Pharmacogenetics of Ketamine Metabolism and Immunopharmacology of Ketamine

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Abstract

Ketamine is an anaesthetic agent that is being increasingly used at sub-anaesthetic doses as an analgesic or co-analgesic in the management of postoperative pain and chronic pain. In most countries, ketamine is administered as a racemic compound consisting of two enantiomers: *(S)*- and *(R)*-ketamine at a ratio of 1:1. Ketamine analgesia is frequently restricted by the low efficacy and large interindividual variability in drug response, which may be associated with the differences in the plasma pharmacokinetics. Previous *in vitro* studies suggested that ketamine is primarily cleared to its active metabolite, norketamine, by two hepatic CYP enzymes: CYP2B6 and CYP3A4, whose expression and catalytic activities show vary large variability in humans due to genetic and environmental influences. Therefore, it is logical that the variability in these enzymes contributes to the variability in ketamine pharmacokinetics. Additional variability in analgesic response may arise from the heterogeneous nature of pain, as ketamine is expected to be more effective against hyperalgesia and allodynia (neuropathic pain). Although these anti-hyperalgesic and anti-allodynic mechanisms have been primarily associated with the non-competitive antagonism of neuronal NMDA receptors, it has been speculated that the attenuation of proinflammatory response may also contribute to ketamine analgesia, since there is evidence to suggest important roles of proinflammatory cytokines in the pathogenesis of neuropathic pain.

Thus, the major aims of this thesis were to examine the influence of variability in enzyme activity, especially that due to *CYP2B6* genetic polymorphisms, on ketamine pharmacokinetics *in vitro* and *ex vivo* in chronic pain patients. The secondary aims of this thesis were to examine the effects of ketamine and norketamine enantiomers on proinflammatory cytokine production *in vitro*, using interleukin-6 (IL-6) as a marker of cytokine production; and to explore the mechanistic characterisation of the drug actions using both *in silico* docking simulations and *in vitro* experiments.

The *in vitro* experiments showed that, at clinically relevant concentrations, CYP2B6 but not CYP3A4 is the major isoform responsible for ketamine metabolism to norketamine in human liver microsomes (HLM). Moreover, the presence of the *CYP2B6*6* allele, the most common allelic variant of the *CYP2B6* gene, reduced the intrinsic clearance of both ketamine enantiomers in HLMs and cDNA-expressed proteins by at least 62%. This substantial *CYP2B6*6* allele-induced decrease in ketamine intrinsic clearance *in vitro* was also observed *ex vivo* in chronic pain patients who received 24 h continuous subcutaneous infusions of 100 mg to 500 mg ketamine. The impact of the *CYP2B6*6* allele, by itself and in combined with the age of the patient, explained approximately 40% and 60% of interindividual variation in plasma ketamine concentrations at steady-state, respectively, whereas sex, disease and other medications had no significant influences. The decrease of ketamine clearance may be associated with the adverse effects of ketamine, as patients who experienced adverse effects showed approximately 15% lower steady-state plasma clearance of ketamine than those who did not. However, no evidence linking the plasma pharmacokinetics of ketamine and norketamine and the analgesic efficacy was found. One possible explanation for this lack of concentration-response relationship is the overwhelming effect of heterogeneous nature of pain on analgesic response, since ketamine analgesic efficacy was higher in patients suffering from neuropathic pain than other pain types. This finding may reflect the fact that the analgesic activity of ketamine is more likely due to the attenuation of pain hypersensitivity rather than the direct suppression of nociceptive transmission.

The *in vitro* experiments on the inhibition of IL-6 by ketamine and norketamine enantiomers showed that pre-incubation with these drugs, at biologically relevant concentrations (1 to 100 μM), stereoselectively attenuated stimulated IL-6 production in recombinant cells in a concentration- and time-dependent manner. *(R)*-ketamine inhibited stimulated IL-6 production by approximately 60% at all exposure duration, an inhibitory effects that were up to 2-fold

greater than *(S)*-ketamine after short term exposure (less than 2 h). However *(S)*-ketamine was as potent as *(R)*-ketamine after long-term exposure (4 to 8 h), as its inhibitory effects were significantly enhanced with exposure duration. In addition, *(S)*-norketamine also attenuated IL-6 response in a time-dependent manner with approximately half the potency of *(S)*-ketamine. Further *in vitro* experiments and *in silico* docking simulation suggested that this time-dependent effects of *(S)*-ketamine and *(S)*-norketamine may indicates a mechanistically-based difference between acute and chronic effects of *(S)*-enantiomers on IL-6 production. This findings extend the current knowledge of the innate immune pharmacology of ketamine that may lead to a new direction for future research into ketamine analgesia.

In summary, this thesis demonstrates a substantial impact of the *CYP2B6*6* allelic variant on the clearance of ketamine, which may contribute to the interindividual variability in drug concentration. However, other factors such as the heterogeneity in the nature of pain and the inflammatory state should be taken into consideration to provide a more accurate prediction on ketamine analgesic response.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree. I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968. The author acknowledges that copyright of published works contained within this thesis resides with the copyright holders of these works.

