaesthetic of choice of mice

In mice, the anaesthetic survey identified methoxyflurane as the inhalation anaesthetic of choice. This is likely due to methoxyflurane being relatively safe use in simple anaesthetic chambers (Flecknell, 1993b, Tarin and Sturdee, 1972, Itah et al., 2004, Saul and Fisher, 1974). It is frequently used for short procedures and has been reported to possess analgesic properties (Arras et al., 2007).

Inadequate anaesthetic depth is the most commonly reported anaesthetic complication

An inadequate depth of anaesthesia following an initial dose of an injectable anaesthetic agent was listed as the most frequently encountered anaesthetic complication. Multiple confounding issues could influence this finding, such as the length of time post injection that the respondent allowed before deeming the anaesthetic inadequate and a possible lack of knowledge of correct anaesthetic dosages. Several survey respondents were unable to supply accurate anaesthetic dose rates but instead provided a recipe for their anaesthetic mixture or a volume administered to each animal without obtaining body weights. This could indicate that many survey respondents were unaware of the exact dosage of an anaesthetic agent each rodent received. Other complicating issues could be associated with differences in anaesthetic requirements for varying strains of rats and mice where protocols may not have been tailored to that particular strain.

Temperature control is difficult in rodents

Maintenance of core temperature is of utmost importance as hypothermia is possibly the most important cause of anaesthetic-related mortality in mice (Flecknell, 1993b). The high surface-to-mass ratio in rodents can lead to rapid heat loss of 4 to10 ^{o}C in core body temperature over 15 to 20 minutes during anaesthesia (Taylor, 2007). Pentobarbital was found to cause profound hypothermia in rats and ketamine-xylazine combinations caused a dose dependent depression in core and surface body temperatures (Wixson et al., 1987c). This is consistent with one investigator's observations that 50% of their rats appeared cold during pentobarbital anaesthesia. Volatile anaesthetics such as isoflurane and methoxyflurane also cause a decrease in body temperature (Hitt et al., 1977). Therefore external heating would be recommended to aid in maintenance of core body temperature. Heat pads or heat blankets with a surface temperature of 37.2-38.9^{o}C provided rats and mice with an effective heat source that could help maintain core body temperature during anaesthesia (Taylor, 2007). In contrast, 4 survey respondents specifically noted that their animals appeared cold regardless of the presence of a heat pad and in this case, these rodents may be experiencing peripheral vasoconstriction rather than actual hypothermia. It must also be mentioned that a heat pad usually only provides a small contact area in which case, would provide little thermoregulatory support to the rat or mouse. In this study, the temperature of the external heating provided by the survey respondent and the body temperature

readings were not ascertained. The respondents were also not asked to define at what point they determined a rodent was cold. So it could only be postulated that if in fact the rodents were hypothermic and the delivery of external heating was inadequate.

Respiratory compromise is frequently associated with rodent anaesthesia

Apnoea was one of the most frequently observed anaesthetic complications in this study. There is a multitude of possible problems that can lead to the development of apnoea. Respiratory depressive drugs, increased anaesthetic depth, and also lack of oxygen supplementation are only a few possible preceding factors leading to apnoea. It is always recommended however, that 100% oxygen supplementation is administered during anaesthesia especially if a respiratory depressive agent.

Unexpected wake up from anaesthesia is not uncommon

Unexpected wake up from anaesthesia was observed during both inhalation and injectable anaesthesia. It is expected that the cause of anaesthetic "wake up" is due to inadequate depth of anaesthesia. This could reflect the lack of training prior to performing anaesthesia with particular anaesthetic agents. Also a lack of expertise in using equipment, such as an inhalation anaesthetic circuit, could in itself be detrimental to the rat or mouse. Additionally, inadequate anaesthesia for injectable agents could likely be due to the IP route of administration as this route is slow to absorb into circulation and is subjected to a high degree of liver metabolism (Lukas et al., 1971) (Flecknell, 1993b). This resultant clinical effect is variability in depth of anaesthesia. Frequently, survey respondents mentioned that their reason for choosing an anaesthetic protocol was due to their familiarity with it and the importance of previous experience with an anaesthetic regimen must not be overlooked.

Research concerns over providing additional analgesia

It is widely accepted that pain and distress should be minimised when performing experimental procedures (Flecknell, 1984, Liles and Flecknell, 1992b, Liles and Flecknell, 1993, Hawkins, 2002). In this survey, 48% of respondents provided analgesia to their rats and 40% for mice. 2 of 12 rats and 1 of 4 mice survey respondents did not provide analgesia for invasive procedures. One common argument against the use of analgesia is that although an analgesic agent could improve the analgesic properties of an anaesthetic regimen, this addition could lead to marked alterations in the physiological effects of an anaesthetic (Wilson et al., 1993). There are some concerns that analgesic agents could potentially affect experimental outcomes, but it is usually possible for an investigator to be able to administer one of several classes of analgesic agents including opioids, non-steroidal anti-inflammatory (NSAID) medications and local anaesthetics. Also pain assessment in rodents can be

subjective and many behavioural markers for pain can be missed (Hawkins, 2002, Roughan and Flecknell, 2003b). This variability in pain assessment methods could ultimately contribute to the lack of pain relief used for particular studies.

Buprenorphine is the most commonly used rodent analgesic agent

Survey results identified buprenorphine as the most frequently used analgesic agent for rats and mice, which is consistent with published data (Richardson and Flecknell, 2005). Buprenorphine is an opioid partial agonist that has a high affinity for the μ receptor, and morphine-like agonist effects at low doses and minimal agonist effects at higher doses (Dum and Herz, 1981, Bishop, 2005, Bourque et al., 2010). This opioid possesses a ceiling effect for respiratory depression but not for its analgesic properties (Guarnieri et al., 2012). Bupenorphine can be administered subcutaneously or orally in rats and mice (Liles and Flecknell, 1992a, Volker et al., 2000, Abelson et al., 2012) and has shown to be effective in treating multiple pain conditions including neuropathic pain (Guarnieri et al., 2012). Pre-anaesthetic subcutaneous (SC) administration of buprenorphine, during ketamine-medetomidine injectable anaesthesia, was associated with respiratory arrest and mortality in rats (Hedenqvist et al., 2000). In contrast, pre-anaesthetic or peri-anaesthetic buprenorphine followed by halothane anaesthesia improved food and water intake post surgically compared with that of control rats (Liles and Flecknell, 1993, Liles and Flecknell, 1994). There is also evidence that opioid medication itself could potentially reduce the stress response associated with surgery and therefore there is a possibility that the effects of buprenorphine could be unrelated to pain (Liles and Flecknell, 1994). Even though buprenorphine administration has associated complications, it appears to have become a prominent analgesic agent for laboratory rodents regardless of the varying opinions on its safety.

NSAIDS provides an alternative analgesic agent to opioid drugs

As a means of reducing opioid associated side effects, alternative analgesics such as the NSAID agents have been employed (Bourque et al., 2010, Roughan and Flecknell, 2003a). The NSAID medications used by survey respondents were meloxicam and carprofen for rats but not mice. Meloxicam and carprofen are cyclo-oxygenase inhibitors that reduce inflammatory pain by reducing or stopping prostaglandin, prostacyclin and thromboxane production (Liles and Flecknell, 1992b). NSAID are sometimes considered weaker analgesic agents than opioid analgesic agents, but due to their anti-inflammatory actions, can suppress pain by reducing pain associated with an inflammatory reaction to surgery (Liles and Flecknell, 1992b, Liles and Flecknell, 1994). In an analgesic study, meloxicam treated rats showed fewer signs of behavioural and physiological changes than buprenorphine treated rats following implantation of radiotelemetery devices (Bourque et al., 2010). There are however potential side effects to the use of NSAID, such as gastric ulceration and other

gastrointestinal complications (Engelhardt et al., 1995a, Engelhardt et al., 1995b, van der Vijver et al., 2012), and these factors should be taken into account when determining the most effective analgesic agent for a particular surgical procedure.
Paracetamol has no anti-inflammatory properties but at the correct doses can provide good analgesia (Liles and Flecknell, 1992b).

Anaesthetic related mortality rates in laboratory rodents are not usually recorded
Mortality rates for laboratory animal anaesthesia are frequently unpublished (Whelan and Flecknell, 1992). In contrast, anaesthetic mortality rates are frequently published in the veterinary and medical professions. In veterinary practice, one study reported a 1 in 679 mortality rate in dogs and cats undergoing anaesthesia and another study recorded 48 in 3546 mortality due to anaesthetic related complications equating to approximately 1.4% (Whelan and Flecknell, 1992, Clarke and Hall, 1990, Bille et al., 2012). Mortality rates as low as 1 in 10,000 was recorded in the medical field (Dyson et al., 1998). In comparison, the Researchers' attitude towards acceptable losses varies from 1.4% as unacceptable to 9% being considered low (Whelan and Flecknell, 1992). Previously published data indicate that mice anaesthetised with ketamine-xylazine or ketamine-diazepam showed the higher mortality rate than pentobarbital and fentanyl in combination with droperidol (Wixson et al., 1987c). High doses of ketamine-xylazine combination given IP to mice above 99/13.2mg.kg^{-1} and rats above 80/12mg.kg^{-1} caused significant mortality (Smith, 1993). This high mortality rate is consistent with findings from other investigators where significant mortalities were noticed with the ketamine-xylazine drug combination (Arras et al., 2001, Wixson et al., 1987a).

In contrast to laboratory mice, the inclusion of ketamine for use in dog and cat anaesthesia was associated with lower than expected complication rates and possibly due to the sympathetic stimulation by ketamine (Dyson et al., 1998). 50% of rat and 41.7% of mice survey respondents did not respond to the question regarding their experience with anaesthetic-related mortality rate. This low response rate could be interpreted in several ways. It is possible that the survey respondents did not experience any mortality with their anaesthetic protocol or they were unwilling to respond to this question. The majority of participants that did not respond used inhalation anaesthesia, which is has been shown to have fewer complications and possibly considered the safer route of anaesthetic delivery. Poor peri-operative care due to inadequately trained anaesthetic administrators could be a major contributor to mortality rates indicated in this survey (Flecknell, 1993b). Hypothermia due to rapid temperature loss could contribute to high mortality rates (Wixson et al., 1987c).

Drug restrictions do not determine anaesthetic regimen

The Health (Drugs and Poisons) Regulation 1996 tightly controls the use of schedule 8 (controlled drugs) drugs of addiction in Australia, therefore increasing the difficulty in obtaining pain relief medication such as buprenorphine as well as anaesthetic agents like ketamine. Even with these restrictions, only 10% of survey respondents chose an alternative anaesthetic protocol because of these restrictions. This could be in part due to the increase in animal welfare requirements for analgesic agents such as opioids. It is likely that many larger research institutions would have access to an authorised person legally able to obtain, store and dispense restricted medications to research staff. With these findings, it would seem that researchers have more freedom for choice of anaesthesia and analgesia protocols than previously speculated.

The rodent anaesthetic survey identified several commonly chosen anaesthetic combinations, analgesic agents and with them their associated anaesthetic complications. The results from the survey show that there is a need for increased anaesthetic training for investigators. At present, only a limited number of anaesthetic agents are being utilised and it appears that these few anaesthetic protocols are still accompanied by a relatively high number of complications. Therefore it is prudent to find a simple and safe anaesthetic protocol for rats and mice that provides a wide margin of safety to ultimately reduce anaesthetic related complications and death. We believe that alfaxalone anaesthesia could possibly fill this gap and provide a safer and easy alternative laboratory rodent anaesthetic to currently available anaesthetic agents.

CHAPTER TWO

INTRODUCTION TO PUBLISHED PAPER

Pilot Study – Determination of alfaxalone concentration

In laboratory rodents, it is not routine to perform anaesthesia via the intravenous route. This is technically difficult in rats and can only be performed by highly trained individuals. Therefore it is important to determine whether other routes of alfaxalone anaesthetic administration are feasible. Results from the rodent anaesthetic survey showed that the most commonly used route of injection in laboratory rodents is by intraperitoneal injection. This route offers ease of administration requiring only simple equipment and minimal handling expertise (Flecknell, 1993b). A pilot experiment was constructed to compare the intraperitoneal, intramuscular and subcutaneous dosing regimen for alfaxalone.

Alfaxalone pilot study - 10 mg.kg^{-1} IM, SC & IP

Personal communication with Jurox Pty Ltd, the manufacturer of Alfaxan®, identified anecdotally that premedication with 2-3 mg.kg^{-1} xylazine followed by 10-11 mg.kg^{-1} Alfaxan® provided adequate anaesthesia for rats. Therefore the starting dose for used in this study was 10 mg.kg^{-1} alfaxalone to provide baseline data. Five adult female Wistar rats weighing 135-157g were each given an IM, SC and IP dose of 10 mg.kg^{-1} alfaxalone (Appendix 2.1). A period of at least 48 hours was allowed between anaesthetic routes to ensure alfaxalone was washed out from previous administration. IM injections were performed in the right quadriceps femoris muscle (Chellman et al., 1994), SC injections were administered into the loose skin on the right dorsal flank and IP injections were given in the left lower quadrant of the ventral abdominal cavity (Smiler et al., 1990). The induction time was recorded as the time from anaesthetic administration to when rats lost their ability to maintain sternal recumbency. Immobilisation time was calculated as the total time from induction to return of sternal recumbency. The twitching score was developed in an attempt to quantitatively assess the degree of twitching observed with each anaesthetic (Table 2.0).

Twitch Score	Description
None (- or 0)	Relaxed body position No spasmodic movements
Mild (+ or 1)	Short-lived minor muscle contractions Relaxed body position Localised to the facial region or extremities
Moderate (++ or 2)	Short-lived minor muscle contractions Multiple regions affected at one time Increased muscle tone
Severe (+++ or 3)	Full body pronounced muscle contractions Increased muscle tone Marked startle response to auditory or physical stimulus

Table 2.0. A descriptive and quantitative measurement for twitching

Twitching was categorised into three categories, where each category shows increasing levels of neuro-excitation

Alfaxalone administration – 10 mg.kg^{-1} IM

The IM route showed the most consistency in inducing anaesthesia, with all five rats injected with alfaxalone showing an induction time of 2-4 minutes. Four of the five rats attained immobilisation for 7-13 minutes, and these same four rats twitched during this time. The twitch was seen as either a full body or facial twitch and all were allocated a twitch score of 2 (Table 2.0).

Alfaxalone administration – 10 mg.kg^{-1} SC

Three out of five rats showed an induction time of four minutes after the SC injection of alfaxalone. Immobilisation time, however, was very short for these rats and only lasted 1-4 minutes. The remaining two rats were not anaesthetised and did not lose their righting reflex. A twitch score of 1 was observed in two rats, one of which was not anaesthetised.

Alfaxalone administration – 10 mg.kg^{-1} IP

Only one of the five rats achieved anaesthetic induction, and the time to induction was long at 10 minutes. This rat was immobilised for two minutes, in which only lateral recumbency was allowed. The remaining 4 rats were not anaesthetised. They appeared sedated and remained responsive to physical and auditory stimulation. Twitching was not observed in any rats receiving IP dosing.

10 mg.kg^{-1} alfaxalone IM, SC or IP

IM appeared to be the most effect route of alfaxalone administration. This was the only route that achieved anaesthetic immobilisation in all rats dosed, albeit short. SC alfaxalone was able to induce three of the five rats for anaesthesia, but immobilisation time was too short to allow for any procedures to be performed. IP alfaxalone at10 mg.kg^{-1} could not be considered an anaesthetic agent, as merely sedation was achieved. This could be attributed to the first pass elimination of alfaxalone through hepatic metabolism after IP dosing (Lukas et al., 1971). Twitching was most apparent with IM alfaxalone and was not observed with IP alfaxalone.

It was obvious that 10 mg.kg^{-1} alfaxalone did not produce adequate anaesthesia via any route. IM alfaxalone appeared promising, however the immobilisation times achieved were short. We therefore decided to increase the alfaxalone dose to 20 mg.kg^{-1}. IP administration at 20 mg.kg^{-1} was shown in our pharmacokinetic data to produce sustained plasma alfaxalone levels up to 60 minutes. The IM route was discontinued at this dose, due to the increased injection volume. Large volumes administered IM is difficult in practice and appears to cause more pain than injections at other sites; it has even been suggested that this route not be used in smaller mammals, including rodents (Morton et al., 2001). Therefore only IP and SC routes were investigated further at 20 mg.kg^{-1} alfaxalone.

Alfaxalone pilot study – 20 mg.kg^{-1} SC & IP

For the SC study, female Wistar rats weighing 156-195g (n=8), were given a single 20 mg.kg^{-1} SC dose of alfaxalone. For the IP study, female Wistar rats at a starting weight of 155-205g (n=4), were given 20 mg.kg^{-1} IP once weekly for 2-4 consecutive weeks.

Alfaxalone administration – 20 mg.kg^{-1} SC

Induction times were variable and ranged between 4-21 minutes. Two of eight rats were not anaesthetised and therefore did not achieve anaesthetic induction or immobilisation. Immobilisation times also varied and ranged from 2-40 minutes (Appendix 2.3.). Twitching occurred in three of eight rats with twitch scores ranging from 1-3.

Alfaxalone administration – 20 mg.kg^{-1} IP

Anaesthetised rats produced induction and immobilisation times ranging from 2-6 minutes and 31-58 minutes respectively. However, on three occasions and in three different rats, anaesthesia was not achieved. These data therefore showed that 75% of rats achieved immobilisation and the remaining 25% were not anaesthetically induced (Appendix 2.2). In addition, moderate to severe twitching was apparent in all rats that were immobilised.

Overall, it appeared that the increased alfaxalone dose was more promising. 20 mg.kg^{-1} alfaxalone was much more effective as an anaesthetic than 10 mg.kg^{-1} alfaxalone. However, as anaesthesia was not achieved in all rats, and the variation in anaesthetic time for the SC route was prevalent, an increase in alfaxalone dose to 30 mg.kg^{-1} was investigated.

Alfaxalone pilot study – 30 mg.kg^{-1} SC & IP

Female Wistar rats (n=3) weighing 182-195g were giving a single dose of alfaxalone SC at 30 mg.kg^{-1}. 30 mg.kg^{-1} IP was administered to male Sprague Dawley rats, weighing 160-220g (n=14).

Alfaxalone administration – 30 mg.kg^{-1} SC

Two out of three rats achieved anaesthetic induction within 3-5 minutes. The last rat however took 20 minutes before the righting reflex was lost. These animals showed a range of 3-20 minutes for induction. Immobilisation times ranged from 39-83 minutes. Twitching occurred in all three rats, with two rats twitching severely (Appendix 2.3).

Alfaxalone administration – 30 mg.kg^{-1} IP

Eleven of fourteen rats achieved anaesthetic induction within 2-4 minutes of alfaxalone administration. Three of fourteen rats did not lose their righting reflex and therefore were not induced for anaesthesia. Of the anaesthetised rats, immobilisation time ranged from 7-33 minutes. The incidence in twitching appeared markedly reduced, and were observed in five of fourteen rats been rated as mild in the majority of these (Appendix 2.4).

Alfaxalone administration – IM route

A starting dose of 10 mg.kg^{-1} alfaxalone was administered. Of the three routes examined, IM appeared the most promising with all five rats immobilised by this route. Unfortunately, increased IM dose rates were not practical, as the injection volume necessary was prohibitive. Increased injection volume following an IM dose could lead to acute pain-on-injection as well as delayed pain associated with deep tissue trauma (Chellman et al., 1994). Rats given an injection volume of 0.16-0.47mL ketamine-xylazine developed gross and microscopic lesions following IM injection, likely causing post-anaesthetic pain and discomfort for at least two weeks (Smiler et al., 1990). Alfaxan® preparation is within a physiological pH of 6.5-7.0 and therefore should be less irritating for IM injections. However, it was shown that a large injection volume will physically distend the muscle, leading to swelling at the injection site and, ultimately, muscle damage and pain (Morton et al., 2001). Also, isotonic solutions of saline administered IM to Sprague Dawley rats at 0.04mL caused myocyte degeneration in the right hind leg musculature and necrosis with histiocytic infiltration from one day post injection (Barrueto et al., 2002). In this study, post-anaesthetic discomfort cannot be ruled out as histopathological examination of the lateral femoral muscles were not examined. It is recommended that the IM route for injections be used only if an alternative route of administration is not possible (Morton et al., 2001).

Alfaxalone administration – SC route

The SC route of anaesthesia showed vast variability in induction times at all doses examined. Through our pilot study trials, the induction times appeared more consistent at 10 mg.kg^{-1}, with three of five rats achieving anaesthetic induction within four minutes, however at the higher doses, an induction time variation of 4-21 minutes was seen with 20 mg.kg^{-1} and 3-20 minutes at 30 mg.kg^{-1}. Due to this variability, the SC route was ruled out as a viable alternative option to IV alfaxalone administration.

Alfaxalone administration – IP route

The administration of 10 mg.kg^{-1} alfaxalone IP was not effective at providing anaesthesia, as several rats were not even induced for anaesthesia. Only one in five rats even developed recumbency and the period to which they were immobilised was short. Following 20 mg.kg^{-1} IP alfaxalone, rats displayed more consistent induction and immobilisation times than that produced from the lower dose, but still 25% rats were not anaesthetised. Additionally an appreciably high frequency of twitching was observed with this dose of alfaxalone (Appendix 2.2). 11 of 14 rats were anaesthetised with 30 mg.kg^{-1} IP alfaxalone, producing short immobilisation times of between seven and 33 minutes. The increase in dosing rate to 30 mg.kg^{-1} was performed with male Sprague Dawley rats (due to availability), in contrast to the other studies, which were performed in female Wistar rats Therefore, a direct comparison between IP doses of alfaxalone at 20 mg.kg^{-1} and 30 mg.kg^{-1} was not made due to the strain and sex differences in rats. However, Wistar and Sprague Dawley rats are outbred rat strains, both more variable than isogenic inbred rats (Festing, 1993). Additionally no published sex differences have been reported in other species with the use of Alfaxan® (Ferre et al., 2006, Goodwin et al., 2011).

The increase in alfaxalone dose to 30 mg.kg^{-1} SC did not appear to improve induction times however immobilisation time was increased. Twitching was severe in two of the three rats administered this dose of alfaxalone SC. As for IP alfaxalone, induction times were consistent, but immobilisation times were shorter. Twitching was severe in one rat given IP alfaxalone. We observed no detrimental effects from a three fold increase in alfaxalone dose from 10 mg.kg^{-1} to 30 mg.kg^{-1} to rats other than twitching, highlighting the large safety margin alfaxalone possesses. The preliminary data showed the IP dose of 20mg.kg^{-1} was most effective at producing immobilisation and therefore was chosen for pharmacokinetic investigation.

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SHORT COMMUNICATION

Full title:

Plasma pharmacokinetics of alfaxalone after a single intraperitoneal or intravenous injection of Alfaxan® in rats

Short running title:

Intravenous and intraperitoneal alfaxalone pharmacokinetics in rats

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Abstract

Alfaxalone (3α-hydroxy-5α-pregnane-11, 20-dione) is a neuroactive steroid with anaesthetic properties and a wide margin of safety. The pharmacokinetic properties of alfaxalone administered intravenously and intraperitoneally in rats (n=28) were investigated. Mean $T_{1/2elim}$ for 2 and 5 $mg.kg^{-1}$ IV was 16.2 and 17.6 min respectively, but could not be estimated for IP dosing, due to sustained plasma levels for up to 60 minutes after injection. Cl_p for IV injection was calculated at 57.8 ± 23.6 and 54.3 ± 6.8 $mL.min^{-1}.kg^{-1}$, which were 24.5% and 23% of cardiac output respectively. The observed C_{max} was 3.0 $mg.L^{-1}$ for IP administration, and 2.2 ± 0.9 and 5.2 ± 1.3 $mg.L^{-1}$ for 2 and 5 $mg.kg^{-1}$ IV administration respectively. AUC_{0-60} was 96.2 $min.mg.L^{-1}$ for IP dosing. The relative bioavailability for IP dosing was 26% and 28% compared to IV dosing. Differences in $t_{1/2elim}$ and Cl_p from previous pharmacokinetic studies in rats are likely due to variations in alfaxalone formulation rather than sex differences. Alfaxan® given IP caused sustained levels of alfaxalone, no apnoea and longer sleep times than IV dosing, although immobilization was not induced in 30% of rats given Alfaxan® IP. A pharmacodynamic study of the effects of combining IP injection of Alfaxan® with other premedication agents is worthwhile, to determine whether improved anaesthesia induction could ultimately provide an alternative anaesthetic regimen for rats.

Keywords

Alfaxalone, rat, pharmacokinetics, intraperitoneal, anaesthesia

Alfaxalone (3α-hydroxy-5α-pregnane-11, 20-dione) is a neuroactive steroid with anaesthetic properties due to its positive allosteric modulation of the gamma aminobutyric acid type A ($GABA_A$) receptor (Visser et al., 2000). Alfaxalone solubilised with the exipient 2-hydroxypropyl-β-cyclodextrin (HPCD) is available as Alfaxan® (Jurox Pty Ltd, Rutherford, NSW, Australia), which is registered for the induction and maintenance of anaesthetic in both dogs and cats in multiple countries. Studies have shown that intravenous Alfaxan® provides satisfactory induction of anaesthesia in dogs (Ferre et al., 2006), cats (Zaki et al., 2009), horses and pigs (Keates, 2003, Goodwin et al., 2011, Goodwin et al., 2012, Whittem et al., 2008), with a five-fold margin of safety at supraclinical doses in dogs (Ferre et al., 2006, Muir et al., 2008) and cats (Whittem et al., 2008). The pharmacokinetic parameters of alfaxalone have been defined in cats (single/multiple doses), dogs (single dose) and horses (single dose) (Ferre et al., 2006, Goodwin et al., 2011, Pasloske et al., 2009, Goodwin et al., 2012, Whittem et al., 2008).

Rodents are commonly anaesthetised for veterinary and biomedical research purposes with a single intraperitoneal (IP) injection. While IP injection is technically simple and requires minimal animal restraint, a single dose of anaesthetic can lead to highly variable anaesthetic effects (Flecknell, 1993a). Therefore it is important for an injectable agent to have a wide margin of safety to account for variation in anaesthetic responses between individuals. Alfaxalone has been investigated in rats in varying formulations including acetonitrile (Visser et al., 2000) and the alfaxalone/alfadalone combination for humans (Althesin®) and animals (Saffan®), equivalent to 9 $mg.mL^{-1}$ alfaxalone in the solvent Cremaphor EL (Child et al., 1972). As yet no work has been performed in rodents with the commercially available formulation Alfaxan®, which is devoid of alfadolone and Cremophor EL. In this study, we investigated the single dose pharmacokinetics of alfaxalone, administered intravenously or intraperitoneally as Alfaxan® in rats.

All experiments were performed after approval by the University of Queensland Animal Ethics Committee (Approval number: 301/10). Adult female Wistar rats (7-10 weeks, 244.2 ± 39.4 g, n = 28) housed in a barrier maintained animal facility with a 12/12 hr light/dark cycle and fed standard rat pellets and water *ad libitum* were used. For IP dosing, a single injection (20 $mg.kg^{-1}$, n=18) of Alfaxan® (Batch # 60091) was administered intraperitoneally into the right lower quadrant of the ventral abdomen. Serial blood samples collected from a cut on the tail tip were taken from 10 rats at 4, 6, 10, 15, 20, 30, 45 and 60 minutes post-injection; samples were not collected from all 10 rats at all times, due to variability in time to recovery from immobilization (Table 2.1) or failure to collect a sufficient volume of blood at different time points. A single intracardiac blood sample was collected at one of these times post-injection in another 8 rats following termination. This sampling

collectively gave sample times of 4, 6, 10, 15, 20, 30, 45 and 60 minutes with varying numbers of animals sampled per time point for IP injection (see Figure 2.1 for number of animals sampled per time point).

For IV dosing, an indwelling IV catheter (polyethylene tubing 50 mm length, 0.50 mm OD, 0.20 mm ID) was introduced into the femoral vein, following anaesthesia with isoflurane gas using a Tec 3 isoflurane vapouriser attached to a rodent circuit and size 1, 6.0cm anaesthetic face mask (all from Vetquip, Castle Hill, NSW, Australia). The catheter was fixed to the skin with cyanoacrylate adhesive. Rats recovered from isoflurane anaesthesia for 30 – 60 minutes post-implantation prior to IV dosing. Alfaxan® (Batch # 60091), diluted to 1 $mg.mL^{-1}$ with sterile water for injection (Norbrook®, Tullamarine, Vic, Australia) was administered by slow, continuous IV infusion over 60 seconds (2 $mg.kg^{-1}$, n=5; 5 $mg.kg^{-1}$, n=5).

For all animals, the time from injection to immobilization (failure of the rat to right itself from lateral or dorsal recumbency) and to recovery (the time at which sternal recumbency was restored) were recorded as induction and recovery times respectively, and the difference between these times was calculated as sleep time. Tail tip blood samples (maximum volume 200 µL, maximum total volume 1.6 mL) were collected from each rat at 2, 4, 6, 10, 15, 20, 30, 45 and 60 minutes, and immediately placed onto wet ice, then centrifuged (4000g, 10 min) within an hour of collection. The minimum effective concentration (MEC) for immobilization was empirically determined by interpolation between plasma concentration before and after the time of recovery from immobilization. Plasma samples were kept frozen at -80 ±10˚C during storage and transport to the Jurox Chemistry Laboratory until analysis. All plasma samples were analysed individually (Ferre et al., 2006, Pasloske et al., 2009, Whittem et al., 2008).

Alfaxalone Analysis

Plasma samples underwent solid phase extraction (25-50 µL sample volume), followed by liquid chromatography-mass spectrometry (10 µL sample volume) over a 24 hour period in the same analytical run using previously described methods (Goodwin et al., 2012). A calibration curve was generated using a standard sample set prepared by spiking known amounts of 11-hydroxyprogesterone as the internal standard and alfaxalone (2 linear standard curves covering 0.02 – 0.5 $mg.L^{-1}$ and 0.5 – 5 $mg.L^{-1}$, $r^2 > 0.99$ for both segments)/ into extracted blank rat plasma. Sample alfaxalone concentration was determined by the alfaxalone;11-hydroxyprogesterone peak area ratio. The assay lower limit of quantification (LLOQ) was 0.02 $mg.L^{-1}$ in extracted plasma.

The intra-assay coefficient of variation at LLOQ was <18.5% and <11.0% for other concentration levels. The limit of detection (LOD) was 0.002 $mg.L^{-1}$. Sample extraction recoveries were >86 % for all concentration levels. Both accuracy and precision were acceptable if residual standard deviation (RSD) was ±<20% at LLOQ and ±<15% for other concentrations. Due to variations in the number of

samples per sample time obtained from rats receiving IP injection, data analysis was performed on pooled data for each time point following IP injection, while data from rats receiving IV injections was analysed for each animal individually (Whittem et al., 2008, Ferre et al., 2006, Pasloske et al., 2009).

Pharmacokinetic analysis was carried out using non-compartmental methods in Phoenix® WinNonLin® v 6.3 (Pharsight Products, Sunnyvale CA, USA) as previously described (Goodwin et al., 2012). The area under the curve (AUC) from time of administration to the last blood sample ($AUC_{0\text{-last}}$) was calculated with the linear trapezoidal rule (Gibaldi, 1982). The terminal slope of the curve (λ_z), estimated by linear regression of ≥ 3 data points using the best fit function, was extrapolated to calculate the total AUC from administration time to infinity ($AUC_{0\text{-inf}}$) as $AUC_{0\text{-last}}$ + the extrapolated AUC. The % AUC extrapolated from the last sample time (AUC_{extrap}%) was calculated as the the extrapolated AUC ÷ $AUC_{0\text{-inf}}$ × 100%. The plasma clearance (Cl_p), volume of distribution (V_D) and elimination half-life ($t_{1/2elim}$) were calculated using standard non-compartmental formulae. For concentration parameters, a best-fit approach was utilised where repeated non-linear regressions were used for a series of terminal points. For each regression, an adjusted R^2 was computed with:

$$Adjusted\ R^2 = \frac{1 - (1 - R^2) \times (n - 1)}{(n - 2)}$$

where *n* are the number of data points in the regression and R^2 is the square of the correlation coefficient. The relative bioavailability (F%) for IP dosing compared to IV dosing was calculated with:

$$F\% = \frac{AUC\ (IP\ route)}{AUC\ (IV\ route)} \times \frac{Dose\ (IV)}{Dose\ (IP)} \times 100$$

(Toutain and Bousquet-Melou, 2004a). All data are expressed as mean ± SD.

The level of anaesthesia achieved by alfaxalone was not tested by evaluating reflex responses or making serial cardiorespiratory measurements. However, a brief description of the immobilization achieved is useful; in particular to note that anaesthesia was sufficient to allow repeated blood sampling from a tail tip cut, which involved manual massage of the tail to expel blood from the wound. Apnoea, lasting 95-170 sec, occurred in 2 of 5 rats at 2 $mg.kg^{-1}$ IV and 4 of 5 rats at 5 $mg.kg^{-1}$ IV dosing, followed by recovery of spontaneous breathing. The mean sleep times for rats given 2 and 5 $mg.kg^{-1}$ IV were 10.4 ± 6.6 and 12.6 ± 3.3 min, respectively, which were not significantly different (P = 0.52, unpaired two tailed t test). In contrast, after IP dosing, immobilization was not induced in 3 of 10 rats, but no apnoea was observed (Table 2.1). The mean sleep time for all rats

given 20 mg.kg^{-1} IP was 29.6 ± 21.4 min, which was not significantly longer than for either IV dose ($P > 0.05$, unpaired two tailed t test); excluding rats in which no immobilization was induced, mean sleep time was 42.3 ± 7.8 min, which was significantly longer than for either IV dose ($P < 0.0001$, unpaired two tailed t test).

Animals which did not become immobilized had plasma concentrations of alfaxalone in the lower end of the range (0.58-0.78 mg.L^{-1}) at 4 minutes after injection; animals which did show immobilization had similar or higher plasma alfaxalone levels at 4 minutes (range 0.47-3.78 mg.L^{-1}). As all animals given alfaxalone IV showed immobilization, we suggest that variable absorption from the site of IP injection are the most likely cause for lack of immobilization. The mean MEC for immobilization of rats receiving 2 or 5 mg.kg^{-1} IV was 1.5 ± 0.8 and 2.1 ± 0.4 mg.L^{-1}, respectively (Figure 2.1); and was estimated from pooled data to be 1.9 mg.L^{-1} for IP dosing. IP injection caused muscular twitching in 8 rats during induction and 7 rats on recovery, with 6 rats twitching during both induction and recovery. Mild facial twitching occurred in 2 rats with 2 mg.kg^{-1} IV alfaxalone. Mean $t_{1/2elim}$ for 2 and 5 mg.kg^{-1} IV were similar (16.2 ± 9.2 and 17.6 ± 3.2 min respectively) as was the mean Cl_p (57.8 ± 23.6 and 54.3 ± 6.8 mL.min^{-}1.kg^{-1} respectively; Table 2.2).

To facilitate comparison of Cl_p between species, Cl_p was calculated at 24.5% and 23% of cardiac output respectively (Toutain and Bousquet-Melou, 2004b). The mean Cl_p in rats following IV injection was similar to that reported for the dog (53 and 59. mL.kg^{-}1.min^{-1} for 2 and 10 mg.kg^{-1} (Ferre et al., 2006)), but was larger than the mean Cl_p reported for cats, horses and foals with (25, 37 and 20 mL.min^{-}1.kg^{-1}, for 5, 1 and 3 mg.kg^{-1} IV respectively (Goodwin et al., 2011, Goodwin et al., 2012, Whittem et al., 2008) In contrast to the high Cl_p of alfaxalone following IV injection, sampling times of ≤60 min were not adequate to reliably determine alfaxalone Cl_p following IP dosing (Figure 2.1).

We observed a C_{max} of 2.99 mg.L^{-1} at 10 minutes after IP administration, a value between the C_{max} of 2.2 ± 0.9 and 5.2 ± 1.3 mg.L^{-1} for 2 and 5 mg.kg^{-1} IV respectively; by comparison, the C_{max} seen in dogs following 2 mg.kg-1 IV was similar (Ferre et al., 2006). In contrast, IP dosing led to sustained alfaxalone plasma levels, with an $AUC_{0\text{-}last}$ of 96.2 min.mg.L^{-1}, in comparison to $AUC_{0\text{-}last}$ of 36.9 and 86.7 for 2 and 5 mg.kg^{-1} IV respectively. Sustained plasma alfaxalone levels are the most likely cause of longer sleep times following IP injection (Fink et al., 1982), compared with IV administration. The relative bioavailability for 20 mg.kg^{-1} alfaxalone given IP was 26% and 28%, compared to the 2 and 5 mg.kg^{-1} doses given IV respectively. This low bioavailability via the IP route is consistent with high first pass elimination of alfaxalone through hepatic metabolism (Sear and McGivan, 1981, Novelli et al., 1975, Child et al., 1972) after IP injection, which predominantly enters the portal circulation (Lukas et al., 1971). The $t_{1/2elim}$ of 16-17 minutes for the IV doses in our study using adult female

Wistar rats is longer than for male Charles River rats (Child et al., 1972) or for male Wistar rats (Visser et al., 2000) (7 minutes for ≈ 9 $mg.kg^{-1}$ alfaxalone as Althesin® IV and 13 minutes for 5 $mg.kg^{-1}$ alfaxalone in acetonitrile IV respectively). The longer $t_{1/2elim}$ observed in our study is consistent with the lower Cl_p we observed, in comparison to a Cl_p of $71mL.min^{-}1.kg^{-1}$ (Visser et al., 2000). While sex could account for differences in $t_{1/2elim}$ and Cl_p in our and other pharmacokinetic studies in rat, and female Wistar rats had a 64% higher C_{max} than males after IP administration of Althesin® (Fink et al., 1982), the pharmacokinetics of alfaxalone given IV are reported to be similar in male and female dogs (Ferre et al., 2006) and cats (Whittem et al., 2008). Methodological differences in analysis of plasma alfaxalone between older (Child et al., 1972, Fink et al., 1982) and more recent (Visser et al., 2000) studies could contribute to the differences between our observations and previous reports; however, both methodologies produce $t_{1/2elim}$ values lower than our observations.

Variation in alfaxalone formulation is therefore the more likely cause of differences in the pharmacokinetics of alfaxalone in rats. Alfaxan® given IP produced some attractive anaesthetic qualities, including sustained plasma levels of alfaxalone, absence of apnoea and longer sleep times, compared to IV dosing. However, immobilization was not induced in 30% of rats given Alfaxan® IP, possibly related to the lower initial plasma concentrations observed in these animals, and muscle twitching was frequently observed. A pharmacodynamic study of the effects of combining IP injection of Alfaxan® with other premedication agents would be useful, to determine whether this would reliably improve anaesthesia induction rates and reduce muscle twitching, and thus provide an alternative anaesthetic regimen for rats with a wide margin of safety.

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Abbreviations

Observed C_{max}: the peak plasma drug concentration for each IV dosing and mean peak plasma concentration for IP dosing.

MEC: Minimum effective concentration, the minimum plasma concentration to maintain immobilization.

Table 2.1. Various pharmacodynamic parameters for Individual adult Wistar rats administered an injection of Alfaxan® (Jurox Pty Ltd, Australia) at 2mg.kg^{-1} and 5mg.kg^{-1} IV, and 20mg.kg^{-1} IP.

Rat ID	Induction time	sleep time	apnoea	twitch on induction	twitch on recovery
2 mg*kg^{-1}IV	(sec)	(min)	(sec)	(y/n)	(y/n)
Rat 4.3	20	22	100	n	n
Rat 5.3	10	7	170	n	n
Rat 6.3	60	8	0	n	n
Rat 7.3	40	6	0	n	y
Rat 12.3	60	9	0	n	y
mean	38	10.4	54		
SD	22.8	6.6	78.0		
Range	10 to 60	6 to 22	0-170	no twitching	1 of 5 twitched

Rat ID	Induction time	sleep time	apnoea	twitch on induction	twitch on recovery
5 mg*kg^{-1}IV	(sec)	(min)	(sec)	(y/n)	(y/n)
Rat 8.3	60	9	95	n	n
Rat 9.3	27	18	108	n	n
Rat 10.3	20	12	100	n	n
Rat 11.3	12	12	113	n	n
Rat 13.3	60	12	0	n	n
mean	35.8	12.6	83.2		
SD	22.7	3.3	47.0		
Range	12 to 60	9 to 12	0 to 113	no twitching	no twitching

Rat ID	Induction time	sleep time	apnoea	twitch on induction	twitch on recovery
20 mg*kg^{-1}IP	(min)	(min)	(sec)	(y/n)	(y/n)
Rat 1	5	35	0	y	n
Rat 2	2	58	0	y	y
Rat 3	3	40	0	y	y
Rat 4	4	35	0	n	y
Rat 5	3	41	0	y	n
Rat 6	4	45	0	y	y
Rat 7	not induced	0	0	y	y
Rat 8	not induced	0	0	n	n
Rat 9	6	42	0	y	y
Rat 10	not induced	0	0	y	y
mean	3.9	29.6	0		
SD	1.3	21.4	0		
Range	3 to 6	0 to 58	0	8 of 10 twitched	7 of 10 twitched

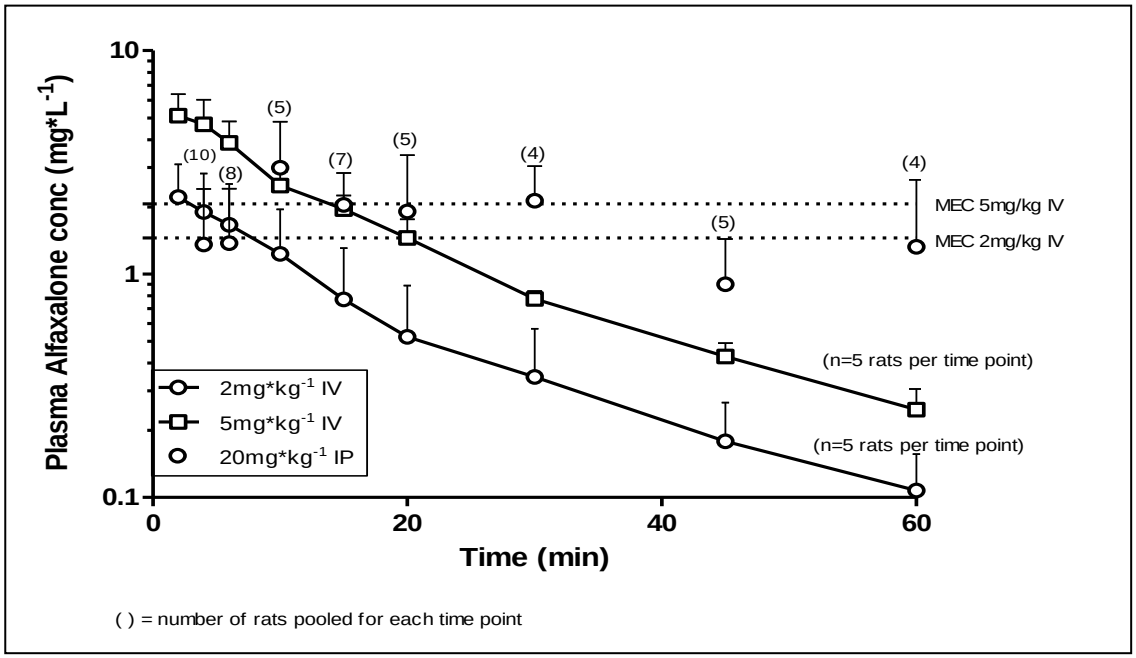
Table 2.2. Pharmacokinetic parameters for ten adult Wistar rats after administration of a 60 sec 'bolus' injection of Alfaxan® (Jurox Pty Ltd, Australia) at 2mg.kg^{-1} and 5mg.kg^{-1}.

Rat ID	$t_{1/2}$elim	Cl	V_z	$AUC_{0\text{-last}}$	$AUC_{0\text{-inf}}$	AUC %	C_{max} observed	C_{max0}extrap
2 mg*kg^{-1}IV	(min)	(mL*min^{-1}*kg^{-1})	(L*kg^{-1})	(min*mg*L^{-1})	(min*mg*L^{-1})	extrap	(mg*L^{-1})	(mg*L^{-1})
Rat 4.3	10.4	68.8	1.0	28.2	29.1	3.0	2.491	3.148
Rat 5.3	11.9	38.9	0.7	50.1	51.4	2.5	2.562	2.676
Rat 6.3	14.6	90.3	1.9	20.2	22.1	8.8	1.402	2.236
Rat 7.3	32.5	59.2	2.8	25.2	33.8	25.3	1.216	1.342
Rat 12.3	11.8	31.7	0.5	60.9	63.1	3.4	3.366	3.679
mean	**16.2**	**57.8**	**1.4**	**36.9**	**39.9**	**8.6**	**2.207**	**2.616**
SD	**9.2**	**23.6**	**0.9**	**17.6**	**16.9**	**9.7**	**0.892**	**0.892**
Range	10.4-32.5	31.7-90.3	0.5-2.8	20.2-60.9	22.1-63.1	2.5-25.3	1.2-3.4	1.3-3.7

Rat ID	$t_{1/2}$elim	Cl	V_z	$AUC_{0\text{-last}}$	$AUC_{0\text{-inf}}$	AUC %	C_{max} observed	C_{max0}extrap
5 mg*kg^{-1}IV	(min)	(mL*min^{-1}*kg^{-1})	(L*kg^{-1})	(min*mg*L^{-1})	(min*mg*L^{-1})	extrap	(mg*L^{-1})	(mg*L^{-1})
Rat 8.3	17.8	53.9	1.4	86.3	92.8	7.0	5.578	6.940
Rat 9.3	15.0	48.8	1.1	98.3	102.5	4.2	6.607	7.380
Rat 10.3	15.8	47.2	1.1	100.9	105.9	4.7	6.224	5.730
Rat 11.3	16.7	63.9	1.5	72.9	78.3	6.9	4.207	5.404
Rat 13.3	23.0	57.6	1.9	75.3	86.8	13.2	3.528	3.427
mean	**17.6**	**54.3**	**1.4**	**86.7**	**93.3**	**7.2**	**5.229**	**5.776**
SD	**3.2**	**6.8**	**0.4**	**12.8**	**11.3**	**3.6**	**1.318**	**1.548**
Range	15.0-23.0	47.2-63.9	1.1-1.9	72.9-100.9	78.3-105.9	4.2-13.2	3.5-6.6	3.4-7.4

Rat ID	$t_{1/2}$elim	Cl	V_z	$AUC_{0\text{-last}}$	$AUC_{0\text{-inf}}$	AUC %	C_{max} observed	C_{max0}extrap
20 mg*kg^{-1}IP	(min)	(mL*min^{-1}*kg^{-1})	(L*kg^{-1})	(min*mg*L^{-1})	(min*mg*L^{-1})	extrap	(mg*L^{-1})	(mg*L^{-1})
Pooled	NA	NA	NA	96.195	NA	NA	2.99	NA

Figure 2.1. Plasma alfaxalone concentration over time in rats administered Alfaxan® at 2 mg.kg^{-1} IV, 5 mg.kg^{-1} IV and 20 mg.kg^{-1} IP doses. Data are mean ± SD. As data for IP injection is derived from different subsets of animals at different sample times (number of animals contributing to each sample time are indicated in parentheses next to the sample time), the symbols are not connected. The mean MEC for each IV dose, defined as the mean minimum plasma alfaxalone concentration required to maintain immobilisation, was calculated by extrapolating the plasma concentrations at the time of recovery from immobilization from individual plasma concentrations, and is presented as a mean, indicated by the dashed lines on the graph.



CHAPTER THREE

The use of premedication agents with alfaxalone anaesthesia for laboratory rats

Abstract

The rodent anaesthetic survey showed that the availability of a safe and reliable injectable anaesthetic is limited for laboratory rats. Although there is an increase in inhalation anaesthetic use, injectable anaesthesia is still a common anaesthetic delivery method. Currently available injectable anaesthetic agents such as ketamine-xylazine were associated with respiratory depression, hypotension, diuresis and prolonged recoveries (Green et al., 1981, Flecknell, 1993b). Alfaxalone has been successfully used in cats and dogs in veterinary medicine and this success could potentially be translated for laboratory rodent use. Intravenous (IV) and intraperitoneal (IP) alfaxalone pharmacokinetic data showed that alfaxalone could potentially be a viable anaesthetic alternative. Pilot data showed that administration of alfaxalone alone produced neuro-excitation, commonly termed twitching, in many rats.

The main study assessed the IP administration of alfaxalone in conjunction with four premedication combinations to abolish the incidence and severity of twitching. Medetomidine ($0.5mg.kg^{-1}$), medetomidine-butorphanol ($0.35mg.kg^{-1}$ -$0.2mg.kg^{-1}$), ACP-methadone ($3mg.kg^{-1}$ -$0.7mg.kg^{-1}$) and ACP-xylazine ($3mg.kg^{-1}$ -$3mg.kg^{-1}$) was injected IP at T = -10 minutes, followed by IP alfaxalone ($20mg.kg^{-1}$, T = 0). Not all rats were induced by the ACP-methadone and ACP-xylazine regimen. Immobilisation times were significantly increased with medetomidine and medetomidine-butorphanol premedication. Surgical anaesthesia was not achieved in un-premedicated rats, and was unreliable in rats premedicated with ACP-methadone and ACP-xylazine. Medetomidine and medetomidine-butorphanol premedication achieved surgical anaesthesia in 14 of 16 rat anaesthetics; however, unexpected mortalities occurred in two rats administered medetomidine premedication.

Twitching severity was significantly reduced with the addition of the premedication agents ACP-xylazine, medetomidine or medetomidine-butorphanol. Mean oxygen saturation was significantly decreased with the addition of medetomidine and medetomidine-butorphanol premedication. ACP-xylazine and, more prominently, medetomidine and medetomidine-butorphanol significantly reduced the respiratory rate of rats undergoing alfaxalone anaesthesia. Medetomidine and medetomidine-butorphanol protocols showed significantly lower skin temperatures than that observed for control rats, whereas ACP-methadone and ACP-xylazine anaesthesia did not show significant mean skin temperature differences from control rats. Medetomidine could not be recommended as a premedication at the current sedation doses, due to anaesthetic-related mortalities. Medetomidine-butorphanol provided surgical anaesthesia without prolonged recovery. Medetomidine-butorphanol

premedication was sufficient to provide an adequate plane of anaesthesia for short painful surgical procedures. The limiting factor for alfaxalone anaesthesia was the presence of twitching. Determining the cause of this neuro-excitation would significantly improve the anaesthetic protocol.