Li Y, Coller JK, Hutchinson MR, Klein K, Zanger UM, Stanley NJ, Abell AD, Somogyi AA. The CYP2B6*6 allele significantly alters the N-demethylation of ketamine enantiomers in vitro. (2013) *Drug Metabolism and Disposition*. 41(6), 1264-1272

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Yibai Li 24 Nov 2014

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Abbreviations

AAG	α_1-acid glycoprotein
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP-1	Activator protein 1
AUC	Area under the concentration-time curve
BPI	Brief Pain Inventory
CaMKII	Calmodulin dependent protein kinase II
CAR	Constitutive androstane receptor
CD	Cluster of differentiation
CL_{int}	Intrinsic clearance
CREB	cAMP response element-binding protein
CRPS	Complex regional pain syndrome
CSCI	Continuous subcutaneous infusion
CYP	Cytochrome P450 enzyme
DMEM	Dulbecco's Modified Eagle Medium
DHNK	Dehydronorketamine
DXO	Dextrorphan
EAAT	Excitatory amino-acid transporters
ERK	Extracellular signal-regulated kinases
GABA	γ-Aminobutyric acid
HEK293	Human embryonic kidney 293 cell line
HLM	Human liver microsome
HNK	Hydroxynorketamine
HPLC	High performance liquid chromatography
IC_{50}	50% inhibitory concentration
IL	Interleukin
i.m.	Intramuscular
IRF	Interferon regulatory factors
it	Intrathecal
i.v.	Intravenous
JNKs	c-Jun N-terminal kinases
K_i	Dissociation constant
K_m	Michaelis constant

LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases
MD-2	Myeloid differentiation protein
mGluRs	Metabotropic glutamate receptors
MK-801	Dizocilpine
NF-κB	Nuclear factor kappa B
NK	Norketamine
NMDA	N-methyl-$_D$-aspartate
NO	Nitric oxide
NRS	Numeric Rating Scale
PBS	Phosphate buffered saline
PCA	Patient controlled analgesia
PCP	Phencyclidine
PKC	Protein kinase C
PXR	Pregnane X receptor
RacK	Racemic ketamine
RCT	Randomised controlled trial
RK	*(R)*-ketamine
RPMI	Roswell Park Memorial Institute medium
s.c.	Subcutaneous
SK	*(S)*-ketamine
SNP	Single nucleotide polymorphism
ThioTEPA	N,N'N'-triethylenethiophosphoramide
TLR	Toll-like receptor
TNF	Tumour necrosis factors
TrkB	Tyrosine receptor kinase B
VAS	Visual analogue scale
UV	Ultraviolet

Chapter 1. Introduction

1.1 A historical overview of ketamine

The story of ketamine begins in 1961. A major US pharmaceutical company at that time, Parke-Davis (now a subsidiary of Pfizer), was searching for alternatives to their problematic anaesthetic product, phencyclidine (PCP), with less severe adverse psychological effects. Prof. Calvin Stevens of Wayne State University, who was a chemical consultant for Parke-Davis, synthesised a series of PCP derivatives for preclinical testing (Jansen, 2001). One of such derivatives was found to be a safer and promising short-acting anaesthetic agent in animals, which was therefore selected for clinical investigations in 1964 and initially named by its assigned clinical investigation number, CI-581 (Domino, 2010). The clinical investigation documented that CI-581 produces a unique anaesthetic state termed "dissociative state", which is characterised by catalepsy, profound amnesia and analgesia while the anaesthetised patient felt disconnected from their environment (Domino *et al.*, 1965). The compound was then renamed ketamine and patented as an anaesthetic for human and veterinary medicine in 1966. Soon after receiving the Food and Drug Administration (FDA) approval in 1970, ketamine was introduced to the market as a sole anaesthetic agent for human use.

Ketamine was first given to American soldiers as a battlefield anaesthetic during the Vietnam War due to its fast-acting and rapid recovery properties (Kelly, 1999). During the 1970s and early 80s, the use of anaesthetic ketamine increased continuously in civilian practice. Although ketamine causes less respiratory and circulatory depression than the concurrent anaesthetics, there were a cumulative number of reports on its undesirable psychotomimetic effects including hallucinations and unpleasant dreams (Lotfy *et al.*, 1970). Consequently, the use of anaesthetic ketamine was restricted to specific clinical scenarios (such as pre-hospital

emergency and remote hospital) since the introduction of other anaesthetics that caused fewer emergency reactions in mid-1980s (Marland *et al.*, 2013). While the popularity of anaesthetic ketamine declined, the recreational use of ketamine has expanded rapidly. The knowledge of ketamine was further spread by the publication of two influential books in 1978: *The Scientist* by John C. Lilly and *Journeys into the Bright World* by Marcia Moore and Howard Alltounian, whereby ketamine-induced dream-like visual sensations and fascinating out-of-body experiences were described in detail. In the following three decades, the recreational use of ketamine has quickly popularised together with the rise of dance culture (Jansen, 2001).