Introduction

The rodent anaesthetic survey provided some insight into what researchers considered important when choosing their anaesthetic regimen. Surprisingly, the regulatory requirements of the schedule 8 restricted drugs were not a major factor influencing their anaesthetic choice. Only a few survey respondents mentioned that their choice of anaesthetic protocol was based on the regulatory limitations of using opioid and dissociative agents. The survey findings indicated that injectable anaesthetics were still commonly used and many complications were still associated with the induction, maintenance and recovery from anaesthesia from these agents. Anaesthetic related mortality rates for rats and mice undergoing recovery surgery were much higher than mortality rates published for veterinary patients and people (Clarke and Hall, 1990, Bille et al., 2012). These survey findings clearly indicate the need for improved anaesthetic protocols that will provide both ease of use, minimal training and predictability of anaesthetic outcomes.

Laboratory rodent anaesthesia has stagnated

Improvements to rodent anaesthetic protocols seem to have stagnated. Agents that were used decades ago are still used today. Ketamine-xylazine anaesthesia was identified as the most commonly used anaesthetic agent for laboratory rodents in rodent anaesthetic. This combination anaesthetic has definitely stood the test of time, as there are publications on its use dating back to the late 1970s. The fact that such agents have been used in rodents for over 30 years, suggests that there are no better anaesthetic combinations available and also highlights the need for a review of current anaesthetic practices (Green et al., 1981). Ketamine is a cyclohexylamine analogue that produces unconsciousness and analgesia, but no muscle relaxation (Green, 1975, Green et al., 1981). Ketamine alone at different drug dose ranges resulted in inconsistent levels of analgesia and xylazine on its own appeared completely ineffective at doses up to 40mg.kg^{-1} (Green, 1975, Green et al., 1981). The addition of xylazine provided muscle relaxation as part of the response to administration of the drug combination, and improved the overall quality of anaesthesia of the two agents alone. However, regardless of the improved sedation and muscle relaxation, many undesirable side effects are apparent, including inconsistent analgesia, respiratory depression, hypotension, diuresis and prolonged anaesthetic recovery (Flecknell, 1993b, Green et al., 1981). Several of these complications were observed by rodent anaesthetic survey respondents. Looking at this drug combination alone highlights the need for improved laboratory rodent protocols.

Alfaxalone as an alternative rodent anaesthetic agent

Alfaxalone is a neuroactive anaesthetic steroid that has been shown to have a high margin of safety in dogs and cats and is currently used extensively in veterinary practice (Ferre et al., 2006, Muir et al.,

2008, Whittem et al., 2008). The formulation of alfaxalone in a lipophilic cyclodextrin excipient is available on the Australian market as Alfaxan® (Jurox Pty Ltd, Rutherford NSW). Limited published data is available for Alfaxan® anaesthesia in rats. In Chapter 2, I undertook a pharmacokinetic study, and established that alfaxalone could be administered IV and IP and that sustained plasma levels are reached with IP administration.

Premedication agents could improve alfaxalone anaesthesia

However, there was a large variation in alfaxalone anaesthetic immobilisation times when alfaxalone was used as a sole agent. In veterinary medicine, anaesthetic drug combinations are commonly utilised to reduce the doses of each medication necessary and reduce potential side effects of each drug (Alves et al.). The addition of premedication agents here would hopefully provide a more predictable anaesthetic. Neuroleptanalgesia was the term coined by human clinical anaesthetists to describe the central nervous depression produced by a combination of a potent sedative analgesic and a tranquilliser (Green, 1975). Neuroleptanalgesia was adapted for use for animals to provide levels of immobilisation, with combinations of phenothiazine, α_2- adrenergic receptor agonists and opioid analgesic agents being the most common (Monteiro et al., 2009, Green, 1975).

Premedication agent combinations that provided neuroleptanalgesia were chosen. Premedication combinations include the opioids methadone and butorphanol, α_2- agonists xylazine and medetomidine, and the phenothiazone acetylpromazine (ACP). Phenothiazines such as ACP have sedative effects with no analgesic actions and are frequently combined with opioids to improve sedation and analgesia (Monteiro et al., 2009, Monteiro et al., 2008, Flecknell, 1993b). Methadone is an opioid analgesic agent commonly administered in combination with ACP in dogs (Flecknell, 1984, Abreu et al., 2012). Methadone analgesia results from its agonist effect on the mu opioid receptor and its N-methyl-D-aspartate (NMDA) glutamate receptor antagonism (Monteiro et al., 2008, Monteiro et al., 2009). Butorphanol is an opioid analgesic agent, which is a kappa opioid receptor agonist and mild mu opioid receptor antagonist (Abreu et al., 2012). Butorphanol enhances the effects of α_2-adrenoreceptor agonists, and therefore is often administered in combination with medetomidine (Hayashi et al., 1994). It has been reported that a synergistic analgesic effect is present when co-administering α_2- agonists with an opioid drug, increasing the potency and efficacy of the opioid analgesia (Valverde, 2012). Medetomidine is a more potent α_2- agonist than xylazine, and is associated with a lower incidence of side effects (Ferrari et al., 2005, Valverde, 2012). Medetomidine has both the sedative and analgesic properties required for a premedication agent. ACP-xylazine combines two sedative agents commonly used in equine anaesthesia (Watney et al., 1988, Nilsfors et al., 1988).

We aim to address some of the current problems associated with rodent anaesthesia by producing an anaesthetic regimen that would allow for a smooth induction, maintenance and recovery from anaesthesia. Medetomidine and ACP-xylazine premedication combinations were included for this purpose. Alongside medetomidine-butorphanol and ACP-methadone, these four combinations of neuroleptanalgesics made up the premedication agents used in combination with IP alfaxalone. Preliminary investigations determined whether alfaxalone anaesthesia is effective when administered IP, IM and SC at varying doses. This was followed by the main study where conjunctive therapies were utilised in an attempt to improve overall alfaxalone anaesthesia. Premedication combinations chosen for this study possess sedation and analgesic properties that work synergistically with alfaxalone to provide a predictable and safe anaesthetic alternative to current injectable anaesthetics available.

From our preliminary investigations, we showed that immobilisation was not always achieved and unfortunately, we could not recommend alfaxalone as a sole agent to reliably induce anaesthesia in rats. The incidence of twitching was observed consistently, and creates a significant drawback to the use of alfaxalone. On the positive side, we observed that a three fold increase in alfaxalone dose from 10 $mg.kg^{-1}$ to 30 $mg.kg^{-1}$ did not cause any adverse effects to rats other than twitching, highlighting the large safety margin alfaxalone possesses. As we cannot use alfaxalone alone, our next step was to consider the addition of premedication agents. It is common practice in veterinary medicine to use combination anaesthesia to reduce the doses of each drug and reduce potential side effects of each drug (Alves et al.). Here we investigated the use of premedication agents with alfaxalone anaesthesia in rats.

The aim of this study was to determine whether premedication agents would help improve alfaxalone anaesthesia by improving overall anaesthetic predictability and reducing twitching in rats. Alfaxalone at 20 $mg.kg^{-1}$ IP was chosen, as this dose induced 75% of rats and the immobilisation time for IP dosing was more consistent than with SC dosing. Also, we noticed that all rats anaesthetised at this dose range via the IP route developed some degree of twitching, and will therefore allow us to see whether alfaxalone reduces this.

Materials and Methods

Female Wistar rats (6-10 weeks old, n = 18) were housed in a barrier maintained animal house (Animal Ethics Approval SBS/115/07/ARC) with a 12-hour/12-hour light and dark cycle. Standard rat pellets and water were available *ad libitum*. Experiments were performed once weekly for four consecutive weeks. Each week rats were given a different premedication agent combination in conjunction with 20 $mg.kg^{-1}$ alfaxalone IP (Table 3.2). Premedication agents investigated were medetomidine (0.5 $mg.kg^{-1}$), medetomidine-butorphanol (0.35 $mg.kg^{-1}$-0.2 $mg.kg^{-1}$), Acetylpromazine-methadone (3 $mg.kg^{-1}$-0.7 $mg.kg^{-1}$) and ACP-xylazine combinations (3 $mg.kg^{-1}$-3 $mg.kg^{-1}$). Doses for these combinations were determined from prior pilot investigations so that the premedication combinations would provide sedation without causing anaesthesia. Throughout the study, the anaesthetic protocols were referred to by their premedication agents. Control rats received sham premedications with injectable 0.9% saline solution. All premedication agent volumes were standardised and mixed with injectable saline to give a total injection volume of 0.6mL.

Rats were premedicated IP ten minutes before (T = -10 min) administration of 20 $mg.kg^{-1}$ alfaxalone IP (T = 0 min). All IP injections were administered into the right lower abdominal quadrant, with the rat upper body tilted downwards to avoid major organs during injection (Smiler et al., 1990). Rats were then placed onto a heat mat and allowed to breathe room air to simulate the usual laboratory setting for rodent anaesthesia. Rats were monitored every minute for five minutes from T = 0 min. The level of sedation achieved from the premedication combination was recorded prior to alfaxalone administration.

Sedation levels were categorised in to light, moderate and deep. Light sedation was recorded when rats showed reduced activity but still ambulatory. Rats displaying moderate sedation were quiet, stationary but responded by moving when provoked. Deep or heavy sedation was achieved when rats were stationary, had reduced muscle tone and was non-ambulatory when provoked. Reflex responses were recorded at five-minute intervals until anaesthetic recovery. The induction time was recorded as the time taken from alfaxalone administration to when rats lost their righting reflex.

Rats were placed into dorsal recumbency to establish the loss of righting reflex and maintained in this position until they could voluntarily right themselves. The time in dorsal recumbency was recorded as the immobilisation time. The cutaneous, pedal and tail pinch reflexes were monitored throughout the procedure as a gauge of anaesthetic depth. Rats-tooth forceps were used to deliver the noxious stimulus by pinching the ventral abdominal skin for the cutaneous skin pinch response, hind paw webbing for the pedal reflex, and the tail tip for the tail pinch reflex. Any avoidance response, such as

tail flicking and limb withdrawal, was recorded as a positive response to noxious stimuli. Surgical anaesthesia time was recorded from immobilised rats that showed negative responses to all noxious stimuli.

A pulse oximeter was applied to the front paw of the rat to obtain oxygen saturation data. Pulse oximetry readings were recorded every five minutes during the immobilisation period. An infrared thermometer was used to non-invasively monitor cutaneous body temperature from the ventral abdominal skin. Twitching score was recorded and quantified according to the twitch score (Table 3.1). Other anaesthetic parameters measured included respiratory rate, respiratory effort, response to auditory stimulus and mucous membrane colour.

Control data for 20 $mg.kg^{-1}$ IP (n =4,) were sourced from the preliminary study to reduce animal numbers (Appendix 2.2). This decision was made for animal welfare grounds as well as to ensure anaesthetised rats stayed within a tight age and weight range. A drug wash out period of seven days was given between anaesthetics to avoid the possibility of drug accumulation in subsequent anaesthetics.

All experiments were performed by one operator in an effort to maintain consistency with response recordings. GraphPad Prism® generated statistics for each premedication anaesthetic protocol. All statistical analyses were compared to control rats from the preliminary study. Rats that failed to achieve anaesthesia were recorded in data tables and not included in the statistical analysis. Twitching score was presented as median and inter-quartile range and statistical significance was achieved using Kruskal-Wallis nonparametric ANOVA. All other data are presented as mean ± SD. A one-way ANOVA was performed on data, followed by Dunnett's Multiple Comparison where a P-value of <0.05 was considered significant.

	Medetomidine	Medetomidine-Butorphanol	ACP-Methadone	ACP-Xylazine
Week 1	Rat 1 Rat 2 Rat 3 Rat 4	Rat 5 Rat 6 Rat 7 Rat 8	Rat 9 Rat 10 Rat 11 Rat 12	Rat 13 Rat 14 Rat 15 Rat 16
Week 2	Rat 13 Rat 14 Rat 15 Rat 16	Rat 1 Rat 2 Rat 3 Rat 4	Rat 5 Rat 6 Rat 7 Rat 8	Rat 9 Rat 10 Rat 11 Rat 12
Week 3	Rat 9 Rat 10 Rat 11 Rat 12	Rat 13 Rat 14 Rat 17 Rat 18	Rat 1 Rat 2 Rat 3 Rat 4	Rat 5 Rat 6 Rat 7 Rat 8
Week 4	Rat 5 Rat 6 Rat 7 Rat 8	Rat 9 Rat 10 Rat 11 Rat 12	Rat 13 Rat 14 Rat 17 Rat 18	Rat 1 Rat 2 Rat 3 Rat 4

Table 3.1. Alfaxalone anaesthesia cross-over design
The cross-over design for anaesthesia shows the time line for which rats received one of four premedication agents in combination with alfaxalone anaesthesia. A one week wash-out period was used between anaesthetics.

Results

Level of sedation following premedication

Premedication agents were allowed 10 minutes to act and levels of sedation were recorded prior to alfaxalone administration. ACP-methadone premedication produced the lightest overall level of sedation with 75% of rats lightly sedated and 25% moderately sedated (Appendix 3.1).
ACP-xylazine produced varying sedation levels, and of the sixteen rats administered this premedication combination, three rats were lightly sedated, eight showed moderate sedation and five were heavily sedated (Appendix 3.2). The majority of rats were moderately sedated with medetomidine: 62.5% of rats were moderately sedated, 12.5% lightly sedated and 25% were heavily sedated (Appendix 3.3). Sedation with medetomidine-butorphanol was varied but the majority of rats achieved light to moderate sedation prior to alfaxalone administration. Of the sixteen rats given medetomidine-butorphanol, six were lightly sedated, seven showed moderate sedation and three rats were deeply sedated. Overall, ACP-methadone showed the lightest average sedation levels followed by medetomidine-butorphanol, ACP-xylazine and with medetomidine the heaviest average sedation levels.

Induction time following alfaxalone administration

Induction times were recorded from the time rats were administered alfaxalone, to the time they lost their righting reflex. From the levels of sedations we achieved, we expected faster inductions with more heavily sedated rats. This did not appear to be the case. There were no significant differences between induction times compared to control rats (Table 3.2). 75% of rats within the control group achieved anaesthetic induction. The mean induction time was 3.9 ± 1.3 minutes.
ACP-methadone induced anaesthesia in 15 of 16 rats. The mean induction time was 4.5 ± 3.6 minutes however most rats were induced within five minutes, with only one rat taking 17 minutes before losing their righting reflex (Appendix 3.1). If this rat was omitted from the data, the mean induction time would be decreased by almost a minute. ACP-xylazine produced anaesthetic induction in 14 of 16 rats. The mean induction time was 2.4 ± 1.3 minutes and similarly to ACP-methadone, one of these 14 rats took much longer to lose their righting reflex than the others (Appendix 3.2). But of the remainder, nine of them were induced within two minutes of alfaxalone administration.
Medetomidine produced a 100% anaesthetic induction rate. One rat was already heavily sedated and allowed placement into dorsal recumbency prior to alfaxalone administration. Induction times ranged between 0 to 6 minutes (Appendix 3.3) and the mean induction time was 2.0 ± 1.5 minutes (Table 3.2). As with medetomidine premedication, medetomidine-butorphanol also achieved anaesthetic

induction in all sixteen rats (Appendix 3.4). One rat took 10 minutes to lose its righting reflex with the remaining 15 rats taking only three minutes or less. The mean induction time was 2.9 ± 2.0 min.

Immobilisation time following alfaxalone administration

The immobilisation time was recorded from the time the rat lost their righting reflex to the time they regained sternal recumbency. We predicted that a longer immobilisation would follow a deeper sedation level. This was apparent as there was no significant difference in immobilisation times of ACP-methadone or ACP-xylazine, but a marked prolongation of immobilisation time with the addition of medetomidine and medetomidine-butorphanol. The mean immobilisation time achieved for control rats was 39.8 ± 7.4 minutes and ranged between 31 to 58 minutes.

ACP-methadone produced a mean immobilisation time of 54.6 ± 15.0 minutes. This was not significantly longer than that achieved by control rats. The addition of ACP-xylazine also did not show a significant increase in immobilisation time. The mean anaesthetic immobilisation time for ACP-xylazine was observed at 66.5 ± 24.2 minutes. Both medetomidine and medetomidine-butorphanol both significantly extended immobilisation time. An immobilisation time of 133 ± 34.1 minutes was achieved by medetomidine with the minimum immobilisation time of 65 minutes (Appendix 3.3). An immobilisation period of 120.2 ± 29.6 was observed for medetomidine-butorphanol anaesthetic. Interestingly, we predicted that the sedation levels achieved would have an impact increasing anaesthetic immobilisation times. This was true to some extent however; ACP-xylazine produced on average a deeper sedation than medetomidine-butorphanol but was much less effective at extending immobilisation time.

Surgical anaesthesia time following alfaxalone administration

Surgical anaesthesia was recorded within the immobilisation period when they were non-responsive to noxious stimuli. Control rats did not develop surgical anaesthesia at any time during alfaxalone anaesthesia. We postulated that premedication agents would improve alfaxalone anaesthesia. For an anaesthetic to be effective, it needs to allow for surgical intervention. We expect the addition of premedication agents to improve the period of surgical anaesthesia.

Only one of 16 rats developed surgical anaesthesia for 20 minutes following ACP-methadone. ACP-xylazine was more effective with 7 of 16 rats reaching a surgical plane of anaesthesia. Medetomidine and medetomidine-butorphanol were the most effective agents with 14 of 16 rats and 13 of 16 rats reaching a surgical plane of anaesthesia respectively. The mean surgical anaesthetic time achieved was 43 ± 29 minutes for medetomidine and 53 ± 37 minutes for medetomidine-butorphanol. Results showed that ACP-methadone and ACP-xylazine were least effective at producing a surgical anaesthetic plane. However it was observed that there were still two rats in each

of the medetomidine and medetomidine-butorphanol groups that did not reach this anaesthetic depth. This suggests inadequate anaesthesia following an initial dose, a common complicating factor with many injectable anaesthetic agents as shown in the rodent anaesthetic survey.

Anaesthetic parameters	Control					
	mean	SD	n			
Induction Time (min)	4.1	1.2	9			
Immobilisation Time (min)	39.8	7.4	9			
Surgical Anaesthetic Time (min)	0	0	12			
Oxygen Saturation (%)	92.6	0.8	11			
Respiratory Rate (bpm)	82	4.1	11			
Cutaneous Temperature (^{o}C)	32.9	0.7	12			
	Median	IQR	n			
Twitch Score	2.0	2.5	12			
Anaesthetic parameters	**ACP-Methadone**			**ACP-Xylazine**		
	mean	SD	n	mean	SD	n
Induction Time (min)	4.5	3.6	15	2.4	1.3	14
Immobilisation Time (min)	54.6	15.0	16	66.5	24.2	16
Surgical Anaesthetic Time (min)	1.3	5	16	8.8	16	16
Oxygen Saturation (%)	94.1	0.8	15	93.3	2.1	16
Respiratory Rate (bpm)	79	6.5	16	72	8.0	16
Cutaneous Temperature (^{o}C)	32.5	0.7	16	31.9	0.3	16
	Median	IQR	n	Median	IQR	n
Twitch Score	1.0	1.7	16	0	1.0	16
Anaesthetic parameters	**Medetomidine**			**Medetomidine-Butorphanol**		
	mean	SD	n	mean	SD	n
Induction Time (min)	2.0	1.5	16	2.9	2	16
Immobilisation Time (min)	133.0	34.1	14	120.2	29.6	16
Surgical Anaesthetic Time (min)	43	29	14	53	37	16
Oxygen Saturation (%)	89.5	3.1	16	87.0	3.6	16
Respiratory Rate (bpm)	63	11.0	16	57	8.4	16
Cutaneous Temperature (^{o}C)	32.7	0.8	16	32.4	0.4	16
	Median	IQR	n	Median	IQR	n
Twitch Score	0	1.0	16	0	1.0	16

Table 3.2. Summary of alfaxalone anaesthetic outcomes
The table shows the anaesthetic parameters for the premedication agents used in conjunction with alfaxalone. Twitch score is presented as Median and Interquartile Range (IQR) and other data is presented as mean ± SD

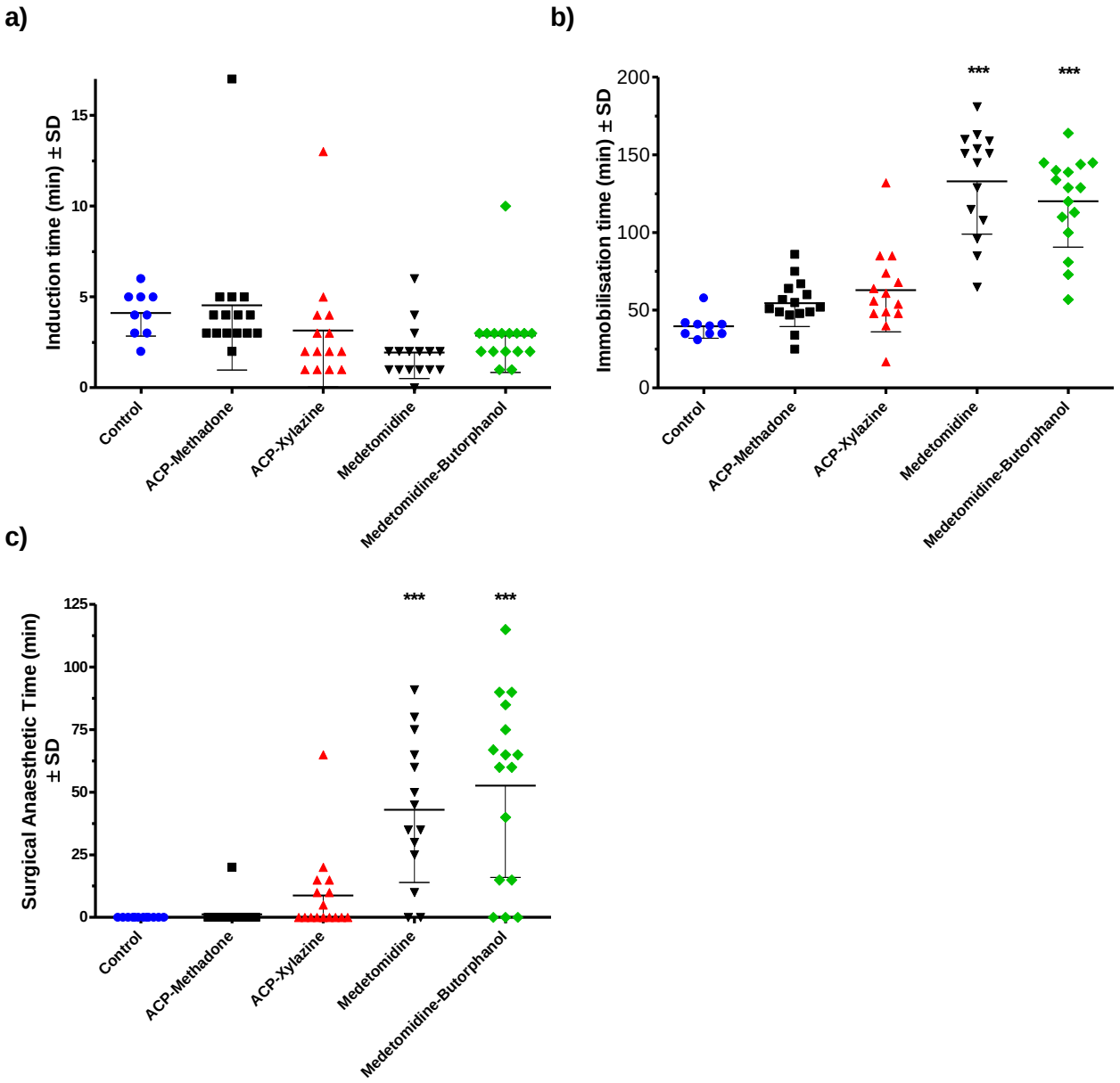


Figure 3.1 Alfaxalone anaesthesia in rats in combination with premedication agents
a) No significant alterations in induction time were observed between premedication agent combinations b) Rats premedicated with medetomidine and medetomidine-butorphanol were immobilised for a significantly longer time period compared to control c) Medetomidine and medetomidine-butorphanol premedication provided longer surgical anaesthetic times but this time was unpredictable. All data is presented as mean ± SD. *** indicates a P value ≤ 0.001

Incidence of twitching following alfaxalone anaesthetic regimen

Control rats showed a 75% incidence of twitching, with most of the twitching considered moderate to severe in nature (Figure 3.2). ACP-methadone was ineffective at decreasing the incidence of twitching and in contrast, actually increased the incidence of twitching to 87.5% and did not appear to decrease the severity of twitching (Appendix 3.1). The other three pre-anaesthetic agents were however much more effective at reducing twitching than ACP-methadone. ACP-xylazine showed a 37.5% incidence of twitching, with more than half of these rats only showing mild twitching (Appendix 3.2). Seven of 16 rats twitched with medetomidine premedication, but all of these rats only showed mild signs of twitching (Appendix 3.3). Six of 16 rats showed mild twitching after medetomidine-butorphanol premedication. It was interesting to note that two rats twitched during all four anaesthetic protocols and one rat did not twitch during any anaesthetic protocol in this cross over study.

Oxygen saturation during alfaxalone anaesthetic protocols

It is not often that oxygen saturation is measured during rodent anaesthesia. However, in the setting of laboratory rodent anaesthesia and surgery, it is uncommon for rats or mice to receive supplementary oxygen during an injectable anaesthetic protocol. It is for this reason we need to determine whether alfaxalone maintains normal blood oxygen saturation while a rat is spontaneously breathing room air.

The mean peripheral arterial oxygen saturation level (SpO_2) for rats premedicated with ACP-methadone and ACP-xylazine were not significantly different from that of control rats (Table 3.2). The SpO_2 for these rats remained above 90% of control levels throughout the procedure (Figure 3.3). SpO_2 for medetomidine and medetomidine-butorphanol, however, were significantly lower than that seen in control. Following administration of alfaxalone, an observed decrease in SpO_2 was apparent at T= 2 minutes and for medetomidine and medetomidine-butorphanol anaesthetic protocols, this decrease in SpO_2 was pronounced by T=3 minutes. During this time the SpO_2 was seen to drop below 80% at T=15 minutes with medetomidine-butorphanol.

Respiration rate during alfaxalone anaesthetic protocols

The mean respiratory rate for control rats during immobilisation was 82 ± 4.1 breaths per minutes. ACP-methadone did not significantly reduce respiration compared with control rats (Figure 3.4). ACP-xylazine, and, even more so, medetomidine and medetomidine-butorphanol significantly depressed respiration rate in rats. The respiration rate decrease observed for medetomidine and medetomidine-butorphanol was most apparent between T= 15-20 minutes, where it then appeared to slowly recover over the anaesthetic period (Figure 3.6). Marked anaesthetic cardiorespiratory

depression was observed with the medetomidine-containing premedication agents, that were not observed with ACP-containing premedications.

Skin temperature drops during alfaxalone anaesthesia

ACP-methadone and ACP-xylazine did not alter mean skin temperature compared with control rats. Medetomidine and medetomidine-butorphanol protocols showed significantly lower skin temperatures than that observed for control rats (Figure 3.5). Medetomidine and medetomidine-butorphanol developed a mean decrease of 0.5 ^{o}C and 0.9 ^{o}C from control rats at 32.9 ± 0.7 ^{o}C.

Mortality with medetomidine protocol

Rat 2.7.14 and Rat 2.7.16 died under the medetomidine protocol despite continuous anaesthetic monitoring. Both rats developed an irregular respiratory rhythm towards the end of anaesthesia, not seen in the other fourteen rats undergoing the same anaesthetic protocol. Unusual signs were observed that were not present during other rat anaesthetics. At T= 70, Rat 2.7.14 developed dull pink mucous membrane colour, hyperaemic ears and cold extremities were apparent and serial cutaneous skin temperature recordings were normal. Rat 2.7.16 was observed to have red urine at T= 24 and a dull cyanotic tail at T= 45. Rat 2.7.14 was lightly sedated and rat 2.7.16 was deeply sedated prior to alfaxalone administration. Rats were breathing room air throughout the procedure and supplementary oxygen was not available. On post mortem examination, no gross pathological findings were evident in either rat. For the remainder of the rats, anaesthetic recovery was uneventful.

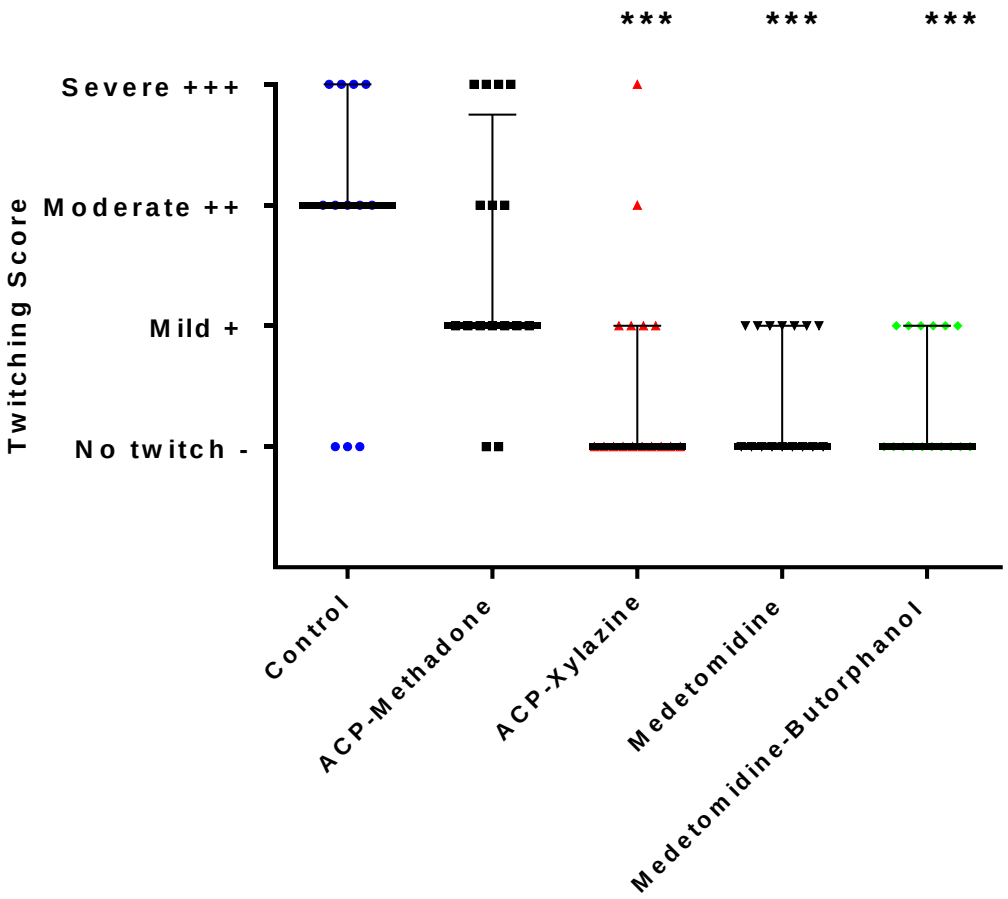
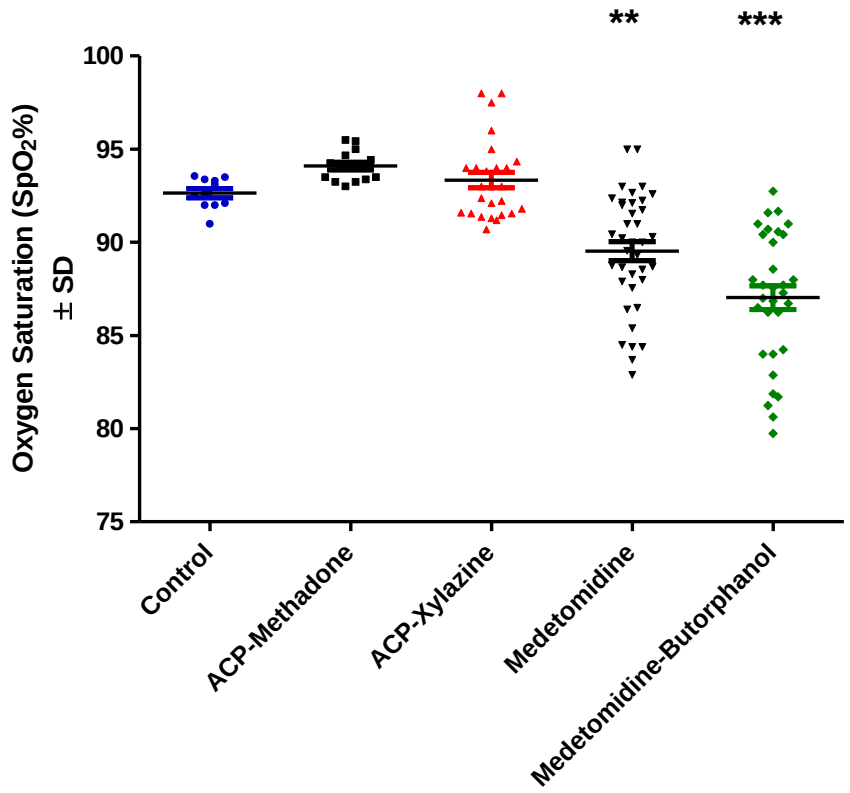


Figure 3.2. Twitching score for rats receiving alfaxalone anaesthesia following various premedication agents

The severity of twitching was reduced in the presence of ACP-xylazine, medetomidine and medetomidine-butorphanol. Data is presented as median with inter-quartile range. *** indicates a P value ≤ 0.001

a)



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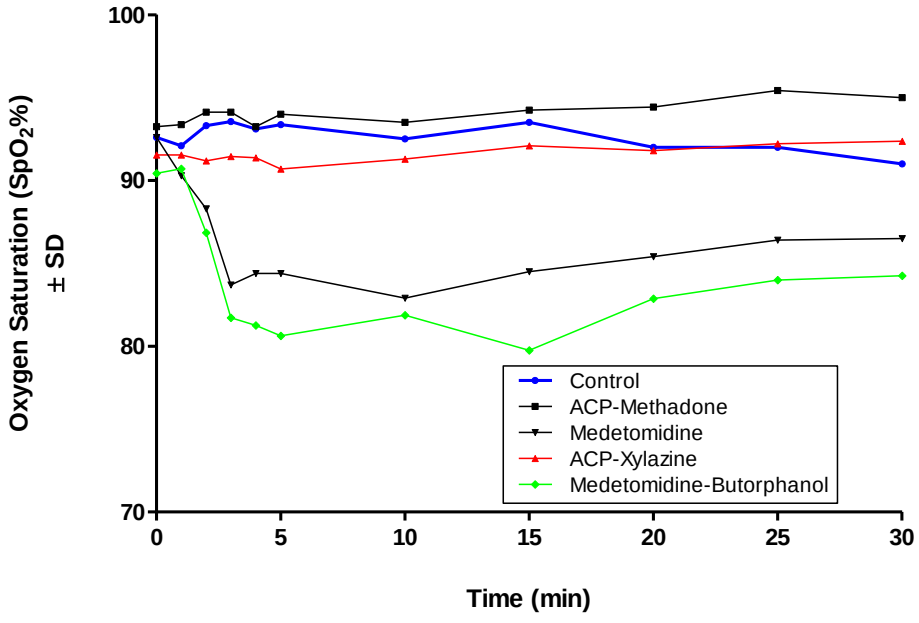
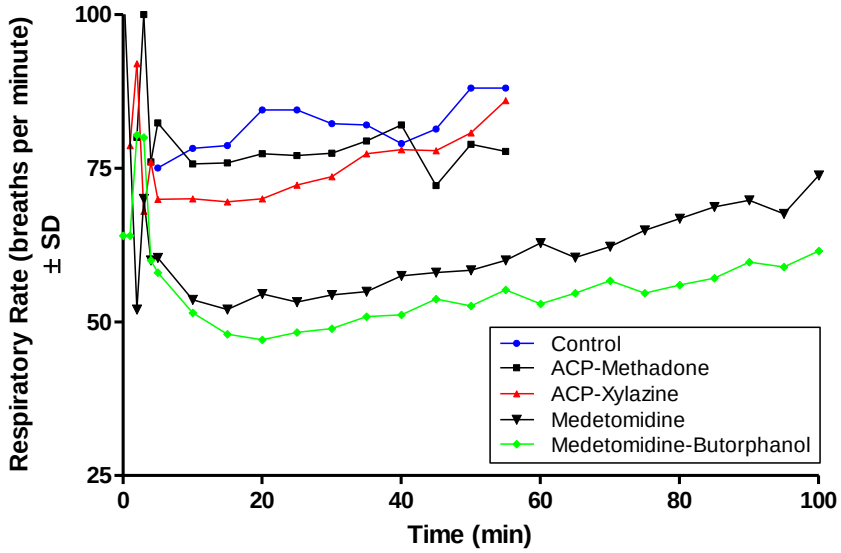


Figure 3.3. Mean oxygen saturation for alfaxalone in the presence of premedication agents
The mean percentage of peripheral blood oxygen saturation (SpO_2) was measured at five-minute intervals during the immobilisation period for each premedication-alfaxalone anaesthetic protocol. A marked reduction in oxygen saturation was observed when medetomidine and medetomidine-butorphanol was administered. Data are presented as a mean. ** indicates a P value ≤ 0.01, *** indicates a P value ≤ 0.001

a)



b)

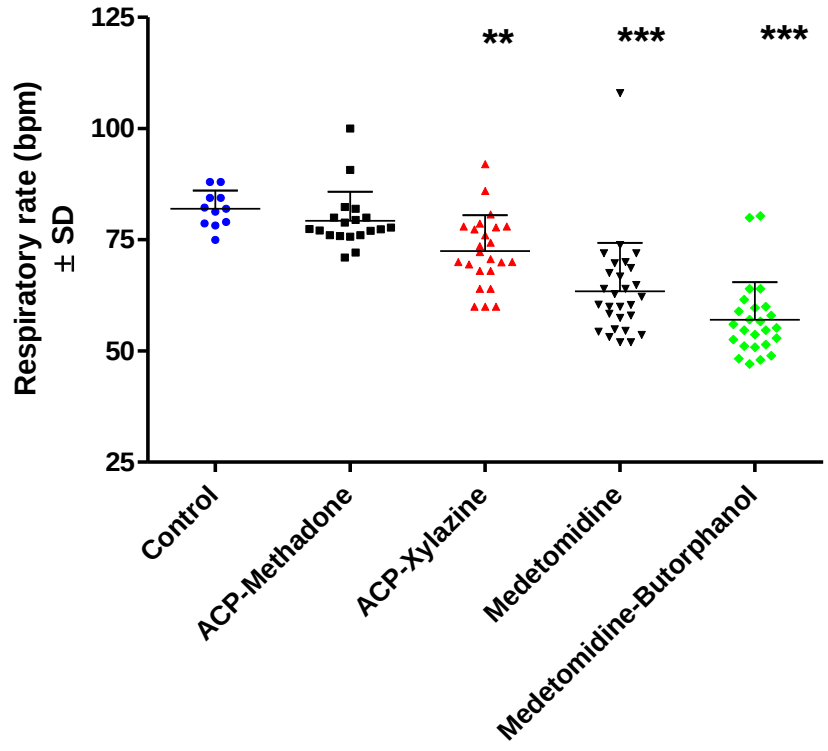


Figure 3.4. Respiratory rate in rats during alfaxalone anaesthesia with premedication agents
This figure depicts the mean respiratory rate of rats from individual premedication groups recorded at five-minute intervals. Medetomidine and medetomidine-butorphanol caused prolonged respiratory depression up to 100 minutes. Note Control, ACP-methadone and ACP-xylazine rat data was recorded until 55 minutes. Recordings were discontinued on anaesthetic recovery a) data is presented as a mean b) data is presented as mean ± SD. ** indicates a P value ≤ 0.01, *** indicates a P value ≤ 0.001

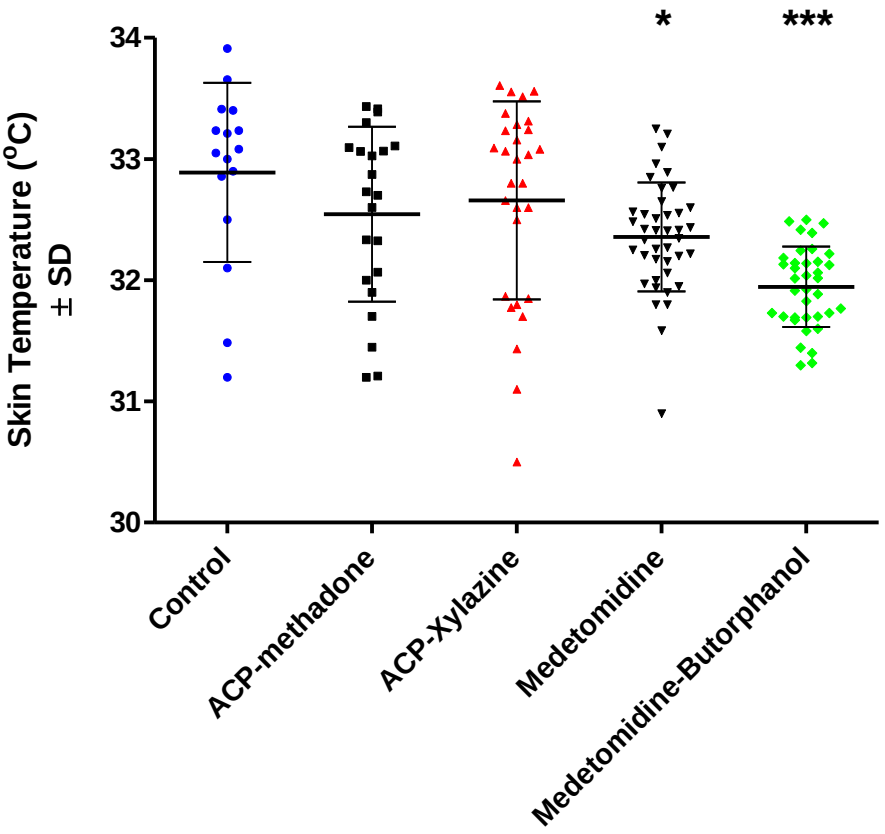


Figure 3.5. Mean skin temperature in rats following alfaxalone anaesthesia with premedication agents

Infrared skin temperature readings were recorded every five minutes during the alfaxalone anaesthetic protocol. Rats premedicated with medetomidine and medetomidine-butorphanol showed significantly lower body temperatures than ACP-methadone and ACP-xylazine premedication agents. Data is presented as mean ± SD. * indicates a P value ≤ 0.05, *** indicates a P value ≤ 0.001

Discussion

Our rat pharmacokinetic study demonstrated that 20 $mg.kg^{-1}$ IP alfaxalone produced sustained plasma levels of alfaxalone for up to 60 minutes (Lau et al., 2013). Due to these sustained alfaxalone levels at 20 $mg.kg^{-1}$, we postulated that an increase in plasma alfaxalone concentration would increase immobilisation duration due to these sustained levels. This proved not to be the case for SC and IP alfaxalone doses; in addition, twitching occurred in 75% of rats receiving IP alfaxalone. An even higher concentration of 30 $mg.kg^{-1}$ alfaxalone was trialled, in which results were essentially similar to those for 20 $mg.kg^{-1}$ alfaxalone. These pilot studies suggested that alfaxalone was not a suitable candidate for sole anaesthetic use. Similar findings were reported in cats showing that singly administered alfaxalone, either IM or SC, was inappropriate for anaesthetic induction and maintenance of anaesthesia (Grubb et al., 2013). Even though the anaesthetic effects of alfaxalone was unpredictable, we could see that alfaxalone had a wide margin of safety, as shown by the three fold increase in dose in these preliminary trials, and we were therefore keen to pursue an effective alfaxalone anaesthetic protocol by combination with other commonly used anaesthetic premedications.

Neuroleptanalgesia

Neuroleptanalgesia is commonly performed in human clinical anaesthesia and we believed that the addition of a sedative analgesic would help us markedly improve alfaxalone anaesthesia. Our premedication choices were based on commonly available veterinary premedications. The doses of these premedication combinations were optimised so that rats were sedated but still able to ambulate when stimulated. ACP-methadone, ACP-xylazine, medetomidine and medetomidine-butorphanol combinations were investigated in conjunction with 20 $mg.kg^{-1}$ IP alfaxalone. Results showed that ACP-methadone did not significantly improve alfaxalone anaesthesia. ACP-xylazine reduced the severity of twitching; however there was no major improvement in immobilisation and surgical anaesthetic times. Medetomidine and medetomidine-butorphanol combinations markedly improved immobilisation times and produced a period of surgical anaesthesia. Additionally there was a reduction in the incidence and severity of twitching. Regardless of these improvements, we did observe two mortalities during the medetomidine protocol that was not expected.

Alfaxalone anaesthetic variability

Pharmacodynamic data from Chapter 2 showed that anaesthetic induction occurred consistently within 60 seconds of IV injection and at 5 $mg.kg^{-1}$ IV alfaxalone, the sleep times achieved were within 9-12 minutes (Lau et al., 2013). Alfaxalone could be due to many different factors associated with the route of administration. The IP route of administration is slow to absorb into the circulation when compared to the IV route and large quantities of drug are required to produce the same effect (Flecknell, 1993b). Compounds administered IP are primarily absorbed by the portal circulation, with a small amount being absorbed through the lymphatic system draining into the systemic circulation (Lukas et al., 1971). The result of this is a high degree of first pass metabolism (Lukas et al., 1971), which in turn decreases the amount of drug available in the systemic circulation and could be a contributor in anaesthetic depth variation in this study. Some drugs are taken up into adipose tissues of the abdominal cavity, and drugs administered IP can diffuse from the abdominal cavity directly into the abdominal tissues (Betschart et al., 1988). This sequestration of drug could lead to variability in the amount of alfaxalone that is absorbed into the systemic circulation.

We cannot discount intraperitoneal injection error as a possible cause of anaesthetic variability. A study using iodinated oily media showed radiographically that errors occurred in 19.6% of 127 Sprague Dawley rats that were administered intraperitoneal injections (Lewis et al., 1966). This error involves a volume of injection partially injected into the gastrointestinal tract, subcutaneously, retro-peritoneally or into the urinary bladder rather than into the peritoneal cavity (Lewis et al., 1966). This Variation in injection technique is inevitable between different people, so all injections were performed by one operator, myself. All IP injections were administered into the right lower ventral abdominal quadrant while rats were tilted forwards to avoid organs such as the caecum. Even with these extra precautionary measures, there is still the possibility that some injections could have been given extra-peritoneally.

Alfaxalone showed a high margin of safety

We postulated that increasing doses of alfaxalone would improve alfaxalone anaesthesia, but one factor that was evident was the fact that a three fold increase in alfaxalone dose from 10 $mg.kg^{-1}$ to 30 $mg.kg^{-1}$ did not increase adverse anaesthetic effects on rats. This shows alfaxalone to have a wide margin of safety in rats, similar to findings reported in other species (Ferre et al., 2006, Muir et al., 2008, Whittem et al., 2008). We know that delivery of a single predetermined dose of an anaesthetic agent can give an individual dose response ranging from inadequate anaesthesia to death (Smith,

1993). So the fact that we see a three fold increase in drug dose without any adverse responses showed alfaxalone to have a high margin of safety.

Surgical anaesthesia with alfaxalone anaesthetic protocols

Surgical anaesthesia was only produced consistently in rats administered medetomidine or medetomidine-butorphanol premedication agents. Surgical anaesthesia was subjectively examined in this study, where a negative response to noxious stimuli was recorded as consistent with surgical anaesthesia. Other investigators used measures for surgical anaesthesia such as the complete loss of the pedal withdrawal reflex (Alves et al.). Others observed that rats could continue to respond to a toe pinch after they stopped responding to the abdominal and tail pinch (Field et al., 1993). The type of surgical stimulus applied would dictate which anaesthetic regimen can provide adequate immobilisation and analgesia. Ultimately, once the responses to nociceptive reflexes are lost, performing surgery is the best gauge of surgical anaesthesia. However, individual rats underwent multiple anaesthetics, so multiple surgical incisions would be necessary to include this in our tests, and so this was not done.

Respiratory parameters with alfaxalone anaesthetic protocols

The pulse oximeter gives a non-invasive estimate of arterial oxygen saturation which correlates well with arterial oxygen saturation measured in arterial blood samples (Hackett, 2002). Therefore pulse oximetry readings gave us a good indicator of general oxygenation in the rats. In our investigation, rats under alfaxalone anaesthesia with premedication maintained voluntary respiration of room air, mucous membrane colours remained visibly pink. Acceptable SpO_2 levels were maintained in ACP-methadone, ACP-xylazine and control rats at above 90%. Medetomidine and medetomidine-butorphanol premedication however dropped SpO_2 levels below 90%. A recent study showed that cats given IM alfaxalone anaesthesia maintained SpO_2 levels above 85% the majority of time whilst breathing room air (Grubb et al., 2013). The addition of the α_2- adrenergic agonists, xylazine or medetomidine appeared to be associated with respiratory depression, consistent with the reduction in SpO_2 levels with these premedications. The addition of capnographic readings would have given further information on blood carbon dioxide saturation; however the reliability of these measurements would be problematic without intubation, which is difficult in rats.

Neuro-excitation (twitching) is a prominent side effect of alfaxalone

The preliminary studies showed that neuro-excitation (twitching) during anaesthesia was consistently produced by IM, SC and IP alfaxalone administration. Additionally, the incidence and severity of this twitching was not greatly reduced with increasing doses of alfaxalone. The addition of premedication

agents was trialled, to attempt to reduce the incidence and severity of twitching. We found that ACP-xylazine, medetomidine and medetomidine-butorphanol, but not ACP-methadone, reduced the incidence and severity of twitching. ACP-methadone was the only premedication agent that did not contain an α_2- adrenergic agonist drug. Therefore the inclusion of an α_2- adrenergic receptor agonist could be one factor contributing to the reduction in the incidence and severity of the alfaxalone-induced twitching. Individual rat variability in evincing this side effect could also potentially play a role in twitching. There was a trend for rats that twitched during one anaesthetic regimen, to twitch during a subsequent anaesthetic regimen. Rat 2.7.2 and Rat 2.7.4 showed signs of twitching following every anaesthetic protocol, while Rat 2.7.10 did not show signs of twitching on any occasion. These observations were consistent with pilot study results where twitching was seen on multiple occasions following alfaxalone anaesthesia on an individual rat (Figure 3.2).

Mortalities following medetomidine-alfaxalone anaesthesia

Published data showed mortality rates with both ketamine-diazepam at 60 $mg.kg^{-1}$-7.5 $mg.kg^{-1}$ and 80 $mg.kg^{-1}$-10 $mg.kg^{-1}$ of 9% and of 6% for fentanyl-droperidol (Wixson et al., 1987a). Wixson *et al* (1987) evaluated the use of ketamine-xylazine, ketamine-diazepam, fentanyl-droperidol and pentobarbital combinations given IP in rats at several dose rates. At high doses of all drugs examined by Wixson *et al,* profound and protracted respiratory and cardiovascular depression was noted. This was discussed as likely inhibiting the return of homeostatic control mechanisms and therefore causing increased mortality (Wixson et al., 1987b). Two mortalities were seen in our current study. The two rats that died were given medetomidine premedication. Medetomidine is a selective α_2- adrenergic agonist drug that markedly potentiates the effects of other anaesthetic agents (Virtanen, 1989, Gopalan et al., 2005, Sladky et al., 2000). α_2- adrenergic receptor agonist activity in the brainstem plays a role in decreasing blood pressure (MacMillan et al., 1996). Similar to the findings by Wixson *et al* (1987), these contributing factors in our study could have inhibited the return of homeostatic control, leading to irreversible morbidity and ultimately mortality. A reversal agent such as atipamezole was not used for medetomidine in this study however; the addition of such an agent at first signs of hyperaemia or respiratory irregularity may have prevented morbidity and, ultimately, mortality of Rat 2.7.14 and Rat 2.7.16. This is of course in making the assumption that medetomidine is the causative agent. The lack of rodent specific monitoring equipment was not available for this study and this scenario would likely be similar for many researchers.

Medetomidine provided a smooth induction, longer anaesthetic time and very consistently provided surgical anaesthesia, but cannot be recommended at the current sedation dose due to anaesthetic-

related mortalities. Medetomidine-butorphanol premedication showed very similar anaesthetic qualities to medetomidine without mortality. This premedication provided surgical anaesthesia without prolonged recovery. The medetomidine-butorphanol protocol was sufficient to provide an adequate plane of anaesthesia for short painful surgical procedures. The ease of administration as well as the minimal equipment required is a huge advantage when choosing an anaesthetic regimen, however finding the correct anaesthetic combination has proven difficult. In rodents, adjustment is not available with injectable agents via SC, IM and IP routes, where a calculated dose is given in a single injection (Roughan et al., 1999). Therefore a safe and predictable anaesthetic agent is preferred.

Alfaxalone could potentially be used as part of an anaesthetic protocol, if premedication agents could be optimised, and if neuro-excitation could be reduced. Veterinarians have anecdotally used premedication agents in an effort to reduce or abolish this neuro-excitation in cats and dogs. In rats, this study showed that the severity of twitching could be significantly reduced with the addition of premedication agents. The use of combination anaesthesia also proved to produce more consistent anaesthetic outcomes between rats. Alfaxalone was safe in rats up to the maximum tested dose of 30 $mg.kg^{-1}$ IP. However, when an anaesthetic is said to be "safe", this provides a false sense of security as there is always a possibility for complications. Pilot studies indicated that alfaxalone could easily be given at three times the starting dose without morbidity or mortality, however mortality occurred with the addition of medetomidine premedication. It must be noted however, that caution should be taken when using medetomidine, at 0.5 $mg.kg^{-1}$ in conjunction with 20 $mg.kg^{-1}$ alfaxalone, and it may be necessary to incorporate a reversal agent into the anaesthetic regimen for α_2 adrenergic agonists to reduce cardiorespiratory depression and recovery time.

The initial hypothesis that premedication agents could potentially improve alfaxalone anaesthetic consistency and possibly abolish twitching was consistent with the findings from this study. However, as twitching was a major undesirable complication of alfaxalone anaesthesia, further investigations to determine the mechanism of twitching are necessary. There are indications that alfaxalone has strychnine-sensitive glycinergic receptor effects (Weir et al., 2004) that could be a contributor to the neuroexcitation. Further examination into the effects of alfaxalone on glycinergic synaptic transmission to motor neurones will give insight into what mechanisms might underlie the neuro-excitation seen with alfaxalone anaesthesia.

CHAPTER FOUR

Alfaxalone Causes Reduction of Glycinergic Inhibitory Inputs and Activates a Depolarising current in Rat Hypoglossal Motor Neurons

Abstract

We investigated the effects of alfaxalone on inhibitory synaptic transmission to hypoglossal motorneurons (HMNs). Whole cell recordings were performed on HMNs in transverse brainstem slices (300µM thickness) prepared from 10-14 day old Wistar rats anaesthetised with sodium pentobarbitone (100 $mg.kg^{-1}$ IP). Spontaneous and evoked inhibitory post-synaptic currents (IPSCs) were recorded at a holding potential of –60mV, using a CsCl-based internal solution, in the presence of the non-NMDA and NMDA glutamate receptor blockers NBQX (10µM) and APV (50µM). Spontaneous data was recorded for a pilot study using bath application of 25µM alfaxalone (n=6 HMNs). The addition of the $GABA_AR$ antagonist bicuculline (5µM) was bath-applied in control solutions for all subsequent studies of the effect of alfaxalone. An alfaxalone dose response study was performed with bath application of alfaxalone at increasing concentrations of 10nM, 30nM, 100nM, 300nM, 1µM, 3µM and 10µM (n=7 HMNs). Both spontaneous and evoked IPSCs were recorded. An inter-stimulus interval of 150ms was set for the evoked IPSC paired pulse recordings to measure paired pulse ratio (PPR). 25µM alfaxalone significantly reduced spontaneous IPSC peak amplitude, half-width and frequency. Alfaxalone did not alter spontaneous IPSC peak amplitude, rise time or half width up to 10µM, but a significant reduction in frequency was noticeable from 3µM and the degree of this reduction increased in a dose-dependent manner. A concentration-dependent reduction in evoked IPSC amplitude was apparent from 30nM, producing a maximal mean difference of –138.1pA, equivalent to 35.8 ±15.4% of control at 10µM. Prolongation of evoked IPSC rise time was significantly noticeable from 1µM and increased evoked IPSC decay time was significant at 10µM alfaxalone. Alfaxalone did not alter evoked IPSC half-width, paired pulse ratio or HMN input resistance. Baseline holding current at -60 mV moved consistently in an inward direction with significant inward changes at 10µM alfaxalone. These results are consistent with alfaxalone-induced glycine receptor (GlyR) modulation, causing a delay in channel opening, and prolongation of channel opening. A decrease in spontaneous IPSC frequency is suggestive of presynaptic depression by alfaxalone, while the decrease in spontaneous and evoked IPSC amplitude may be either pre- or postsynaptically mediated. These results show that alfaxalone reduces glycinergic inhibitory transmission to rat HMNs via a combination of pre- and postsynaptic mechanisms, and activates an inward current, which remains to be identified. These responses to alfaxalone are likely to make HMNs more excitable, and may therefore underlie neuro-excitation during alfaxalone anaesthesia.

Introduction

During alfaxalone anaesthetic, exaggerated neuro-excitatory motor responses, commonly referred to as twitching, have been reported in cats, dogs, horses and pigs during recovery (Keates, 2003, Ferre et al., 2006, Goodwin et al., 2011, Mathis et al.). This neuro-excitation was also consistently observed during our clinical studies in rats (Chapter 3), when alfaxalone was administered IV, IM and SC. Neuro-excitation is an unwelcome side effect of alfaxalone anaesthesia and limits its use as a viable alternative to other injectable rodent anaesthetic agents. The use of various premedication agents reduced the incidence and severity of twitching, however none of the trialled agents were able to abolish the neuro-excitation and/or had significant adverse effects on the quality of anaesthesia.

Alfaxalone is a positive allosteric modulator of the γ-aminobutyric acid type A receptor ($GABA_AR$) and it is through this action that it is thought to exert its anaesthetic effects (Lambert et al., 1996). As $GABA_AR$ activation is the major contributor to alfaxalone's anaesthetic effects, we looked at effects of alfaxalone on other receptors or ion channels that could potentially cause neuro-excitation. Alfaxalone, and other neurosteroids, also modulate voltage-activated calcium (Ca^{2+}) channels and glycine-activated chloride channels (Prince and Simmonds, 1992, Lambert et al., 1996, ffrench-Mullen et al., 1994). The glycine receptor (GlyR) is of particular interest, as glycinergic neurotransmission is a major regulator of muscle tone (Biro and Maksay, 2004, Maksay et al., 2002, Wu et al., 1997). GlyR activity mediates inhibitory synaptic transmission between interneurons and motor neurons (MN) and suppression of the GlyR will produce undesirable consequences (Lynch, 2009). An abnormal reduction in the conductance of glycine-gated chloride channels cause startle disease, hyperkeplexia, that manifests as temporary muscle rigidity to unexpected stimuli (Lynch, 2004). Abnormal GlyR suppression also occurs with tetanus toxicity and strychnine poisoning. Tetanus neurotoxin inhibits presynaptic exocytosis of glycine, causing muscle rigidity and paroxysmal muscle contractions (Shin et al., 2012, Williamson et al., 1992, Bergey et al., 1987). Strychnine is a heterocyclic alkaloid that potently and specifically blocks GlyRs, thus inhibiting postsynaptic glycinergic activity, and producing a characteristic hyper-reflexia, progressing to intense muscle spasms and convulsions (Gundlach, 1990, Parker et al., 2011). These presynaptic and postsynaptic inhibitory effects on the GlyR lead us to believe that the neuro-excitation caused by alfaxalone could also be of due to suppression of GlyR activation on motor neurons.

Different steroid compounds have vastly different effects on the GlyR, where neurosteroids have been shown to cause subunit composition dependent bi-directional modulation of GlyRs (Laube et al., 2002). The synthetic steroid RU5135 possesses convulsant properties and has been identified as a

strychnine sensitive GlyR antagonist (Paul and Purdy, 1992). In contrast, alfaxalone was reported to have no effect on GlyRs in cultured neurons (Hill-Venning et al., 1996, Simmonds, 1983). Other studies have shown that alfaxalone weakly potentiated strychnine-sensitive GlyR activity elicited by glycine application, but was unable to elicit a current response in the absence of glycine (Weir et al., 2004, Mascia et al., 1996). This weak potentiation of GlyR responses to glycine occurred at a mean EC_{50} of 27.8μM alfaxalone (Weir et al., 2004). Similarly, homomeric α1 GlyR expressed in *Xenopus laevis* oocytes were weakly potentiated by 6μM alfaxalone on addition of glycine (Mascia et al., 1996). The neurosteroid pregnenolone 3β-sulphate directly modulated GlyRs in the chick embryonic spinal cord (Wu et al., 1997) and bi-directional allosteric modulation of the GlyR has been observed with minaxolone (Biro and Maksay, 2004).

Previous electrophysiological studies of the effects of neurosteroids were performed on GlyRs in cultured neurons, *Xenopus laevis* oocytes and chick embryos, but not in the mammalian neuromotor system. Different neurosteroids also have been shown to produce markedly different modulatory effects on the GlyR (Laube et al., 2002). It is therefore important to directly determine the effect of alfaxalone on mammalian motor neurons and identify whether alfaxalone produces GlyR inhibition or potentiation at clinical dose ranges. Here we investigated the effects of alfaxalone on hypoglossal motor neurons (HMNs) of Wistar rats, using whole cell patch clamp recording techniques under voltage clamp conditions in *in vitro* brainstem slices. We hypothesise that alfaxalone causes inhibition of the GlyR and of inhibitory neurotransmission at clinically relevant doses and could likely cause alfaxalone induced neuroexcitation.

Materials and Methods

Slice Preparation

Whole cell recordings were performed using in vitro brain stem slices from juvenile Wistar rats of either sex (10-14 days old). Rats were anaesthetised using sodium pentobarbitone (100 $mg.kg^{-1}$ IP). When deep anaesthesia was established, the rat was swiftly decapitated. The skull, cerebrum, cerebellum and the neck muscles were removed to expose the brainstem. The brainstem was then placed into an icy-cold bath of artificial cerebrospinal fluid (aCSF) for cutting (see solutions), which was bubbled with carbogen (95%O_2, 5%CO2). Transverse slices at a thickness of 300μM were cut with a DSK Microslicer DTK-1000 (TED Pella Inc) and incubated for 35-50 min in the same cutting aCSF at 35^{o}C. The slices were then maintained at room temperature (19-21^{o}C) in a maintenance aCSF (see solutions), bubbled with carbogen.

Solutions

The aCSF solution used for cutting and initial incubation of slices contained (in mM) 130 NaCl, 26 $NaHCO_3$, 3 KCl, 5 $MgCl_2$, 1 $CaCl_2$, 1.25 $NaPO_4$,10 Glucose. The maintenance aCSF solution, was a similar solution with the exception of 2 $CaCl_2$ and 1 $MgCl_2$.

The patch pipette internal solution contained (in mM) 120CsCl, 4 NaCl, 4 $MgCl_2$, 0.001 $CaCl_2$, 10 Cs *N*-2-hydroxyethyl-piperazine-N'-2ethanesulfonic acid (HEPES), 10 Cesium ethylene glycol-bis(β-aminoethyl ether)-N,N,N,N-tetra-acetic acid (EGTA), pH adjusted to 7.2 with CsOH and osmolarity was adjusted to 290-300mOsM with sucrose. Osmolarity was measured with a vapour osmometer. 3 adenosine 5'-triphosphate (ATP-Mg) and 0.3 guanosine 5-triphosphate-tris (hydroxymethyl) aminomethane (GTP-Tris) was added to the internal solution before use.

DL-2-amino-5-phosphonopentanoic acid (APV, Sigma, 50μM) and NBQX disodium salt hydrate (Sigma, 10μM) were added into the external bath solutions to block N-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptor activity (both DL-α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA) and kainate receptors.1(S),9(R)-(-)-Bicuculline methchloride (Sigma, 5μM) and Strychnine (Sigma, 500nM) solutions were added to the external bath solution to block $GABA_A$ receptor and Glycine receptor activity respectively.

Drugs

3-α-hydroxy-5-α-pregna-11, 20-dione (Alfaxalone, a generous gift of Jurox Pty Ltd) was dissolved in hydroxypropyl substituted β-cyclodextrin (HPCD, a gift of Jurox Pty Ltd) to a ratio of 1:8 to make a stock concentration of 10mM alfaxalone, then diluted to the required bath concentration of 10nM, 30nM, 100nM, 300nM, 1μM, 3μM, 10μM and 25μM. 50μM APV and 10μM NBQX were added into

all control baths to block NMDA and non-NMDA glutamate receptors, isolating IPSC activity. 1(S),9(R)-(-)-Bicuculline methchloride (Sigma, 5μM) and strychnine hydrochloride (Sigma, 500nM) solutions were added to the external bath solution to block $GABA_AR$ and GlyR activity respectively. The stock solution solvent (DMSO, ethanol or HPCD) was always diluted by a factor of 1000 or > in the external bathing solution, and had no effect when applied alone at these concentrations. Application of drugs via the bathing fluid was always for >2min; the time taken to completely exchange the recording chamber solution was typically <40s. The alfaxalone dose response study was only applied to one HMN per slice.

Electrophysiological recordings

Brainstem slices were submerged in a mounted microscope chamber with a volume of ~ 0.5 mL and were continuously superfused with maintenance aCSF at a rate of 1.5-2mL/min. Patch electrodes were pulled from thin-walled borosilicate glass capillary tubes without a filament (Vitrex Medical) on a two-stage electrode puller (PP-83, Narishige); patch electrodes had a final DC resistance of 2-3MΩ when filled with the internal solution and a tip diameter of 1-2 μm. Recordings were performed at room temperature (19-21^oC) with the patch electrode connected to the headstage of an Axopatch 1D patch-clamp amplifier (Axon instruments). HMNs were visually identified by their size, shape, location in the hypoglossal motor nucleus (nXII), and whole cell capacitance (>20pF). Whole cell recordings were obtained by the "blow and seal" method (Stuart et al., 1993), where positive pressure (10-15kPa) was maintained in the pipette to allow surrounding neuropil to be cleaned away as the pipette tip is guided onto the surface of the target HMN (Bellingham and Berger, 1996).
The program pCLAMP 8 (Axon Instruments) was used to apply voltage commands and record whole cell currents and measure responses. Spontaneous and evoked inhibitory postsynaptic current (IPSC) activity was recorded with the HMN voltage clamped at a membrane potential of –60mV. For evoked IPSC recordings, a bipolar concentric stimulation electrode (Frederick Haer Company) was placed in the reticular formation ventrolateral to the border of the hypoglossal motor nucleus, and a stimulus current of 0.5-1.1mA and 0.1 ms duration was applied to reliably evoked an IPSC with consistent first pulse IPSC amplitudes. The recorded signal was amplified (2-20x) and low pass filtered with a cut-off frequency of 2kHz by the Axopatch 1D amplifier before digitization with a 16-bit digitizer (Digidata 1320A, Axon Instruments) and recording on a PC hard disk (Dell Optiplex, running Windows XP Professional). Data was acquired as episodic sweeps of 1.04sec duration for evoked IPSCs, or as continuous blocks of data of 2 min duration for spontaneous IPSCs. For evoked IPSCs, the first stimulus pulse was preceded by a short (20 ms) voltage step of -10 mV, to monitor input resistance (Rn) and series resistance.

Alfaxalone Pilot Study

Alfaxalone was bath applied at 25μM (n=6) to rat HMNs in the presence of excitatory glutamate receptor antagonists APV and NBQX. Only spontaneous IPSC waveform parameters were recorded.

Inhibitory Neurotransmission Pilot Study

Control IPSCs recordings were performed in the presence of APV and NBQX to determine the proportions of $GABA_AR$ and GlyR contributing to IPSCs recorded from rat HMNs. After control recordings, 5μM bicuculline was then bath applied to selectively block $GABA_AR$, and both spontaneous and evoked IPSC activity was recorded. This was followed by the addition of 500nM strychnine to selectively block GlyRs and both spontaneous and evoked IPSCs were recorded. Under the latter conditions, no spontaneous or evoked synaptic activity was present.

Alfaxalone Dose Response Study

Control recordings were performed in the presence of APV, NBQX and bicuculline to isolate glycinergic IPSCs. A dose response study was performed with alfaxalone bath-applied at 10nM, 30nM, 100nM, 300nM, 1μM, 3μM and 10μM (n=7) to rat hypoglossal motor neurones. Both spontaneous and evoked IPSCs were recorded.

Data analysis

Data measurements were made with Clampfit 10 (Axon Instruments). Waveform parameters (amplitude, 10-90% rise time, half-width, decay time constant) and baseline holding current (I_{hold}) were measured, as well as the interval time between IPSCs for spontaneous recordings and input resistance (R_n) for evoked recordings. The IPSC paired pulse ratio was calculated as the mean peak amplitude of the averaged second evoked IPSC divided by the mean peak amplitude of the averaged first evoked IPSC. The decay phase of individual evoked IPSCs were fitted with a mono-exponential function ($f(t) = Ae^{-t/\tau} + C$) where A = amplitude, t = time, τ= decay time constant. This data was imported into Excel 2007 (Microsoft) and further analysed with custom written Visual Basic for Applications routines as described in Bellingham & Berger (1996). Drug induced changes were determined by finding the maximal change of 10 to 12 consecutive responses as compared with the same number of responses for a control average immediately before drug application (Bellingham and Berger, 1996). Statistical significance was shown in graphs by using asterisks. * depicts a P value ≤ 0.05, ** depicts a P value ≤ 0.01, and *** depicts a P value ≤ 0.001. Data are shown as Mean ± SD and statistical significance was determined by a one-way ANOVA or paired two-tailed T-test followed by dunnett's multiple comparison post test and accepted at $P < 0.05$ using Graphpad Prism 5 software.

Results

Alfaxalone pilot study

First we determined whether alfaxalone at a high clinical dose could modulate spontaneous inhibitory transmission in HMNs. An initial dose of 25μM alfaxalone was applied to HMNs in the presence of APV (50μM) and NBQX (10μM).

Alfaxalone caused spontaneous IPSC size, shape and frequency augmentation

Alfaxalone significantly decreased spontaneous IPSC peak amplitude and half-width, but not rise time. IPSC peak amplitude decreased to 71 ± 15.8% of control and IPSC half-width was reduced from 4.6 ± 2.6 to 2.7 ± 2.0 ms on bath application of alfaxalone (Figure 4.1). Bath application of alfaxalone markedly increased IPSC interval time by 346 ± 111% of control. The cause of spontaneous IPSC amplitude depression could be presynaptic or postsynaptic in origin. Increased IPSC interval suggests direct presynaptic depression of inhibitory synaptic release by alfaxalone. The reduction in spontaneous IPSC halfwidth, however is consistent with postsynaptic $GABA_AR$ or GlyR channel modulation.

Alfaxalone produced an inward current shift

The baseline holding current was significantly shifted by three fold (2.99 ± 0.76 increase) in an inward direction (Table 4.1). The production of an inward holding current suggests activation of postsynaptic ion channels by alfaxalone.

Inhibitory Neurotransmission Pilot Study

These pilot results showed 25μM alfaxalone caused suppression of inhibitory neurotransmission to rat HMNs. To determine whether these suppressive effects were acting on glycinergic or $GABA_AR$ mediated synaptic responses, an additional pilot study was performed to determine the contribution of these two receptors to spontaneous and evoked IPSCs in rat HMNs. The $GABA_AR$ blocker bicuculline, followed by the GlyR blocker strychnine, were bath applied to identify the proportion of inhibitory activity attributable to the $GABA_AR$ and GlyR.

Bicuculline did not significantly alter IPSC size, shape or frequency

Bath application of the GABA blocker bicuculline (5μM) did not significantly alter spontaneous or evoked IPSC peak amplitude, rise time or half width (Table 4.2). This shows that there is minimal GABAergic influence in rat HMNs in our recording conditions.

Strychnine abolished spontaneous and evoked IPSC activity

As the addition of bicuculline did not significantly alter IPSC activity, we expected inhibitory synaptic transmission to be glycinergic in nature. The addition of the GlyR blocker strychnine (500nM) completely blocked evoked IPSC pulses and almost all spontaneous IPSCs (Figure 4.2). This cessation of IPSC activity confirmed that the majority of the inhibitory transmission in rat HMNs were attributable to strychnine-sensitive GlyRs. For all future experiments, bicuculline was applied to definitively exclude any contribution of $GABA_A$Rs to inhibitory synaptic transmission.

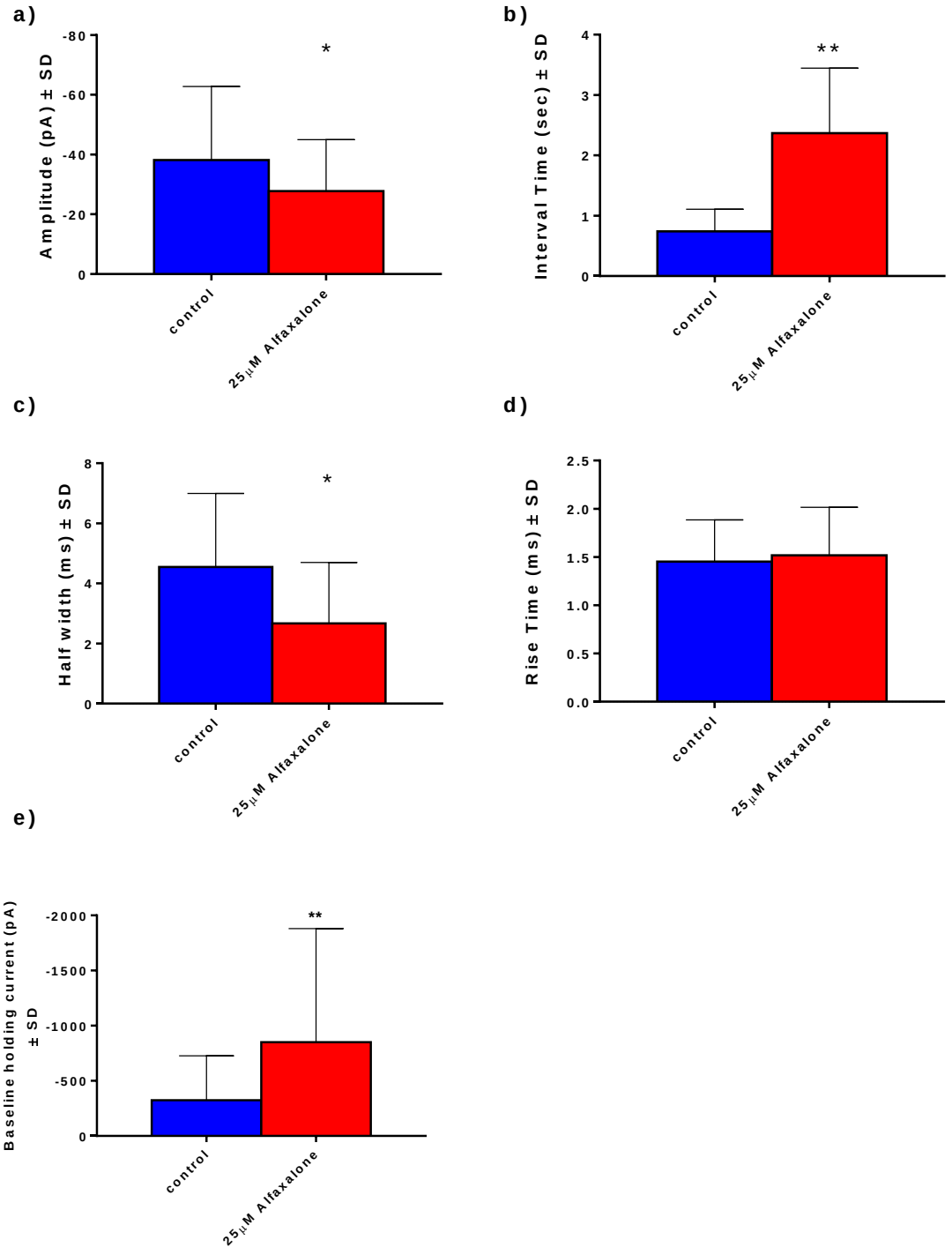


Figure 4.1. Effects of 25μM Alfaxalone on the spontaneous IPSC amplitude
Spontaneous IPSC activity from HMNs (n=6) of 9-11 day Wistar rats. 50μM APV and 10μM NBQX were added to block NMDA and non-NMDA glutamate receptors. a) alfaxalone caused significant IPSC amplitude reduction, b) and decreased IPSC frequency. c) A reduction in IPSC half width was noted, however d) alfaxalone did not alter IPSC rise time, e) A significant inward shift in baseline holding current was observed. All data is presented as mean ± SD. Significance was determined from normalised control data. * indicates a P value ≤ 0.05, ** indicates a P value ≤ 0.01

Parameters	Control			25µM Alfaxalone			Statistical
Spontaneous	mean	SD	n	mean	SD	n	Significance
Amplitude (pA)	-39.2	17.2	6	-26.9	11.0	6	*
Rise Time (ms)	1.5	0.5	6	1.4	0.9	6	ns
Half Width (ms)	4.5	2.7	6	2.7	2.0	6	*
Interval Time (ms)	0.7	0.4	6	2.4	1.1	6	**
Baseline Current (pA)	-323.6	403.5	6	-850.8	1029.0	6	**

Table 4.1. Effects of 25 µM alfaxalone on spontaneous IPSC activity
Whole cell patch clamping was performed to record spontaneous IPSC activity from HMNs of 9-11 day Wistar rats. 50µM APV and 10µM NBQX were added to block NMDA and non-NMDA glutamate receptors. 25µM alfaxalone significantly altered spontaneous IPSC amplitude, half width and frequency but not rise time. An inward shift in current was also detected. All data is presented as mean ± SD. * indicates a P value ≤ 0.05, ** indicates a P value ≤ 0.01

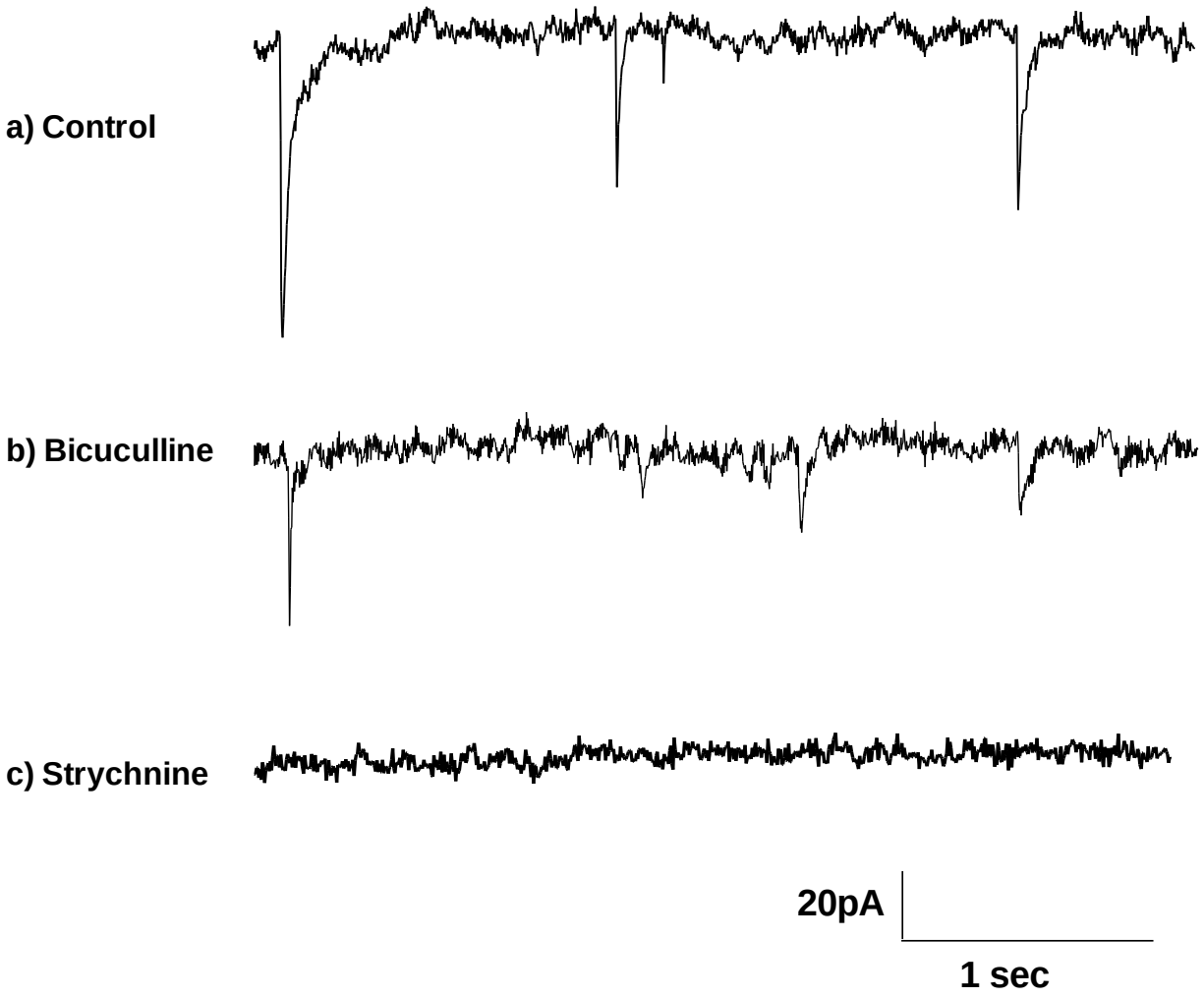


Figure 4.2. Representative traces showing the effects of bicuculline and strychnine on spontaneous IPSC activity

Spontaneous IPSC activity was recorded in a hypoglossal motor neurone of a 10-day-old Wistar rat. a) Spontaneous IPSC activity in the presence of 50μM APV and 10μM NBQX. b) There were no significant changes in spontaneous IPSC waveform parameters or frequency in the presence of 5μM bicuculline. c) 500nM Strychnine caused complete cessation of spontaneous IPSC activity in two of four cells, increasing the interval time to a maximum of two IPSC events within a two-minute period.

Parameters	Control			5µM Bicuculline			500nM Strychnine		
Spontaneous	mean	SD	n	mean	SD	n	mean	SD	n
Amplitude (pA)	-46.1	6.44	4	-40.9	13.6	4	No IPSCs detected		4*
Rise Time (ms)	1.3	0.6	4	1.5	0.8	4	No IPSCs detected		4*
Half Width (ms)	5.6	3.35	4	4.9	4.6	4	No IPSCs detected		4*
Interval time (ms)	2.2	0.95	4	3.7	2.3	4	No IPSCs detected		4*
Evoked	mean	SD	n	mean	SD	n	mean	SD	n
Amplitude (pA)	-103.9	37.97	4	-70.5	63.9	4	no response		4*
Rise Time (ms)	3.4	1.794	4	3.7	1.4	4	no response		4*
Half Width (ms)	8.9	3.8	4	7.5	1.5	4	no response		4*
Baseline Current (pA)	-265.4	106.8	4	-191.8	125.7	4	no response		4*
Paired Pulse Ratio	1.7	0.33	4	1.5	0.2	4	no response		4*

Table 4.2. Effects of bicuculline and strychnine on spontaneous and evoked IPSC activity
No significant changes in spontaneous or evoked IPSC waveform changes were noted with the addition of 5µM bicuculline. However, a marked reduction in IPSC activity was observed on addition of strychnine.
* Strychnine caused complete cessation of evoked and spontaneous IPSC activity in 2 cells. Only one IPSC appeared in one cell and two IPSCs in another cell within a 2-minute file recording period. This is in comparison to 61 to 83 observed events for 2 minute files recorded from HMNs in the presence of bicuculline.

Alfaxalone dose response study

Pilot recordings showed that a high concentration of alfaxalone induced suppression of GlyR-mediated inhibitory synaptic activity. Following on from this finding, a dose response study of the effects of alfaxalone on glycinergic synaptic transmission was carried out to determine at what dose the suppressive effects of alfaxalone began at, and whether these changes occurred at clinically relevant doses. Our rat pharmacokinetic studies revealed the MEC of alfaxalone in a rat as 1.45 ± 0.79 and 2.06 ± 0.38mg/L for 2 $mg.kg^{-1}$ and 5 $mg.kg^{-1}$ dose ranges respectively. With a molar mass of alfaxalone at 332.5g/mol, the MEC equivalent in molar concentrations is 4.36 and 6.02µM respectively. We expected suppression of glycinergic neurotransmission to occur at or below these concentrations, as most neuro-excitation was observed during anaesthetic recovery when alfaxalone concentrations should be declining.

Alfaxalone did not significantly alter spontaneous IPSC size

Alfaxalone did not significantly alter spontaneous IPSC size and shape at concentrations up to 10µM. However, the spontaneous IPSC peak amplitude size ranged markedly, from –8.1pA to –653.5pA. A paradoxical increase in spontaneous IPSC amplitude was also observed at alfaxalone concentrations of 1µM and 3µM, increasing by an observed 107 ± 42% and 105 ± 56% from control respectively. This increase was consistent, as it was noticed in 5 of the 7 cell recordings (Table 4.3).

Alfaxalone caused a dose-dependent decrease in IPSC frequency

A dose dependent increase in spontaneous IPSC interval was apparent from 3µM alfaxalone and IPSC interval increased to 3.44 ± 5.63-fold longer than control IPSC interval at 10µM alfaxalone (Figure 4.3.3). This reduction in frequency indicates a decrease in presynaptic excitability may reduce glycinergic transmission.

Alfaxalone did not change spontaneous IPSC shape

Spontaneous IPSC rise time was not significantly changed by alfaxalone concentrations up to 10µM. A maximal reduction was observed at 1µM with IPSC rise time at 61.4 ± 22.78% of control (Figure 4.3.3). Spontaneous IPSC half width was unaltered at all concentrations up to 10µM alfaxalone.

Alfaxalone caused a dose dependent reduction in evoked IPSC amplitude

Reduction in the evoked IPSC peak amplitude was apparent from 30nM alfaxalone; evoked EPSC amplitude decreased in a dose-dependent manner, to a maximal mean difference of –138.1pA from control at 10µM alfaxalone (Figure 4.3.3). Similar to spontaneous IPSC peak amplitude findings, evoked IPSC size also varied markedly and ranged from –8.1pA to –658.1pA. This reduction in

evoked IPSC size suggests that alfaxalone causes a direct decrease in postsynaptic sensitivity of GlyR to glycine.

Alfaxalone cause dose dependent alterations in evoked IPSC shape

Alfaxalone increased the evoked IPSC rise time in a dose dependent manner. Significantly longer rise times were observed from 1µM alfaxalone, with a maximal increase of 2.3 ± 0.64 fold longer at 10µM (Figure 4.3.3). Overall, evoked IPSC half width was not significantly altered by alfaxalone concentrations up to 10µM; however half width increased in a dose dependent manner at low alfaxalone concentrations, with a maximal increase of 127.4 ± 15.6% of control at 300nM. Evoked IPSC half width then decreased, to a minimum of 77.5 ± 44.28% of control at 10µM (Figure 4.3.3). Alfaxalone produced a dose dependent increase in evoked IPSC decay time, with a significant 2.7 ± 1.1 fold increase from control occurring at 10µM (Figure 4.3.4). These results show modulation of postsynaptic GlyR channel activity, starting from low micromolar concentrations of alfaxalone.

Alfaxalone did not affect paired pulse ratio (PPR)

PPR was unaltered at up to 10µM alfaxalone; paired pulse facilitation (PPF) was consistent throughout the alfaxalone dose range (Figure 4.3.3). This shows that alfaxalone does not significantly alter Ca^{2+} dependent neurotransmitter release of glycine, and suggests that the decrease in spontaneous IPSC frequency was due to a decrease in presynaptic excitability rather than modulation of presynaptic terminal Ca^{2+} influx.

Alfaxalone produced an inward current without altering input resistance

A dose dependent inward shift in baseline holding current was observed up to 10µM alfaxalone. A significant inward shift of 173.3 ± 50.2% of control was apparent at 10µM alfaxalone (Figure 4.3.6). R_n however was not affected by alfaxalone (Figure 4.3.6). One would expect an increase in R_n concurrent with the activation of an inward current, however no significant R_n changes were observed.

Alfaxalone caused an increase in baseline current noise at high concentrations

An increase in baseline noise was observed in some recordings, this increase in noise was more pronounced at high micromolar concentrations of applied alfaxalone. This increased current noise may suggest a tonic effect of alfaxalone but these effects were not investigated further.

Alfaxalone caused glycinergic IPSC inhibition at clinically relevant doses

These results confirmed that alfaxalone caused suppression of glycinergic synaptic transmission in a dose dependent manner. A decrease in evoked IPSC amplitude was observed from 30nM, increased

evoked IPSC rise time from 1µM, and decreased IPSC frequency from 3µM. These changes fall below the plasma concentrations of 4.36 to 6.02µM required for anaesthesia with alfaxalone. In addition, alfaxalone elicited an inward current shift and increased evoked IPSC decay time constant at 10µM. Therefore these data shows that the concentrations at which glycinergic transmission is suppressed in rat HMNs are below those necessary to produce anaesthetic immobilisation, consistent with neuro-excitation occurring during recovery from anaesthesia with alfaxalone.

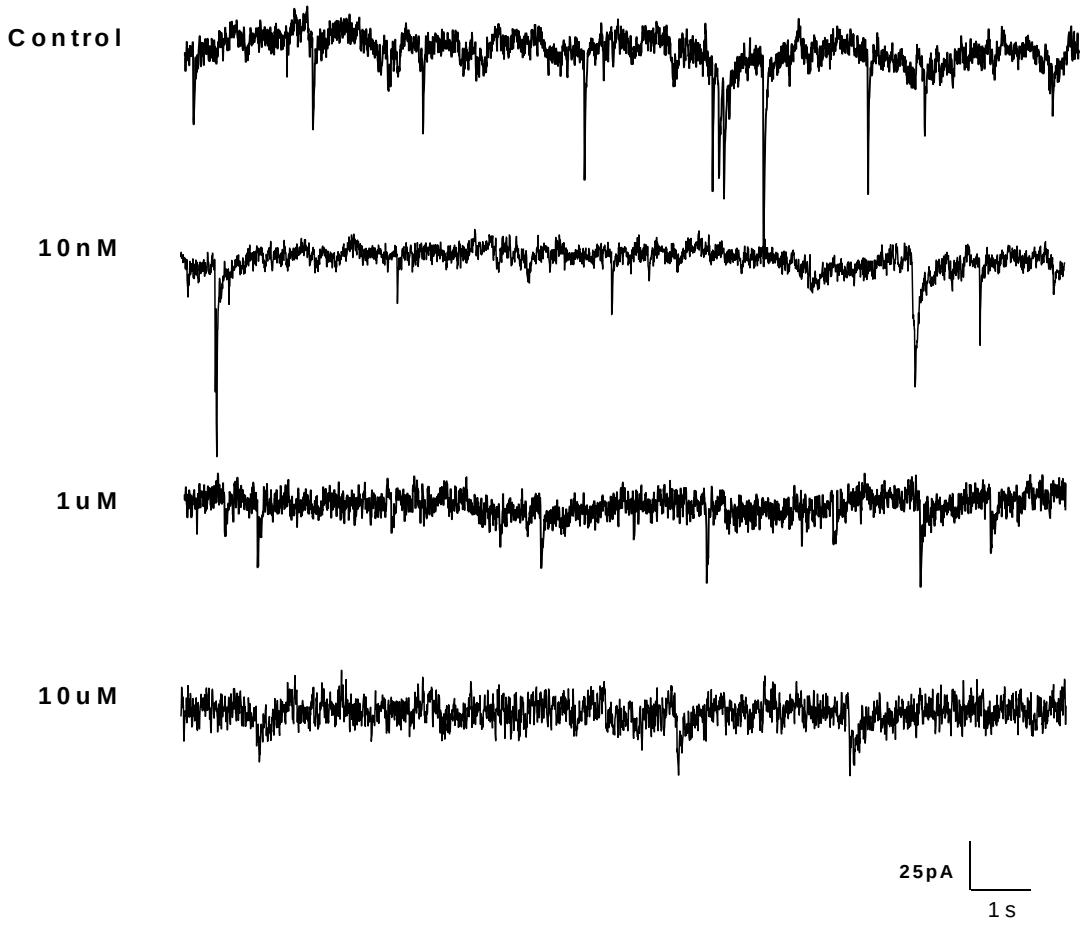


Figure 4.3.1. Representative spontaneous IPSC activity in rat hypoglossal motor neurones at increasing concentrations of Alfaxalone
Spontaneous IPSC recordings were performed under voltage clamp conditions at a membrane potential of –60mV. Note the reduction in spontaneous IPSC frequency and amplitude at increasing concentrations of alfaxalone.

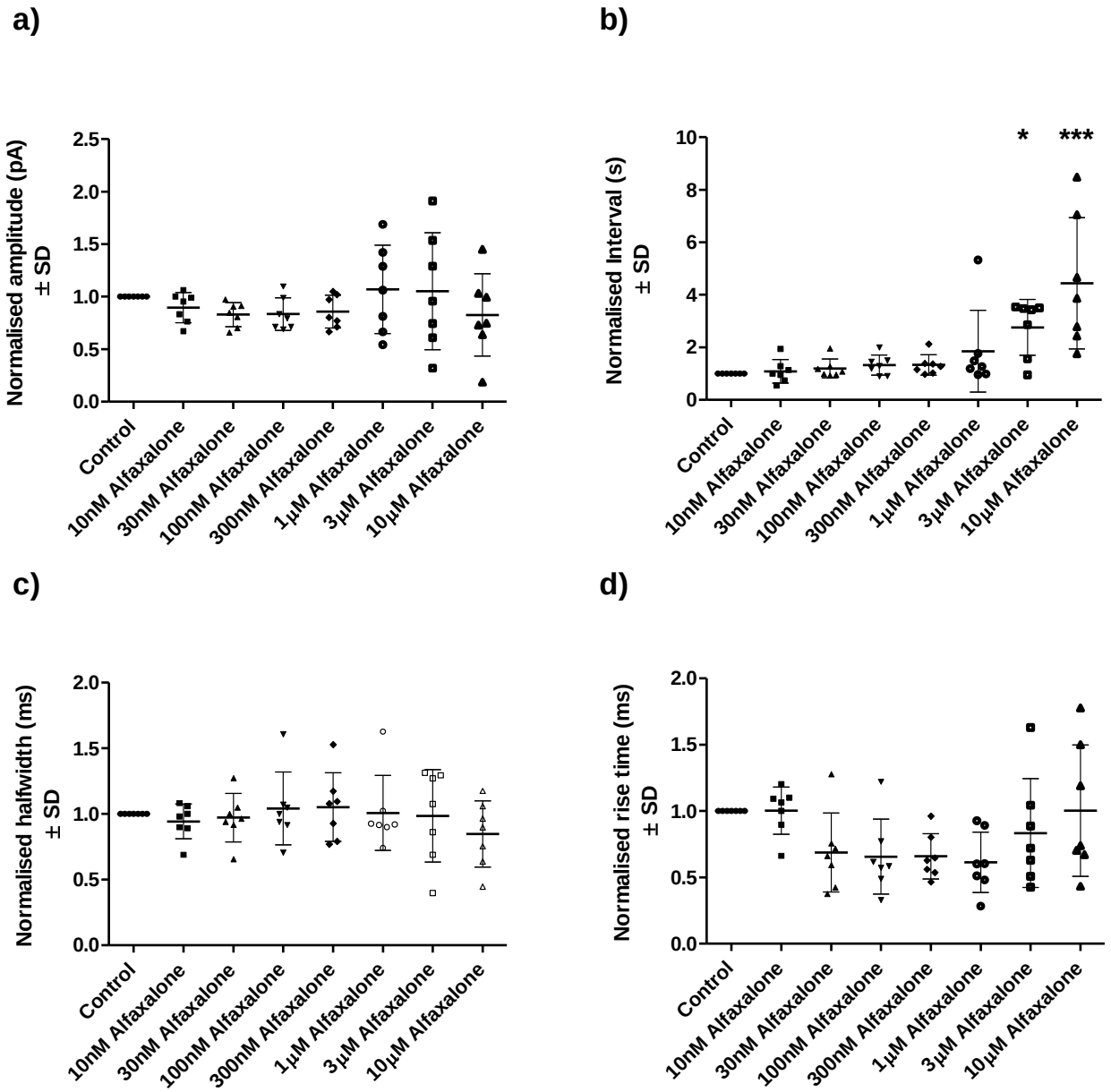


Figure 4.3.2. Spontaneous IPSC parameters with increasing concentrations of alfaxalone
a) Spontaneous IPSC peak amplitude was unaltered by alfaxalone. However b) spontaneous IPSC frequency was significantly reduced at micromolar concentrations of alfaxalone. c) Alfaxalone did not change spontaneous IPSC half width d) or rise time. All data is represented as mean ± SD.
* indicates a P value ≤ 0.05, *** indicates a P value ≤ 0.001

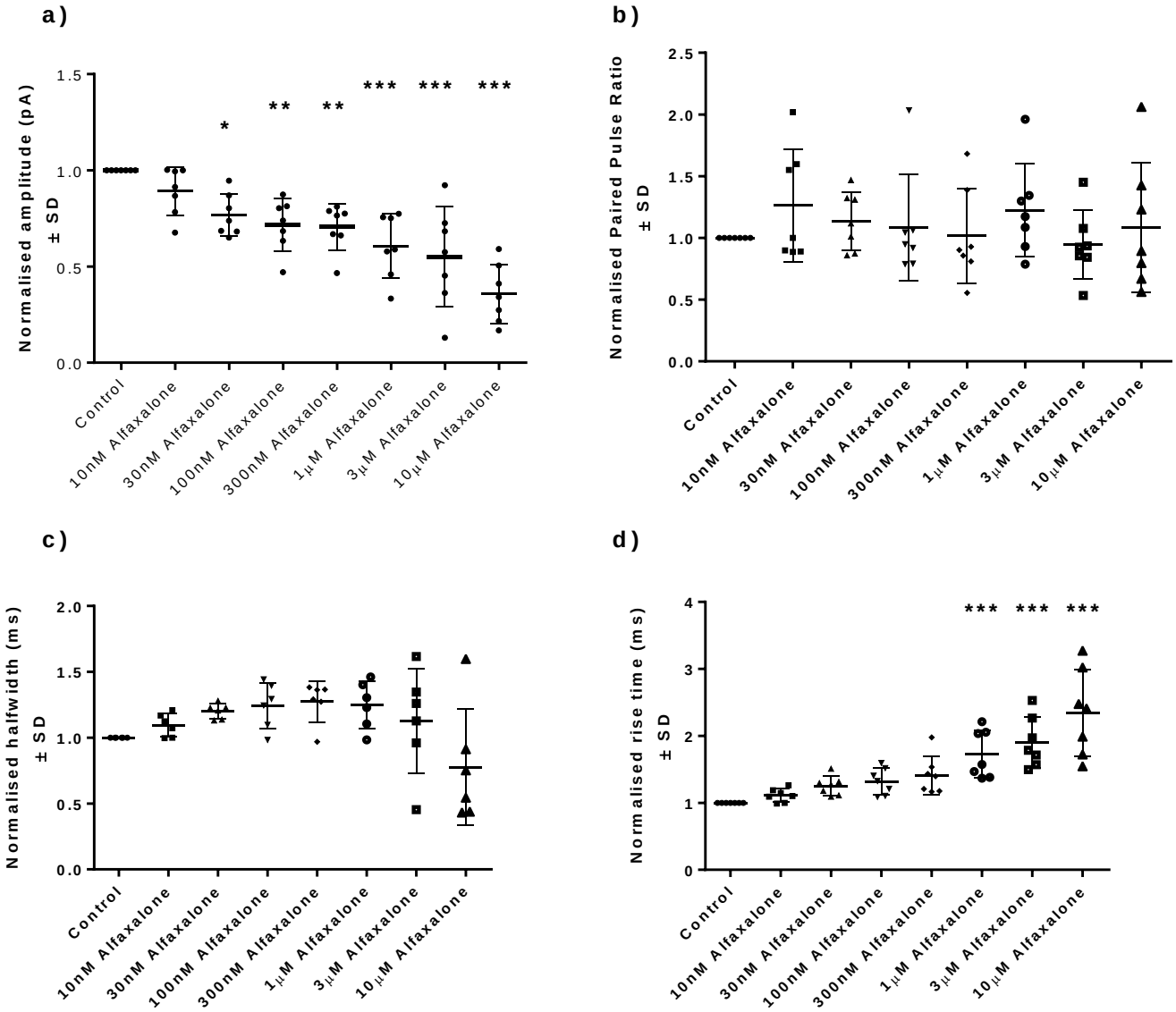
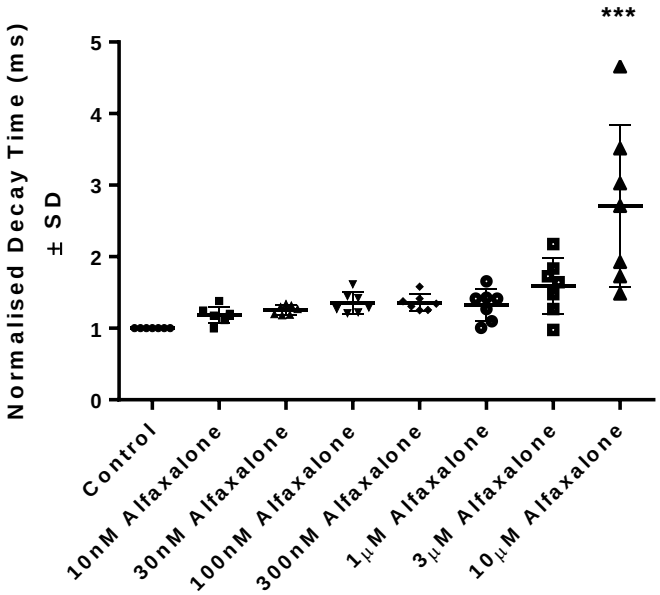


Figure 4.3.3. Evoked IPSC parameters with increasing concentrations of alfaxalone
a) A dose-dependent reduction in evoked IPSC amplitude was apparent from 10nM and was significant for all concentrations from 300 nM upwards. b) PPR was unchanged by alfaxalone. c) There was no change in evoked IPSC half width, although half width appeared to increase at low conentrations and then decrease at higher concentrations of alfaxalone. d) evoked IPSC rise time was significantly prolonged from 1μM alfaxalone. All data is represented as mean ± SD.
* indicates a P value ≤ 0.05, ** indicates a P value ≤ 0.01, *** indicates a P value ≤ 0.001

a)



b)

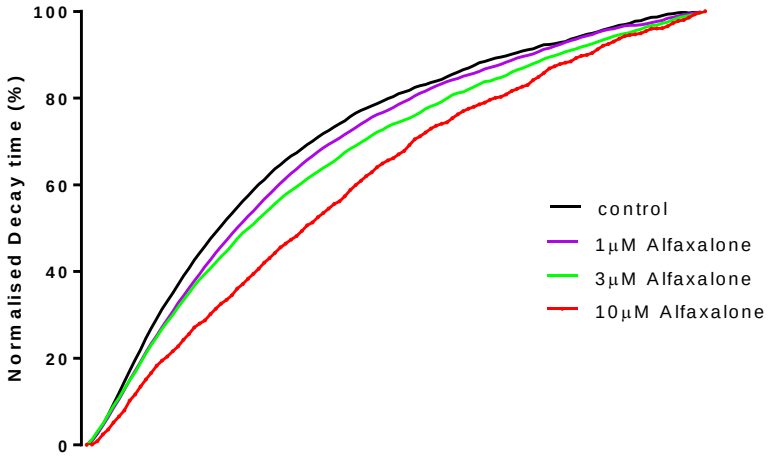


Figure 4.3.4. Evoked IPSC decay time increased with increasing concentrations of alfaxalone
a) Significant prolongation of IPSC decay time was observed at 10μM alfaxalone, and was apparent at lower concentrations b) Superimposed traces show the extended decay time of evoked IPSCs, after scaling to peak amplitude. The concentration of bath-applied alfaxalone is indicated in the colour legend to the right of the trace. *** indicates a P value ≤ 0.001

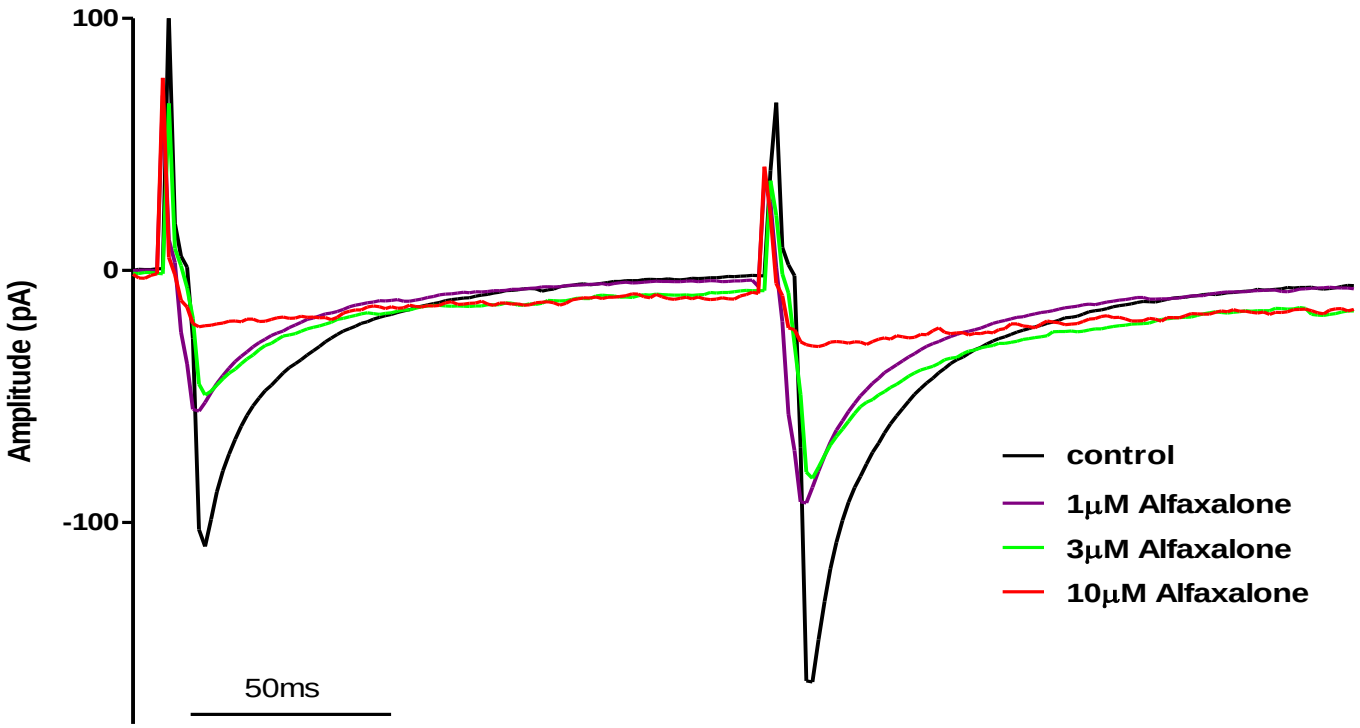


Figure 4.3.5. Representative paired pulse evoked IPSCs show equivalent decreases in IPSC amplitude at micromolar concentrations of alfaxalone, without change in PPR
Superimposed traces showing glycinergic IPSCs evoked by paired pulse stimulation with an interstimulus interval of 150ms. The concentration of bath-applied alfaxalone is indicated in the colour legend to the right of the trace. Increasing concentrations of alfaxalone up to10μM did not significantly alter the paired pulse ratio. Note the proportional decrease in both first and second pulse evoked IPSC peak amplitude at micromolar concentrations of alfaxalone.

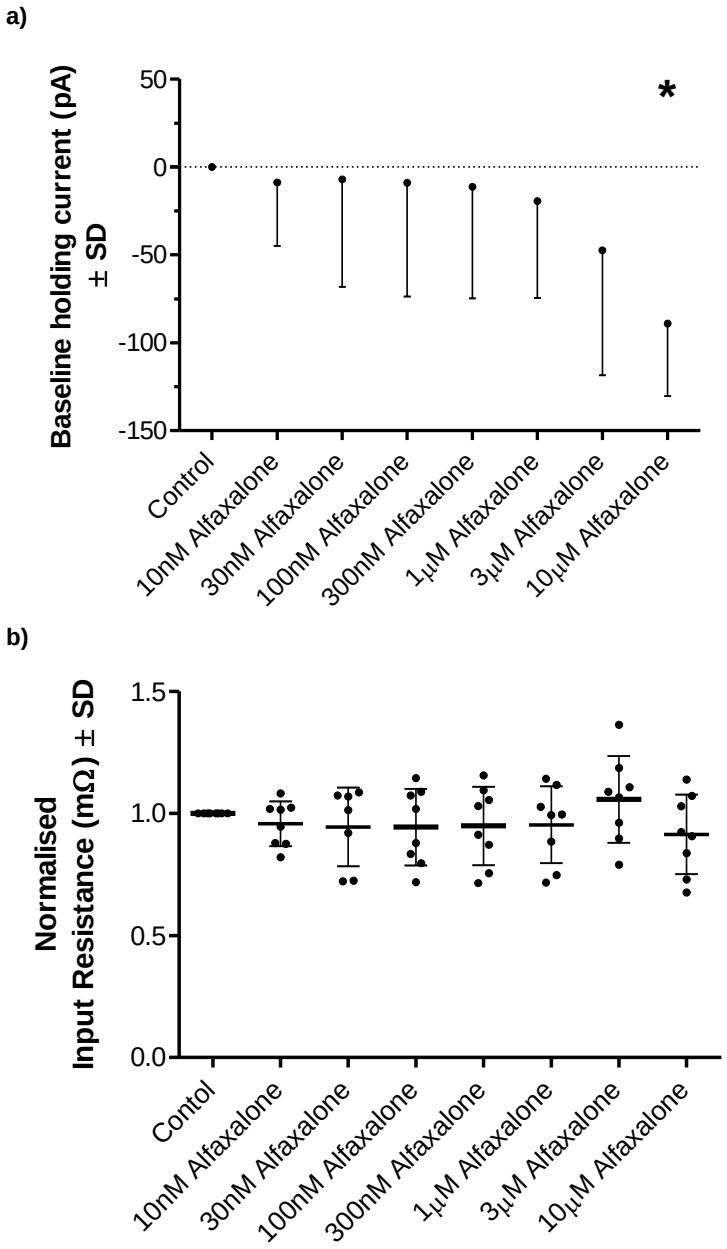


Figure 4.3.6. Effects of increasing concentrations of alfaxalone on baseline holding current and input resistance

a) Baseline holding current was unaffected by increasing doses of alfaxalone up to 10μM where a significant inward shift in holding current was produced. The baseline current in alfaxalone has been normalized to the baseline current in control recording conditions. b) Alfaxalone did not change HMN input resistance at any concentration of alfaxalone. All data is presented as mean ± SD.
* indicates a P value ≤ 0.05

Spontaneous Parameters									
		Control	10nM	30nM	100nM	300nM	1μM	3μM	10μM
Amplitude (pA)	Mean	-56.26	-49.74	-45.5	-47.33	-45.94	-54.36	-52.34	-40.61
	SD	21.75	20.83	16.59	24.76	13.48	20.13	26.74	17.22
	n	7	7	7	7	7	7	7	7
	Significance (<0.05)		ns	ns	ns	ns	ns	ns	ns
Rise Time (ms)	Mean	8.386	7.986	5.271	5.071	5.157	4.571	6.357	7.129
	SD	3.877	2.774	2.193	2.227	1.655	1.208	2.418	1.743
	n	7	7	7	7	7	7	7	7
	Significance (<0.05)		ns	ns	ns	ns	ns	ns	ns
Half Width (ms)	Mean	6.129	5.786	5.914	6.3	6.329	6.114	5.843	5.029
	SD	1.688	1.797	1.832	2.007	1.884	2.048	2.521	1.678
	n	7	7	7	7	7	7	7	7
	Significance (<0.05)		ns	ns	ns	ns	ns	ns	ns
Interval Time (s)	Mean	0.97	0.99	1.16	1.3	1.3	1.75	2.33	3.67
	SD	0.52	0.56	0.62	0.72	0.72	1.48	1.24	2.3
	n	7	7	7	7	7	7	7	7
	Significance (<0.05)		ns	ns	ns	ns	ns	*	***
Evoked Parameters									
		Control	10nM	30nM	100nM	300nM	1μM	3μM	10μM
Amplitude (pA)	Mean	-217.1	-183.5	-164.8	-155.2	-156.4	-135.6	-118.1	-79.02
	SD	132.9	96.44	99.18	102.8	108	110.3	105.6	66.89
	n	7	7	7	7	7	7	7	7
	Significance (<0.05)		ns	*	**	**	***	***	***
Rise Time (ms)	Mean	2.484	2.68	3.107	3.413	3.712	4.44	4.855	5.802
	SD	1.153	1.059	1.47	1.966	2.444	2.73	2.709	2.983
	n	7	7	7	7	7	7	7	7
	Significance (<0.05)		ns	ns	ns	ns	***	***	***
Half Width (ms)	Mean	13.48	14.55	16.13	16.39	16.92	16.29	14.28	9.767
	SD	4.16	3.691	4.771	4.454	4.767	3.462	4.498	4.722
	n	7	7	7	7	7	7	7	7
	Significance (<0.05)		ns	ns	ns	ns	ns	ns	ns
Decay Time (ms)	Mean	18.23	21.07	22.58	24.05	24.51	23.05	28.06	45.42
	SD	6.631	5.844	7.171	7.193	8.501	4.983	10.39	13.26
	n	7	7	7	7	7	7	7	7
	Significance (<0.05)		ns	ns	ns	ns	ns	ns	***
Paired Pulse Ratio	Mean	1.62	2.11	1.79	1.69	1.55	1.98	1.5	1.81
	SD	0.42	1.13	0.39	0.56	0.4	0.78	0.5	1.06
	n	7	7	7	7	7	7	7	7
	Significance (<0.05)		ns	ns	ns	ns	ns	ns	ns
Baseline Current (pA)	Mean	-148.5	-157.5	-155.6	-157.5	-159.8	-167.9	-196	-237.7
	SD	60.65	52.87	53.39	53.76	53.1	51.79	62.48	58.97
	n	7	7	7	7	7	7	7	7
	Significance (<0.05)		ns	ns	ns	ns	ns	ns	*
Input Resistance (mΩ)	Mean	159.4	150.5	143.8	146.2	145.5	145.6	163.1	144.7
	SD	68.79	61.23	64.64	55.08	52.21	51.66	59.86	61.28
	n	7	7	7	7	7	7	7	7
	Significance (<0.05)		ns	ns	ns	ns	ns	ns	ns

Table 4.3. Alfaxalone Spontaneous and Evoked IPSC Data Parameters

See previous page

A significant increase in IPSC frequency was apparent from 3μM. Evoked IPSC peak amplitude was depressed from 30nM. Evoked IPSC rise time and decay time were prolonged.

* indicates a P value ≤ 0.05, ** indicates a P value ≤ 0.01, *** indicates a P value ≤ 0.001

Discussion

Alfaxalone induced neuro-excitation regularly during anaesthetic recovery in rats; neuro-excitation was characterised by spontaneous muscle contractions and muscle rigidity. These signs are similar to those that manifest from diseases such as hyperekplexia or tetanus or strychnine poisoning, all of which occur due to abnormal suppression of synaptic inhibition of motor neurons by GlyRs. Here we hypothesised that alfaxalone produces suppression of glycinergic inhibition of rat HMNs at clinically relevant doses. Different neurosteroids have been reported to produce vastly different effects on GlyRs, including positive or negative GlyR modulation, so it was firstly important to determine whether alfaxalone produced any suppressive effects on GlyR-mediated synaptic transmission. A pilot study was performed at a supra-clinical dose of 25μM alfaxalone, and results showed that alfaxalone did indeed suppress glycinergic activity. Secondly, we aimed to determine whether alfaxalone produced suppression of GlyR inhibition at clinically relevant doses. Our previous pharmacokinetic study in rats determined the minimum plasma level to produce anaesthetic immobilisation in rats was 4.36μM for 2mg.kg^{-1} IV and 6.02μM at 5mg.kg^{-1} IV. Results presented in this chapter showed that alfaxalone suppressed GlyR inhibition in a dose dependent manner. Reduced amplitude of evoked glycinergic IPSCs was apparent from 30nM, with the majority of postsynaptic changes in glycinergic IPSCs being observed within low micromolar alfaxalone concentrations. These data are in agreement with our initial hypothesis that alfaxalone produces suppression of GlyR inhibition at clinically relevant doses, and that this suppression could likely be the cause of neuro-excitation during alfaxalone anaesthesia.

Juvenile rats possess adult inhibitory glycinergic function

Juvenile rats with an age range of 10 to 14 days old were used for the electrophysiological experiments. The brainstems in younger rats are less myelinated and therefore provide fewer complications to securing a motor neuron seal. Neonatal rats possess a different proportion of GlyR subunits than adult animals. In situ hybridisation studies in rats showed a dramatic increase in expression of adult α_1 GlyR subunit mRNA within the first two weeks of life (Singer et al., 1998). Moreover, they also noted that the level of neonatal α_2 GlyR subunit mRNA decreased to background levels by day 18. Motor neurones change markedly in the first two weeks and have acquired adult-like electrophysiological and morphological properties by approximately 16 days of age (Berger et al., 1996, Singer and Berger, 2000). There is the potential that α_2 GlyR subunits could contribute to some of the glyicinergic activity, however we would expect the majority of glycinergic activity to be mediated by the adult α_1 GlyR.

Have Alfaxalone suppresses glycinergic synaptic transmission to rat HMNs by both presynaptic and postsynaptic effects.

The addition of 25µM alfaxalone caused a marked reduction in both spontaneous IPSC amplitude and frequency, which is suggestive of presynaptic suppression of inhibitory neurotransmission. However, spontaneous IPSC half width changes at 25µM alfaxalone indicated postsynaptic modulation of GlyRs by alfaxalone. Therefore the pilot study of the effects of alfaxalone showed both presynaptic and postsynaptic effects on glycinergic IPSCs by alfaxalone. Due to the absence of $GABA_AR$ blockers, it was unclear what proportion of IPSC suppression could be attributed to effects on GABAergic or glycinergic components of the IPSC.

The predominant component of the IPSC in juvenile rat HMNs is due to glycinergic inhibitory transmission

The hypoglossal motor nucleus contains the highest GlyR density of any area within the CNS (Singer and Berger, 2000, Singer and Berger, 1999). This suggests that the great majority of IPSC activity of HMNs would be attributable to glycinergic synaptic transmission, and it was for this reason our experiments were performed in HMNs. However, previous studies have shown that glycinergic synaptic transmission to HMNs undergoes changes during postnatal development (Singer et al 1999). In addition, others have shown that co-transmission of GABA and glycine from the same presynaptic vesicle was possible in brainstem and spinal cord MNs, where dual component miniature IPSCs with GABAergic and glycinergic components were observed (O'Brien and Berger, 1999, Mitchell et al., 2007, Jonas et al., 1998). It has also been shown that 5µM bicuculline blocked 96.7 ± 1.1% of $GABA_AR$ responses and only 5.7 ± 7.5% of GlyR responses, while 500nM strychnine blocked 97.2 ± 2.4% of GlyR responses and only 11.3 ±10.5% of $GABA_AR$ mediated responses in one to five day old Sprague Dawley rat HMNs (O'Brien and Berger, 1999). To confirm that GlyRs were the major inhibitory influence in the juvenile rat HMN, a pilot study with the selective antagonists bicuculline and strychnine was performed. The addition of 5µM bicuculline to control external solutions did not significantly change spontaneous or evoked IPSC waveform parameters, suggesting minimal GABAergic influence in juvenile rat HMNs. The addition of 500 nM strychnine in the presence of bicuculline abolished evoked IPSCs, and abolished or markedly reduced spontaneous IPSC activity, confirming that the great majority of inhibitory synaptic activity in juvenile rat HMNs was glycinergic in nature. In order to rule out effects of alfaxalone on $GABA_AR$ as a confounding factor in our alfaxalone dose response study, 5µM bicuculline was applied to the bath solutions for the main study to block $GABA_ARs$.

Alfaxalone causes a decrease in spontaneous IPSC amplitude and frequency at higher concentration

In our pilot study, alfaxalone at 25µM decreased spontaneous IPSC amplitude, increased spontaneous IPSC half width and increased the interval between spontaneous IPSCs. These effects are consistent with a presynaptic reduction in glycinergic transmission and postsynaptic modulation of GlyR activity. However, in the main dose response study, alfaxalone at up to 10µM did not have significant effects on spontaneous IPSC amplitude or half width, but significantly increased IPSC interval at 3 and 10µM. As we observed depression of evoked IPSC amplitude at much lower concentrations (from 30 nM upwards), one reason for an absence of depression of spontaneous IPSC amplitude at low alfaxalone concentration may be the large variability in spontaneous IPSC amplitude, which masked small changes in average amplitude. Other investigators have noted that spontaneous and miniature glycinergic IPSC amplitude size are highly variable (Mitchell et al., 2007, Singer and Berger, 1999, Lim et al., 2003, Lim et al., 1999). However, spontaneous IPSC amplitude depression may also occur through presynaptic mechanisms which depress presynaptic excitability; one test for future studies of the effect of alfaxalone would be to see if miniature IPSC amplitude and frequency are decreased in the presence of presynaptic activity blockers such as tetrodotoxin. While the decrease in spontaneous IPSC frequency at alfaxalone concentration $\geq 3\mu M$ is consistent with a decrease in presynaptic glycine release, the absence of a change in PPR of evoked IPSCs suggests that the presynaptic actions of alfaxalone are less prominent than postsynaptic modulation of GlyRs. Our data does not give insight into how alfaxalone might cause a reduction in presynaptic excitability, and whether alfaxalone directly decreases Ca^{2+} transients within the presynaptic terminal or indirectly regulates presynaptic excitability by other pathways is yet to be determined.

Alfaxalone causes dose dependent reduction in evoked IPSC amplitude and shape.

In the dose response study of evoked IPSCs, alfaxalone concentration started at 10nM and increased incrementally logwise to 10µM. A reduction in evoked IPSC peak amplitude was first observed at 30nM alfaxalone concentrations, and this reduction progressively increased in size to a maximal reduction to 36% of control amplitude at 10µM alfaxalone. This reduction in evoked IPSC size could be due to either a presynaptic depression of glycine release, or postsynaptic down regulation of GlyR activity.

However, other changes in the evoked IPSC are consistent with the latter interpretation rather than the former. Evoked IPSC rise time was increased from 1µM alfaxalone and evoked IPSC decay time at 10µM alfaxalone concentrations. Glycinergic IPSC decay phases are reportedly limited by GlyR channel opening and closure rather than glycine transmitter rebinding or GlyR desensitisation (Singer and Berger, 1999, Singer et al., 1998, Legendre, 1998). The rise time of glycine-evoked currents

resulting from recombinant mammalian α1 and $\alpha_1\beta$ GlyR channel openings or native GlyRs in Zebrafish brain neurons is glycine concentration-dependent, in that rise time decreased with increasing glycine concentrations (Mohammadi et al., 2003, Legendre, 1998). A presynaptic reduction in glycine release therefore might result in delayed GlyR channel opening and therefore an increase in IPSC rise time, consistent with a shift to a "reluctant" gating state which has been reported to contribute to the kinetics miniature IPSCs mediated by GlyRs in Zebrafish brain neurons (Legendre, 1998). However, the amplitude of miniature IPSCs is independent of their rise time, consistent with a saturating concentration of glycine at postsynaptic GlyRs (Legendre, 1998). Increases in evoked IPSC rise time and decay time prolongation are also consistent with postsynaptic modulation of the GlyRs, suggesting that alfaxalone increases the latency to opening of GlyRs by synaptically released glycine, and either delays GlyR closure or increases the probability of channel opening.

Alfaxalone did not alter PPR.

Alfaxalone did not alter PPR of evoked IPSCs, which showed stable PPF up to 10µM alfaxalone concentration (Figure 4.3.3). The addition of increasing concentrations of alfaxalone led to a dose-dependent decrease in both first and second IPSC peak amplitude but did not alter the PPR. PPF is indicative of a low overall probability of glycine release from the presynaptic membrane. As there was no change significant change in PPR throughout the alfaxalone dose response, this is a significant piece of evidence that alfaxalone does not directly influence the Ca^{2+} dependent release of glycine from pre-synaptic terminals.

Taken together, changes in the evoked EPSC amplitude and time course, and a lack of change in PPR are strongly suggestive of postsynaptic GlyR modulation by alfaxalone, leading to amplitude reduction and prolongation of IPSC rise time and decay time. Our results at this stage are compatible with a direct modulation of GlyRs by alfaxalone, but we cannot be rule out the possibility that application of alfaxalone causes an indirect modulation of GlyR by activating other receptors, ion channels or signalling pathways.

Alfaxalone suppresses glycinergic transmission at clinically relevant doses.

The minimum plasma concentration to maintain sleep in rats was calculated at between 4.36 to 6.02µM alfaxalone. These results are consistent with previous studies where the concentration of alfaxalone associated with surgical anaesthesia in humans is within the low micromolar concentrations (Harrison and Simmonds, 1984). Neuro-excitation was observed most frequently during anaesthetic recovery in rats. This observation suggests that neuroexcitation would typically occur at or below concentrations of alfaxalone needed to maintain sleep. Our data demonstrate that alfaxalone caused suppression of glycinergic inhibition from nanomolar concentrations; however most of the inhibitory effects of alfaxalone on glycinergic IPSCs were seen at low micromolar concentrations. Evoked IPSC peak amplitude was significantly depressed at 30nM alfaxalone, IPSC rise time was prolonged from 1µM alfaxalone and spontaneous IPSC frequency reduction was apparent from 3µM. Our results are compatible with a postsynaptic modulation of GlyR channel activity starting at low alfaxalone concentrations, with a decrease in presynaptic release of glycine at higher concentrations.

High alfaxalone concentrations potentially activates multiple channels

Higher doses of alfaxalone caused a noticeable increase in baseline noise in some cells. This effect was usually observed at concentrations above 1µM alfaxalone. This increase in baseline noise was also reported in an alfaxalone dose range study where doses of above 100µM were applied to transfected HEK 293 cell lines (Foadi et al., 2012). Increased membrane noise was detected when alfaxalone (1-100µM) was applied to bovine chromaffin cells under voltage clamp conditions at negative potentials (Cottrell et al., 1987). These results are consistent with our present alfaxalone findings in HMNs and could potentially be due to activation of other channels by alfaxalone.

Alfaxalone produced an inward current without altering input resistance

Alfaxalone elicited an inward current in rat HMNs at 10µM, but the cause of this inward shift is still not clear, as it was not accompanied by a significant change in input resistance. The application of alfaxalone (1-100µM) to bovine chromaffin cells was reported to cause simultaneous activation of $GABA_A$ channels and inhibition of nicotinic acetylcholine channels, in whole cell patch clamp recording with a CsCl pipette solution that produced a net inward current through chloride-permeable channels (Cottrell et al., 1987). Although we used roughly equivalent recording conditions in these experiments, it is unlikely that an inward current was generated by activation of $GABA_A$ channels, due to the presence of bicuculline in our external recording solutions.

Alternate explanations for the inward current include activation of the hyperpolarisation-activated cation (IH) current or modulation of the transient receptor potential (TRP) channels. The IH current is an anomalous rectifying cationic current that produces an increased inward membrane conductance during hyperpolarisation from resting membrane potential, and, importantly, has been reported in juvenile rat HMNs (Bayliss et al., 1994). The IH current has been shown to play an important role in setting the resting membrane potential of HMNs as deletion of the HCN1 gene, which encodes for IH, resulted in significant hyperpolarisation of resting membrane potential (Chen et al., 2009, Chen et al., 2005, Bellingham, 2013). IH automatically inactivates at depolarised membrane potentials. A positive shift in the voltage dependent IH will lead to a more positive membrane potential, provided there is no change in leak K^+ conductance (Ireland et al., 2012, Wenker et al., 2012). The inward current caused by high alfaxalone concentrations could thus be due to a positive shift in the voltage activation of the IH current; this mechanism would also be compatible with a lack of change in R_n, as the slowly activating IH current is not directly assessed by the brief membrane pulses which were used to measure R_n.

However, the lack of change in R_n may simply be due to the inability of a somatic recording pipette to measure changes in R_n originating from remote dendritic membrane. Evidence for this is seen in the largely dendritic excitatory synaptic inputs from the respiratory pattern generator, which produce a large change in conductance that is not associated with a change in input resistance. Other possible sources of an inward current elicited by neurosteroids include activation of TRP channels, a large family of non-selective cation permeable channels that are found in most mammalian cell types and are known to have a role in modulation of intracellular Ca^{2+} (Clapham et al., 2001, Desai and Clapham, 2005, Pedersen et al., 2005, Moran et al., 2004). TRP channels are often functionally associated with G protein-coupled receptors such as cannabinoid receptors (Moran et al., 2004). Further investigation is necessary to help identify whether activation of TRP channels might be a source of this alfaxalone induced inward current, and this is part of the investigations carried out in the final chapter of this thesis.

The overall results presented in this chapter suggest that alfaxalone produces a reduction in GlyR inhibition by both presynaptic and postsynaptic mechanisms. Alfaxalone leads to a decrease in glycine release from the presynaptic terminal and causes postsynaptic GlyR channel modulation, which together reduce the strength of glycinergic inhibition received by rat HMNs. Alfaxalone also induced an inward current without significant change in input resistance. These effects of alfaxalone occurred at or below clinically relevant anaesthetic doses. It is still unclear however as to how alfaxalone mediates these changes; however, we hypothesise that there are possibly multiple mechanisms involved. We are aware of the important role that cannabinoid receptors (CBR) can directly modulate both glycine release and GlyR function (Mukhtarov et al., 2005, Lozovaya et al., 2005). CBR are G protein-coupled receptors and a functional association has been identified between these receptors and TRP channels (Moran et al., 2004). We therefore propose that alfaxalone induces endocannabinoid synthesis by increased intracellular Ca^{2+} from TRP channel activation, resulting in endocannabinoid release which modulates glycine release in the presynaptic terminal and/or postsynaptic GlyR activity by activation of CBRs.

CHAPTER FIVE

The role of cannabinoid receptors in the electrophysiological effects of alfaxalone on the rat hypoglossal motor neurons

Abstract

Alfaxalone was shown to cause inhibition of glycinergic transmission to rat HMNs. This reduction in inhibitory activity has also been postulated as the cause of neuro-excitation during alfaxalone anaesthesia. It is hypothesised that the reduction in glycinergic synaptic transmission caused by alfaxalone is induced partly by activation of TRP channels and the endocannabinoid synthesis pathway. Here we investigated the effects of alfaxalone on HMNs in the presence of the cannabinoid 1 receptor (CB_1R) agonist WIN55,212-2, the CB_1R inverse agonist AM251 and the CB_1R antagonist NESS0327, using whole cell patch clamp techniques under voltage clamp conditions. Whole cell recordings were performed on HMNs in transverse brainstem slices (300µM thickness) prepared from 10-14 day old Wistar rats anaesthetised with sodium pentobarbitone (100 $mg.kg^{-1}$ IP). Spontaneous and evoked inhibitory post-synaptic currents (IPSC) were recorded at a holding potential of –60mV, using a CsCl-based internal solution, in the presence of the non-NMDA and NMDA glutamate receptor blockers NBQX (10µM), APV (50µM) and the $GABA_AR$ antagonist bicuculline (5µM) was bath-applied in control solutions for all studies. A WIN55,212-2 dose response study was performed with bath application of 10nM, 30nM, 100nM, 300nM, 1µM and 3µM WIN55,212-2. Subsequent studies involved pre-application of WIN55,212-2 (300nM), AM251 (1µM), NESS0327 (100pM) or 2-APB (100µM) to a control bath, followed by application of 1µM, 3µM and 10µM of alfaxalone. WIN55,212-2 significantly decreased evoked IPSC amplitude to 50.85 ± 17.20% of control at 10nM showing a dose dependent decrease to 41.41 ± 18.85% of control at 3µM concentration. Non-linear fitting of a dose response curve yielded an EC_{50} value of 18 nM. Significant spontaneous IPSC amplitude depression was apparent from 3µM alfaxalone and reduced IPSC frequency was apparent from 10µM alfaxalone in the presence of WIN55,212-2. AM251 (1µM) and NESS0327 (100pM) blocked evoked IPSC amplitude reductions and evoked IPSC event prolongation. No significant changes in spontaneous IPSC peak amplitude, frequency, half width and decay time or baseline holding current were apparent on co-application of 2-APB and alfaxalone. Results of this study support our hypothesis that alfaxalone causes a reduction in glycinergic transmission via the endocannabinoid signalling pathway and produces an inward current through activation of TRP channels. The resultant increase in neuronal excitation would clinically manifest as neuro-excitation and could underlie the mechanism to which causes the alfaxalone twitch. A combination anaesthetic

comprising of alfaxalone with a CB_1R antagonist, could potentially stop the alfaxalone twitch and markedly improve alfaxalone anaesthesia for laboratory rodents but also other veterinary species.

Introduction

Most neurosteroids are active at a wide range of receptors (Webb and Lynch, 2007), making it difficult to determine *a priori* what receptor mechanism mediates suppression of glycinergic inputs by alfaxalone. Glycinergic inputs to HMNs can be modulated presynaptically by a wide variety of G-protein coupled receptors (metabotropic Glutamate Rs, aminergic Rs, muscarinic Rs, purinergic Rs, peptidergic Rs, $GABA_BR$ and cannabinoid Rs) and by steroidal compounds (Rekling et al., 2000, Pagnotta et al., 2005). Postsynaptic modulation of glycinergic Rs by cannabinoid Rs has also been reported (Mukhtarov et al., 2005, Lozovaya et al., 2011). Cannabinoid receptors (CBR) are of particular interest, as they are known to reside on the presynaptic GlyR terminal and are involved in modulation of glycine release (Lozovaya et al., 2005, Lozovaya et al., 2011).
The *Cannabis sativa* plant has been long known for its mood-altering effects and has long been used as a recreational drug (Hejazi et al., 2006, Ledent et al., 1999). Cannabinoids modulates many areas of the brain and spinal cord to produce modulatory effects on a multitude of actions through the CB_1R including learning and memory, movement, food intake and antinociception (Hejazi et al., 2006, Pertwee, 1999).

The synthesis of endocannabinoids anandamide and 2-arachidonoyl glycerol (2-AG) is triggered by an increase in cytoplasmic Ca^{2+} (Wilson and Nicoll, 2001, Bardell and Barker, 2010, Kreitzer and Regehr, 2002) and depolarisation-dependent release from neurons (Pertwee, 1999, Mukhtarov et al., 2005). The resultant endogenously synthesised cannabinoids then function as neuromodulators or neurotransmitters (Pertwee, 1999, Alger, 2002, Lozovaya et al., 2011, Wilson and Nicoll, 2001). These lipophilic molecules can mediate effects that are not associated with CBRs and can modulate voltage-gated Ca^{2+}, Na^+ and various K^+ channels (Oz, 2006, Hejazi et al., 2006, Oliver et al., 2004, Kreitzer and Regehr, 2002). However, the endocannabinoid activation of the CB_1R is of particular interest, as CB_1R activation has been shown to reduce glycinergic inputs to HMNs, and could thus be the cause of alfaxalone induced inhibition of presynaptic glycinergic transmission.

CBRs are G protein-coupled receptors with two known receptor types with CB_1R predominantly found in the CNS and CB_2R mainly in peripheral tissues (Pertwee, 1997, Pertwee, 2005). Therefore it is thought that the majority of cannabinoid effects within the brain is mediated by CB_1Rs of neurons (Navarrete and Araque). Retrograde cannabinoid signalling involves the activation of CB_1R and consequently decreases glycine release by modulating intracellular Ca^{2+} transients (Mukhtarov et al., 2005, Navarrete and Araque). In HMNs, glycinergic synaptic currents are partially modulated presynaptically by retrograde cannabinoid signalling (Mukhtarov et al., 2005) and also by directly

modulating the postsynaptic GlyR (Lozovaya et al., 2011, Webb and Lynch, 2007). We hypothesise that alfaxalone has direct effects on the production of endocannabinoids thereby reducing glycinergic transmission via CB_1Rs.

Since their discoveries, there have been numerous developments of CB_1R and CB_2R selective drugs (Pertwee, 2005). CB_1R agonists produce therapeutically beneficial properties such as analgesic, anticonvulsant, and antiemetic effects (Ledent et al., 1999). Specifically, the synthetic cannabinoid, WIN55,212-2 reversed hyperalgesia and allodynia in a rat model of neuropathic pain (Bridges et al., 2001). The CB_1R antagonist AM251 has inverse agonist activity and possesses significant antinociceptive properties at low doses (McMahon and Koek, 2007, Chambers et al., 2006) (Naderi et al.), potentially making it a useful adjunct for anaesthesia. However, the CB_1R inverse agonist SR141716 possesses significant psychiatric side effects including anxiety, depression and suicidality due to its constitutive CB_1R activity (Meye et al., 2012). The potent CB_1R competitive antagonist NESS0327 has no antinociceptive activity and lacks the negative constitutive effects of CB_1R inverse agonists (Ruiu et al., 2003, Meye et al., 2012). For this study, we examined both CB_1R inverse agonist AM251 and CB_1R competitive antagonist NESS0327 for their differential effects at the CB_1R.

Transient receptor potential (TRP) channels are a family of ligand-gated cation channels, where many of these channels are permeable to Ca^{2+} (Pedersen et al., 2005). These non-selective cation channels are involved in a diverse range of regulatory mechanisms including thermoregulation, olfaction, osmoregulation, pressure and acid sensation, stretch-evoked responses and many more (Clapham, 2007, Desai and Clapham, 2005, Clapham et al., 2001, Di Marzo and De Petrocellis, 2010, Venkatachalam and Montell, 2007). Few TRP channel endogenous ligands have been identified but the result of TRP channel activation is cell depolarisation and Ca^{2+} influx (Wu et al., 2010, Bardell and Barker, 2010). Many TRP channels are potentiated by intracellular phospholipase C activation and also by activation of G protein-coupled receptors and tyrosine kinase receptors (Wu et al., 2010, Clapham et al., 2001). Additionally, elevations in intracellular Ca^{2+} modulates almost all TRP channels (Wu et al., 2010, Bardell and Barker, 2010). Evidence of direct activation of a TRP channel (TRPM3) by an endogenous neurosteroid pregnenalone sulphate was shown in pancreatic β cells (Wagner et al., 2008, Nilius and Voets, 2008). This highlights the possibility of direct activation of TRP channels within the brain by other neurosteroids such as alfaxalone. Furthermore, activation of TRP channels may be associated with heightening the startle response by elevating sensations related to pain and thermoregulatory responses (Clapham et al., 2001). 2-APB is a non-specific blocker of most TRP channel activity, with the exception of TRPV2, which is activated by 2-APB . We hypothesise that alfaxalone induces an inward current by activating TRP channels. We also propose

that the depolarisation and Ca^{2+} influx due to TRP channel activation is likely the mechanism that triggers endocannabinoid synthesis and retrograde cannabinoid signalling.

Here we investigated the effects of alfaxalone on HMNs in the presence of CB_1R agonist WIN55,212-2, CB_1R inverse agonist AM251 and CB_1R antagonist NESS0327, using whole cell patch clamp techniques under voltage clamp conditions. We expect the application of CB_1R agonist WIN55,212-2 to mimick the inhibition of glycinergic neurotransmission in a manner similar to that of alfaxalone, and to occlude alfaxalone effects. We also expect CB_1R antagonists AM251 and NESS0327 to suppress alfaxalone-induced inhibition of glycinergic transmission. Finally, we expect that the addition of a TRP channel blocker, 2-APB, will block the inward current produced by alfaxalone and thereby reduce alfaxalone induced inhibition of glycinergic transmission.

Materials and Methods

Slice Preparation

Slice preparation was performed as per Chapter 4 Materials and Methods.

Solutions

Solutions were prepared as per Chapter 4 Materials and Methods. Variations to this involved the addition of (S),9(R)-(-)-Bicuculline methchloride (Sigma, 5μM) to the external control bath solution to block GABA receptor activity.

Drugs

3-α-hydroxy-5-α-pregna-11, 20-dione (Alfaxalone, a gift of Jurox Pty Ltd) was dissolved in hydroxypropyl substituted β-cyclodextrin (HPCD, a gift of Jurox Pty Ltd) to a ratio of 1:8 to make a stock concentration of 10mM, then diluted to the required bath concentration of 10nM, 30nM, 100nM, 300nM, 1μM, 3μM and 10μM.

[R]-[+]-[2,3-Dihydro-5-methyl-3-[4-morpholinylmethly]pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate (WIN 55, 212-2) was dissolved in DMSO at concentration of 100mM to produce a stock concentration of 10mM/L, then diluted to the required bath concentration of 10nM, 30nM, 100nM, 300nM, 1μM and 3μM. N-[piperidin-1-yl]-5-[4-iodophenyl]-1-[2,4-dichlorophenyl]-4-methyl-1 *H*-pyrazole-3-carboxamide (AM251) was dissolved in DMSO at concentration of 10mg/mL to produce a stock concentration of 10mM/L, then diluted to the required bath concentration of 1μM. N-piperidinyl-[8-chloro-1-(2,4-dichlorophenyl)-1,4,5,6-tetrahydrobenzo[6,7] cyclohepta[1,2-c]pyrazole-3-carboxamide] (NESS0327) was dissolved in DMSO at concentration of 0.25mg/mL while bubbled in carbogen for 10 minutes. A final stock concentration of 100μM/L was diluted to the required bath concentration of 100pM. 2-aminoethoxydiphenyl borate (2-APB) was dissolved in 95% ethanol to make a stock solution of 100mM/L and diluted to the required bath concentration of 100μM.

The stock solution solvent (DMSO, ethanol or HPCD) was always diluted by a factor of 1000 or >, and had no effect when applied at these concentrations. Application of drug via the bathing fluid was always for >2min; the time taken to completely exchange the recording chamber solution was typically <40s. The alfaxalone dose response study was only applied to one HMN per slice.

Electrophysiological recordings

Brain slice preparation and recordings were performed as per Chapter 4 Materials and Methods.

Data analysis

Data measurements were analysed as per Chapter 4 Materials and Methods. Data are shown as Mean ± SD and statistical significance was determined by a one-way ANOVA or paired two-tailed T-test followed by dunnett's multiple comparison post test and accepted at $P<0.05$ using Graphpad Prism 5 software.

Results

We hypothesise that alfaxalone causes inhibition of glycinergic transmission by activation of CB_1R. Therefore, we first needed to determine whether direct activation of CB_1Rs caused modulatory effects on glycinergic transmission similar to those of alfaxalone in rat HMNs. We predict that the application of a potent CB_1R agonist WIN55,212-2 will mimic the effects of alfaxalone. Control data for spontaneous and evoked recordings were obtained in the presence of APV (50µM), NBQX (10µM) and bicuculline methchloride (5µM) to exclude excitatory and GABAergic synaptic input, and therefore isolate glycinergic synaptic activity.

WIN55,212-2 dose response study

Spontaneous IPSC recordings were only recorded in two cells and therefore statistical analysis was not possible. We could however see a trend towards a dose dependent decrease in IPSC size and frequency (Table 5.1). IPSC amplitude decreased from -96.9 ± 4.4pA at control to 32.9 ± 13.3pA at 3µM WIN55,212-2. IPSC interval time was lengthened from 0.3 ± 0.1 sec at control to 4.4 ± 2.3 sec at 3µM (Table 5.1).

WIN55,212-2 reduces evoked IPSC size but did not alter evoked IPSC shape

WIN55,212-2 at 10 nM significantly reduced evoked IPSC amplitude to 50.85 ± 17.20% of control (Figure 5.1.1). This decrease in evoked IPSC amplitude was slightly larger at higher concentrations of WIN55,212-2, reaching 41.41 ± 18.85% of control at 3µM concentration. Non-linear fitting of a dose response curve yielded an EC_{50} value of 18 nM. We also observe within the WIN55,212-2 dose response study, we see maximal amplitude reduction. The decrease in IPSC amplitude however was not accompanied by a significant change in IPSC shape parameters, as rise time, half width and decay time were not changed at any concentration of WIN55,212-2. The reduction of evoked IPSC amplitude could be caused by either a decrease in presynaptic glycine release or postsynaptic inhibition of the GlyR.

WIN55,212-2 had no effect on PPR

PPR was not altered by increasing concentrations of WIN55,212-2 up to 3µM. This effect shows that WIN55,212-2 does not significantly change Ca^{2+} dependent glycine release from the presynaptic terminal.

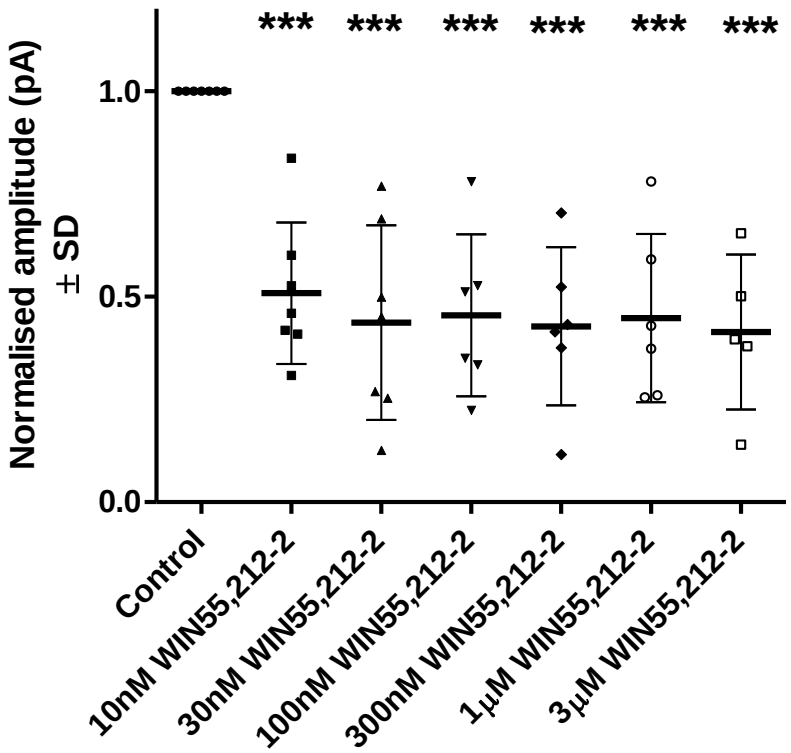
WIN55,212-2 did not alter baseline holding current or input resistance

WIN55,212-2 did not change baseline current at concentrations up to 3µM. In contrast, there was a possible trend towards the development of an outward current at 3µM WIN55,212-2, although this trend did not reach significance. HMN R_n also remained steady throughout the WIN55,212-2 dose response study. This lack of change in baseline holding current and R_n implies that the development of an inward current is unlikely due to CB_1R activation.

Both WIN55,212-2 and alfaxalone caused GlyR inhibition

Although no statistical analysis was performed during spontaneous recordings, the reduction in spontaneous IPSC amplitude and frequency are consistent with presynaptic depression of glycine release, similarly to the results from the alfaxalone dose response study of spontaneous IPSCs. A reduction in evoked IPSC amplitude was observed in both WIN55,212-2 and alfaxalone dose response studies. Neither WIN55,212-2 nor alfaxalone affected PPR or R_n. Alfaxalone did however cause evoked IPSC rise time prolongation and development of an inward current that was not apparent with application of WIN55,212-2.

a)



b)

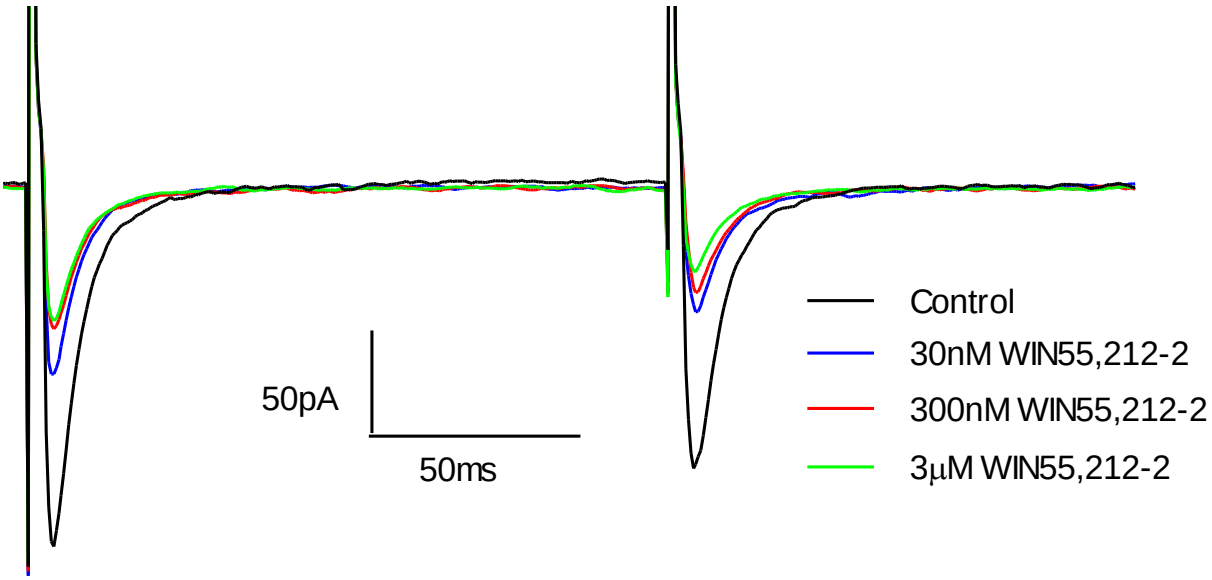


Figure 5.1.1. Evoked IPSC amplitude is reduced by increasing concentrations of WIN55,212-2
a) WIN55,212-2 significantly decreased IPSC amplitude starting at the lowest bath applied concentration of 10nM, and continuing up to 3μM. No other alterations in evoked IPSC waveform parameters were observed with bath application of WIN55,212-2 to HMNs. Significance was calculated relative to normalised control data (1 way ANOVA and Dunnett's multiple comparison) and all data is presented as mean ± SD. *** indicates a P value ≤ 0.001
b) Representative traces of evoked IPSCs highlights the marked decrease in IPSC amplitude in the presence of different concentrations of WIN55,212-2, without change in IPSC shape or PPR.

Spontaneous Parameters								
		Control	WIN55,212-2					
			10nM	30nM	100nM	300nM	1μM	3μM
Amplitude (pA)	Mean	-96.9	-78.2	-63.5	-35.5	-31.4	-32.9	
	SD	4.4	16.3	11.4	21.3	16.3	13.3	
	n	2	2	2	2	2	2	
Rise Time (ms)	Mean	6.7	6.4	2.3	3.2	4.1	4.5	
	SD	5.4	4.9	1.4	0.4	0.7	2.3	
	n	2	2	2	2	2	2	
Half Width (ms)	Mean	6.4	6.1	6.5	6.5	7.6	7.8	
	SD	3.8	3.3	3.3	3.5	2.3	4.3	
	n	2	2	2	2	2	2	
Interval Time (s)	Mean	0.31	0.4	0.6	0.9	3.6	4.4	
	SD	0.1	0.2	0.2	0.4	2.6	2.3	
	n	2	2	2	2	2	2	
Evoked Parameters								
		Control	WIN55,212-2					
			10nM	30nM	100nM	300nM	1μM	3μM
Amplitude (pA)	Mean	-111.7	-49.5	-40.5	-38.4	-36.2	-37.9	-37.6
	SD	67.4	21.1	24.5	17.2	21.6	15.9	22.4
	n	7	7	7	6	6	6	5
	Significance (<0.05)		***	***	***	***	***	***
Rise Time (ms)	Mean	2.4	3.0	3.5	3.0	3.5	3.2	3.0
	SD	0.7	0.6	1.2	0.7	1.6	1.7	0.9
	n	7	7	6	6	6	6	5
	Significance (<0.05)		ns	ns	ns	ns	ns	ns
Half Width (ms)	Mean	8.3	8.5	8.5	8.7	8.9	9.4	7.9
	SD	2.3	1.7	4.1	3.5	4.1	5.1	4.0
	n	7	7	7	6	6	6	5
	Significance (<0.05)		ns	ns	Ns	ns	ns	ns
Decay Time (ms)	Mean	8.1	6.7	6.7	6.0	9.9	6.6	12.6
	SD	3.1	2.7	3.3	4.2	7.1	3.1	6.7
	n	7	7	7	6	6	6	5
	Significance (<0.05)		ns	ns	ns	ns	ns	ns
Paired Pulse Ratio	Mean	1.9	2.0	1.8	2.2	1.6	1.8	1.8
	SD	0.7	0.7	0.8	0.5	0.5	0.8	0.7
	n	7	7	7	6	6	6	5
	Significance (<0.05)		ns	ns	ns	ns	ns	ns
Baseline Current (pA)	Mean	-158.8	-191.3	-200.8	-181.2	-166.8	-180.1	-117.4
	SD	112.8	133.5	126.2	132.8	125.7	134.4	55.4
	n	7	7	7	6	6	6	5
	Significance (<0.05)		ns	ns	ns	ns	ns	ns
Input Resistance (mO)	Mean	196.1	188.6	181.8	194.2	192	196.8	207.7
	SD	124.9	134.4	135.3	149.2	152.1	151.1	132.4
	n	7	7	7	6	6	6	5
	Significance (<0.05)		ns	ns	ns	ns	ns	ns

Table 5.1. Spontaneous and evoked IPSC data for WIN 55,212-2 dose response study
Spontaneous IPSC recordings were performed on two cells and therefore significant statistical analysis was not performed. Evoked IPSC amplitude reduction was observed from 10nM.
*** indicates a P value ≤ 0.001

The effects of alfaxalone in the presence of CB_1R agonist WIN55,212-2

We postulated that alfaxalone caused inhibition of glycinergic transmission by activation of CB_1R receptors. We therefore expected the WIN55,212-2 dose response study to mimick the effects of alfaxalone on GlyR synaptic activity. Results showed that there were similarities between the two dose response studies; however, to substantiate this effect, we would need to next see the effects of alfaxalone in the presence of WIN55,212-2. As WIN55,212-2 is a highly potent CB_1R ligand, we would predict that it will partially occlude effects of alfaxalone at low concentration by competition for the CB_1R binding site. Significant suppression of GlyR activity was apparent from 10nM WIN55,212-2, and a half maximal effective concentration (EC_{50}) for evoked IPSC amplitude reduction was calculated to be 18nM WIN55,121-2. To ensure a full CB_1R agonist effect, the WIN55,212-2 concentration in this study was chosen to be 300nM. Alfaxalone was applied at micromolar concentrations as most of the effects of alfaxalone were apparent at these concentrations. Control recordings were performed in the presence of presence of APV (50µM), NBQX (10µM), bicuculline methchloride (5µM) and WIN55,212-2 (300nM).

Alfaxalone reduced spontaneous IPSC size without altering IPSC shape or frequency

Alfaxalone significantly reduced spontaneous IPSC peak amplitude from 3µM where a reduction of 65.5 ± 12.7% from control was observed. This decrease in IPSC amplitude was not accompanied by spontaneous IPSC rise time or half width changes (Figure 5.2.1). IPSC frequency decreased in a dose dependent manner, but this effect was not significantly apparent until 10µM alfaxalone. IPSC interval time was increased from 1.0 ± 0.5 sec to 9.8 ± 4.0 sec at 10µM alfaxalone.

Alfaxalone reduced evoked IPSC size

A significant decrease in evoked IPSC peak amplitude was apparent at 3µM alfaxalone and a further decrease to 58.7 ± 23.8% of control was seen at 10µM alfaxalone (Figure 5.2.2).

Alfaxalone prolonged evoked IPSC decay time

Evoked IPSC decay time prolongation was observed at 10µM alfaxalone, increasing to 176.9 ± 79.9% of control. Evoked IPSC rise time and half width also appeared to increase in a dose dependent manner, but these changes were not statistically significant.

Alfaxalone did not alter PPR

The addition of alfaxalone in the presence of WIN55,212-2 did not affect PPR (Table 5.2)

Alfaxalone produced an inward current without altering input resistance

An inward current was observed on application of 10µM alfaxalone where a 2.5 ± 1.1 fold increase in inward holding current was observed compared to control. R_n was unchanged by alfaxalone in the presence of WIN55,212-2.

WIN55,212-2 delayed the suppressive effects of alfaxalone on glycinergic IPSCs

We expected the pre-application of the CB_1R with WIN55,212-2 would reduce the inhibitory effects of alfaxalone by competitive binding with CB1Rs causing reduction in glycinergic transmission and therefore reduce the effectiveness of alfaxalone to indirectly activate CB_1Rs.

There were several comparable differences between the alfaxalone dose response study and the effects of alfaxalone with WIN55,212-2 pre-application. Alfaxalone alone did not alter spontaneous IPSC amplitude, but in the presence of WIN55,212-2, a noticeable amplitude reduction was apparent from 3µM alfaxalone. IPSC frequency was decreased by alfaxalone in both studies but a less pronounced reduction was noticed with pre-application of WIN55,212-2.

Evoked IPSC amplitude reduction was noticeable from 30nM for alfaxalone applied alone; however a decrease in evoked IPSC amplitude was only observed at 3 and 10µM alfaxalone in the presence of WIN55,212-2. Pre-application of WIN55,212-2 blocked the evoked IPSC rise time prolongation caused by alfaxalone. IPSC decay times were prolonged from 10µM alfaxalone with or without the pre-application of WIN55,212. In addition, the development of an inward current was still observed at 10µM alfaxalone when WIN55,212-2 was present. Overall, this comparison shows that pre-application of the CB_1R agonist WIN55,212-2 reduced the effects of alfaxalone on glycinergic transmission, but had no influence on the development of a depolarising current. This indicates that both WIN55,212-2 and alfaxalone are likely to activate CB_1Rs which depress glycinergic transmission to HMNs.

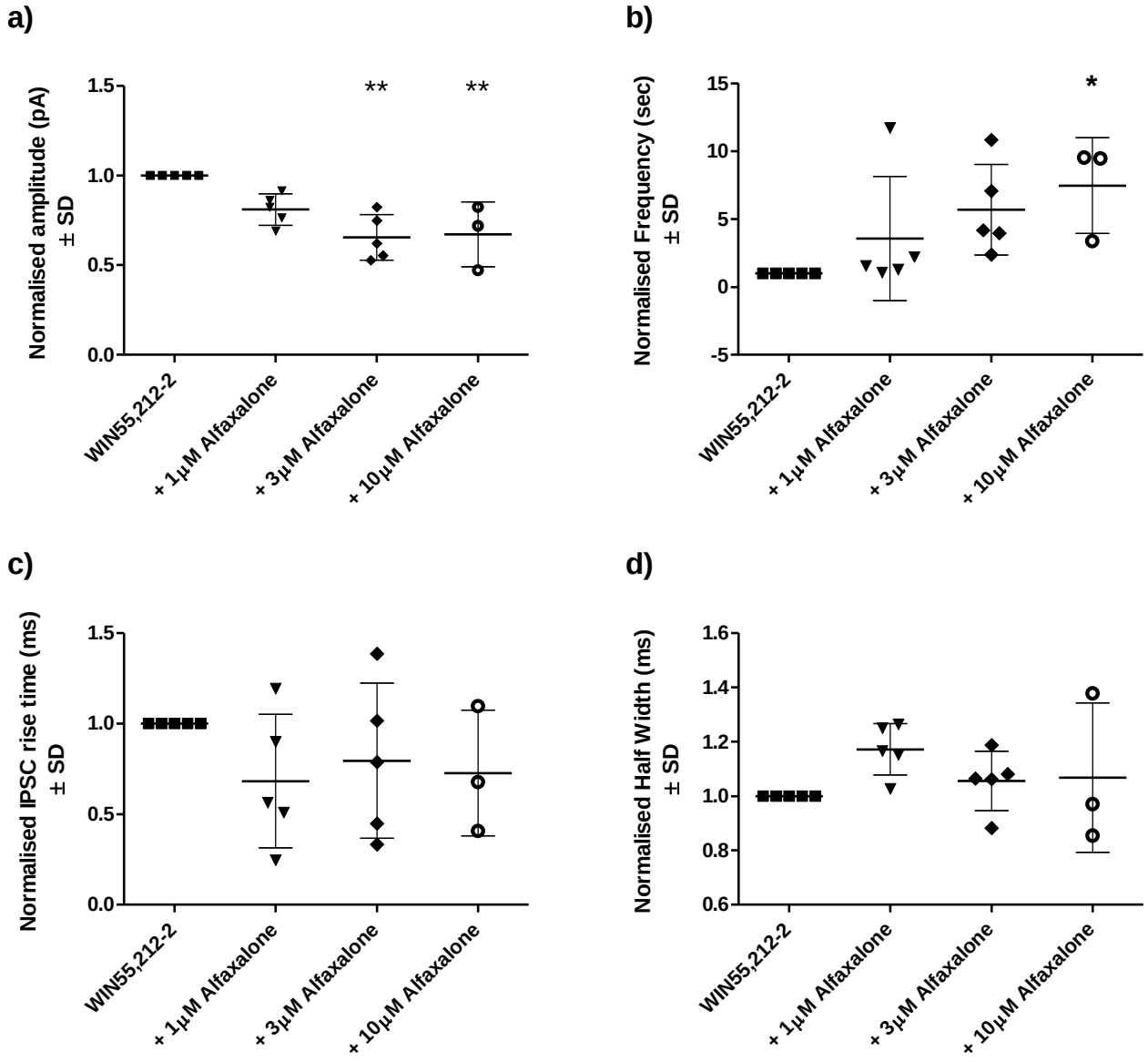


Figure 5.2.1. Spontaneous IPSC parameters for increasing concentrations of alfaxalone in the presence of 300nM WIN55,212-2

a) Pre-application of WIN55,212-2 caused significant spontaneous IPSC amplitude depression at 3µM and 10µM alfaxalone. b) A increase in IPSC interval was apparent at 3µM and was significant at 10µM alfaxalone c) there were no significant changes in IPSC rise time d) or IPSC halfwidth on coapplication of WIN55,212-2 and alfaxalone. Significance was calculated from normalised data and all data is presented as mean ± SD. * indicates a P value ≤ 0.05, ** indicates a P value ≤ 0.01

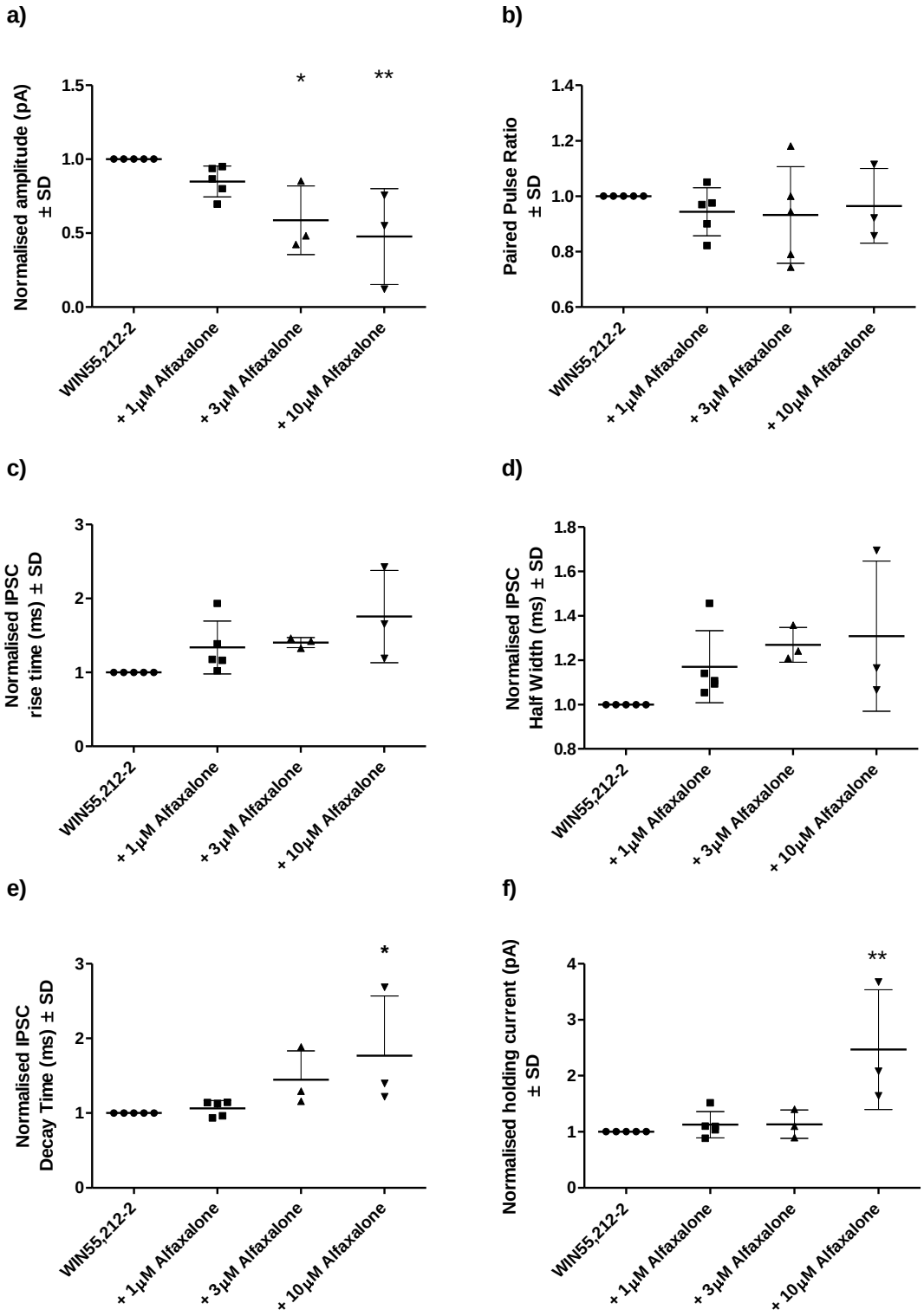


Figure 5.2.2. Evoked IPSC parameters for increasing concentrations of alfaxalone in the presence of 300nM WIN55,212-2

a) In the presence of WIN55,212-2, alfaxalone caused IPSC amplitude depression at 3 and 10 µM. b) PPR was not affected by alfaxalone c) nor was rise time, d) or halfwidth e) but decay time was prolonged from 10µM alfaxalone with pre-application of WIN55,212-2f) A significant inward shift in baseline holding current was observed at 10µM alfaxalone in the presence of WIN55,212-2,. Significance was calculated from normalised data and all data is presented as mean ± SD.
* indicates a P value ≤ 0.05, ** indicates a P value ≤ 0.01

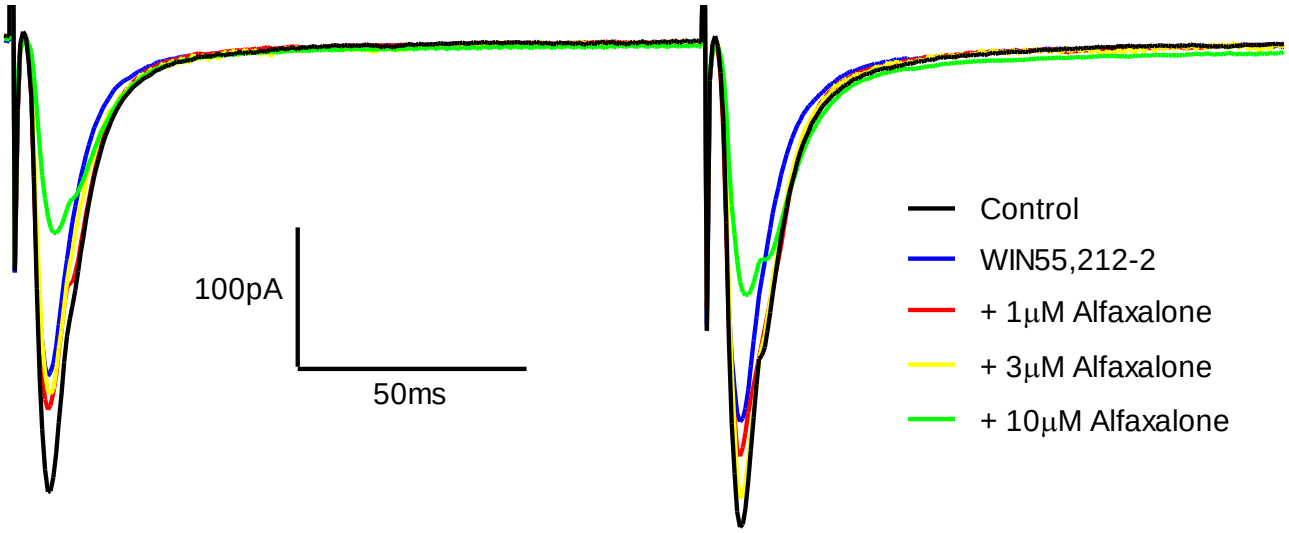


Figure 5.2.3. Representative trace of evoked IPSC recordings of increasing alfaxalone concentrations in the presence of 300nM WIN55,212-2
Pre-administration of WIN55,212-2 to alfaxalone caused a dose dependent decrease in evoked IPSC amplitude of both first and second pulses. IPSC rise time was unaltered, while decay time was slightly prolonged following application of 10μM alfaxalone with WIN55,212-2. The PPR was unaltered in the presence of either WIN55,212-2 alone or with micromolar concentrations of alfaxalone.

Spontaneous Parameters					
		WIN55,212-2	Alfaxalone		
		300nM	1μM	3μM	10μM
Amplitude (pA)	Mean	-45.0	-36.5	-29.3	-27.5
	SD	8.1	7.2	6.8	9.3
	n	5	5	5	3
	P <0.05		ns	**	**
Rise Time (ms)	Mean	6.3	3.8	4.3	4.2
	SD	2.2	1.4	1.2	1.5
	n	5	5	5	3
	P <0.05		ns	ns	ns
Half Width (ms)	Mean	4.6	5.4	4.9	4.2
	SD	1.2	1.6	1.7	0.9
	n	5	5	5	3
	P <0.05		ns	ns	ns
Interval Time	Mean	1.9	4.3	6.2	9.8
	SD	0.5	6.4	5.3	4.0
	n	5	5	5	3
	P <0.05		ns	ns	*
Evoked Parameters					
		WIN55,212-2	Alfaxalone		
		300nM	1μM	3μM	10μM
Amplitude (pA)	Mean	-295.2	-248.7	-158.9	-140.6
	SD	61.84	48.05	42.72	112.5
	n	5	5	3	3
	P <0.05		ns	*	**
Rise Time (ms)	Mean	2.0	2.3	3.1	4.0
	SD	0.5	0.5	0.9	2.3
	n	5	5	3	3
	P <0.05		ns	ns	ns
Half Width (ms)	Mean	7.8	9.2	10.7	10.9
	SD	0.9	1.7	0.9	2.2
	n	5	5	3	3
	P <0.05		ns	ns	ns
Decay Time (ms)	Mean	6.0	6.5	9.5	11.4
	SD	1.3	1.8	1.1	3.2
	n	5	5	3	3
	P <0.05		ns	ns	*
Paired Pulse Ratio	Mean	1.4	1.3	1.2	1.2
	SD	0.4	0.2	0.1	0.2
	n	5	5	3	3
	P <0.05		ns	ns	ns
Baseline Current (pA)	Mean	-118.5	-127.2	-106.7	-215.0
	SD	39.0	29.9	35.0	4.0
	n	5	5	3	3
	P <0.05		ns	ns	**
Input Resistance (mΩ)	Mean	156.1	153.7	157.2	134.4
	SD	77.4	63.9	65.9	35.7
	n	5	5	3	3
	P <0.05		ns	ns	ns

Table 5.2. Spontaneous and evoked IPSC parameters for increasing concentrations of alfaxalone in the presence of 300nM WIN 55,212-2

* indicates a P value ≤ 0.05, ** indicates a P value ≤ 0.01, *** indicates a P value ≤ 0.001

The effects of alfaxalone in the presence of CB_1R inverse agonist AM251

These data showed that WIN55,212-2 blocked many of the inhibitory effects of alfaxalone on glycinergic transmission without altering the inward current elicited by alfaxalone. These results strongly suggest that alfaxalone may act through CBR activation to suppress glycinergic transmission. To provide further evidence to support this hypothesis, we would expect the pre-application of a CB_1R inverse agonist AM251 to block the inhibitory effects of alfaxalone on glycinergic transmission. As an inverse agonist, we would also expect to see antagonism of any constitutive CB_1R activity in the absence of an agonist, and therefore we may potentially see an increase in glycinergic transmission with application of AM251 alone.

AM251 alone did not alter spontaneous or evoked glycinergic transmission

1µM AM251 was bath-applied in a control aCSF containing APV (50µM), NBQX (10µM) and bicuculline methchloride (5µM). No significant changes in spontaneous or evoked IPSC parameters were observed (Table 5.3). A slight increase in IPSC frequency was observed between AM251 and control conditions, but this increase was not significant. This slight increase in IPSC frequency could be due to a reduction in constitutive CB_1R activity produced by this inverse agonist. However, the lack of any significant effects o application of AM251 alone suggests that constitutive CB_1R activity does not modulate glycinergic transmission to HMNs.

Alfaxalone did not alter spontaneous IPSC size or frequency in the presence of AM251

Alfaxalone did not alter spontaneous IPSC peak amplitude at concentrations up to 10µM following pre-application of AM251 (Figure 5.3.2). Spontaneous IPSC frequency also remained constant at all concentrations of alfaxalone and did not significantly differ from that seen in AM251 alone.

Alfaxalone caused reduction in spontaneous IPSC rise time but did not change half width in the presence of AM251

A significant reduction in spontaneous IPSC rise time was observed at all micromolar concentrations of alfaxalone (Figure 5.3.2) in the presence of AM251. A maximal reduction to 51.5 ± 13.4% was seen at 3µM alfaxalone compare to rise time in AM251 alone. In contrast, alfaxalone did not alter spontaneous IPSC half width at any concentration in the presence of AM251.

Alfaxalone did not alter evoked IPSC amplitude or shape in the presence of AM251

Evoked IPSC amplitude and shape were not significantly altered by alfaxalone following pre-application of AM251. Mean evoked IPSC peak amplitude showed increased variability at high concentrations of alfaxalone; amplitudes ranging from -38.7 to -136.0pA at 10µM alfaxalone (Figure

5.3.3). Evoked IPSC rise time showed similar variability at 10µM alfaxalone. Evoked IPSC half width and decay time were unaltered through the dose range.

PPR was unaffected by either AM251 or alfaxalone in the presence of AM251

The addition of AM251 alone did not significantly change evoked IPSC PPR, and nor did co-application of AM251 and alfaxalone.

Baseline holding current and input resistance was unaltered by alfaxalone

Baseline holding current was not significantly altered by the addition of AM251 alone, or by alfaxalone in the presence of AM251. However, there were large variations in holding current following addition of 10µM alfaxalone in the presence of AM251, with a mean change ± SD of -85.6 ± 99.1 (Figure 5.3.4). An observed decrease in R_n at 10µM alfaxalone was seen, although this decrease was not significant. This decrease in R_n could be a result of activation of more channels and could account for increased baseline noise observed in some of the recordings at 10µM alfaxalone (Figure 5.3.1)

AM251 blocked the effects of alfaxalone on glycinergic IPSCs and on holding current

The effects of alfaxalone on glycinergic transmission were significantly reduced in the presence of the CB_1R inverse agonist AM251. A reduction in spontaneous IPSC frequency was not seen following alfaxalone application with AM251 present, while alfaxalone applied alone produced a marked reduction in IPSC frequency from 3µM. Evoked IPSC amplitude was not altered by alfaxalone with pre-application of AM251. This too was in contrast to the alfaxalone dose response study, in the absence of AM251, where we saw marked evoked IPSC amplitude reduction from 30nM alfaxalone concentration. Alfaxalone reduced spontaneous IPSC rise time with AM251 present, an effect which was not seen with alfaxalone applied alone. The pre-application of AM251 blocked changes in the evoked IPSC rise and decay time caused by alfaxalone applied alone. Interestingly, a depolarising current was not elicited by alfaxalone in the presence of AM251. Overall, the results show that CB_1R antagonism blocked presynaptic and postsynaptic effects of alfaxalone on glycinergic transmission, but also blocked the development of an inward current at 10µM alfaxalone.

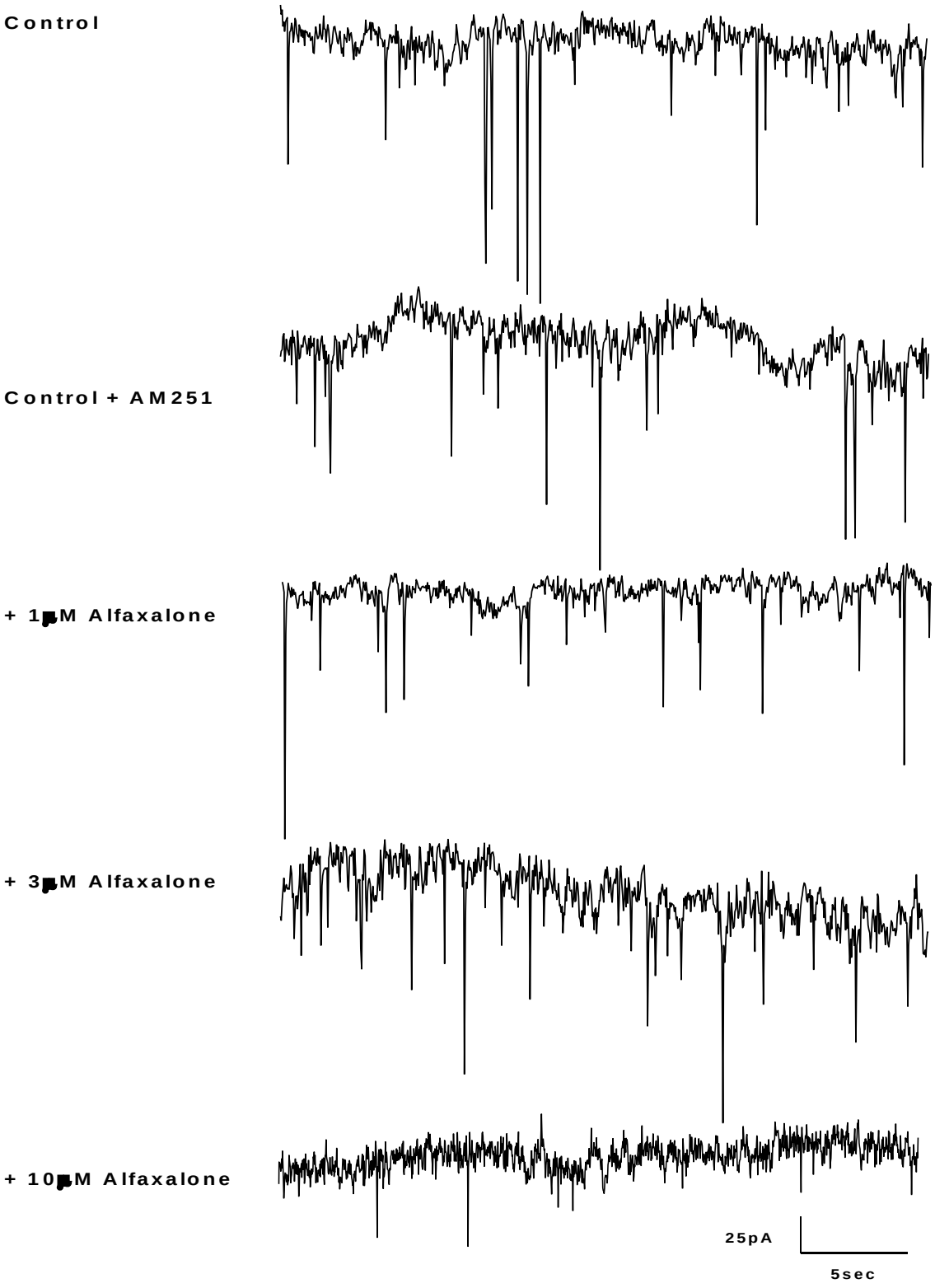


Figure 5.3.1. Representative traces of spontaneous IPSC activity with micromolar alfaxalone concentrations in the presence of AM251
AM251 alone did not alter control spontaneous IPSC activity. No significant alterations in IPSC size or frequency were observed on addition of increasing concentrations of alfaxalone with AM251 present. Note increased baseline noise was apparent in the recording on addition of 10µM alfaxalone.

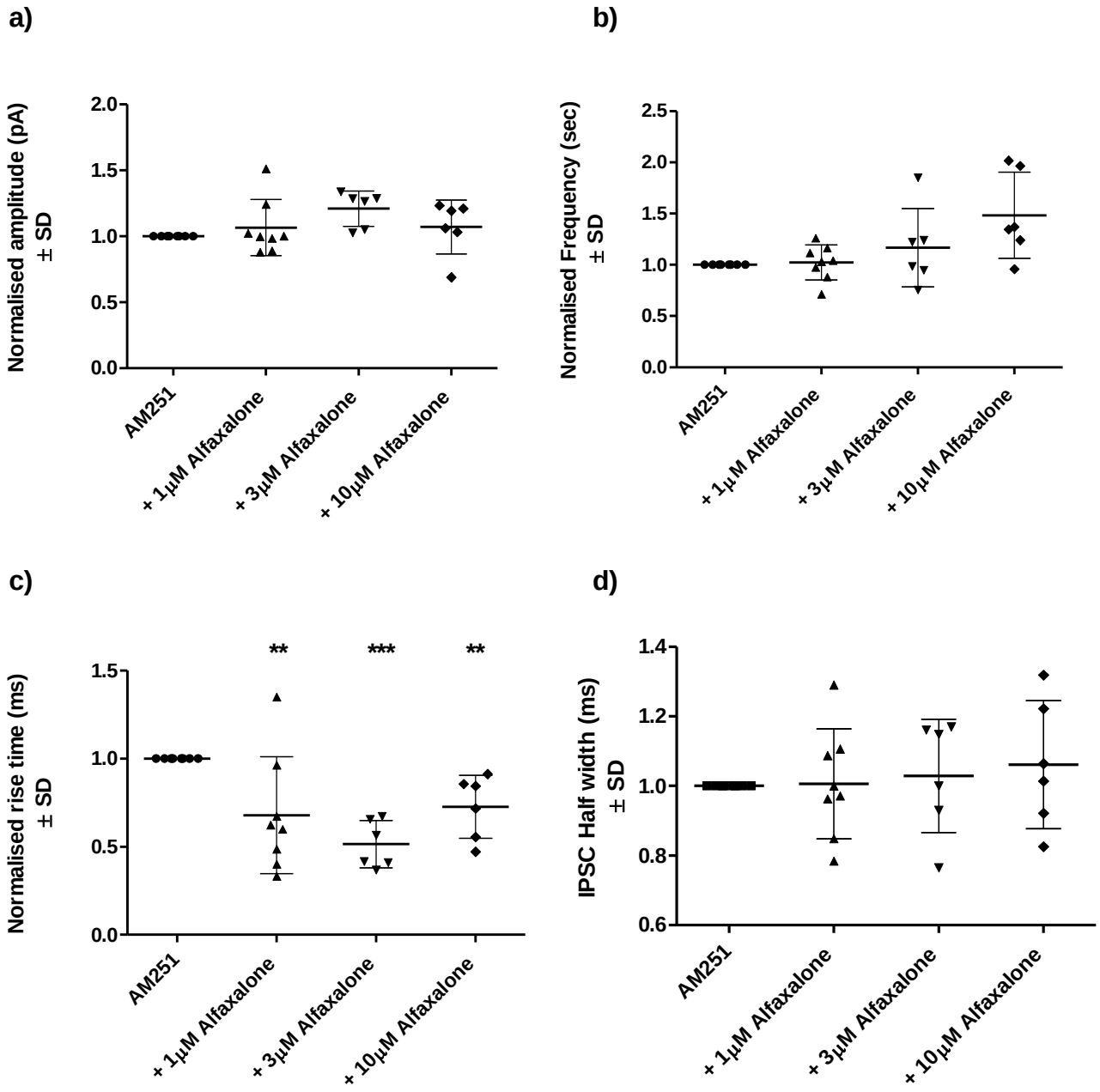


Figure 5.3.2. Spontaneous IPSC amplitude for increasing concentrations of alfaxalone in the presence of 1µM AM251

a) Spontaneous IPSC amplitude was unaltered by micromolar concentrations of alfaxalone, following the addition of 1µM AM251. b) AM251 also blocked the reduction in spontaneous IPSC frequency caused by alfaxalone applied alone. c) A significant decrease in spontaneous IPSC rise time was observed with application of micromolar concentrations of alfaxalone, following the pre-application of 1µM AM251; this effect was not present with alfaxalone applied without AM251. Significance was calculated from normalised data and all data is presented as mean ± SD. ** indicates a P value ≤ 0.01, *** indicates a P value ≤ 0.001

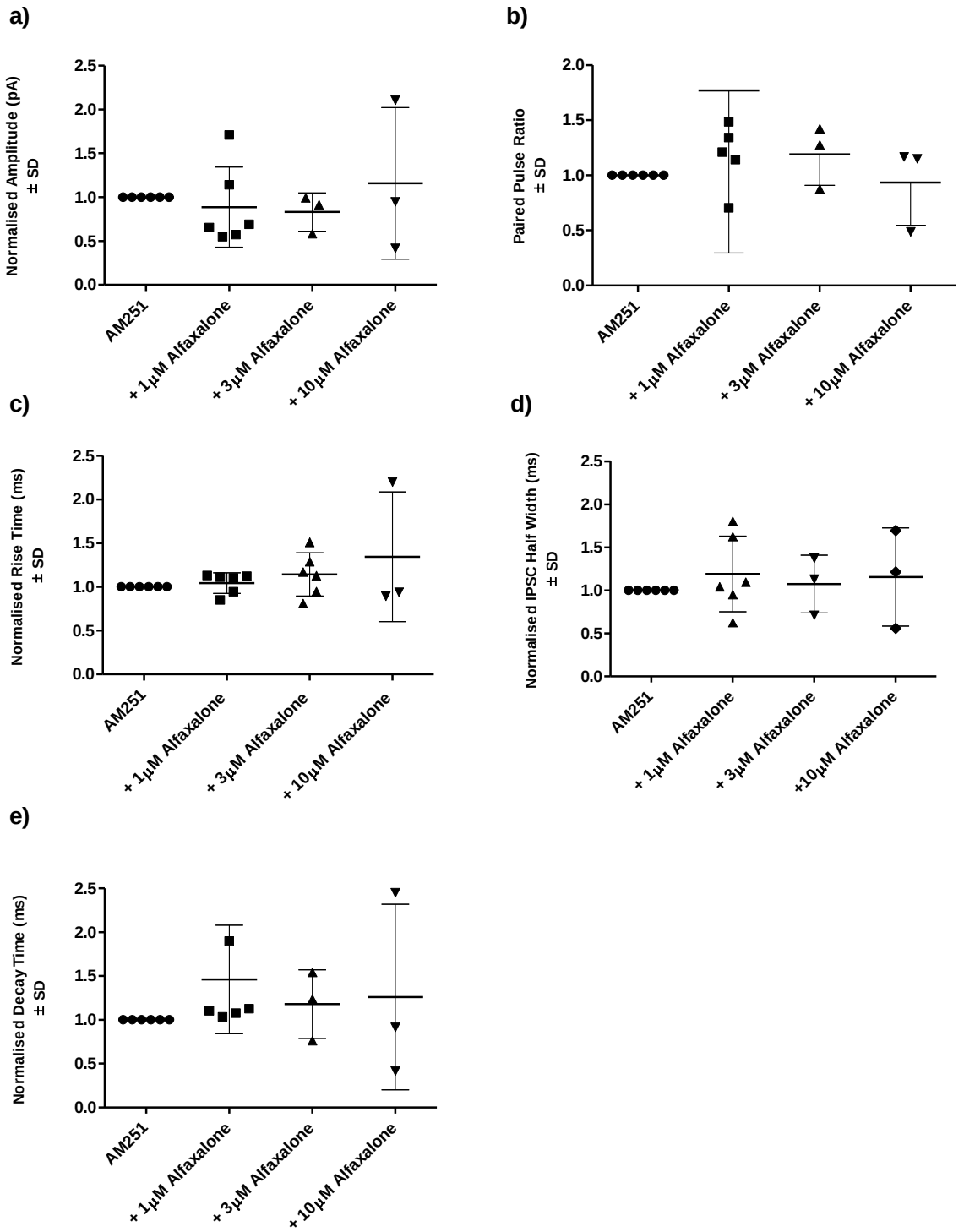


Figure 5.3.3. Evoked IPSC parameters for increasing micromolar concentrations of alfaxalone with pre-application of 1µM AM251

a) The pre-application of 1µM AM251 blocked the evoked IPSC amplitude reduction caused by micromolar concentrations of alfaxalone. b) PPR was unaltered by alfaxalone in the presence of AM251. c) The marked reduction in evoked IPSC rise time reduction seen from 1µM alfaxalone was abolished by prior application of 1 µM AM251. (d) Evoked IPSC half-width and e) IPSC decay time were not altered by micromolar alfaxalone after the addition of AM251 Significance was calculated from normalised data and all data is presented as mean ± SD.

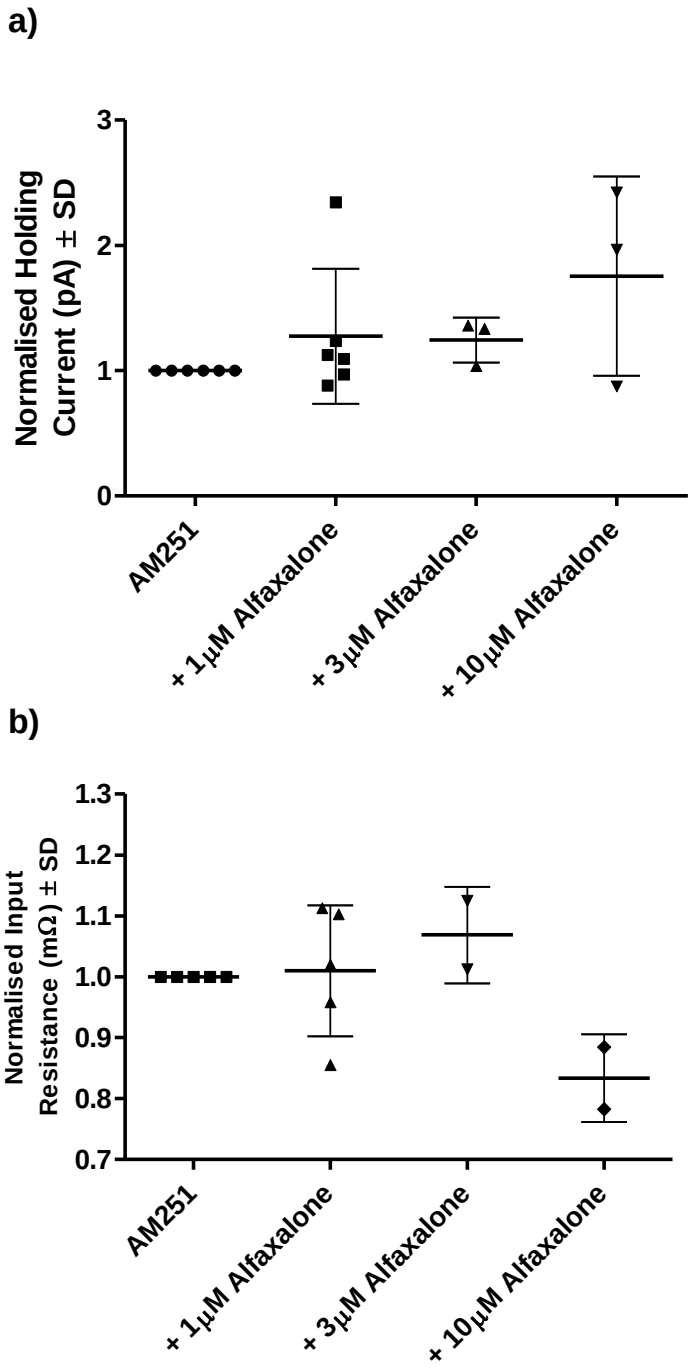


Figure 5.3.4. HMN holding current at -60 mV and R_n following micromolar concentrations of alfaxalone co-applied with 1µM AM251

a) Baseline holding current was altered by alfaxalone following pre-application of AM251 b) There was no significant change in R_n with micromolar alfaxalone in the presence of AM251; however there was a trend towards R_n reduction with 10µM alfaxalone

Spontaneous Parameters						
		Control	AM251	Alfaxalone		
			1μM	1μM	3μM	10μM
Amplitude (pA)	Mean	-53.3	-49.5	-53.2	-62.5	-55.4
	SD	23.2	16.1	22.9	25.6	25.7
	n	8	8	8	6	6
		ns	P <0.05	ns	ns	ns
Rise Time (ms)	Mean	4.9	4.6	2.7	2.7	3.8
	SD	2.7	2.3	1.0	1.0	1.5
	n	8	8	8	6	6
		ns	P <0.05	**	***	**
Half Width (ms)	Mean	4.9	5.1	5.0	5.2	5.3
	SD	1.7	2.	1.8	2.0	1.8
	n	8	8	8	6	6
		ns	P <0.05	ns	ns	ns
Interval Time	Mean	1.4	1.1	1.2	0.7	0.8
	SD	1.5	1.2	1.3	0.2	0.2
	n	8	8	8	6	6
		ns	P <0.05	ns	ns	ns
Evoked Parameters						
		Control	AM251	Alfaxalone		
			1μM	1μM	3μM	10μM
Amplitude (pA)	Mean	-97.9	-103.8	-93.9	-53.9	-72.7
	SD	19.9	48.2	61.2	14.6	54.9
	n	6	6	6	3	3
		ns	P <0.05	ns	ns	ns
Rise Time (ms)	Mean	3.1	3.1	3.3	4.8	6.2
	SD	1.6	1.4	1.1	1.6	3.5
	n	6	6	6	3	3
		ns	P <0.05	ns	ns	ns
Half Width (ms)	Mean	11.6	9.9	11.1	12.5	13.9
	SD	8.8	4.6	4.6	3.4	8.9
	n	6	6	6	3	3
		ns	P <0.05	ns	ns	ns
Decay Time (ms)	Mean	10.9	9.9	14.6	12.7	13.3
	SD	9.0	5.1	9.0	5.1	11.9
	n	6	6	6	3	3
		ns	P <0.05	ns	ns	ns
Paired Pulse Ratio	Mean	1.6	1.7	1.9	1.7	1.5
	SD	0.4	0.5	0.8	0.2	0.1
	n	6	6	6	3	3
		ns	P <0.05	ns	ns	ns
Baseline Current (pA)	Mean	-117.2	-118.6	-186.0	-61.2	-85.6
	SD	38.3	96.3	242.1	57.6	99.1
	n	6	6	6	3	3
		ns	P <0.05	ns	ns	ns
Input Resistance (mO)	Mean	183.5	202.9	205.1	332.6	254.6
	SD	92.0	103.3	103.2	103.8	39.7
	n	5	5	5	2	2
		ns	P <0.05	ns	ns	ns

Table 5.3. Spontaneous and evoked IPSC parameters for micromolar concentrations of alfaxalone in the presence of 1μM AM251

The effects of alfaxalone in the presence of CB_1R antagonist NESS0327 on glycinergic transmission

These data show that the presence of AM251 blocked effects of alfaxalone on glycinergic transmission and holding current. These effects strongly suggest that the reduction of glycinergic transmission by alfaxalone, are mediated by activation of CB_1Rs. However, it is possible that the inverse agonist action of AM251 may partially counteract effects of alfaxalone, complicating the interpretation of the effects of AM251. In this study, we used NESS0327, a potent competitive CB_1R without inverse agonist activity, to determine where its pre-application produced similar outcomes to that of AM251 on the actions of alfaxalone. The control aCSF contained APV (50µM), NBQX (10µM) and bicuculline methchloride (5µM). NESS0327 (100pM) was applied to determine the effects of NESS0327 alone, prior to addition of alfaxalone at micromolar concentrations.

NESS0327 alone did not affect spontaneous or evoked glycinergic transmission

Application of NESS0327 alone did not significantly alter spontaneous or evoked IPSC amplitude and shape. Mean spontaneous IPSC frequency was unaltered; however the interval between spontaneous IPSCs was more variable in the presence of NESS0327 (Table 5.4). The PPR of evoked IPSCs remained unchanged, and no effects on baseline holding current or input resistance were seen.

Alfaxalone did not alter spontaneous IPSC amplitude or frequency with NESS0327

In the presence of NESS0327, spontaneous IPSC amplitude was not significantly altered by alfaxalone up to 10µM. There was no significant change in spontaneous IPSC frequency following alfaxalone in the presence of NESS0327; however the interval range varied widely at all doses of alfaxalone. Spontaneous IPSC interval time ranged from 0.21 - 1.04 sec with NESS0327 alone, and consistently varying from 0.22 – 4.50 sec at 10µM alfaxalone in the presence of NESS0327 (Table 5.4).

Alfaxalone caused decreased spontaneous IPSC rise time in the presence of NESS0327

Spontaneous IPSC half-width remained altered with increasing doses of alfaxalone following application of NESS0327. However, a significant reduction in spontaneous IPSC rise time was observed (Figure 5.4.4); a marked reduction in rise time was noticed at 1µM alfaxalone with a decrease to 52.8 ± 14.5% of control rise time and this effect was apparent up to 10µM alfaxalone (Figure 5.4.1).

Evoked IPSC amplitude and shape were not altered by alfaxalone in the presence of NESS0327

Evoked IPSC amplitude was not altered by alfaxalone in the presence of NESS0327. An observed increase in IPSC amplitude variability was observed, with IPSC amplitudes ranging from -50.1 ± 375.5pA for NESS0327 alone, to -30.0 ± 334.3pA at 10µM alfaxalone after NESS0327. Similarly, no change in evoked IPSC shape was seen with alfaxalone in the presence of NESS0327. Evoked IPSC rise time, half width and decay time remained relatively constant with the addition of increasing micromolar concentrations of alfaxalone (Figure 5.4.2).

Alfaxalone did not affect PPR in the presence of NESS0327

No alterations in PPR were observed with micromolar alfaxalone in the presence of NESS0327 (Figure 5.4.2). This is consistent with neither NESS0327 nor alfaxalone altering the Ca^{2+} dependent presynaptic release of glycine.

Baseline holding current and input resistance was unchanged by alfaxalone

Baseline holding current was not significantly altered by alfaxalone up to 10µM in the presence of NESS0327 (Figure 5.4.3). R_n also remained unchanged up to 10µM alfaxalone (Figure 5.3.1)

NESS0327 mimicked the effects of AM251 in blocking the effects of alfaxalone

We predicted a similar blockade of the inhibitory effects of alfaxalone following NESS0327 application, as previously seen following AM251 application. These results indeed showed a close similarity in the actions of AM251 and NESS0327. Spontaneous IPSC frequency reduction caused by alfaxalone was blocked by both AM251 and NESS0327. Dose dependent evoked IPSC amplitude reductions caused by alfaxalone were blocked by both AM251 and NESS0327. Alfaxalone alone caused marked prolongation in both IPSC rise and decay times, an effect which the prior application of either NESS0327 or AM251 was able to block. In addition to this, pre-application of AM251 or NESS0327 revealed a reduction in spontaneous IPSC rise time with the application of alfaxalone. The presence of NESS0327 blocked the inward current observed with 10µM alfaxalone, as did AM251. Overall, the results show that CB_1R antagonism with NESS0327 blocked presynaptic and postsynaptic effects of alfaxalone in a manner identical to that of AM251. These results undoubtedly provide strong evidence for CB_1R activation by alfaxalone in causing suppression of glycinergic transmission.

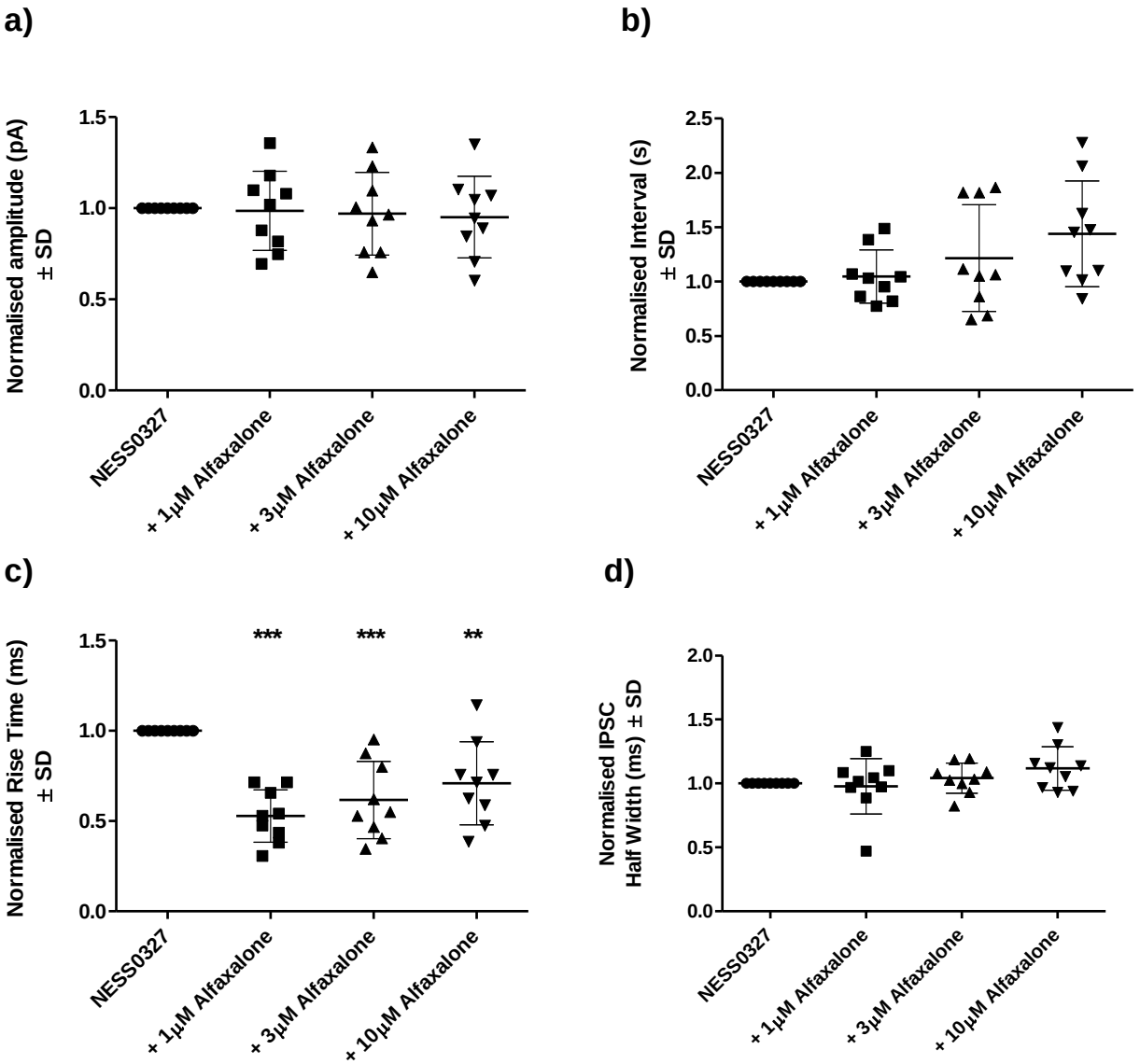


Figure 5.4.1. Spontaneous IPSC parameters for increasing concentrations of alfaxalone in the presence of 100pM NESS0327

a) Spontaneous IPSC amplitude were unaltered by alfaxalone following the addition of 100pM NESS0327. b) NESS0327 also blocked the reduction in spontaneous IPSC frequency caused by alfaxalone. c) A significant decrease in spontaneous IPSC rise time was observed with the addition alfaxalone in the presence of 100pM NESS0327, which was not presence with alfaxalone applied by itself d) Spontaneous IPSC half width was not altered by alfaxalone with NESS0327 present. All data is presented as mean ± SD. ** indicates a P value ≤ 0.01, *** indicates a P value ≤ 0.001

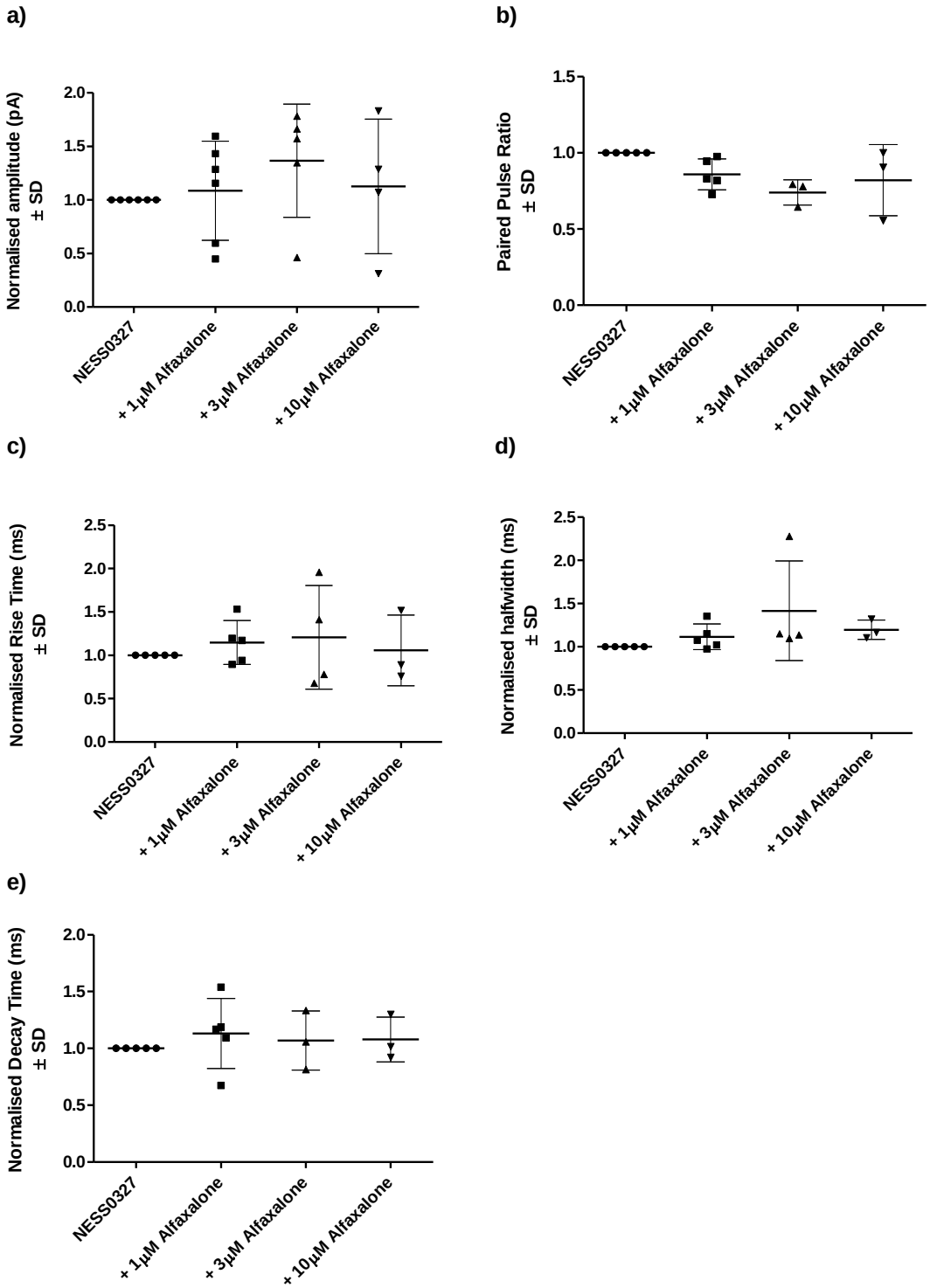


Figure 5.4.2. Evoked IPSC parameters were not altered by increasing concentrations of alfaxalone in the presence of 100pM NESS0327

a) The addition of 100pM NESS0327 blocked reduction in evoked IPSC amplitude caused by alfaxalone alone b) PPR was unaltered. c) The marked reduction in evoked IPSC rise time with micromolar alfaxalone was abolished by prior application of 100pM NESS0327. (d) Evoked IPSC half-width was unchanged by alfaxalone when NESS0327 was present. e) Evoked IPSC decay time prolongation caused by alfaxalone at 10µM was abolished by prior application of 100pM NESS0327. All data is presented as mean ± SD.

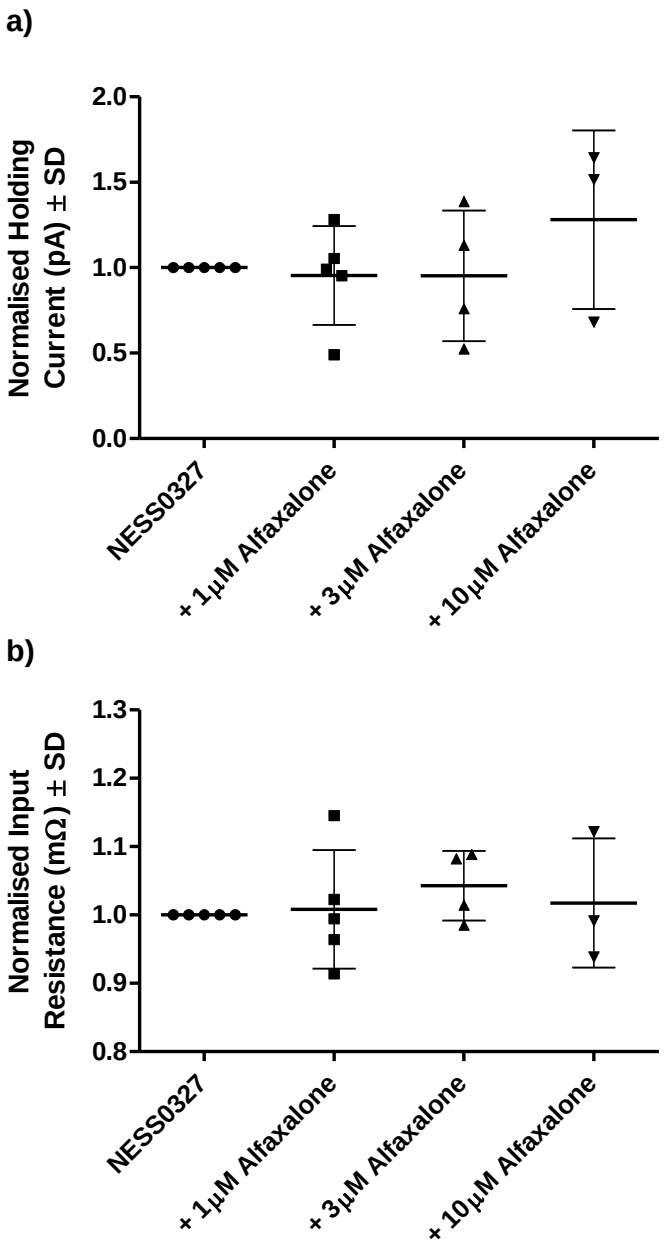


Figure 5.4.3. Baseline holding current and input resistance data for alfaxalone in the presence of NESS0327

a) Baseline holding current changes caused by alfaxalone alone was not apparent in the presence of NESS0327. b) Input resistance remained unaltered by micromolar alfaxalone in the presence of NESS0327. All data is presented as mean ± SD.

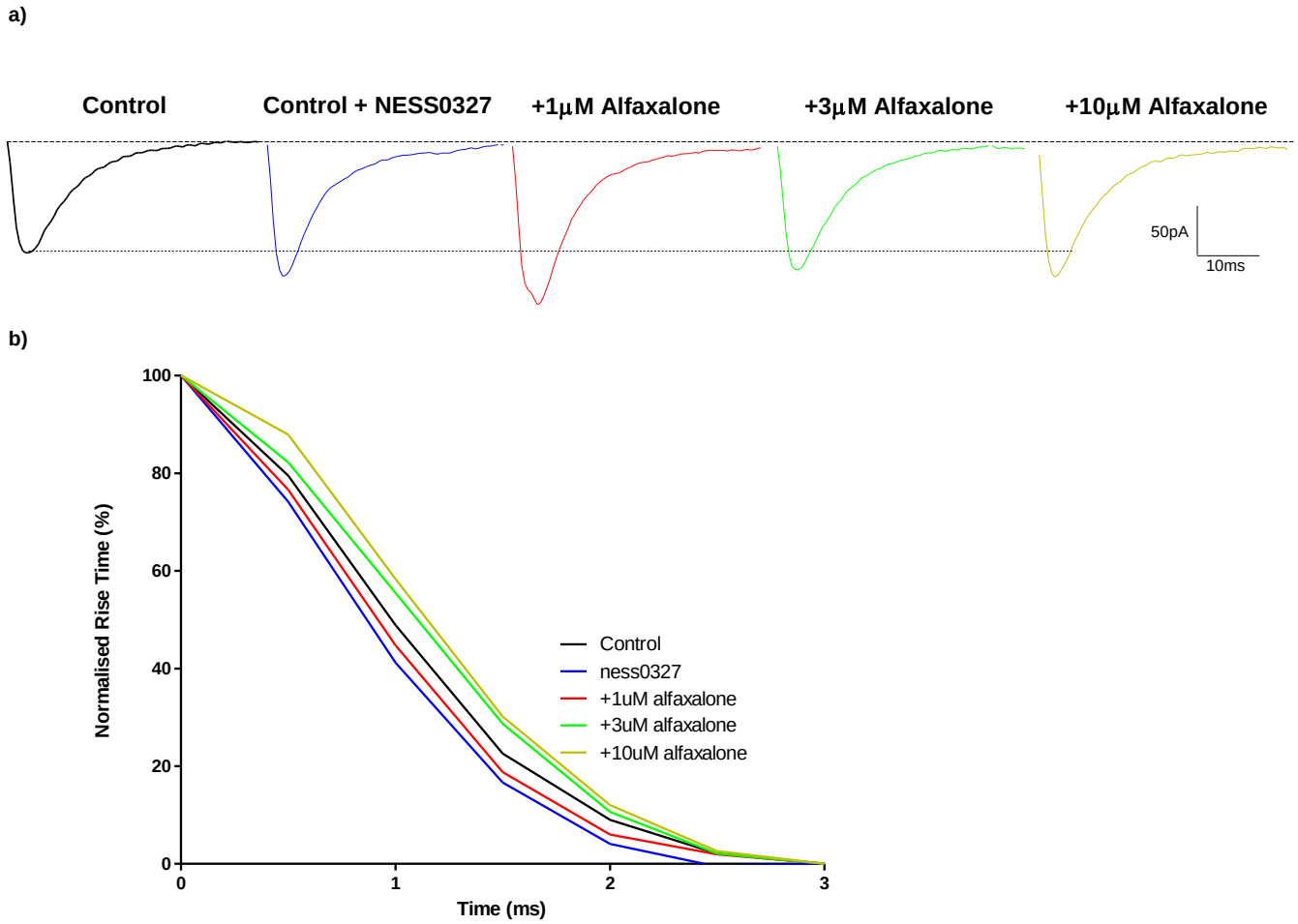


Figure 5.4.4. Representative traces of evoked IPSCs exposed to increasing alfaxalone concentrations in the presence of 100pM NESS0327

a) There were no significant alterations in evoked IPSCs parameters following alfaxalone application in the presence of NESS0327. Pre-application of 100pM NESS0327 blocked evoked IPSC amplitude reductions caused by alfaxalone. Evoked IPSC decay time prolongation, apparent with 10μM alfaxalone applied alone, were abolished by the pre-addition of NESS0327. b) Evoked IPSC rise time changes caused by alfaxalone were also removed with the pre-addition of NESS0327.

Spontaneous Parameters						
		Control	NESS0327	Alfaxalone		
			100pM	1μM	3μM	10μM
Amplitude (pA)	Mean	-51.97	-50.64	-49.66	-47.43	-47.27
	SD	16.88	14.26	15.8	11.38	14.87
	n	9	9	9	9	9
		ns	P <0.05	ns	ns	ns
Rise Time (ms)	Mean	4.544	4.433	2.211	2.567	3.022
	SD	1.363	1.45	0.454	0.691	1.041
	n	9	9	9	9	9
		ns	P <0.05	***	***	**
Half Width (ms)	Mean	4.578	4.333	4.367	4.556	4.833
	SD	1.818	1.396	1.623	1.519	1.525
	n	9	9	9	9	9
		ns	P <0.05	ns	ns	ns
Interval Time	Mean	0.4167	0.8503	0.8619	0.898	1.078
	SD	0.1696	1.22	1.167	1.07	1.321
	n	9	9	9	9	9
		ns	P <0.05	ns	ns	ns
Evoked Parameters						
		Control	NESS0327	Alfaxalone		
			100pM	1μM	3μM	10μM
Amplitude (pA)	Mean	-245.8	-232.4	-246.4	-228.3	-204.8
	SD	95.44	131.8	117.3	140.2	122.1
	n	5	5	5	3	3
		ns	P <0.05	ns	ns	ns
Rise Time (ms)	Mean	3.285	3.07	3.45	3.05	3.38
	SD	1.322	1.19	1.30	1.04	1.37
	n	5	5	5	3	3
		ns	P <0.05	ns	ns	ns
Half Width (ms)	Mean	8.057	7.41	8.18	9.67	8.96
	SD	1.577	1.15	1.00	2.52	1.06
	n	5	5	5	3	3
		ns	P <0.05	ns	ns	ns
Decay Time (ms)	Mean	6.947	5.88	6.41	6.59	6.70
	SD	1.954	1.10	1.00	0.47	0.13
	n	5	5	5	3	3
		ns	P <0.05	ns	ns	ns
Paired Pulse Ratio	Mean	1.488	1.525	1.292	1.153	1.239
	SD	0.1471	0.3098	0.1575	0.1633	0.08061
	n	5	5	5	3	3
		ns	P <0.05	ns	ns	ns
Baseline Current (pA)	Mean	-107.6	-159.4	-156.2	-141.8	-177.7
	SD	27.08	43.13	67.81	66.07	101
	n	5	5	5	3	3
		ns	P <0.05	ns	ns	ns
Input Resistance (mΩ)	Mean	119.4	112.1	112	121.6	107.6
	SD	39.67	28.66	25.04	32.27	21.97
	n	5	5	5	4	3
		ns	P <0.05	ns	ns	ns

Table 5.4. Spontaneous and evoked IPSC parameters for micromolar concentrations of alfaxalone in the presence of 100pM NESS0327

** indicates a P value ≤ 0.01, *** indicates a P value ≤ 0.001

Effects of alfaxalone on glycinergic transmission in the presence of a non-specific TRP Channel antagonist, 2-APB

We hypothesised that the inward current elicited by alfaxalone was due to TRP channel activation. We also hypothesised that the activation of TRP channels and the subsequent Ca^{2+} influx is the trigger for endocannabinoid synthesis and retrograde cannabinoid signalling. We therefore expect pre-application of the non-specific TRP channel blocker 2-APB to eliminate the inward current shift caused by alfaxalone alone. In addition, we expect a decrease in CB_1R activity due to blockade of endocannabinoid synthesis following 2-APB. Spontaneous IPSC and holding current recordings were performed with a control bath containing APV (50µM), NBQX (10µM) and bicuculline methchloride (5µM) and compared to recordings on addition of 2-APB (100pM).

2-APB did not affect spontaneous glycinergic transmission

The addition of 2-APB did not significantly affect spontaneous IPSC amplitude, shape or frequency. Holding current and input resistance was also unchanged by 2-APB application. This suggests that endogenous TRP channel activity is minimal in the control recording conditions (Table 5.5).

Alfaxalone did not affect spontaneous IPSC amplitude or frequency in the presence of 2-APB

Neither spontaneous IPSC amplitude nor frequency were significantly altered by alfaxalone in the presence of 2-APB. There was however a trend towards an increase in IPSC frequency up to 10µM alfaxalone (Table 5.5).

Alfaxalone caused spontaneous IPSC shape augmentations

1µM and 3µM but not 10µM alfaxalone caused a marked reduction in spontaneous IPSC rise time with 2-APB present (Table 5.5). Spontaneous IPSC half width or decay time however were unaffected by alfaxalone in the presence of 2-APB.

Alfaxalone did not alter baseline holding current in the presence of 2-APB

The baseline holding current was not significantly altered at up to 10µM alfaxalone with 2-APB present (Table 5.5). A large variation in baseline holding current was seen between cells, which ranged from -174.2pA to -814.1pA with 2-APB application alone and -144.5pA to -831.0pA with 3µM alfaxalone applied after 2-APB. At 10µM alfaxalone, a range from -180.4pA to -306.8pA was noted however these data represents n=2.

2-ABP blocked the effects of alfaxalone on glycinergic inhibition

Although only spontaneous IPSC activity was recorded, it was clear that the TRP channel blocker 2-APB blocked presynaptic and postsynaptic effects of alfaxalone on glycinergic transmission and holding current. 2-APB prevented the spontaneous IPSC frequency reduction caused by alfaxalone along, as well as abolishing spontaneous IPSC amplitude by alfaxalone. Interestingly, 2-APB also revealed a reduction in spontaneous IPSC rise time following alfaxalone application, a similar effect to that seen with pre-application of AM251 and NESS0327. Alfaxalone did not cause an inward change in baseline holding current in the presence of 2-APB; in contrast, a trend towards an outward current shift at 10µM alfaxalone was observed. Here we showed that a non-specific TRP channel blocker produced effects very similar to those seen after CB_1R blockade. These data suggests a signalling inter-relationship between TRP channels and CB_1R activity. These results are supportive of our hypothesis that alfaxalone modulates TRP channels to elicit an inward current that can then modulate the cannabinoid signalling pathway.

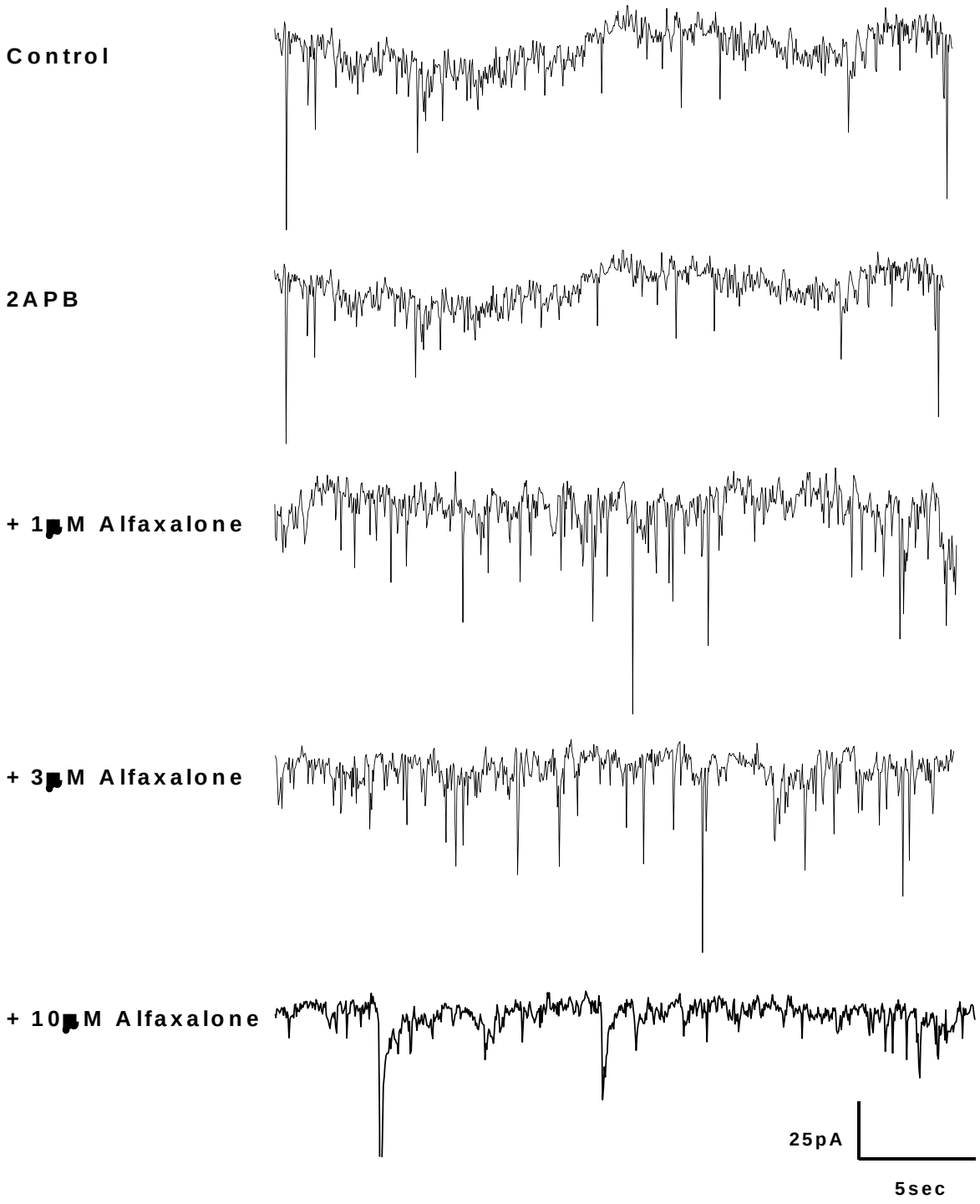


Figure 5.5.1. Representative spontaneous IPSC traces in the presence of 2-APB and in combination micromolar concentrations of alfaxalone
Ten-second traces of spontaneous IPSC recordings in rat hypoglossal motor neurons showed no significant changes in spontaneous IPSC peak amplitude, frequency, half width and decay time or baseline holding current with application of 2-APB alone, and prior to micromolar concentrations of alfaxalone. Note the variations in spontaneous IPSC peak amplitudes evident with all traces.

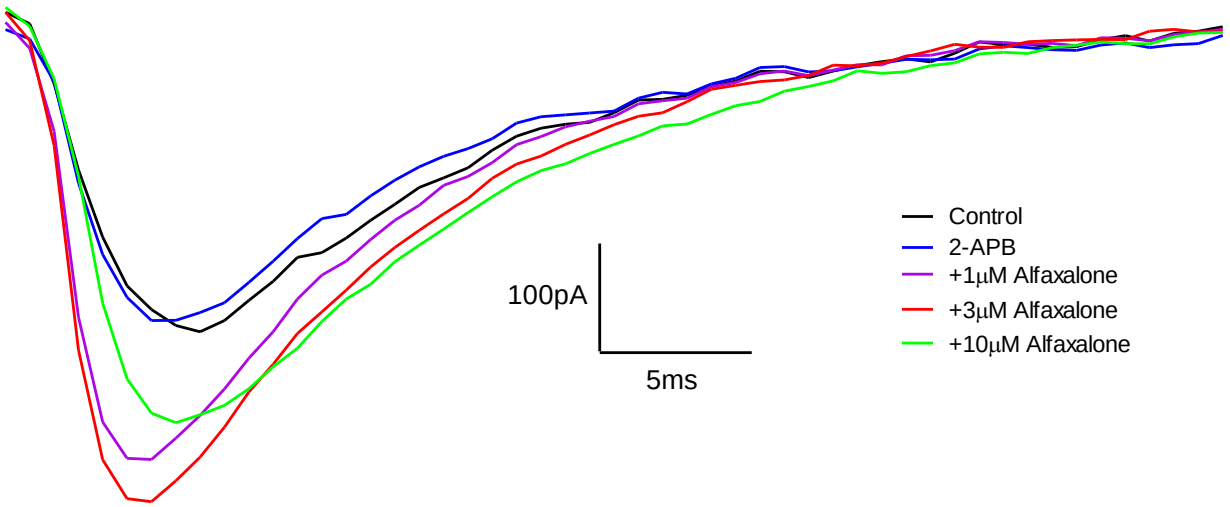


Figure 5.5.2. Representative average spontaneous IPSC trace during co-application of 2-APB and alfaxalone
Representative spontaneous IPSC traces depict a reduction in spontaneous IPSC rise time regardless of peak amplitude variations with administration of alfaxalone in the presence of 2-APB.

Spontaneous Parameters						
		Control	2-APB	Alfaxalone		
			100pM	1μM	3μM	10μM
Amplitude (pA)	Mean	-251.8	-276.7	-257.5	-273.5	-262.7
	SD	217.2	238.7	215.9	240.4	302.1
	n	4	4	4	4	2
		ns	P <0.05	ns	ns	ns
Rise Time (ms)	Mean	5.8	5.4	3.1	3.1	4.1
	SD	1.8	1.2	0.6	0.6	1.0
	n	4	4	4	4	2
		ns	P <0.05	***	**	ns
Half Width (ms)	Mean	5.4	5.3	5.6	5.8	6.6
	SD	1.5	0.5	0.8	1.2	0.8
	n	4	4	4	4	2
		ns	P <0.05	ns	ns	ns
Decay Time (ms)	Mean	13.6	13.5	13.8	14.0	13.8
	SD	1.3	1.9	0.9	1.8	1.5
	n	4	4	4	4	2
		ns	P <0.05	ns	ns	ns
Interval Time (sec)	Mean	1.6	2.4	3.2	2.5	0.4
	SD	0.9	3.3	5.5	4.2	0.2
	n	4	4	4	4	2
		Ns	P <0.05	ns	ns	ns
Holding Current (pA)	Mean	-444.0	-478.6	-408.1	-396.4	-245.6
	SD	325.8	334.1	296	303.2	86.55
	n	4	4	4	4	2
		ns	P <0.05	ns	ns	ns

Table 5.5. Spontaneous IPSC kinetics for micromolar concentrations of alfaxalone in the presence of 2-APB
** indicates a P value ≤ 0.01, *** indicates a P value ≤ 0.001

Discussion

W have here investigated the hypothesis that alfaxalone modulates glycinergic transmission to rat HMNs by activating the cannabinoid receptor signalling pathway. We tested the effects of alfaxalone on glycinergic transmission in rat HMNs in the presence of a potent CB_1R agonist WIN55,212-2, a CB_1R inverse agonist AM251, a potent CB_1R antagonist NESS0327 and a non-specific TRP channel antagonist 2-APB. First, we saw that the inhibitory effects of the CB_1R agonist WIN55,212-2 on glycinergic transmission were similar to those caused by alfaxalone, which was suggestive of CB_1R activation by alfaxalone; WIN55,212-2 also partly occluded the effects of alfaxalone. We then saw that antagonism of the CB_1R by either AM251 or NESS0327 blocked the inhibitory effects of alfaxalone on glycinergic transmission. These results showed that alfaxalone indeed causes activation of the CB_1R, thereby reducing glycinergic transmission. However, we also saw that CB_1R antagonism blocked some of the postsynaptic effects of alfaxalone, including evoked IPSC amplitude reduction without changes in PPR and changes in IPSC shape parameters. Additionally, we showed that both CB_1R blockers abolished the inward shift in holding current elicited by alfaxalone. Finally, the addition of 2-APB blocked not only the inward shift in holding current elicited by alfaxalone, but also blocked the reduction in spontaneous IPSC amplitude and frequency caused by and decreased spontaneous IPSC rise time. As 2-APB has no direct effects on CB_1Rs, we interpret this data as showing that alfaxalone directly modulates TRP channels to produce an inward current which triggers the endocannabinoid signalling pathway to ultimately activate CB_1Rs which reduce glycinergic transmission to HMNs. Our results are consistently supportive of this hypothesis.

WIN55,212-2, a CB_1R agonist, potently suppressed glycinergic transmission to HMNs

Previous studies have shown that endocannabinoids generated within HMNs by strong depolarization can reduce glycinergic transmission to HMNs by activating presynaptic CB_1Rs (Mukhtarov et al., 2005). Key evidence for this mechanism included a change in PPR and blockade of reduction of glycinergic IPSCs by application of WIN55,212-2. We therefore expected to see a depression of glycinergic transmission with the application of the CB_1R agonist WIN55,212-2. The results from the WIN55,212-2 dose response study were consistent with this expected presynaptic depression in some aspects but not in others. The decrease in spontaneous IPSC amplitude and frequency seen on addition of WIN55,212-2 are consistent with a decrease in glycine release from the presynaptic terminal, but could also be due to a reduction in the spontaneous activity of presynaptic neurons.. However, the PPR of evoked IPSCs was not altered by WIN55,212-2, which suggests that the CB_1Rs activated by WIN55,212-2 are not presynaptic. In contrast to alfaxalone alone, the application of WIN55,212-2 did not produce postsynaptic effects on glycinergic IPSCs caused by alfaxalone, as WIN55,212-2 did not cause increases in either IPSC rise time and decay time prolongation. This suggests that these

effects of alfaxalone may not be mediated by CB_1Rs. Finally, the marked suppression of both spontaneous and evoked IPSC amplitude was evident at low nanomolar concentrations of WIN55,212-2, with a calculated EC_{50} of 18 nM. This value is similar to that for binding to CB_1Rs, suggesting that off target effects on other receptors or channels do not produce the effects of WIN55,212-2 (Pertwee et al., 2010).

WIN55,212-2 markedly reduced effects of alfaxalone on glycinergic transmission to HMNs

As WIN55,212-2 by itself induced a reduction in IPSC amplitude and frequency similar to that of alfaxalone alone, suggesting that both drugs act through a common mechanism to cause inhibition of glycinergic transmission. A test of this hypothesis is to show that WIN55,212-2 occludes the effects of alfaxalone at lower doses of alfaxalone. We therefore performed an alfaxalone dose response study in the presence of WIN55,212-2 at 300 nM to largely saturate CB_1R binding. Under these conditions, effects of alfaxalone on glycinergic transmission were not seen at until 3µM alfaxalone. As alfaxalone applied alone caused inhibition of glycinergic IPSCs at nanomolar concentrations, these results suggest that the pre-application of WIN55,212-2 occupied CB_1R and prevented the actions of alfaxalone until sufficiently high concentrations of alfaxalone were achieved.

Both a CB_1R inverse agonist and a CB_1R antagonist had little effect on glycinergic transmission to HMNs indicating a lack of endocannabinoid modulation.

Application of either 1µM AM251 or 100pM NESS0327 alone caused no significant changes in spontaneous or evoked IPSC amplitude and shape. These results suggest that endogenous activation of CB_1R do not contribute significantly to modulation of glycinergic activity in the rat HMN in control conditions. As an inverse agonist, we expected AM251 would increase spontaneous IPSC frequency and IPSC amplitude, by reducing constitutive CB_1R activity. We observed only a slight and insignificant increase in IPSC frequency following AM251 application compared to control. This suggests that little constitutive CB_1Rs activity is present under our experimental conditions. As NESS0327 is a neutral competitive antagonist, we did not expect any change in IPSC activity in the absence of an agonist.

CB_1R antagonism markedly reduced the effects of alfaxalone on glycinergic transmission to HMNs

We predicted that both AM251 and NESS0327 would block the effects of alfaxalone on glycinergic inhibition. Pre-application of AM251 or NESS0327 indeed blocked the reduction in spontaneous IPSC frequency, and the reduction in amplitude of both spontaneous and evoked IPSC amplitude, therefore strongly implicating CB_1R activation as a mechanism for alfaxalone induced reduction of glycinergic transmission. Interestingly, when CB_1Rs were blocked by these antagonists, the

application of alfaxalone caused reductions in IPSC rise time and decay time; this was in marked contrast to the effect of alfaxalone applied alone, which caused increased IPSC rise time and decay time. It has been reported that an increase in postsynaptic Ca^{2+} not only can lead to endocannabinoid synthesis, but also direct modulation of the GlyR seen as increased IPSC amplitude and prolongation of decay time (Mukhtarov et al., 2005). In addition, it was reported that the endocannabinoid 2-AG caused a reduction in rise time in CB_1R knockout mice, suggesting that endocannabinoids also possessed direct GlyR modulatory effects (Lozovaya et al., 2011). In this case, the activation of endocannabinoid synthesis by alfaxalone could lead to direct endocannabinoid or Ca^{2+} modulation of the GlyR accounting for these differential effects on IPSC shape parameters when CB_1Rs are blocked. Further experiments with intracellular Ca^{2+} chelators or modulators of endocannabinoid synthesis are needed to unravel these possible mechanisms.

CB_1R antagonists reduced the inward current activated by alfaxalone

Alfaxalone applied alone consistently elicited a depolarising inward current, which was largely absent when alfaxalone was applied in the presence of the CB_1R blockers AM251 or NESS0327. Interestingly, an inward current shift was not seen in the WIN55,212-2 dose response study, but was elicited by a high concentration (10 μM) of alfaxalone in the presence of WIN55,212-2. These findings show that blockade of CB_1Rs prevented an inward current caused by alfaxalone. However, the CB_1R agonist WIN55,212-2 alone did not elicit the inward current seen with alfaxalone. One potential explanation is that postsynaptic CB_1Rs are rapidly internalized following the binding of WIN55,212-2 (Hsieh et al., 1999), while internalization is much slower or absent with endogenous endocannabinoids. This suggests that WIN55,212-2 alone does not evoke an inward current but CB_1R antagonists can block an inward current generated by endogenous endocannabinoids acting via CB_1Rs. Evidence for the existence of postsynaptic CB_1Rs have been reported. CB_1R radioligand binding shows sparse binding in the rat hypoglossal nucleus and spinal cord ventral horn, while immunohistochemical studies identified postsynaptic CB_1Rs in the rat lamina II islet cells of the spinal cord (Salio et al., 2002) and in the dendrites of large ventral horn neurones of monkeys (Ong and Mackie, 1999). Our results here also suggest the existence of postsynaptic CB_1Rs in the rat brainstem, but further investigation is necessary before these claims can be substantiated.

2-APB, a non-specific TRP channel blocker, markedly reduced the inward current activated by alfaxalone

The inward current elicited by alfaxalone may contribute to motor neuron depolarization and thus neuro-excitation; in addition, it may play a permissive role in generating endogenous endocannabinoids which typically require a depolarization-mediated rise in intracellular Ca^{2+} for their synthesis and release. We hypothesised that the inward current shift caused by alfaxalone was due to activation of TRP channels, which are Ca^{2+} permeable cationic channels modulated by a wide variety of stimuli, including neurosteroids, endogenous endocannabinoids and G-protein coupled receptors. To test this hypothesis, we predicted that non-specific blockade of TRP channel activity would remove this inward current shift, hindering endocannabinoid production and therefore all the downstream effects of the cannabinoid signalling pathway. 2-APB is a small molecule that inhibits most TRP channels, although it activates TRPV channels. The application of 100pM 2-APB alone did not alter spontaneous IPSC event kinetics or change holding current. This indicated that there was no significant endogenous TRP channel activity present on rat HMNs in the absence of alfaxalone.

Alfaxalone applied after 2-APB elicited a marked decrease in spontaneous IPSC rise time, consistent the effect of alfaxalone applied after CB_1R antagonists. Other effects of alfaxalone on holding current, spontaneous IPSC amplitude, frequency and shape parameters were blocked by prior application of 2-APB, consistent with the results seen with the application of alfaxalone in the presence of CB_1R antagonists. As 2-APB does not block CB_1Rs, this data strongly suggests that TRP channel activation is the upstream mechanism for endocannabinoid production and subsequent CB_1R acttivation.

Neurosteroids themselves have also been implicated in direct activation of TRP channels, in particular the TRPM3 channel (Majeed et al., 2010, Nilius and Voets, 2008). The neurosteroid pregnenolone sulphate rapidly activated TRPM3 channels in pancreatic β cells, indicative of direct neurosteroid binding to TRPM3 (Nilius and Voets, 2008, Wagner et al., 2008, Majeed et al., 2010, Majeed et al., 2012). It was also reported that stimulation of TRPC6 channels in cath.a differentiated (CAD) neuronal cells increased 2-AG release, which suggests that TRPC6 channels could activate endocannabinoid synthesis (Bardell and Barker, 2010). From these findings, it could be postulated that the neurosteroid alfaxalone could also potentially directly bind to and modulate TRP channels thereby increasing intracellular Ca^{2+} and facilitating endocannabinoid synthesis.

More recently, there have been also several reports of interactions between endocannabinoids, CB_1Rs and TRP channels. The co-expression of TRPV1 channels and CB_1Rs has been reported in the

midbrain dorsal periaqueductal gray neurons (Casarotto et al., 2012). This study described panic responses produced by the TRPV1 antagonists capsazepine and SB366791, which were prevented by AM251, suggesting that the endocannabinoid, anandamide may exert effects via actions at both CB_1R and TRPV1 (Casarotto et al., 2012). In addition, the cannabinoid cannabichromene (CBC), naturally derived from the *Cannabis sativa* plant, is known to inhibit endocannabinoid reuptake and to activate TRP ankyrin 1-type (TRPA1) channels (Romano et al., 2013).

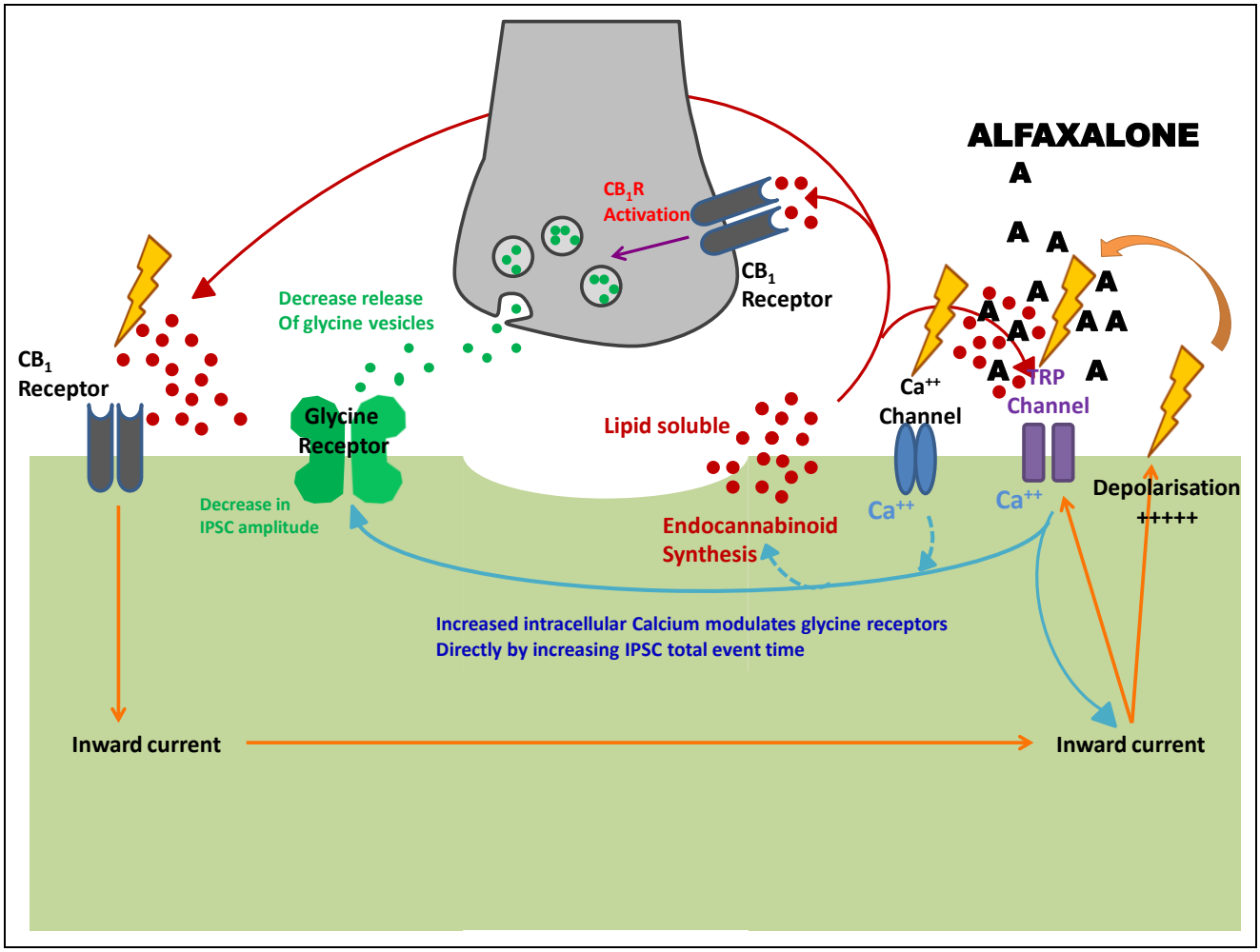


Figure 5.6. Stylistic view of the proposed mechanism of alfaxalone action on HMN synaptic terminal.
Alfaxalone activates TRP channels which results in an inward current created by an influx of cations, resulting in a increase in intracellular Ca^{2+} in the postsynaptic neuron. This increase in intracellular Ca^{2+} triggers endocannabinoid synthesis. Endocannabinoids are retrograde messengers decrease neurotransmitter release by binding to presynaptic G-protein-coupled cannabinoid receptors (CB_1R) and in turn decreases IPSC amplitude and frequency. CB1Rs located on the postsynaptic membrane may also interact with the GlyRs to change IPSC amplitude and shape parameters. Modulation of the GlyR from the Ca^{2+} influx or via endocannabinoids causes prolongation of total IPSC event times.

Results of this study support our hypothesis that alfaxalone activates the endocannabinoid signalling pathway in HMNs and produces an inward current through modulation of TRP channels. We also have raised the possibility of postsynaptic CB_1R localisation, as our results provided evidence that alfaxalone produced both presynaptic and postsynaptic GlyR modulation as well as causing a depolarising current. The resultant changes involve a reduction in glycinergic inhibitory transmission and increased neuronal excitation, and would clinically manifest as increased muscle tone, twitchiness and neuro-excitation. These effects could underlie the mechanism behind the neuro-excitation seen with alfaxalone anaesthesia. We reported in this study that antagonism of the CB_1R receptor or of TRP channels blocked alfaxalone-induced reductions in glycinergic inhibition and development of a depolarising current. This exciting finding shows the potential for developing a combination anaesthetic regimen, comprising of alfaxalone with a CB_1R antagonist or TRP channel blocker, which could reduce or prevent the prevalence of alfaxalone neuro-excitation and markedly improve alfaxalone anaesthesia for laboratory rodents and other species.

GENERAL DISCUSSION

Rodent anaesthesia is performed routinely for husbandry and research purposes and the use of rodents has helped pave the way for new advancements in biomedical research. However, it appears the development of improved anaesthesia has been left behind. Anaesthetics such as ketamine-xylazine combination, were first administered over 30 years ago and are still commonly used today (Stickrod, 1979, Green et al., 1981, Saha et al., 2007). One would assume that this continued use of an anaesthetic combination means that it provides safe and predictable anaesthesia but this is not the case. This lack of improvement in the safety and efficacy of anaesthetic regimens indicates no better anaesthetic agents have been investigated and a review of current anaesthetic protocols is necessary (Green et al., 1981).

Need for a new anaesthetic protocol for laboratory rodents

The rodent anaesthetic survey we conducted (Chapter 2) showed that only a limited number of anaesthetic agent combinations appear to be utilised for rodent anaesthesia, each associated with complications. Unpredictable levels of anaesthesia, hypothermia, apnoea and prolonged recovery times were repeatedly mentioned in the survey and highlighted the need for improved anaesthetic agents. The rodent anaesthetic survey identified several complications associated with ketamine-xylazine, including persistent reflexes, prolonged recovery and high mortality rates (Smith, 1993, Wixson et al., 1987c, Wixson et al., 1987a, Arras et al., 2001). The shift towards increased use of inhalation anaesthesia has brought with it many benefits, in that supplementary oxygen is co-administered, but also it has brought with it a false sense of security. Inhalation agents are delivered in conjunction with 100% oxygen but often rodents are not intubated and the amount of anaesthetic delivered is dependent on the animal spontaneously breathing (Flecknell, 1993a). Alfaxalone provides a high margin of safety for veterinary anaesthesia, so it was prudent to determine whether alfaxalone was an effective anaesthetic agent for rodent anaesthesia.

Alfaxalone pharmacokinetics for rats

An IV and IP pharmacokinetic study was performed using the commercially available HPCD formulation of alfaxalone, Alfaxan® in rats. The mean elimination half-life for alfaxalone was short at 16.2 and 17.6 minutes for 2mg.kg^{-1} and 5mg.kg^{-1} respectively. The rat alfaxalone Cl_p was fast at 54.3 ± 6.8mL.min^{-1}kg^{-1}, which is double the speed of clearance in the cat, at 25.1 ± 7.6mL.min^{-1}kg^{-1} for the same IV dose (Whittem et al., 2008). In a clinical setting, this would mean that a constant infusion of alfaxalone needs to be given if IV alfaxalone anaesthesia is used in rats. Fortunately, IP

pharmacokinetics showed that sustained plasma levels were maintained up to 60 minutes after dosing with 20mg.kg^{-1} alfaxalone, although 30% of rats did not become induced for anaesthesia.

Singly applied alfaxalone does not provide optimal anaesthesia

The IM dose most predictably produced a loss of righting reflex and anaesthetic induction. Unfortunately, the large injection volume necessary to produce this effect precluded the use of alfaxalone via this route. SC administration was the least predictable route of administration for alfaxalone producing highly variable induction times at 20mg.kg^{-1}. On top of this, all anaesthetic routes examined produced neuromuscular twitching. This neuro-excitatory event was pronounced and in itself could potentially rule out the use of alfaxalone anaesthesia in rodents. Following on from these findings, it was clear that alfaxalone could not be used as an anaesthetic agent alone in rats and the administration of alfaxalone via different routes confirmed this finding.

The addition of premedication agent combinations with alfaxalone anaesthesia

It was then hypothesised that the use of added premedication agents would increase anaesthetic efficacy of alfaxalone and reduce the incidence and severity of twitching. Anaesthetic immobilisation times and surgical anaesthetic times were markedly increased with the use of medetomidine and medetomidine-butorphanol premedication agents. Acetylpromazine-xylazine, medetomidine or medetomidine-butorphanol premedications reduced the incidence of twitching by at least 50% and when twitching occurred, a marked reduction in the severity of twitching was observed. Acetylpromazine-methadone premedication, on the other hand was unable to produce these effects and even appeared to increase the incidence of twitching. These results showed that the presence of an α_2-adrenergic agonist drug suppressed the neuro-excitatory effects of alfaxalone but not in all rats. Specifically, medetomidine is a potent presynaptic and postsynaptic α_2-adrenergic agonist that decreases the release of noradrenaline, dopamine and serotonin in a dose dependent manner (Virtanen, 1989). Interestingly, it has been reported that alfaxalone can also suppress norepinephrine uptake by interfering with desipramine binding and norepinephrine recognition in cultured adrenal medullary cells (Horishita et al., 2002). The fact that alfaxalone also interferes with noradrenaline depression is suggestive that a reduction in noradrenaline by medetomidine will aid in the sedative effects of alfaxalone but it is unlikely to eliminate neuro-excitation, as alfaxalone itself already possesses adrenergic activity.

Premedication agents reduced the incidence and severity of twitching

The use of premedication to reduce the muscular twitching caused by alfaxalone showed promising results. However, it became clear that the potential mechanisms behind the alfaxalone induced neuro-excitation needed to be established, in order to identify possible targets for premedication.

The anaesthetic actions of alfaxalone have been attributed to its positive allosteric modulation of the $GABA_AR$ (Lambert et al., 1996, Lambert et al., 1995, Harrison and Simmonds, 1984). The GlyR also plays an important role in fast inhibitory neurotransmission and is important for coordination of reflex responses and muscle tone regulation (Mukhtarov et al., 2005, Maksay et al., 2002, Biro and Maksay, 2004). The GlyR is a ligand-gated chloride channel that causes postsynaptic hyper-polarisation which mediates synaptic inhibition in the brainstem and spinal cord (Zhou et al., 2002). The impairment of postsynaptic α_1 GlyR activity and reduction in the efficacy and potency of glycine has clinically manifested as hyperekplexia (Maksay et al., 2008). The enhancement of GlyR activity has the potential to suppress nociceptive signals and many studies have focussed on whether alfaxalone possesses this property (Ahrens et al., 2008, Betz and Laube, 2006, Laube et al., 2002). However, previous studies have shown that the ability of alfaxalone to potentiate GlyR activity was minimal and the concentration of alfaxalone necessary to potentiate α_1 GlyR in HEK 293 cells was outside the clinically relevant dose ranges (Ahrens et al., 2008). By contrast, the inhibition of α_1 glycinergic transmission causes exaggerated startle responses, as seen with hyperekplexia and strychnine poisoning (Zhou et al., 2002) and we hypothesised alfaxalone might produce similar suppressive effects on glycinergic transmission.

Alfaxalone inhibits glycinergic transmission at clinically relevant doses

To investigate this in the context of motor output, we chose to make electrophysiological recordings from HMNs, a group of brainstem motor neurons which innervate the tongue muscles. HMN are easily identified by their location and size, and the HMN pool contains the highest GlyR density of any area within the CNS (Singer and Berger, 2000, Singer and Berger, 1999). We therefore focused on this area for electrophysiological recordings. We investigated the possible inhibitory activity of alfaxalone on IPSCs mediated by GlyRs, by performing an alfaxalone dose response study. Alfaxalone caused a dose dependent decrease in IPSC size and caused IPSC rise time and decay time prolongation at 10µM. IPSC peak amplitude changes were noticeable from 30nM alfaxalone and a reduction in IPSC frequency at 3µM alfaxalone. These findings were suggestive of both presynaptic and postsynaptic modulation of GlyR activity by alfaxalone. Rat plasma alfaxalone levels calculated from the IV pharmacokinetic study showed the minimum effective plasma concentration of alfaxalone necessary to maintain sleep was between 4.36 and 6.02µM. As most of the twitching was experienced during the anaesthetic recovery period, we would expect the suppression of GlyR activity

to occur around these low micromolar concentrations. These data confirms that alfaxalone could indeed cause GlyR inhibition at clinically relevant doses in rats.

The alfaxalone dose response study found alfaxalone reduced GlyR inhibition through both presynaptic and postsynaptic mechanisms, and also produced a depolarising current without significantly altering the input resistance of HMNs. It is unclear, however, how alfaxalone mediates these changes, and there is the potential for a multitude of mechanisms being involved in producing these inhibitory effects on GlyR neurotransmission. Intracellular Ca^{2+} plays an important role in glycinergic synaptic transmission and Ca^{2+} modulation in the presynaptic nerve terminals releasing glycine has been linked to presynaptic CB_1R activity (Mukhtarov et al., 2005). Presynaptic CB_1Rs are said to reduce Ca^{2+} transients and thereby reduce glycine vesicular release. We therefore proposed that alfaxalone activated CB_1R through endocannabinoid synthesis within HMNs, which then led to CB_1R activation. Due to the inward current changes caused by alfaxalone, we also proposed that alfaxalone activated TRP channel receptors that, when activated, produce a depolarising current and increases in intracellular Ca^{2+}. This increase in intracellular Ca^{2+} in turn triggers the production of endocannabinoids and activates the cannabinoid signalling pathway.

WIN55,212-2 shifted alfaxalone induced glycinergic inhibition to higher concentrations of alfaxalone

The first experiments to help us determine whether these hypothesises are true involved the application of WIN55,212-2 , a potent CB_1R agonist. If alfaxalone causes inhibition of glycinergic transmission through activation of the CB_1R, then we would expect the addition of WIN55,212-2 to mimick the effects of alfaxalone. WIN55,212-2 indeed produced inhibition of glycinergic transmission similar to that caused by alfaxalone, which was supportive of our claims of CB_1R activation by alfaxalone. Following on from this, an alfaxalone dose response study was performed in the presence of WIN55,212-2. Pre-saturation of the CB_1R with 300nM of WIN55,212-2 should cause activation of CB_1R, and therefore we expected to see a reduction in the inhibitory effects of alfaxalone on glycinergic transmission. Results of this study indeed showed a shift in the development of alfaxalone induced glycinergic inhibition to higher concentrations of alfaxalone.

Alfaxalone causes CB_1R activation

WIN55,212-2 alone mimicked the effects of alfaxalone and caused reduction of glycinergic inhibition. We therefore also expected a CB_1R antagonist would block reduction of glycinergic IPSCs caused by alfaxalone. Antagonism of the CB_1R by either AM251 or NESS0327 blocked the inhibitory effects of alfaxalone on glycinergic transmission. These results showed that alfaxalone indeed causes activation of the CB_1R, thereby reducing glycine release from the presynaptic terminal.

However, we noted that neither CB_1R modulation nor alfaxalone caused a change in PPR, which is not consistent with a presynaptic change in evoked release. In addition, we also saw that CB_1R antagonism blocked some of the postsynaptic effects of alfaxalone, such as evoked IPSC size reduction and changes in IPSC shape.

Alfaxalone produces an inward current

The alfaxalone dose response study identified a significant inward current shift at 10µM or greater. This depolarising current was elicited by WIN55,212-2 up to 3µM in the dose response study, but was seen on co-application of 10µM alfaxalone in the presence of WIN55,212-2. We showed that both CB_1R blockers prevented the depolarising current changes seen with alfaxalone application. Furthermore, the addition of 2-APB, a non-specific blocker of many TRP channels, prevented not only the depolarising current shift caused by alfaxalone, but also blocked the presynaptic reduction in spontaneous IPSC frequency and decreased spontaneous IPSC rise time.

These results suggest that the inward current elicited by alfaxalone does not directly involve the CB_1R. Early studies reported the application of alfaxalone likely activated several other channels, simultaneous with $GABA_A$Rs. An inward current was observed during voltage clamp experiments associated with increases in membrane noise in bovine chromaffin cells (Cottrell et al., 1987). However, on pre-application of AM251 and NESS0327, we noticed no such depolarisation effect up to 10µM alfaxalone. This suggested, while direct activation of CB_1Rs did not elicit an inward current, that the blockade of CB_1Rs removed the inward current, and CB_1Rs therefore somehow involved. There is strong evidence that CB_1Rs are functionally associated with TRP channels (Moran et al., 2004). We postulated that the alfaxalone-activated inward current was due to activation of postsynaptically located TRP channels which might be modulated by CB_1Rs.

Alfaxalone activates TRP channels

TRP channels are a large family of stimulus-activated cationic ion channel proteins expressed in low numbers per cell, but which yield small net inward currents (Clapham et al., 2001) with a Ca^{2+} ion component. For this reason, an additional study was performed to determine whether these TRP channels were involved in the production of the alfaxalone induced inward current. The non-specific TRP channel blocker 2-APB was applied to the control bath and did not alter control IPSC parameters. Spontaneous IPSC recordings showed no change in baseline holding current up to 10µM alfaxalone. The blockade of TRP channels also blocked spontaneous IPSC amplitude reduction as well as changes in rise time and decay time of glycinergic IPSCs. These responses to TRP channel blockage were consistent with the results seen with CB_1R blockade by application of both AM251 or

NESS0327. These results are strongly supportive of TRP channel activation by alfaxalone, which then leads to activation of the cannabinoid signalling pathway, activation of CB_1Rs and reduction of glycinergic transmission through pre- and postsynaptic mechanisms.

TRP channel activation

TRP channels are a large and diverse family of ion channels, whose pharmacology is still poorly developed. The determination of which specific TRP channels are likely to be involved in alfaxalone-induced changes in HMNs is beyond the scope of this thesis, but would likely yield invaluable insights. 2-APB will inhibit members of the TRPC, TRPM and TRPA sub-families, but has been reported to selectively activate several members of the TRPV sub-family, including TRPV1-3, but not TRPV4-6. We tentatively conclude that it is less likely for TRPV channels to cause the inward current elicited by alfaxalone. There is evidence that neurosteroids can directly activate TRP channels; for example, direct activation of TRPM3 by the endogenous neurosteroid pregnenalone sulphate has been reported in pancreatic β cells (Wagner and Hellyer, 2000, Nilius and Voets, 2008, Clapham, 2007). Additionally, almost all TRP channels are permeable to Ca^{2+}, causing elevation in intracellular Ca^{2+}, which in turn modulates TRP channel activity (Bardell and Barker, 2010, Wu et al., 2010, Pedersen et al., 2005). Endocannabinoids also modulate many TRP channels, particularly the TRPV sub-family. Activation of TRPV1 by the endocannabinoid anandamide showed the possibility of off target activation of TRV1 channels in the brain, and these actions could precipitate anxiogenic-like behavioural responses produced by cannabinergic compounds (McLaughlin and Gobbi, 2012, Saito et al., 2010). There is also evidence suggesting that TRPV4 channels, which are not activated by 2-APB, can be activated by anandamide and arachidonic acid via a complex intracellular signalling pathway (Watanabe et al., 2003).There are thus several possible candidates for further study in the future.

The production of an inward current by alfaxalone in the presence of WIN55,212-2, but not WIN55,212-2 alone poses several questions. We could hazard a guess that WIN55,212-2 does not directly activate TRP channels, but the production of the endocannabinoids mediated by alfaxalone could account for modulation of TRP channel activity. However, we have yet to determine whether blocking endocannabinoid synthesis would prevent the activation of TRP channels by alfaxalone. Another possible explanation is the induction of CB_1R internalisation by WIN55,212-2; this would also require experimental verification.

From our current findings, we have begun to understand the potential mechanism behind alfaxalone induced neuro-excitation. There are still many questions that remain unanswered, but we believe we

have enough evidence to suggest that CB_1R or TRP channel blockade will reduce or prevent the inhibitory effects of alfaxalone on glycinergic transmission, thereby reducing or removing neuroexcitation during alfaxalone anaesthesia. Surprisingly, any of the four drugs investigated here could potentially block the effects of alfaxalone. However, there may be legal drug restrictions associated with the use of WIN55,212-2, due to its similarity in effect to psychoactive components of *Cannibis sativa*. Also, clinical side effects of CB_1R activation have been reported. CB_1R agonists have reportedly produced anxiolytic, anxiogenic or even ineffective effects clinically, with these biphasic effects likely associated with ranging doses causing differential effects on different regions of the brain (Cagni and Barros, 2013). Local stimulation of CB_1R in the basolateral amygdala has induced anxiogenesis: however stimulation of the central amygdala can promote anxiolytic effects (McLaughlin and Gobbi, 2012). Therefore it is more difficult to justify using a CB_1R agonist, if a CB_1R antagonist or TRP channel antagonist provide the same effect without the complications associated with psychoactive drug molecules. The next step therefore involves the use of AM251, NESS0327 and 2-APB as premedications with alfaxalone anaesthesia in a clinical setting to block neuro-excitation in alfaxalone anaethesia.

Future directions

The progression from *in vitro* to *in vivo* studies will ultimately be the next step. This work could potentially validate the current electrophysiological work performed and provide clinical evidence of the potential use of either TRP channel antagonists or CB_1R antagonists to ameliorate the alfaxalone induced hyperexcitatory side effects. Clinical trials of the CB_1R antagonists and TRP channel antagonists as premedication adjuncts however may in themselves cause some side effects as previously indicated. We must therefore look further into whether one of these drugs examined, or drugs with similar pharmacological activity, are more likely to give us additional benefits, eg antinociceptive actions of some CB_1R inverse agonists. Ultimately the drug of choice will be determined by their clinical efficacy.

Conclusion

Regardless of what is mediating the changes caused by alfaxalone in our in vitro neurophysiological tests, we need to validate whether the blockade of CB_1Rs or TRP channels will clinically remove the detrimental side effects of alfaxalone anaesthesia. Electrophysiological studies have identified potential targets for *in vivo* clinical testing that would hopefully reduce or remove the neuro-excitatory side effects of alfaxalone.

My main aim for this thesis was to develop a safe and easy to administer anaesthetic regimen for use in laboratory rodents. In doing so, I have been able to identify many pitfalls associated with currently available rodent anaesthetic regimens. Alfaxalone showed a lot of promise in filling this market, as seen with the large margin of safety it has shown in veterinary anaesthesia. I therefore endeavoured to develop alfaxalone anaesthesia for laboratory rodent anaesthesia in the hope to ultimately improve currently available anaesthetic regimen. I believe that, through these investigations, we are on the right path to improving alfaxalone anaesthesia. Ultimately, continually improving anaesthetic protocols for laboratory rodents will aid both researchers and laboratory animal technicians, but most importantly, it will help improve animal welfare for laboratory rodents undergoing anaesthesia.

REFERENCES

ABELSON, K. S., JACOBSEN, K. R., SUNDBOM, R., KALLIOKOSKI, O. & HAU, J. (2012) Voluntary ingestion of nut paste for administration of buprenorphine in rats and mice. *Lab Anim,* 46**,** 349-51.

ABREU, M., AGUADO, D., BENITO, J. & GOMEZ DE SEGURA, I. A. (2012) Reduction of the sevoflurane minimum alveolar concentration induced by methadone, tramadol, butorphanol and morphine in rats. *Lab Anim,* 46**,** 200-6.

AHRENS, J., LEUWER, M., DEMIR, R., KRAMPFL, K., FOADI, N. & HAESELER, G. (2008) The anaesthetic steroid alphaxalone positively modulates alpha1-glycine receptor function. *Pharmacol,* 82**,** 228-32.

ALGER, B. E. (2002) Retrograde signaling in the regulation of synaptic transmission: focus on endocannabinoids. *Prog Neurobiol,* 68**,** 247-86.

ALVES, H. N., DA SILVA, A. L., OLSSON, I. A., ORDEN, J. M. & ANTUNES, L. M. (2012) Anesthesia with intraperitoneal propofol, medetomidine, and fentanyl in rats. *J Am Assoc Lab Anim Sci,* 49**,** 454-9.

ARRAS, M., AUTENRIED, P., RETTICH, A., SPAENI, D. & RULICKE, T. (2001) Optimization of intraperitoneal injection anesthesia in mice: drugs, dosages, adverse effects, and anesthesia depth. *Comp Med,* 51**,** 443-56.

ARRAS, M., RETTICH, A., SEIFERT, B., KASERMANN, H. P. & RULICKE, T. (2007) Should laboratory mice be anaesthetized for tail biopsy? *Lab Anim,* 41**,** 30-45.

AVSAROGLU, H., VAN DER SAR, A. S., VAN LITH, H. A., VAN ZUTPHEN, L. F. & HELLEBREKERS, L. J. (2007) Differences in response to anaesthetics and analgesics between inbred rat strains. *Lab Anim,* 41**,** 337-44.

BARDELL, T. K. & BARKER, E. L. (2010) Activation of TRPC6 channels promotes endocannabinoid biosynthesis in neuronal CAD cells. *Neurochem Int,* 57**,** 76-83.

BARRUETO, F., JR., SALLENG, K., SAHNI, R. & BREWER, K. L. (2002) Histopathologic effects of the single intramuscular injection of ketamine, atropine and midazolam in a rat model. *Vet Hum Toxicol,* 44**,** 306-10.

BAYLISS, D. A., VIANA, F., BELLINGHAM, M. C. & BERGER, A. J. (1994) Characteristics and postnatal development of a hyperpolarization-activated inward current in rat hypoglossal motoneurons in vitro. *J Neurophysiol,* 71**,** 119-28.

BELELLI, D. & LAMBERT, J. J. (2005) Neurosteroids: endogenous regulators of the GABA(A) receptor. *Nat Rev Neurosci,* 6**,** 565-75.

BELLINGHAM, M. C. (2013) Pre- and postsynaptic mechanisms underlying inhibition of hypoglossal motor neuron excitability by riluzole. *J Neurophysiol***,** aheadofprint.

BELLINGHAM, M. C. & BERGER, A. J. (1996) Presynaptic depression of excitatory synaptic inputs to rat hypoglossal motoneurons by muscarinic M2 receptors. *J Neurophysiol,* 76**,** 3758-70.

BERGER, A. J., BAYLISS, D. A. & VIANA, F. (1996) Development of hypoglossal motoneurons. *J Appl Physiol (1985),* 81**,** 1039-48.

BERGEY, G. K., BIGALKE, H. & NELSON, P. G. (1987) Differential effects of tetanus toxin on inhibitory and excitatory synaptic transmission in mammalian spinal cord neurons in culture: a presynaptic locus of action for tetanus toxin. *J Neurophysiol,* 57**,** 121-31.

BETSCHART, H. R., JONDORF, W. R. & BICKEL, M. H. (1988) Differences in adipose tissue distribution of basic lipophilic drugs between intraperitoneal and other routes of administration. *Xenobiotica,* 18**,** 113-21.

BETZ, H. & LAUBE, B. (2006) Glycine receptors: recent insights into their structural organization and functional diversity. *J Neurochem,* 97**,** 1600-10.

BILLE, C., AUVIGNE, V., LIBERMANN, S., BOMASSI, E., DURIEUX, P. & RATTEZ, E. (2012) Risk of anaesthetic mortality in dogs and cats: an observational cohort study of 3546 cases. *Vet Anaesth Analg,* 39**,** 59-68.

BIRO, T. & MAKSAY, G. (2004) Allosteric modulation of glycine receptors is more efficacious for partial rather than full agonists. *Neurochem Int,* 44**,** 521-7.
BISHOP, Y. (Ed.) (2005) *The Veterinary Formulary,* London, the Pharmaceutical Press.
BOURQUE, S. L., ADAMS, M. A., NAKATSU, K. & WINTERBORN, A. (2010) Comparison of buprenorphine and meloxicam for postsurgical analgesia in rats: effects on body weight, locomotor activity, and hemodynamic parameters. *J Am Assoc Lab Anim Sci,* 49**,** 617-22.
BREWSTER, M. E., ESTES, K. S. & BODOR, N. (1989) Development of a Non-Surfactant Formulation for Alfaxalone Through the Use of Chemically-Modified Cyclodextrins. *J Parenter Sci Technol,* 43**,** 262-265.
BRIDGES, D., AHMAD, K. & RICE, A. S. (2001) The synthetic cannabinoid WIN55,212-2 attenuates hyperalgesia and allodynia in a rat model of neuropathic pain. *Br J Pharmacol,* 133**,** 586-94.
BRITTON, K. T., PAGE, M., BALDWIN, H. & KOOB, G. F. (1991) Anxiolytic activity of steroid anesthetic alphaxalone. *J Pharmacol Exp Ther,* 258**,** 124-9.
CAGNI, P. & BARROS, M. (2013) Cannabinoid type 1 receptor ligands WIN 55,212-2 and AM 251 alter anxiety-like behaviors of marmoset monkeys in an open-field test. *Behav Brain Res,* 240**,** 91-4.
CARD, B., MCCULLOCH, R. J. & PRATT, D. A. (1972) Tissue distribution of CT 1341 in the rat; an autoradiographic study. *Postgrad Med J,* 48**,** Suppl 2:34-7.
CARLSON, B. X., ENGBLOM, A. C., KRISTIANSEN, U., SCHOUSBOE, A. & OLSEN, R. W. (2000) A single glycine residue at the entrance to the first membrane-spanning domain of the gamma-aminobutyric acid type A receptor beta(2) subunit affects allosteric sensitivity to GABA and anesthetics. *Mol Pharmacol,* 57**,** 474-84.
CASAROTTO, P. C., TERZIAN, A. L., AGUIAR, D. C., ZANGROSSI, H., GUIMARAES, F. S., WOTJAK, C. T. & MOREIRA, F. A. (2012) Opposing roles for cannabinoid receptor type-1 (CB(1)) and transient receptor potential vanilloid type-1 channel (TRPV1) on the modulation of panic-like responses in rats. *Neuropsychopharmacol,* 37**,** 478-86.
CHAMBERS, A. P., KOOPMANS, H. S., PITTMAN, Q. J. & SHARKEY, K. A. (2006) AM 251 produces sustained reductions in food intake and body weight that are resistant to tolerance and conditioned taste aversion. *Br J Pharmacol,* 147**,** 109-16.
CHELLMAN, G. J., LOLLINI, L. O., DORR, A. E. & DEPASS, L. R. (1994) Comparison of ketorolac tromethamine with other injectable nonsteroidal anti-inflammatory drugs for pain-on-injection and muscle damage in the rat. *Hum Exp Toxicol,* 13**,** 111-7.
CHEN, X., SHU, S., KENNEDY, D. P., WILLCOX, S. C. & BAYLISS, D. A. (2009) Subunit-specific effects of isoflurane on neuronal Ih in HCN1 knockout mice. *J Neurophysiol,* 101**,** 129-40.
CHEN, X., SIROIS, J. E., LEI, Q., TALLEY, E. M., LYNCH, C., 3RD & BAYLISS, D. A. (2005) HCN subunit-specific and cAMP-modulated effects of anesthetics on neuronal pacemaker currents. *J Neurosci,* 25**,** 5803-14.
CHILD, K. J., GIBSON, W., HARNBY, G. & HART, J. W. (1972) Metabolism and excretion of Althesin (CT 1341) in the rat. *Postgrad Med J,* 48**,** Suppl 2:37-43.
CHING, M. (1984) Comparison of the effects of Althesin, chloralose-urethane, urethane, and pentobarbital on mammalian physiologic responses. *Can J Physiol Pharmacol,* 62**,** 654-7.
CHO, Y. J., LEE, Y. A., LEE, J. W., KIM, J. I. & HAN, J. S. (2011) Kinetics of proinflammatory cytokines after intraperitoneal injection of tribromoethanol and a tribromoethanol/xylazine combination in ICR mice. *Lab Anim Res,* 27**,** 197-203.
CLAPHAM, D. E. (2007) SnapShot: mammalian TRP channels. *Cell,* 129**,** 220.
CLAPHAM, D. E., RUNNELS, L. W. & STRUBING, C. (2001) The TRP ion channel family. *Nat Rev Neurosci,* 2**,** 387-96.
CLARKE, K. W. & HALL, L. W. (1990) A survey of anaesthesia in small animal practice: AVA/BSAVA report. *J Ass vet Anaesth,* 17**,** 4-10.

COMPAGNONE, N. A. & MELLON, S. H. (2000) Neurosteroids: biosynthesis and function of these novel neuromodulators. *Front Neuroendocrinol,* 21**,** 1-56.
COTTRELL, G. A., LAMBERT, J. J. & PETERS, J. A. (1987) Modulation of GABAA receptor activity by alphaxalone. *Br J Pharmacol,* 90**,** 491-500.
DANNEMAN, P. J. & MANDRELL, T. D. (1997) Evaluation of five agents/methods for anesthesia of neonatal rats. *Lab Anim Sci,* 47**,** 386-95.
DAVIS, B. & PEARCE, D. (1972) An introduction to Althesin (CT 1341). *Postgrad Med J,* June Suppl.**,** 13-17.
DE LA PENA, J. B., LEE, H. C., DE LA PENA, I. C., WOO, T. S., YOON, S. Y., LEE, H. L., HAN, J. S., LEE, J. I., CHO, Y. J., SHIN, C. Y. & CHEONG, J. H. (2012) Rewarding and reinforcing effects of the NMDA receptor antagonist-benzodiazepine combination, Zoletil(R): difference between acute and repeated exposure. *Behav Brain Res,* 233**,** 434-42.
DESAI, B. N. & CLAPHAM, D. E. (2005) TRP channels and mice deficient in TRP channels. *Pflugers Arch,* 451**,** 11-8.
DI MARZO, V. & DE PETROCELLIS, L. (2010) Endocannabinoids as regulators of transient receptor potential (TRP) channels: A further opportunity to develop new endocannabinoid-based therapeutic drugs. *Curr Med Chem,* 17**,** 1430-49.
DUM, J. E. & HERZ, A. (1981) In vivo receptor binding of the opiate partial agonist, buprenorphine, correlated with its agonistic and antagonistic actions. *Br J Pharmacol,* 74**,** 627-33.
DYSON, D. H., MAXIE, M. G. & SCHNURR, D. (1998) Morbidity and mortality associated with anesthetic management in small animal veterinary practice in Ontario. *J Am Anim Hosp Assoc,* 34**,** 325-35.
ENGELHARDT, G., HOMMA, D., SCHLEGEL, K., UTZMANN, R. & SCHNITZLER, C. (1995a) Anti-inflammatory, analgesic, antipyretic and related properties of meloxicam, a new non-steroidal anti-inflammatory agent with favourable gastrointestinal tolerance. *Inflamm Res,* 44**,** 423-33.
ENGELHARDT, G., HOMMA, D. & SCHNITZLER, C. (1995b) Meloxicam: a potent inhibitor of adjuvant arthritis in the Lewis rat. *Inflamm Res,* 44**,** 548-55.
ESTES, K. S., BREWSTER, M. E., WEBB, A. I. & BODOR, N. (1990) A non-surfactant formulation for alfaxalone based on an amorphous cyclodextrin: Activity studies in rats and dogs. *Int J Pharm,* 65**,** 101-107.
FERRARI, L., TURRINI, G., ROSTELLO, C., GUIDI, A., CASARTELLI, A., PIAIA, A. & SARTORI, M. (2005) Evaluation of two combinations of Domitor, Zoletil 100, and Euthatal to obtain long-term nonrecovery anesthesia in Sprague-Dawley rats. *Comp Med,* 55**,** 256-64.
FERRE, P. J., KIRBY., P., TED., W., MG., R., QIANG., L. & P., L. H. (2006) Plasma pharmacokinetics of alfaxalone in dogs after an intravenous bolus of Alfaxan-CD RTU. *Vet Anaesth Analg,* 33**,** 229-236.
FESTING, M. F. (1993) Genetic variation in outbred rats and mice and its implications for toxicological screening. *J Exp Anim Sci,* 35**,** 210-20.
FFRENCH-MULLEN, J. M., DANKS, P. & SPENCE, K. T. (1994) Neurosteroids modulate calcium currents in hippocampal CA1 neurons via a pertussis toxin-sensitive G-protein-coupled mechanism. *J Neurosci,* 14**,** 1963-77.
FIELD, K. J., WHITE, W. J. & LANG, C. M. (1993) Anaesthetic effects of chloral hydrate, pentobarbitone and urethane in adult male rats. *Lab Anim,* 27**,** 258-69.
FINK, G., SARKAR, D. K., DOW, R. C., DICK, H., BORTHWICK, N., MALNICK, S. & TWINE, M. (1982) Sex difference in response to alphaxalone anaesthesia may be oestrogen dependent. *Nature,* 298**,** 270-2.
FLECKNELL, P. A. (1984) The relief of pain in laboratory animals. *Lab Anim,* 18**,** 147-60.
FLECKNELL, P. A. (1993a) Anaesthesia of animals for biomedical research. *Br J Anaesth,* 71**,** 885-94.
FLECKNELL, P. A. (1993b) Anesthesia and perioperative care. *Methods Enzymol,* 225**,** 16-33.

FOADI, N., PILAWSKI, I., BUCHHOLZ, V., DE LA ROCHE, J., WEGNER, F., LEFFLER, A., AHRENS, J. & KASTNER, S. (2012) Interaction of alfaxalone with the neuronal and the skeletal muscle sodium channel. *Pharmacol,* 89**,** 295-302.

GIBALDI, M. P., D (1982) Estimation of Areas. *Pharmacokinetics.* Basel, Marcel Dekker.

GOODWIN, W., KEATES, H., PASLOSKE, K., PEARSON, M., SAUER, B. & RANASINGHE, M. G. (2012) Plasma pharmacokinetics and pharmacodynamics of alfaxalone in neonatal foals after an intravenous bolus of alfaxalone following premedication with butorphanol tartrate. *Vet Anaesth Analg,* 39**,** 503-10.

GOODWIN, W. A., KEATES, H. L., PASLOSKE, K., PEARSON, M., SAUER, B. & RANASINGHE, M. G. (2011) The pharmacokinetics and pharmacodynamics of the injectable anaesthetic alfaxalone in the horse. *Vet Anaesth Analg,* 38**,** 431-8.

GOPALAN, C., HEGADE, G. M., BAY, T. N., BROWN, S. R. & TALCOTT, M. R. (2005) Tribromoethanol-medetomidine combination provides a safe and reversible anesthetic effect in Sprague-Dawley rats. *Contemp Top Lab Anim Sci,* 44**,** 7-10.

GOPALAN, C., SIMMONS, A. S., MUNGAL, G. K., WILSON, T. J., SIEVE, M., SALEM, D. K. & RIVKIN, M. A. (2007) Effects of tribromoethanol-medetomidine combination on cycling and ovariectomized Sprague-Dawley rats. *Lab Anim (NY),* 36**,** 36-40.

GREEN, C. J. (1975) Neuroleptanalgesic drug combinations in the anaesthetic management of small laboratory animals. *Lab Anim,* 9**,** 161-78.

GREEN, C. J., KNIGHT, J., PRECIOUS, S. & SIMPKIN, S. (1981) Ketamine alone and combined with diazepam or xylazine in laboratory animals: a 10 year experience. *Lab Anim,* 15**,** 163-70.

GRUBB, T. L., GREENE, S. A. & PEREZ, T. E. (2013) Cardiovascular and respiratory effects, and quality of anesthesia produced by alfaxalone administered intramuscularly to cats sedated with dexmedetomidine and hydromorphone. *J Feline Med Surg***,** aheadofprint.

GUARNIERI, M., BRAYTON, C., DETOLLA, L., FORBES-MCBEAN, N., SARABIA-ESTRADA, R. & ZADNIK, P. (2012) Safety and efficacy of buprenorphine for analgesia in laboratory mice and rats. *Lab Anim (NY),* 41**,** 337-43.

GUNDLACH, A. L. (1990) Disorder of the inhibitory glycine receptor: inherited myoclonus in Poll Hereford calves. *Faseb J,* 4**,** 2761-6.

HACKETT, T. B. (2002) Pulse oximetry and end tidal carbon dioxide monitoring. *Vet Clin North Am Small Anim Pract,* 32**,** 1021-9.

HARRISON, N. L. & SIMMONDS, M. A. (1984) Modulation of the GABA receptor complex by a steroid anaesthetic. *Brain Res,* 323**,** 287-92.

HAWKINS, P. (2002) Recognizing and assessing pain, suffering and distress in laboratory animals: a survey of current practice in the UK with recommendations. *Lab Anim,* 36**,** 378-95.

HAYASHI, K., NISHIMURA, R., YAMAKI, A., KIM, H., MATSUNAGA, S., SASAKI, N. & TAKEUCHI, A. (1994) Comparison of sedative effects induced by medetomidine, medetomidine-midazolam and medetomidine-butorphanol in dogs. *J Vet Med Sci,* 56**,** 951-6.

HEDENQVIST, P., ROUGHAN, J. V. & FLECKNELL, P. A. (2000) Effects of repeated anaesthesia with ketamine/medetomidine and of pre-anaesthetic administration of buprenorphine in rats. *Lab Anim,* 34**,** 207-11.

HEIMBURG, T. & JACKSON, A. D. (2007) The thermodynamics of general anesthesia. *Biophys J,* 92**,** 3159-65.

HEJAZI, N., ZHOU, C., OZ, M., SUN, H., YE, J. H. & ZHANG, L. (2006) Delta9-tetrahydrocannabinol and endogenous cannabinoid anandamide directly potentiate the function of glycine receptors. *Mol Pharmacol,* 69**,** 991-7.

HILL-VENNING, C., PETERS, J. A., CALLACHAN, H., LAMBERT, J. J., GEMMELL, D. K., ANDERSON, A., BYFORD, A., HAMILTON, N., HILL, D. R., MARSHALL, R. J. & CAMPBELL, A. C. (1996) The anaesthetic action and modulation of GABAA receptor activity by the novel water-soluble aminosteroid Org 20599. *Neuropharmacology,* 35**,** 1209-22.

HITT, B. A., MAZZE, R. I., COOK, T. L., BEPPU, W. J. & KOSEK, J. C. (1977) Thermoregulatory defect in rats during anesthesia. *Anesth Analg,* 56**,** 9-15.
HORISHITA, T., MINAMI, K., YANAGIHARA, N., SHIRAISHI, M., OKAMOTO, T., SHIGA, Y., UENO, S. & SHIGEMATSU, A. (2002) Alphaxalone, a neurosteroid anesthetic, inhibits norepinephrine transporter function in cultured bovine adrenal medullary cells. *Anesth Analg,* 95**,** 1661-6, table of contents.
HOSIE, A. M., WILKINS, M. E., DA SILVA, H. M. & SMART, T. G. (2006) Endogenous neurosteroids regulate GABAA receptors through two discrete transmembrane sites. *Nature,* 444**,** 486-9.
HSIEH, C., BROWN, S., DERLETH, C. & MACKIE, K. (1999) Internalization and recycling of the CB1 cannabinoid receptor. *J Neurochem,* 73**,** 493-501.
HUBBELL, J. A., BEDNARSKI, R. M. & MUIR, W. W. (1989) Xylazine and tiletamine-zolazepam anesthesia in horses. *Am J Vet Res,* 50**,** 737-42.
IRELAND, M. F., FUNK, G. D. & BELLINGHAM, M. C. (2012) Muscarinic acetylcholine receptors enhance neonatal mouse hypoglossal motoneuron excitability in vitro. *J Appl Physiol,* 113**,** 1024-39.
ITAH, R., GITELMAN, I. & DAVIS, C. (2004) A replacement for methoxyflurane (Metofane) in open-circuit anaesthesia. *Lab Anim,* 38**,** 280-5.
JONAS, P., BISCHOFBERGER, J. & SANDKUHLER, J. (1998) Corelease of two fast neurotransmitters at a central synapse. *Science,* 281**,** 419-24.
KEATES, H. (2003) Induction of anaesthesia in pigs using a new alphaxalone formulation. *Vet Rec,* 153**,** 627-8.
KREITZER, A. C. & REGEHR, W. G. (2002) Retrograde signaling by endocannabinoids. *Curr Opin Neurobiol,* 12**,** 324-30.
LAMBERT, J. J., BELELLI, D., HILL-VENNING, C., CALLACHAN, H. & PETERS, J. A. (1996) Neurosteroid modulation of native and recombinant GABAA receptors. *Cell Mol Neurobiol,* 16**,** 155-74.
LAMBERT, J. J., BELELLI, D., HILL-VENNING, C. & PETERS, J. A. (1995) Neurosteroids and GABAA receptor function. *Trends Pharmacol Sci,* 16**,** 295-303.
LAU, C., RANASINGHE, M. G., SHIELS, I., KEATES, H., PASLOSKE, K. & BELLINGHAM, M. C. (2013) Plasma pharmacokinetics of alfaxalone after a single intraperitoneal or intravenous injection of Alfaxan in rats. *J Vet Pharmacol Ther***,** aheadofprint.
LAUBE, B., MAKSAY, G., SCHEMM, R. & BETZ, H. (2002) Modulation of glycine receptor function: a novel approach for therapeutic intervention at inhibitory synapses? *Trends Pharmacol Sci,* 23**,** 519-27.
LEDENT, C., VALVERDE, O., COSSU, G., PETITET, F., AUBERT, J. F., BESLOT, F., BOHME, G. A., IMPERATO, A., PEDRAZZINI, T., ROQUES, B. P., VASSART, G., FRATTA, W. & PARMENTIER, M. (1999) Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice. *Science,* 283**,** 401-4.
LEGENDRE, P. (1998) A reluctant gating mode of glycine receptor channels determines the time course of inhibitory miniature synaptic events in zebrafish hindbrain neurons. *J Neurosci,* 18**,** 2856-70.
LEWIS, R. E., KUNZ, A. L. & BELL, R. E. (1966) Error of intraperitoneal injections in rats. *Lab Anim Care,* 16**,** 505-9.
LILES, J. H. & FLECKNELL, P. A. (1992a) The effects of buprenorphine, nalbuphine and butorphanol alone or following halothane anaesthesia on food and water consumption and locomotor movement in rats. *Lab Anim,* 26**,** 180-9.
LILES, J. H. & FLECKNELL, P. A. (1992b) The use of non-steroidal anti-inflammatory drugs for the relief of pain in laboratory rodents and rabbits. *Lab Anim,* 26**,** 241-55.
LILES, J. H. & FLECKNELL, P. A. (1993) The influence of buprenorphine or bupivacaine on the post-operative effects of laparotomy and bile-duct ligation in rats. *Lab Anim,* 27**,** 374-80.

LILES, J. H. & FLECKNELL, P. A. (1994) A comparison of the effects of buprenorphine, carprofen and flunixin following laparotomy in rats. *J Vet Pharmacol Ther,* 17**,** 284-90.
LIM, R., ALVAREZ, F. J. & WALMSLEY, B. (1999) Quantal size is correlated with receptor cluster area at glycinergic synapses in the rat brainstem. *J Physiol,* 516 (Pt 2)**,** 505-12.
LIM, R., OLESKEVICH, S., FEW, A. P., LEAO, R. N. & WALMSLEY, B. (2003) Glycinergic mIPSCs in mouse and rat brainstem auditory nuclei: modulation by ruthenium red and the role of calcium stores. *J Physiol,* 546**,** 691-9.
LORENZ, W., REIMANN, H. J., SCHMAL, A., DORMANN, P., SCHWARZ, B., NEUGEBAUER, E. & DOENICKE, A. (1977) Histamine release in dogs by Cremophor E1 and its derivatives: oxethylated oleic acid is the most effective constituent. *Agents Actions,* 7**,** 63-7.
LOZOVAYA, N., MUKHTAROV, M., TSINTSADZE, T., LEDENT, C., BURNASHEV, N. & BREGESTOVSKI, P. (2011) Frequency-Dependent Cannabinoid Receptor-Independent Modulation of Glycine Receptors by Endocannabinoid 2-AG. *Front Mol Neurosci,* 4**,** 13.
LOZOVAYA, N., YATSENKO, N., BEKETOV, A., TSINTSADZE, T. & BURNASHEV, N. (2005) Glycine receptors in CNS neurons as a target for nonretrograde action of cannabinoids. *J Neurosci,* 25**,** 7499-506.
LUKAS, G., BRINDLE, S. D. & GREENGARD, P. (1971) The route of absorption of intraperitoneally administered compounds. *J Pharmacol Exp Ther,* 178**,** 562-4.
LYNCH, J. W. (2004) Molecular structure and function of the glycine receptor chloride channel. *Physiol Rev,* 84**,** 1051-95.
LYNCH, J. W. (2009) Native glycine receptor subtypes and their physiological roles. *Neuropharmacology,* 56**,** 303-9.
MACMILLAN, L. B., HEIN, L., SMITH, M. S., PIASCIK, M. T. & LIMBIRD, L. E. (1996) Central hypotensive effects of the alpha2a-adrenergic receptor subtype. *Science,* 273**,** 801-3.
MAJEED, Y., AGARWAL, A. K., NAYLOR, J., SEYMOUR, V. A., JIANG, S., MURAKI, K., FISHWICK, C. W. & BEECH, D. J. (2010) Cis-isomerism and other chemical requirements of steroidal agonists and partial agonists acting at TRPM3 channels. *Br J Pharmacol,* 161**,** 430-41.
MAJEED, Y., TUMOVA, S., GREEN, B. L., SEYMOUR, V. A., WOODS, D. M., AGARWAL, A. K., NAYLOR, J., JIANG, S., PICTON, H. M., PORTER, K. E., O'REGAN, D. J., MURAKI, K., FISHWICK, C. W. & BEECH, D. J. (2012) Pregnenolone sulphate-independent inhibition of TRPM3 channels by progesterone. *Cell Calcium,* 51**,** 1-11.
MAKSAY, G., BIRO, T. & LAUBE, B. (2002) Hyperekplexia mutation of glycine receptors: decreased gating efficacy with altered binding thermodynamics. *Biochem Pharmacol,* 64**,** 285-8.
MAKSAY, G., BIRO, T., LAUBE, B. & NEMES, P. (2008) Hyperekplexia mutation R271L of alpha1 glycine receptors potentiates allosteric interactions of nortropeines, propofol and glycine with [3H]strychnine binding. *Neurochem Int,* 52**,** 235-40.
MASCIA, M. P., MACHU, T. K. & HARRIS, R. A. (1996) Enhancement of homomeric glycine receptor function by long-chain alcohols and anaesthetics. *Br J Pharmacol,* 119**,** 1331-6.
MATHIS, A., PINELAS, R., BRODBELT, D. C. & ALIBHAI, H. I. (2012) Comparison of quality of recovery from anaesthesia in cats induced with propofol or alfaxalone. *Vet Anaesth Analg,* 39**,** 282-90.
MAZZE, R. I. (1985) Fertility, reproduction, and postnatal survival in mice chronically exposed to isoflurane. *Anesthesiology,* 63**,** 663-7.
MCLAUGHLIN, R. J. & GOBBI, G. (2012) Cannabinoids and emotionality: a neuroanatomical perspective. *Neuroscience,* 204**,** 134-44.
MCMAHON, L. R. & KOEK, W. (2007) Differences in the relative potency of SR 141716A and AM 251 as antagonists of various in vivo effects of cannabinoid agonists in C57BL/6J mice. *Eur J Pharmacol,* 569**,** 70-6.

MEYE, F. J., TREZZA, V., VANDERSCHUREN, L. J., RAMAKERS, G. M. & ADAN, R. A. (2012) Neutral antagonism at the cannabinoid 1 receptor: a safer treatment for obesity. *Mol Psychiatry***,** aheadofprint.

MITCHELL, E. A., GENTET, L. J., DEMPSTER, J. & BELELLI, D. (2007) GABAA and glycine receptor-mediated transmission in rat lamina II neurones: relevance to the analgesic actions of neuroactive steroids. *J Physiol,* 583**,** 1021-40.

MOHAMMADI, B., KRAMPFL, K., CETINKAYA, C., MOSCHREF, H., GROSSKREUTZ, J., DENGLER, R. & BUFLER, J. (2003) Kinetic analysis of recombinant mammalian alpha(1) and alpha(1)beta glycine receptor channels. *Eur Biophys J,* 32**,** 529-36.

MONTEIRO, E. R., FIGUEROA, C. D., CHOMA, J. C., CAMPAGNOL, D. & BETTINI, C. M. (2008) Effects of methadone, alone or in combination with acepromazine or xylazine, on sedation and physiologic values in dogs. *Vet Anaesth Analg,* 35**,** 519-27.

MONTEIRO, E. R., JUNIOR, A. R., ASSIS, H. M., CAMPAGNOL, D. & QUITZAN, J. G. (2009) Comparative study on the sedative effects of morphine, methadone, butorphanol or tramadol, in combination with acepromazine, in dogs. *Vet Anaesth Analg,* 36**,** 25-33.

MORAN, M. M., XU, H. & CLAPHAM, D. E. (2004) TRP ion channels in the nervous system. *Curr Opin Neurobiol,* 14**,** 362-9.

MORTON, D. B., JENNINGS, M., BUCKWELL, A., EWBANK, R., GODFREY, C., HOLGATE, B., INGLIS, I., JAMES, R., PAGE, C., SHARMAN, I., VERSCHOYLE, R., WESTALL, L. & WILSON, A. B. (2001) Refining procedures for the administration of substances. Report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement. British Veterinary Association Animal Welfare Foundation/Fund for the Replacement of Animals in Medical Experiments/Royal Society for the Prevention of Cruelty to Animals/Universities Federation for Animal Welfare. *Lab Anim,* 35**,** 1-41.

MUIR, W., LERCHE, P., WIESE, A., NELSON, L., PASLOSKE, K. & WHITTEM, T. (2008) Cardiorespiratory and anesthetic effects of clinical and supraclinical doses of alfaxalone in dogs. *Vet Anaesth Analg,* 35**,** 451-62.

MUIR, W., LERCHE, P., WIESE, A., NELSON, L., PASLOSKE, K. & WHITTEM, T. (2009) The cardiorespiratory and anesthetic effects of clinical and supraclinical doses of alfaxalone in cats. *Vet Anaesth Analg,* 36**,** 42-54.

MUKHTAROV, M., RAGOZZINO, D. & BREGESTOVSKI, P. (2005) Dual Ca2+ modulation of glycinergic synaptic currents in rodent hypoglossal motoneurones. *J Physiol,* 569**,** 817-31.

NADERI, N., MAJIDI, M., MOUSAVI, Z., KHORAMIAN TUSI, S., MANSOURI, Z. & KHODAGHOLI, F. (2012) The interaction between intrathecal administration of low doses of palmitoylethanolamide and AM251 in formalin-induced pain related behavior and spinal cord IL1-beta expression in rats. *Neurochem Res,* 37**,** 778-85.

NADESON, R. & GOODCHILD, C. S. (2000) Antinociceptive properties of neurosteroids II. Experiments with Saffan and its components alphaxalone and alphadolone to reveal separation of anaesthetic and antinociceptive effects and the involvement of spinal cord GABA(A) receptors. *Pain,* 88**,** 31-9.

NADESON, R. & GOODCHILD, C. S. (2001) Antinociceptive properties of neurosteroids III: experiments with alphadolone given intravenously, intraperitoneally, and intragastrically. *Br J Anaesth,* 86**,** 704-8.

NAVARRETE, M. & ARAQUE, A. (2010) Endocannabinoids potentiate synaptic transmission through stimulation of astrocytes. *Neuron,* 68**,** 113-26.

NILIUS, B. & VOETS, T. (2008) A TRP channel-steroid marriage. *Nat Cell Biol,* 10**,** 1383-4.

NILSFORS, L., KVART, C., KALLINGS, P., CARLSTEN, J. & BONDESSON, U. (1988) Cardiorespiratory and sedative effects of a combination of acepromazine, xylazine and methadone in the horse. *Equine Vet J,* 20**,** 364-7.

NOLAN, M. F., GIBSON, I. C. & LOGAN, S. D. (1997) Actions of the anaesthetic Saffan on rat sympathetic preganglionic neurones in vitro. *Br J Pharmacol,* 121**,** 324-30.

NOVELLI, G. P., MARSILI, M. & LORENZI, P. (1975) Influence of liver metabolism on the actions of althesin and thiopentone. *Br J Anaesth,* 47**,** 913-6.
O'BRIEN, J. A. & BERGER, A. J. (1999) Cotransmission of GABA and glycine to brain stem motoneurons. *J Neurophysiol,* 82**,** 1638-41.
OLIVER, D., LIEN, C. C., SOOM, M., BAUKROWITZ, T., JONAS, P. & FAKLER, B. (2004) Functional conversion between A-type and delayed rectifier K+ channels by membrane lipids. *Science,* 304**,** 265-70.
ONG, W. Y. & MACKIE, K. (1999) A light and electron microscopic study of the CB1 cannabinoid receptor in primate brain. *Neuroscience,* 92**,** 1177-91.
OZ, M. (2006) Receptor-independent actions of cannabinoids on cell membranes: focus on endocannabinoids. *Pharmacol Ther,* 111**,** 114-44.
PAGNOTTA, S. E., LAPE, R., QUITADAMO, C. & NISTRI, A. (2005) Pre- and postsynaptic modulation of glycinergic and gabaergic transmission by muscarinic receptors on rat hypoglossal motoneurons in vitro. *Neuroscience,* 130**,** 783-95.
PARKER, A. J., LEE, J. B., REDMAN, J. & JOLLIFFE, L. (2011) Strychnine poisoning: gone but not forgotten. *Emerg Med J,* 28**,** 84.
PASLOSKE, K., SAUER, B., PERKINS, N. & WHITTEM, T. (2009) Plasma pharmacokinetics of alfaxalone in both premedicated and unpremedicated Greyhound dogs after single, intravenous administration of Alfaxan at a clinical dose. *J Vet Pharmacol Ther,* 32**,** 510-3.
PAUL, S. M. & PURDY, R. H. (1992) Neuroactive steroids. *Faseb J,* 6**,** 2311-22.
PEDERSEN, S. F., OWSIANIK, G. & NILIUS, B. (2005) TRP channels: an overview. *Cell Calcium,* 38**,** 233-52.
PERTWEE, R. G. (1997) Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacol Ther,* 74**,** 129-80.
PERTWEE, R. G. (1999) Cannabis and cannabinoids: pharmacology and rationale for clinical use. *Forsch Komplementarmed,* 6 Suppl 3**,** 12-5.
PERTWEE, R. G. (2005) Inverse agonism and neutral antagonism at cannabinoid CB1 receptors. *Life Sci,* 76**,** 1307-24.
PERTWEE, R. G., HOWLETT, A. C., ABOOD, M. E., ALEXANDER, S. P., DI MARZO, V., ELPHICK, M. R., GREASLEY, P. J., HANSEN, H. S., KUNOS, G., MACKIE, K., MECHOULAM, R. & ROSS, R. A. (2010) International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB(1) and CB(2). *Pharmacol Rev,* 62**,** 588-631.
PRINCE, R. J. & SIMMONDS, M. A. (1992) Steroid modulation of the strychnine-sensitive glycine receptor. *Neuropharmacology,* 31**,** 201-5.
REKLING, J. C., FUNK, G. D., BAYLISS, D. A., DONG, X. W. & FELDMAN, J. L. (2000) Synaptic control of motoneuronal excitability. *Physiol Rev,* 80**,** 767-852.
RICHARDSON, C. A. & FLECKNELL, P. A. (2005) Anaesthesia and post-operative analgesia following experimental surgery in laboratory rodents: are we making progress? *Altern Lab Anim,* 33**,** 119-27.
ROMANO, B., BORRELLI, F., FASOLINO, I., CAPASSO, R., PISCITELLI, F., CASCIO, M., PERTWEE, R., COPPOLA, D., VASSALLO, L., ORLANDO, P., DI MARZO, V. & IZZO, A. (2013) The cannabinoid TRPA1 agonist cannabichromene inhibits nitric oxide production in macrophages and ameliorates murine colitis. *Br J Pharmacol,* 169**,** 213-29.
ROUGHAN, J. V. & FLECKNELL, P. A. (2003a) Evaluation of a short duration behaviour-based post-operative pain scoring system in rats. *Eur J Pain,* 7**,** 397-406.
ROUGHAN, J. V. & FLECKNELL, P. A. (2003b) Pain assessment and control in laboratory animals. *Lab Anim,* 37**,** 172.
ROUGHAN, J. V., OJEDA, O. B. & FLECKNELL, P. A. (1999) The influence of pre-anaesthetic administration of buprenorphine on the anaesthetic effects of ketamine/medetomidine and pentobarbitone in rats and the consequences of repeated anaesthesia. *Lab Anim,* 33**,** 234-42.

RUIU, S., PINNA, G. A., MARCHESE, G., MUSSINU, J. M., SABA, P., TAMBARO, S., CASTI, P., VARGIU, R. & PANI, L. (2003) Synthesis and characterization of NESS 0327: a novel putative antagonist of the CB1 cannabinoid receptor. *J Pharmacol Exp Ther,* 306**,** 363-70.

SAHA, D. C., SAHA, A. C., MALIK, G., ASTIZ, M. E. & RACKOW, E. C. (2007) Comparison of cardiovascular effects of tiletamine-zolazepam, pentobarbital, and ketamine-xylazine in male rats. *J Am Assoc Lab Anim Sci,* 46**,** 74-80.

SAITO, V. M., WOTJAK, C. T. & MOREIRA, F. A. (2010) [Pharmacological exploitation of the endocannabinoid system: new perspectives for the treatment of depression and anxiety disorders?]. *Rev Bras Psiquiatr,* 32 Suppl 1**,** S7-14.

SALIO, C., FISCHER, J., FRANZONI, M. F. & CONRATH, M. (2002) Pre- and postsynaptic localizations of the CB1 cannabinoid receptor in the dorsal horn of the rat spinal cord. *Neuroscience,* 110**,** 755-64.

SAUL, P. B. & FISHER, J. C. (1974) Preliminary report: the use of methoxyflurane for small animal anesthesia. *J Surg Res,* 17**,** 271-3.

SEAR, J. W. & MCGIVAN, J. D. (1981) Metabolism of alphaxalone in the rat: evidence for the limitation of the anaesthetic effect by the rate of degradation through the hepatic mixed function oxygenase system. *Br J Anaesth,* 53**,** 417-24.

SHIN, M. C., NONAKA, K., WAKITA, M., YAMAGA, T., TORII, Y., HARAKAWA, T., GINNAGA, A., ITO, Y. & AKAIKE, N. (2012) Effects of tetanus toxin on spontaneous and evoked transmitter release at inhibitory and excitatory synapses in the rat SDCN neurons. *Toxicon,* 59**,** 385-92.

SHIRAISHI, M., MINAMI, K., SHIBUYA, I., UEZONO, Y., OGATA, J., OKAMOTO, T., MURASAKI, O., KAIBARA, M., UETA, Y. & SHIGEMATSU, A. (2003) The inhibitory effects of alphaxalone on M1 and M3 muscarinic receptors expressed in Xenopus oocytes. *Anesth Analg,* 97**,** 449-55, table of contents.

SIMMONDS, M. A. (1983) Depolarizing responses to glycine, beta-alanine and muscimol in isolated optic nerve and cuneate nucleus. *Br J Pharmacol,* 79**,** 799-806.

SINGER, J. H. & BERGER, A. J. (1999) Contribution of single-channel properties to the time course and amplitude variance of quantal glycine currents recorded in rat motoneurons. *J Neurophysiol,* 81**,** 1608-16.

SINGER, J. H. & BERGER, A. J. (2000) Development of inhibitory synaptic transmission to motoneurons. *Brain Res Bull,* 53**,** 553-60.

SINGER, J. H., TALLEY, E. M., BAYLISS, D. A. & BERGER, A. J. (1998) Development of glycinergic synaptic transmission to rat brain stem motoneurons. *J Neurophysiol,* 80**,** 2608-20.

SLADKY, K. K., KELLY, B. T., LOOMIS, M. R., STOSKOPF, M. K. & HORNE, W. A. (2000) Cardiorespiratory effects of four alpha2-adrenoceptor agonist-ketamine combinations in captive red wolves. *J Am Vet Med Assoc,* 217**,** 1366-71.

SMILER, K. L., STEIN, S., HRAPKIEWICZ, K. L. & HIBEN, J. R. (1990) Tissue response to intramuscular and intraperitoneal injections of ketamine and xylazine in rats. *Lab Anim Sci,* 40**,** 60-4.

SMITH, W. (1993) Responses of laboratory animals to some injectable anaesthetics. *Lab Anim,* 27**,** 30-39.

STICKROD, G. (1979) Ketamine/xylazine anesthesia in the pregnant rat. *J Am Vet Med Assoc,* 175**,** 952-3.

STOKES, E. L., FLECKNELL, P. A. & RICHARDSON, C. A. (2009) Reported analgesic and anaesthetic administration to rodents undergoing experimental surgical procedures. *Lab Anim,* 43**,** 149-54.

STUART, G. J., DODT, H. U. & SAKMANN, B. (1993) Patch-clamp recordings from the soma and dendrites of neurons in brain slices using infrared video microscopy. *Pflugers Arch,* 423**,** 511-8.

SUTTON, J. (1972) A brief history of steroid anaesthesia before Althesin (CT1341). *Postgrad Med J,* June Suppl.**,** 9-13.

TARIN, D. & STURDEE, A. (1972) Surgical anaesthesia of mice: evaluation of tribromo-ethanol, ether, halothane and methoxyflurane and development of a reliable technique. *Lab Anim,* 6**,** 79-84.
TAYLOR, D. K. (2007) Study of two devices used to maintain normothermia in rats and mice during general anesthesia. *J Am Assoc Lab Anim Sci,* 46**,** 37-41.
THOMPSON, S. A. & WAFFORD, K. (2001) Mechanism of action of general anaesthetics--new information from molecular pharmacology. *Curr Opin Pharmacol,* 1**,** 78-83.
TOUTAIN, P. L. & BOUSQUET-MELOU, A. (2004a) Bioavailability and its assessment. *J Vet Pharmacol Ther,* 27**,** 455-66.
TOUTAIN, P. L. & BOUSQUET-MELOU, A. (2004b) Plasma clearance. *J Vet Pharmacol Ther,* 27**,** 415-25.
VALVERDE, A. (2012) Alpha-2 agonists as pain therapy in horses. *Vet Clin North Am Equine Pract,* 26**,** 515-32.
VAN DER VIJVER, R. J., VAN LAARHOVEN, C. J., LOMME, R. M. & HENDRIKS, T. (2012) Carprofen for perioperative analgesia causes early anastomotic leakage in the rat ileum. *BMC Vet Res,* 8**,** 247.
VENKATACHALAM, K. & MONTELL, C. (2007) TRP channels. *Annu Rev Biochem,* 76**,** 387-417.
VIRTANEN, R. (1989) Pharmacological profiles of medetomidine and its antagonist, atipamezole. *Acta Vet Scand Suppl,* 85**,** 29-37.
VISSER, S. A., SMULDERS, C. J., GLADDINES, W. W., IRTH, H., VAN DER GRAAF, P. H. & DANHOF, M. (2000) High-performance liquid chromatography of the neuroactive steroids alphaxalone and pregnanolone in plasma using dansyl hydrazine as fluorescent label: application to a pharmacokinetic-pharmacodynamic study in rats. *J Chromatogr B Biomed Sci Appl,* 745**,** 357-63.
VISSER, S. A., SMULDERS, C. J., REIJERS, B. P., VAN DER GRAAF, P. H., PELETIER, L. A. & DANHOF, M. (2002) Mechanism-based pharmacokinetic-pharmacodynamic modeling of concentration-dependent hysteresis and biphasic electroencephalogram effects of alphaxalone in rats. *J Pharmacol Exp Ther,* 302**,** 1158-67.
VOLKER, D., BATE, M., GENTLE, R. & GARG, M. (2000) Oral buprenorphine is anti-inflammatory and modulates the pathogenesis of streptococcal cell wall polymer-induced arthritis in the Lew/SSN rat. *Lab Anim,* 34**,** 423-9.
WAGNER, A. E. & HELLYER, P. W. (2000) Survey of anesthesia techniques and concerns in private veterinary practice. *J Am Vet Med Assoc,* 217**,** 1652-7.
WAGNER, T. F., LOCH, S., LAMBERT, S., STRAUB, I., MANNEBACH, S., MATHAR, I., DUFER, M., LIS, A., FLOCKERZI, V., PHILIPP, S. E. & OBERWINKLER, J. (2008) Transient receptor potential M3 channels are ionotropic steroid receptors in pancreatic beta cells. *Nat Cell Biol,* 10**,** 1421-30.
WATANABE, H., VRIENS, J., PRENEN, J., DROOGMANS, G., VOETS, T. & NILIUS, B. (2003) Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels. *Nature,* 424**,** 434-8.
WATNEY, G. C., HALL, L. W., JORDAN, C. & NOLAN, A. M. (1988) Effects of xylazine and acepromazine on bronchomotor tone of anaesthetised ponies. *Equine Vet J,* 20**,** 185-8.
WEBB, T. I. & LYNCH, J. W. (2007) Molecular pharmacology of the glycine receptor chloride channel. *Curr Pharm Des,* 13**,** 2350-67.
WEIR, C. J., LING, A. T., BELELLI, D., WILDSMITH, J. A., PETERS, J. A. & LAMBERT, J. J. (2004) The interaction of anaesthetic steroids with recombinant glycine and GABAA receptors. *Br J Anaesth,* 92**,** 704-11.
WEISS, J. & ZIMMERMANN, F. (1999) Tribromoethanol (Avertin) as an anaesthetic in mice. *Lab Anim,* 33**,** 192-3.
WENKER, I. C., BENOIT, J. P., CHEN, X., LIU, H., HORNER, R. L. & MULKEY, D. K. (2012) Nitric oxide activates hypoglossal motoneurons by cGMP-dependent inhibition of TASK channels and cGMP-independent activation of HCN channels. *J Neurophysiol,* 107**,** 1489-99.

WHELAN, G. & FLECKNELL, P. A. (1992) The assessment of depth of anaesthesia in animals and man. *Lab Anim,* 26**,** 153-62.
WHITTEM, T., PASLOSKE, K. S., HEIT, M. C. & RANASINGHE, M. G. (2008) The pharmacokinetics and pharmacodynamics of alfaxalone in cats after single and multiple intravenous administration of Alfaxan at clinical and supraclinical doses. *J Vet Pharmacol Ther.*
WILLIAMSON, L. C., FITZGERALD, S. C. & NEALE, E. A. (1992) Differential effects of tetanus toxin on inhibitory and excitatory neurotransmitter release from mammalian spinal cord cells in culture. *J Neurochem,* 59**,** 2148-57.
WILSON, R. I. & NICOLL, R. A. (2001) Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses. *Nature,* 410**,** 588-92.
WILSON, R. P., ZAGON, I. S., LARACH, D. R. & LANG, C. M. (1993) Cardiovascular and respiratory effects of tiletamine-zolazepam. *Pharmacol Biochem Behav,* 44**,** 1-8.
WINTER, L., NADESON, R., TUCKER, A. P. & GOODCHILD, C. S. (2003) Antinociceptive properties of neurosteroids: a comparison of alphadolone and alphaxalone in potentiation of opioid antinociception. *Anesth Analg,* 97**,** 798-805.
WIXSON, S. K., WHITE, W. J., HUGHES, H. C., JR., LANG, C. M. & MARSHALL, W. K. (1987a) A comparison of pentobarbital, fentanyl-droperidol, ketamine-xylazine and ketamine-diazepam anesthesia in adult male rats. *Lab Anim Sci,* 37**,** 726-30.
WIXSON, S. K., WHITE, W. J., HUGHES, H. C., JR., LANG, C. M. & MARSHALL, W. K. (1987b) The effects of pentobarbital, fentanyl-droperidol, ketamine-xylazine and ketamine-diazepam on arterial blood pH, blood gases, mean arterial blood pressure and heart rate in adult male rats. *Lab Anim Sci,* 37**,** 736-42.
WIXSON, S. K., WHITE, W. J., HUGHES, H. C., JR., LANG, C. M. & MARSHALL, W. K. (1987c) The effects of pentobarbital, fentanyl-droperidol, ketamine-xylazine and ketamine-diazepam on core and surface body temperature regulation in adult male rats. *Lab Anim Sci,* 37**,** 743-9.
WU, F. S., CHEN, S. C. & TSAI, J. J. (1997) Competitive inhibition of the glycine-induced current by pregnenolone sulfate in cultured chick spinal cord neurons. *Brain Res,* 750**,** 318-20.
WU, L. J., SWEET, T. B. & CLAPHAM, D. E. (2010) International Union of Basic and Clinical Pharmacology. LXXVI. Current progress in the mammalian TRP ion channel family. *Pharmacol Rev,* 62**,** 381-404.
ZAKI, S., TICEHURST, K. & MIYAKI, Y. (2009) Clinical evaluation of Alfaxan-CD(R) as an intravenous anaesthetic in young cats. *Aust Vet J,* 87**,** 82-7.
ZELLER, W., MEIER, G., BURKI, K. & PANOUSSIS, B. (1998) Adverse effects of tribromoethanol as used in the production of transgenic mice. *Lab Anim,* 32**,** 407-13.
ZHOU, L., CHILLAG, K. L. & NIGRO, M. A. (2002) Hyperekplexia: a treatable neurogenetic disease. *Brain Dev,* 24**,** 669-74.

APPENDIX ONE

The University of Queensland

School of Veterinary Science St Lucia

RODENT ANAESTHESIA SURVEY

Dear Researcher,

The National Health and Medical Research Council (NHMRC) Guidelines for laboratory animal anaesthesia provide a comprehensive list of anaesthetic agent combinations. Our survey aims to identify the most frequently used anaesthetic regimens for rats and mice and to determine whether there were specific factors that have influenced your choice.

Your first hand experiences in using these anaesthetic agents will collectively provide invaluable information on the efficacy and ease of use of specific anaesthetics.
This in turn will help us to improve our understanding of rodent anaesthesia and therefore the welfare of laboratory animals undergoing anaesthesia.
The information provided will remain anonymous. We greatly appreciate the time you have taken to complete this survey.

With Kind Regards,

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The University of Queensland

School of Veterinary Science St Lucia

RODENT ANAESTHESIA SURVEY

What age/sex/strain/colony of rat/mouse were used?

Rat :

……

Mouse:

……

What procedure/s was the anaesthetic required for? Please list

……

……

……

……

What length of anaesthesia was required to perform the procedure/s?
Please list

……

……

……

……

Do you use gaseous or injectable anaesthesia? Or both?

……

Number of animals:

……

What injectable anaesthetic combinations are you commonly using?

Anaesthetic agent 1 : …….

Route of administration:……

Dose:……..
(Eg. mg/kg, μg/kg or mL per animal)

Anaesthetic agent 2 : …….

Route of administration:……

Dose:……..
(Eg. mg/kg, μg/kg or mL per animal)

Anaesthetic agent 3 :……..……..

Route of administration:……

Dose:……
(Eg. mg/kg, μg/kg or mL per animal)

Do you use an anaesthetic reversal agent with this regimen?

……

Do you use an additional analgesic agent?

……

What is the usual recovery time (eg. time taken to wake up)?

……

Have you encountered any complications from this anaesthetic regimen?
Please indicate the number of animals in the box below
(please provide a percentage if an accurate number is not known- please indicate with %)

- ☐ Inadequate depth of anaesthetic from initial dose
- ☐ Needed top up of anaesthetic during procedure
- ☐ Stopped breathing
- ☐ Unexpected wake up
- ☐ Took a long time to recover from anaesthetic
- ☐ Animal was cold (if yes, was heating provided? ……..)
- ☐ Death
- ☐ Other ……

(please comment)

……

……

What do you feel are the advantages and disadvantages of this anaesthetic regimen?
…….

…….

…….

…….

In your opinion, what factors would you like improved from this anaesthetic regimen?
Eg. Faster anaesthetic recovery

…….

…….

…….

……..

Is your choice of anaesthetic related to the schedule of the drug and/or availability?
…….

…….

…….

……..

Any comments?

…….

…….

…….

Thank you for your participation in this survey. If you have any questions in regards to this survey, please feel free to contact me by email: c.lau1@uq.edu.au or mob: 0413 120 931

Cora Lau BVSc (Hons)
University of Queensland
School of Veterinary Science
PhD Candidate

APPENDIX TWO

A copy of the published article from Chapter Two is attached to the end of this document

Title: Plasma pharmacokinetics of alfaxalone after a single intraperitoneal or intravenous injection of Alfaxan® in rats

	Intramuscular		
	Induction time (minutes)	Immobilisation time (minutes)	Twitch Score
Rat 2.1.1	2	7	++
Rat 2.1.2	2	12	++
Rat 2.1.3	3	7	++
Rat 2.1.4	2	13	++
Rat 2.1.5	4	1	-

	Subcutaneous		
	Induction time (minutes)	Immobilisation time (minutes)	Twitch Score
Rat 2.1.1	4	1	-
Rat 2.1.2	4	4	-
Rat 2.1.3	Not Induced		+
Rat 2.1.4	Not Induced		-
Rat 2.1.5	4	1	+

	Intraperitoneal		
	Induction time (minutes)	Immobilisation time (minutes)	Twitch Score
Rat 2.1.1	Not Induced		-
Rat 2.1.2	Not Induced		-
Rat 2.1.3	10	2*	-
Rat 2.1.4	Not Induced		-
Rat 2.1.5	Not Induced		-

Appendix 2.1. Anaesthetic parameters for adult female Wistar rats administered a single dose of 10mg.kg^{-1} Alfaxalone IM, SC and IP.

*A loss of righting reflex allowed a laterally recumbent position.

All other rats were maintained dorsal recumbency during the immobilisation period.

		20mg/kg Intraperitoneal Alfaxan®		
		Induction time (minutes)	Immobilisation time (minutes)	Twitch Score
Rat 2.2.1	week 1	5	35	+++
	week 2	3	41	++
	week 3	6	42	+++
	week 4	5	31	+++
Rat 2.2.2	week 1	2	58	++
	week 2	4	41	++
	week 3	Not Induced		-
	week 4	5	35	+++
Rat 2.2.3	week 1	3	40	++
	week 2	Not Induced		-
	week 3			
	week 4			
Rat 2.2.4	week 1	4	35	++
	week 2	Not Induced		-
	week 3			
	week 4			
	Range	2 to 6	31 to 58	Total twitching
	Mean	4.11	39.78	9 of 12
	SD	1.20	7.35	75%

Appendix 2.2. Anaesthetic parameters for adult female Wistar rats administered weekly injections of 20mg.kg^{-1} IP Alfaxalone.

Anaesthetic injections were administered once weekly for four consecutive weeks. Induction time, immobilisation time and twitching was recorded. Results are recorded as mean ± SD and range. compare

a)

	20 mg.kg^{-1} Subcutaneous Alfaxan®		
	Induction time (minutes)	Immobilisation time (minutes)	Twitch Score
Rat 2.3.1	Not Induced		-
Rat 2.3.2	4	2	-
Rat 2.3.3	9	30	++
Rat 2.3.4	20	25	-
Rat 2.3.5	4	6	+
Rat 2.3.6	10	40	-
Rat 2.3.7	21	9	+++
Rat 2.3.8	Not Induced		-
Range	**4 to 21**	**2 to 40**	**Total twitching**
Mean	**11.33**	**18.67**	**3 of 8**
SD	**7.53**	**15.20**	**37.5%**

b)

	30 mg.kg^{-1} Subcutaneous Alfaxan®		
	Induction time (minutes)	Immobilisation time (minutes)	Twitch Score
Rat 2.3.6	3	83	+++
Rat 2.3.7	5	80	+++
Rat 2.3.8	20	39	+
Range	**3 to 20**	**39 to 83**	**100%**

Appendix 2.3. Anaesthetic parameters for adult female Wistar rats administered a single dose of SC Alfaxalone.

a) The increase in variability in induction and immobilisation times in comparison to the previous 10 mg.kg^{-1} SC Alfaxan® study was not expected. Following on from this study, Rats 6, 7 and 8 were given dose of Alfaxan® a week later at 30 mg.kg^{-1}.

b) The immobilisation time increased with the increase in dose from 20 mg.kg^{-1} to 30 mg.kg^{-1} SC however the variability in induction time and immobilisation time between animals was still pronounced.

	30 mg.kg^{-1} Intraperitoneal Alfaxan®		
	Induction time (minutes)	Immobilisation time (minutes)	Twitch Score
SD 1	2	31	+
SD 2	3	33	-
SD 3	2	12	-
SD 4	3	22	-
SD 5	3	7	-
SD 6	3	17	-
SD 7	3	13	+
SD 8	3	11	-
SD 9	4	13	-
SD 10	Not induced		+
SD 11	Not induced		-
SD 12	2	20	+
SD 13	Not induced		-
SD 14	2	24	+++
Range	**2 to 4**	**7 to 33**	**Total twitching**
Mean	**2.73**	**18.45**	**5 of 14**
SD	**0.65**	**8.39**	**33.7%**

Appendix 2.4. Anaesthetic parameters for adult male Sprague Dawley rats administered a single dose of 30 mg.kg^{-1} SC Alfaxalone.

Fourteen Sprague Dawley rats were administered a single dose of anaesthetic. Induction time, immobilisation time and twitching was recorded as mean ± SD and range. Three of fourteen rats were not induced for anaesthesia.

APPENDIX THREE

	ACP-Methadone (3 mg.kg^{-1}-0.7 mg.kg^{-1} IP)			
	Sedation Level	**Induction time (minutes)**	**Immobilisation time (minutes)**	**Twitch Score**
Rat 2.7.1	Light	Not Induced		+++
Rat 2.7.2	Light	2	86	+
Rat 2.7.3	Moderate	3	64	++
Rat 2.7.4	Light	5	52	+
Rat 2.7.5	Light	3	67	+++
Rat 2.7.6	Light	3	57	+
Rat 2.7.7	Light	4	49	+++
Rat 2.7.8	Light	5	47	++
Rat 2.7.9	Light	3	51	+
Rat 2.7.10	Light	5	49	-
Rat 2.7.11	Light	3	60	+
Rat 2.7.12	Moderate	17	25	-
Rat 2.7.13	Moderate	4	55	+++
Rat 2.7.15	Light	3	34	+
Rat 2.7.17	Moderate	4	75	+
Rat 2.7.18	Light	4	48	++
	Range	**2 to 17**	**25 to 86**	**Total twitching**
	Mean	**4.5**	**54.6**	**14 of 16**
	SD	**3.6**	**15.0**	**87.50%**

Appendix 3.1. Alfaxalone Anaesthesia with ACP-Methadone Premedication

Rats were premedicated with ACP-methadone followed by 20 mg.kg^{-1} alfaxalone. Sedation level, induction time, immobilisation time and twitching was recorded as mean ± SD and range.

	ACP-Xylazine (3 mg.kg^{-1}-3 mg.kg^{-1} IP)			
	Sedation Level	**Induction time (minutes)**	**Immobilisation time (minutes)**	**Twitch Score**
Rat 2.7.1	Moderate	13	17	+
Rat 2.7.2	Moderate	1	132	++
Rat 2.7.3	Heavy	2	85	+++
Rat 2.7.4	Moderate	3	54	+
Rat 2.7.5	Moderate	4	48	-
Rat 2.7.6	Light	Not Induced		-
Rat 2.7.7	Moderate	4	74	+
Rat 2.7.8	Heavy	2	85	+
Rat 2.7.9	Heavy	2	68	-
Rat 2.7.10	Light	5	40	-
Rat 2.7.11	Moderate	1	61	-
Rat 2.7.12	Heavy	1	64	-
Rat 2.7.13	Moderate	2	48	-
Rat 2.7.14	Light	Not Induced		-
Rat 2.7.15	Moderate	1	56	-
Rat 2.7.16	Heavy	3	49	-
	Range	**1 to 13**	**17 to 132**	**Total twitching**
	Mean	**2.4**	**66.5**	**6 of 16**
	SD	**1.3**	**24.2**	**37.50%**

Appendix 3.2. Alfaxalone Anaesthesia with ACP-Xylazine Premedication

Rats were premedicated with ACP-xylazine followed by 20 mg.kg^{-1} alfaxalone. Sedation level, induction time, immobilisation time and twitching was recorded as mean ± SD and range.

	Medetomidine (0.5 mg.kg^{-1} IP)			
	Sedation Level	**Induction time (minutes)**	**Immobilisation time (minutes)**	**Twitch Score**
Rat 2.7.1	Moderate	4	96	+
Rat 2.7.2	Moderate	6	85	+
Rat 2.7.3	Moderate	3	108	-
Rat 2.7.4	Moderate	2	129	+
Rat 2.7.5	Moderate	1	159	+
Rat 2.7.6	Moderate	2	151	+
Rat 2.7.7	Heavy	1	163	+
Rat 2.7.8	Moderate	1	160	-
Rat 2.7.9	Heavy	0	181	-
Rat 2.7.10	Moderate	1	145	-
Rat 2.7.11	Moderate	2	154	-
Rat 2.7.12	Light	2	151	+
Rat 2.7.13	Moderate	2	65	-
Rat 2.7.14	Light	1*	prolonged	-
Rat 2.7.15	Heavy	1	115	-
Rat 2.7.16	Heavy	2*	prolonged	-
	Range	**0 to 6**	**65 to 181**	**Total twitching**
	Mean	**2.0**	**133**	**7 of 16**
	SD	**1.5**	**34.1**	**43.75%**

Appendix 3.3. Alfaxalone Anaesthesia with Medetomidine Premedication
Rats were premedicated with medetomidine followed by 20 mg.kg^{-1} alfaxalone. Sedation level, induction time, immobilisation time and twitching was recorded as mean ± SD and range.
* indicates rat 2.7.14 and 2.7.16 died during anaesthesia. Note rat 2.7.14 was not deeply sedated following premedication.

	Medetomidine-Butorphanol (0.35 mg.kg^{-1}-0.2 mg.kg^{-1} IP)			
	Sedation Level	**Induction time (minutes)**	**Immobilisation time (minutes)**	**Twitch Score**
Rat 2.7.1	Light	3	113	-
Rat 2.7.2	Heavy	3	140	+
Rat 2.7.3	Moderate	1	145	+
Rat 2.7.4	Moderate	3	100	+
Rat 2.7.5	Moderate	3	73	+
Rat 2.7.6	Heavy	3	110	-
Rat 2.7.7	Light	3	81	-
Rat 2.7.8	Light	1	164	+
Rat 2.7.9	Moderate	2	145	-
Rat 2.7.10	Moderate	2	120	-
Rat 2.7.11	Light	2	139	-
Rat 2.7.12	Heavy	3	134	-
Rat 2.7.13	Moderate	2	144	-
Rat 2.7.15	Moderate	3	129	-
Rat 2.7.17	Light	2	129	-
Rat 2.7.18	Light	10	57	+
	Range	**1 to 10**	**57 to 164**	**Total twitching**
	Mean	**2.9**	**120.2**	**6 of 16**
	SD	**2.0**	**29.6**	**37.50%**

Appendix 3.4. Alfaxalone Anaesthesia with Medetomidine-Butorphanol Premedication

Rats were premedicated with medetomidine-butorphanol followed by 20 mg.kg^{-1} alfaxalone. Sedation level, induction time, immobilisation time and twitching was recorded as mean ± SD and range.

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Veterinary Pharmacology and Therapeutics

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Plasma pharmacokinetics of alfaxalone after a single intraperitoneal or intravenous injection of Alfaxan® in rats

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Alfaxalone (3α-hydroxy-5α-pregnane-11, 20-dione) is a neuroactive steroid with anaesthetic properties and a wide margin of safety. The pharmacokinetic properties of alfaxalone administered intravenously and intraperitoneally in rats (n = 28) were investigated. Mean $t_{1/2elim}$ for 2 and 5 mg/kg i.v. was 16.2 and 17.6 min, respectively, but could not be estimated for IP dosing, due to sustained plasma levels for up to 60 min after injection. Cl_p for i.v. injection was calculated at 57.8 ± 23.6 and 54.3 ± 6.8 mL/min/kg, which were 24.5% and 23% of cardiac output, respectively. The observed C_{max} was 3.0 mg/L for IP administration, and 2.2 ± 0.9 and 5.2 ± 1.3 mg/L for 2 and 5 mg/kg i.v. administration, respectively. $AUC_{0–60}$ was 96.2 min·mg/L for IP dosing. The relative bioavailability for IP dosing was 26% and 28% compared to i.v. dosing. Differences in $t_{1/2elim}$ and Cl_p from previous pharmacokinetic studies in rats are likely due to variations in alfaxalone formulation rather than sex differences. Alfaxan® given IP caused sustained levels of alfaxalone, no apnoea and longer sleep times than i.v. dosing, although immobilization was not induced in 30% of rats given Alfaxan® IP. A pharmacodynamic study of the effects of combining IP injection of Alfaxan® with other premedication agents is worthwhile, to determine whether improved anaesthesia induction could ultimately provide an alternative anaesthetic regimen for rats.

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INTRODUCTION

Alfaxalone (3α-hydroxy-5α-pregnane-11, 20-dione) is a neuroactive steroid with anaesthetic properties due to its positive allosteric modulation of the gamma aminobutyric acid type A ($GABA_A$) receptor (Visser *et al.*, 2000). Alfaxalone solubilized with the excipient 2-hydroxypropyl-β-cyclodextrin (HPCD) is available as Alfaxan® (Jurox Pty Ltd, Rutherford, NSW, Australia), which is registered for the induction and maintenance of anaesthetic in both dogs and cats in multiple countries. Studies have shown that intravenous Alfaxan® provides satisfactory induction of anaesthesia in dogs (Ferre *et al.*, 2006), cats (Zaki *et al.*, 2009), horses and pigs (Keates, 2003; Whittem *et al.*, 2008; Goodwin *et al.*, 2011, 2012), with a fivefold margin of safety at supraclinical doses in dogs (Ferre *et al.*, 2006; Muir *et al.*, 2008) and cats (Whittem *et al.*, 2008). The pharmacokinetic parameters of alfaxalone have been defined in cats (single/multiple doses), dogs (single dose) and horses (single dose) (Ferre *et al.*, 2006; Whittem *et al.*, 2008; Pasloske *et al.*, 2009; Goodwin *et al.*, 2011, 2012).

Rodents are commonly anaesthetized for veterinary and biomedical research purposes with a single intraperitoneal (IP) injection. While IP injection is technically simple and requires minimal animal restraint, a single dose of anaesthetic can lead to highly variable anaesthetic effects (Flecknell, 1993). Therefore, it is important for an injectable agent to have a wide margin of safety to account for variation in anaesthetic responses between individuals. Alfaxalone has been investigated in rats in varying formulations including acetonitrile (Visser *et al.*, 2000) and the alfaxalone/alfadolone combination for humans (Althesin®, GlaxoSmithKline, UK) and animals (Saffan®, Pitman-Moore, Terre Haute, IN, USA), equivalent to 9 mg/mL alfaxalone in the solvent Cremophor EL (Child *et al.*, 1972). As yet no work has been performed in rodents with the commercially available formulation Alfaxan®, which is devoid of alfadolone and Cremophor EL. In this study, we investigated the single-dose pharmacokinetics of

alfaxalone, administered intravenously or intraperitoneally as Alfaxan® in rats.

All experiments were performed after approval by the University of Queensland Animal Ethics Committee (Approval number: 301/10). Adult female Wistar rats (7–10 weeks, 244.2 ± 39.4 g, $n = 28$) housed in a barrier-maintained animal facility with a 12/12 h light/dark cycle and fed standard rat pellets and water *ad libitum* were used. For IP dosing, a single injection (20 mg/kg, $n = 18$) of Alfaxan® (Batch # 60091) was administered intraperitoneally into the right lower quadrant of the ventral abdomen. Serial blood samples collected from a cut on the tail tip were taken from 10 rats at 4, 6, 10, 15, 20, 30, 45 and 60 min postinjection; samples were not collected from all 10 rats at all times, due to variability in time to recovery from immobilization (Table 1) or failure to collect a sufficient volume of blood at different time points. A single intracardiac blood sample was collected at one of these times postinjection in another eight rats following termination. This sampling collectively gave sample times of 4, 6, 10, 15, 20, 30, 45 and 60 min with varying numbers of animals sampled per time point for IP injection (see Fig. 1 for number of animals sampled per time point). For i.v. dosing, an indwelling i.v. catheter (polyethylene tubing 50 mm length, 0.50 mm OD, 0.20 mm ID) was introduced into the femoral vein, following anaesthesia with isoflurane gas using a Tec 3 isoflurane vapourizer attached to a rodent circuit and size 1, 6.0 cm anaesthetic face mask (all from Vetquip, Castle Hill, NSW, Australia). The catheter was fixed to the skin with cyanoacrylate adhesive. Rats recovered from isoflurane anaesthesia for 30–60 min postimplantation prior to i.v. dosing. Alfaxan® (Batch # 60091), diluted to 1 mg/mL with sterile water for injection (Norbrook®, Tullamarine, Vic, Australia), was administered by slow, continuous i.v. infusion over 60 sec (2 mg/kg, $n = 5$; 5 mg/kg, $n = 5$). For all animals, the time from injection to immobilization (failure of the rat to right itself from lateral or dorsal recumbency) and to recovery (the time at which sternal recumbency was restored) was recorded as induction and recovery times, respectively, and the difference between these times was calculated as sleep time. Tail tip blood samples (maximum volume 200 μL, maximum total volume 1.6 mL)

Table 1. Various pharmacodynamic parameters for individual adult Wistar rats administered an injection of Alfaxan® (Jurox Pty Ltd) at 2 and 5 mg/kg i.v., and 20 mg/kg IP

Rat ID	Induction time (sec)	Sleep time (min)	Apnoea (sec)	Twitch on induction (y/n)	Twitch on recovery (y/n)
2 mg/kg i.v.					
Rat 4.3	20	22	100	n	n
Rat 5.3	10	7	170	n	n
Rat 6.3	60	8	0	n	n
Rat 7.3	40	6	0	n	y
Rat 12.3	60	9	0	n	y
Mean	38	10.4	54		
SD	22.8	6.6	78.0		
Range	10–60	6–22	0–170	No twitching	2 of 5 twitched
5 mg/kg i.v.					
Rat 8.3	60	9	95	n	n
Rat 9.3	27	18	108	n	n
Rat 10.3	20	12	100	n	n
Rat 11.3	12	12	113	n	n
Rat 13.3	60	12	0	n	n
Mean	35.8	12.6	83.2		
SD	22.7	3.3	47.0		
Range	12–60	9–12	0–113	No twitching	No twitching

Rat ID	Induction time (min)	Sleep time (min)	Apnoea (sec)	Twitch on induction (y/n)	Twitch on recovery (y/n)
20 mg/kg IP					
Rat 1	5	35	0	y	n
Rat 2	2	58	0	y	y
Rat 3	3	40	0	y	y
Rat 4	4	35	0	n	y
Rat 5	3	41	0	y	n
Rat 6	4	45	0	y	y
Rat 7	Not induced	0	0	y	y
Rat 8	Not induced	0	0	n	n
Rat 9	6	42	0	y	y
Rat 10	Not induced	0	0	y	y
Mean	3.9	29.6	0		
SD	1.3	21.4	0		
Range	3–6	0–58	0	8 of 10 twitched	7 of 10 twitched

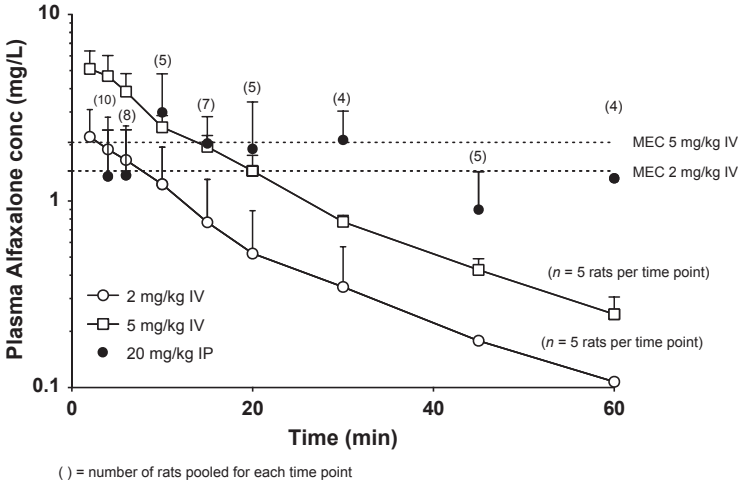

Fig. 1. Plasma alfaxalone concentration over time in rats administered Alfaxan® at 2 mg/kg i.v., 5 mg/kg i.v. and 20 mg/kg IP doses. Data are mean ± SD. As data for IP injection are derived from different subsets of animals at different sample times (number of animals contributing to each sample time are indicated in parentheses next to the sample time), the symbols are not connected. The mean minimum effective concentration (MEC) for each i.v. dose, defined as the mean minimum plasma alfaxalone concentration required to maintain immobilization, was calculated by extrapolating the plasma concentrations at the time of recovery from immobilization from individual plasma concentrations and is presented as a mean, indicated by the dashed lines on the graph.

were collected from each rat at 2, 4, 6, 10, 15, 20, 30, 45 and 60 min and immediately placed onto wet ice and then centrifuged (4000 ***g***, 10 min) within an hour of collection. The minimum effective concentration (MEC) for immobilization was empirically determined by interpolation between plasma concentration before and after the time of recovery from immobilization. Plasma samples were kept frozen at −80 ± 10 °C during storage and transport to the Jurox Chemistry Laboratory until analysis. All plasma samples were analysed individually (Ferre *et al.*, 2006; Whittem *et al.*, 2008; Pasloske *et al.*, 2009).

ALFAXALONE ANALYSIS

Plasma samples underwent solid phase extraction (25–50 μL sample volume), followed by liquid chromatography–mass spectrometry (10 μL sample volume) over a 24-h period in the same analytical run using previously described methods (Goodwin *et al.*, 2012). A calibration curve was generated using a standard sample set prepared by spiking known amounts of 11-hydroxyprogesterone as the internal standard and alfaxalone (two linear standard curves covering 0.02–0.5 and 0.5–5 mg/L, $r^2 > 0.99$ for both segments) into extracted blank rat plasma. Sample alfaxalone concentration was determined by the alfaxalone/11-hydroxyprogesterone peak area ratio. The assay lower limit of quantification (LLOQ) was 0.02 mg/L in extracted plasma. The intra-assay coefficient of variation at LLOQ was <18.5% and <11.0% for other concentration levels. The limit of detection (LOD) was 0.002 mg/L. Sample extraction recoveries were >86% for all concentration levels. Both accuracy and precision were acceptable if residual standard deviation (RSD) was ±<20% at LLOQ and ±<15% for other concentrations. Due to variations in the number of samples per sample time collected from rats receiving IP injection, data analysis was performed on pooled data for each time point following IP injection, while data from rats receiving i.v. injections were analysed for each animal individually (Ferre *et al.*, 2006; Whittem *et al.*, 2008; Pasloske *et al.*, 2009). Pharmacokinetic analysis was carried out using noncompartmental methods in Phoenix® WinNonLin® v 6.3 (Pharsight Products, Sunnyvale, CA, USA) as previously described (Goodwin *et al.*, 2012). The area under the curve (AUC) from time of administration to the last blood sample ($AUC_{0–last}$) was calculated with the linear trapezoidal rule (Gibaldi & Perrier, 1982). The terminal slope of the curve (λ_z), estimated by linear regression of ≥ 3 data points using the best-fit function, was extrapolated to calculate the total AUC from administration time to infinity ($AUC_{0–inf}$) as $AUC_{0–last}$ + the extrapolated AUC. The% AUC extrapolated from the last sample time (AUC_{extrap}%) was calculated as the extrapolated AUC ÷ $AUC_{0–inf}$ × 100%. The plasma clearance (Cl_p), volume of distribution (V_D) and elimination half-life ($t_{1/2elim}$) were calculated using standard noncompartmental formulae. For concentration parameters, a best-fit approach was utilized where repeated nonlinear regressions were used for a series of terminal points. For each regression, an adjusted R^2 was computed with:

$$\text{Adjusted } R^2 = \frac{1-(1-R^2)\times(n-1)}{(n-2)}$$

where n are the number of data points in the regression and R^2 is the square of the correlation coefficient. The relative bioavailability (F%) for IP dosing compared to i.v. dosing was calculated with:

$$\text{F}\% = \frac{\text{AUC (IP route)}}{\text{AUC (i.v. route)}} \times \frac{\text{Dose (i.v.)}}{\text{Dose (IP)}} \times 100$$

(Toutain & Bousquet-Melou, 2004a). All data are expressed as mean ± SD.

The level of anaesthesia achieved by alfaxalone was not tested by evaluating reflex responses or making serial cardiorespiratory measurements. However, a brief description of the immobilization achieved is useful, in particular, to note that anaesthesia was sufficient to allow repeated blood sampling from a tail tip cut, which involved manual massage of the tail to expel blood from the wound. Apnoea, lasting 95–170 sec, occurred in two of five rats at 2 mg/kg i.v. and four of five rats at 5 mg/kg i.v. dosing, followed by recovery of spontaneous breathing. The mean sleep times for rats given 2 and 5 mg/kg i.v. were 10.4 ± 6.6 and 12.6 ± 3.3 min, respectively, which were not significantly different ($P = 0.52$, unpaired two-tailed t test). In contrast, after IP dosing, immobilization was not induced in three of 10 rats, but no apnoea was observed (Table 1). The mean sleep time for all rats given 20 mg/kg IP was 29.6 ± 21.4 min, which was not significantly longer than for either i.v. dose ($P > 0.05$, unpaired two-tailed t test); excluding rats in which no immobilization was induced, mean sleep time was 42.3 ± 7.8 min, which was significantly longer than for either i.v. dose ($P < 0.0001$, unpaired two-tailed t test). Animals that did not become immobilized had plasma concentrations of alfaxalone in the lower end of the range (0.58–0.78 mg/L) at 4 min after injection; animals that did show immobilization had similar or higher plasma alfaxalone levels at 4 min (range 0.47–3.78 mg/L). As all animals given alfaxalone i.v. showed immobilization, we suggest that variable absorption from the site of IP injection is the most likely cause for lack of immobilization. The mean MEC for immobilization of rats receiving 2 or 5 mg/kg i.v. was 1.5 ± 0.8 and 2.1 ± 0.4 mg/L, respectively, (Fig. 1) and was estimated from pooled data to be 1.9 mg/L for IP dosing. IP injection caused muscular twitching in eight rats during induction and seven rats on recovery, with six rats twitching during both induction and recovery. Mild facial twitching occurred in two rats with 2 mg/kg i.v. alfaxalone. Mean $t_{1/2elim}$ for 2 and 5 mg/kg i.v. was similar (16.2 ± 9.2 and 17.6 ± 3.2 min, respectively) as was the mean Cl_p (57.8 ± 23.6 and 54.3 ± 6.8 mL/min/kg, respectively; Table 2). To facilitate comparison of Cl_p between species, Cl_p was calculated at 24.5% and 23% of cardiac output, respectively (Toutain & Bousquet-Melou, 2004b). The mean Cl_p in rats following i.v. injection was similar to that reported for the dog [53 and 59 mL/kg/min for 2 and 10 mg/kg (Ferre *et al.*, 2006)], but was larger than the mean Cl_p reported for cats, horses and foals with 25, 37 and 20 mL/min/kg, for 5, 1 and 3 mg/kg i.v., respectively, (Whittem *et al.*, 2008; Goodwin *et al.*, 2011, 2012). In contrast to the high Cl_p of alfaxalone following i.v. injection, sampling times of $\leq$60 min were not adequate to reliably determine alfaxalone Cl_p following IP dosing (Fig. 1). We observed a C_{max} of 2.99 mg/L at 10 min after IP administration, a value between the C_{max} of 2.2 ± 0.9 and 5.2 ± 1.3 mg/L for 2 and 5 mg/kg i.v., respectively; by comparison, the C_{max} seen in dogs following 2 mg/kg i.v. was similar (Ferre *et al.*, 2006). In contrast, IP dosing led to sustained alfaxalone plasma levels, with an AUC_{0-last} of 96.2 min/mg/L, in comparison with AUC_{0-last} of 36.9 and 86.7 for 2 and 5 mg/kg i.v., respectively. Sustained plasma alfaxalone levels are the most likely cause of longer sleep times following IP injection (Fink *et al.*, 1982), compared with i.v. administration. The relative bioavailability for 20 mg/kg alfaxalone given IP was 26% and 28%, compared to the 2 and 5 mg/kg doses given i.v., respectively. This low bioavailability via the IP route is consistent with high first-pass elimination of alfaxalone through hepatic metabolism (Child *et al.*, 1972; Novelli *et al.*, 1975; Sear & McGivan, 1981) after IP

Table 2. Pharmacokinetic parameters for 10 adult Wistar rats after administration of a 60-sec 'bolus' injection of Alfaxan® (Jurox Pty Ltd) at 2 and 5 mg/kg

Rat ID	$t_{1/2elim}$ (min)	Cl_p (mL/min/kg)	V_Z (L/kg)	AUC_{0-last} (min mg/L)	AUC_{0-inf} (min mg/L)	AUC% extrap	C_{max} observed (mg/L)	C_{max0} extrap (mg/L)
2 mg/kg i.v.								
Rat 4.3	10.4	68.8	1.0	28.2	29.1	3.0	2.491	3.148
Rat 5.3	11.9	38.9	0.7	50.1	51.4	2.5	2.562	2.676
Rat 6.3	14.6	90.3	1.9	20.2	22.1	8.8	1.402	2.236
Rat 7.3	32.5	59.2	2.8	25.2	33.8	25.3	1.216	1.342
Rat 12.3	11.8	31.7	0.5	60.9	63.1	3.4	3.366	3.679
Mean	16.2	57.8	1.4	36.9	39.9	8.6	2.207	2.616
SD	9.2	23.6	0.9	17.6	16.9	9.7	0.892	0.892
Range	10.4–32.5	31.7–90.3	0.5–2.8	20.2–60.9	22.1–63.1	2.5–25.3	1.2–3.4	1.3–3.7
5 mg/kg i.v.								
Rat 8.3	17.8	53.9	1.4	86.3	92.8	7.0	5.578	6.940
Rat 9.3	15.0	48.8	1.1	98.3	102.5	4.2	6.607	7.380
Rat 10.3	15.8	47.2	1.1	100.9	105.9	4.7	6.224	5.730
Rat 11.3	16.7	63.9	1.5	72.9	78.3	6.9	4.207	5.404
Rat 13.3	23.0	57.6	1.9	75.3	86.8	13.2	3.528	3.427
Mean	17.6	54.3	1.4	86.7	93.3	7.2	5.229	5.776
SD	3.2	6.8	0.4	12.8	11.3	3.6	1.318	1.548
Range	15.0–23.0	47.2–63.9	1.1–1.9	72.9–100.9	78.3–105.9	4.2–13.2	3.5–6.6	3.4–7.4
20 mg/kg IP								
Pooled	NA	NA	NA	96.195	NA	NA	2.99	NA

injection, which predominantly enters the portal circulation (Lukas *et al.*, 1971). The $t_{1/2elim}$ of 16–17 min for the i.v. doses in our study using adult female Wistar rats is longer than for male Charles River rats (Child *et al.*, 1972) or for male Wistar rats (Visser *et al.*, 2000) (7 min for ≈ 9 mg/kg alfaxalone as Althesin® i.v. and 13 min for 5 mg/kg alfaxalone in acetonitrile i.v., respectively). The longer $t_{1/2elim}$ observed in our study is consistent with the lower Cl_p we observed, in comparison with a Cl_p of 71 mL/min/kg (Visser *et al.*, 2000). While sex could account for differences in $t_{1/2elim}$ and Cl_p in our and other pharmacokinetic studies in rat, and female Wistar rats had a 64% higher C_{max} than males after IP administration of Althesin® (Fink *et al.*, 1982), the pharmacokinetics of alfaxalone given i.v. are reported to be similar in male and female dogs (Ferre *et al.*, 2006) and cats (Whittem *et al.*, 2008). Methodological differences in analysis of plasma alfaxalone between older (Child *et al.*, 1972; Fink *et al.*, 1982) and more recent (Visser *et al.*, 2000) studies could contribute to the differences between our observations and previous reports; however, both methodologies produce $t_{1/2elim}$ values lower than our observations. Variation in alfaxalone formulation is therefore the more likely cause of differences in the pharmacokinetics of alfaxalone in rats. Alfaxan® given IP produced some attractive anaesthetic qualities, including sustained plasma levels of alfaxalone, absence of apnoea and longer sleep times, compared to i.v. dosing. However, immobilization was not induced in 30% of rats given Alfaxan® IP, possibly related to the lower initial plasma concentrations observed in these animals, and muscle twitching was frequently observed. A pharmacodynamic study of the effects of combining IP injection of Alfaxan® with other premedication agents would be useful, to determine whether this would reliably improve anaesthesia induction rates and reduce muscle twitching, and thus provide an alternative anaesthetic regimen for rats with a wide margin of safety.

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REFERENCES

Child, K.J., Gibson, W., Harnby, G. & Hart, J.W. (1972) Metabolism and excretion of Althesin (CT 1341) in the rat. *Postgraduate Medical Journal*, **48** (Suppl. 2), 37–43.

Ferre, P.J., Pasloske, K., Whittem, T., Ranasinghe, M.G., Li, Q. & Lefebvre, H.P. (2006) Plasma pharmacokinetics of alfaxalone in dogs after an intravenous bolus of Alfaxan-CD RTU. *Veterinary Anaesthesia and Analgesia*, **33**, 229–236.

Fink, G., Sarkar, D.K., Dow, R.C., Dick, H., Borthwick, N., Malnick, S. & Twine, M. (1982) Sex difference in response to alphaxalone anaesthesia may be oestrogen dependent. *Nature*, **298**, 270–272.

Flecknell, P.A. (1993) Anesthesia and perioperative care. *Methods in Enzymology*, **225**, 16–33.

Gibaldi, M. & Perrier, D. (1982) Estimation of Areas. *Pharmacokinetics*, pp. 445–449. Marcel Dekker, Basel.

Goodwin, W.A., Keates, H.L., Pasloske, K., Pearson, M., Sauer, B. & Ranasinghe, M.G. (2011) The pharmacokinetics and pharmacodynamics of the injectable anaesthetic alfaxalone in the horse. *Veterinary Anaesthesia and Analgesia*, **38**, 431–438.

Goodwin, W., Keates, H., Pasloske, K., Pearson, M., Sauer, B. & Ranasinghe, M.G. (2012) Plasma pharmacokinetics and pharmacodynamics of alfaxalone in neonatal foals after an intravenous bolus of alfaxalone following premedication with butorphanol tartrate. *Veterinary Anaesthesia and Analgesia*, **39**, 503–510.

Keates, H. (2003) Induction of anaesthesia in pigs using a new alphaxalone formulation. *The Veterinary Record*, **153**, 627–628.

Lukas, G., Brindle, S.D. & Greengard, P. (1971) The route of absorption of intraperitoneally administered compounds. *Journal of Pharmacology and Experimental Therapeutics*, **178**, 562–564.

Muir, W., Lerche, P., Wiese, A., Nelson, L., Pasloske, K. & Whittem, T. (2008) Cardiorespiratory and anesthetic effects of clinical and supraclinical doses of alfaxalone in dogs. *Veterinary Anaesthesia and Analgesia*, **35**, 451–462.

Novelli, G.P., Marsili, M. & Lorenzi, P. (1975) Influence of liver metabolism on the actions of althesin and thiopentone. *British Journal of Anaesthesia*, **47**, 913–916.

Pasloske, K., Sauer, B., Perkins, N. & Whittem, T. (2009) Plasma pharmacokinetics of alfaxalone in both premedicated and unpremedicated Greyhound dogs after single, intravenous administration of Alfaxan at a clinical dose. *Journal of Veterinary Pharmacology and Therapeutics*, **32**, 510–513.

Sear, J.W. & McGivan, J.D. (1981) Metabolism of alphaxalone in the rat: evidence for the limitation of the anaesthetic effect by the rate of degradation through the hepatic mixed function oxygenase system. *British Journal of Anaesthesia*, **53**, 417–424.

Toutain, P.L. & Bousquet-Melou, A. (2004a) Bioavailability and its assessment. *Journal of Veterinary Pharmacology and Therapeutics*, **27**, 455–466.

Toutain, P.L. & Bousquet-Melou, A. (2004b) Plasma clearance. *Journal of Veterinary Pharmacology and Therapeutics*, **27**, 415–425.

Visser, S.A., Smulders, C.J., Gladdines, W.W., Irth, H., van der Graaf, P.H. & Danhof, M. (2000) High-performance liquid chromatography of the neuroactive steroids alphaxalone and pregnenolone in plasma using dansyl hydrazine as fluorescent label: application to a pharmacokinetic-pharmacodynamic study in rats. *Journal of Chromatography B: Biomedical Sciences and Applications*, **745**, 357–363.

Whittem, T., Pasloske, K.S., Heit, M.C. & Ranasinghe, M.G. (2008) The pharmacokinetics and pharmacodynamics of alfaxalone in cats after single and multiple intravenous administration of Alfaxan at clinical and supraclinical doses. *Journal of Veterinary Pharmacology and Therapeutics*, **31**, 571–579.

Zaki, S., Ticehurst, K. and Miyaki, Y. (2009) Clinical evaluation of Alfaxan-CD(R) as an intravenous anaesthetic in young cats. *Australian Veterinary Journal*, **87**, 82–87.