However, the drug did not completely fade from the clinical setting. Despite being less frequently used as an anaesthetic in humans, ketamine is being increasingly used at sub-anaesthetic doses as an adjuvant to opioid treatment for chronic pain in the past decade (Hirota and Lambert, 2011). Clinical experiments have demonstrated that supplemental ketamine potentiates the quality of opioid analgesic effects, reduces opioid consumption and prevents the development of opioid tolerance (a decrease of opioid potency over time) and hyperalgesia (increased sensitivity to painful stimuli) (Subramaniam *et al.*, 2004). Additionally, recent studies also showed that a single sub-anaesthetic dose of ketamine produces anti-depressant effects within hours after administration in patients with major depressive disorder or bipolar disorder, including those who are treatment-resistant (Katalinic *et al.*, 2013). More notably, ketamine has been found to suppress suicidal thoughts within one hour (DiazGranados *et al.*, 2010). Such a fast-acting effect of ketamine gives it advantages over the conventional anti-depressant medications, which generally require weeks to be effective. Although, in comparison with the anaesthetic application, the uses of ketamine at sub-anaesthetic doses are associated with less psychotomimetic adverse effects, they are limited by the considerable variability in drug responses between individuals (see section *1.5 The applications of sub-anaesthetic ketamine in pain management*, page 32-39).

1.2 Structure and Chemistry

Structurally similar to PCP and other PCP derivatives, ketamine (2-(o-chlorophenyl)-2-(methylamino)-cyclohexanone) is an arylcycloalkylamine. Ketamine exists as two enantiomers: *(S)*- and *(R)*-, due to the presence of a chiral centre at the second carbon position of the cyclohexanone ring (structures of ketamine hydrochloride are shown in Figure 1-1A, page 5). A study using X-ray crystallographic analysis showed that *(S)*-configuration has a negative (-) optical rotation sign as the free base and positive (+) optical rotation sign as the hydrochloride salt. Accordingly, *(R)*-configuration has the opposite optical rotation signs (Chankvetadze *et al.*, 2002). In most countries including Australia, the commercially available pharmaceutical form of ketamine is a 1:1 racemic mixture (hereafter referred to as racemic ketamine) of *(S)*-(+)-ketamine hydrochloride salt (hereafter referred to as *(S)*-ketamine) and *(R)*-(-)-ketamine hydrochloride salt (hereafter referred to as *(R)*-ketamine).

The two enantiomers of ketamine differ considerably in their pharmacodynamic and pharmacokinetic properties (summarised in Table 1-1, page 6). *(S)*-ketamine has been described as the pharmacologically more active enantiomer, which has 3-4 times the analgesic potency of *(R)*-ketamine and twice that of racemic ketamine in humans (Arendt-Nielsen *et al.*, 1996; Mathisen *et al.*, 1995). At an equianalgesic dose, both ketamine enantiomers induced less psychotomimetic effects and cognitive impairment compared with racemic ketamine (Mathisen *et al.*, 1995; Pfenninger *et al.*, 2002). Moreover, *(S)*-ketamine is associated with less decline in concentration capacity and primary memory (Pfenninger *et al.*, 2002). Because of these advantages, *(S)*-ketamine has already replaced the racemic formulation in several European countries including Denmark, Finland, Germany, Iceland and The Netherlands (Mann *et al.*, 2013).

Ketamine hydrochloride is manufactured as a water-soluble white crystalline powder and generally marketed in a packaged injectable liquid form. The commercially available form of ketamine normally contains benzethonium chloride as a preservative, which may also contribute to the toxicity of ketamine (Malinovsky *et al.*, 1993). Ketamine has a pKa value of 7.5 and a molecular weight of 237.7 and 274.2 $g.mol^{-1}$ as the free base and the hydrochloride, respectively. Ketamine has an experimental octanol/buffer partition coefficient of 60 (Wachtel *et al.*, 1992), and a predicted octanol/buffer partition coefficient of 490 (estimated using ALOGPS 2.1 software, Tetko *et al.*, 2005). This high lipid solubility allows the compound to rapidly distribute in the body and cross the blood brain barrier (Cohen and Trevor, 1974).

Norketamine, the primary and weakly active metabolite of ketamine, may contribute to ketamine anaesthesia and analgesia (see section *1.4.1.1 Metabolism*, page 14-31, for more information regarding ketamine metabolites). Like its parent, norketamine also exists as *(S)*- and *(R)*-configurations (structures are shown in Figure 1-1B, next page), which differ in their analgesic activities (see section *1.3.1.2 Antagonism of NMDA receptors by ketamine*, page 7-8). Norketamine has a pKa value of 6.65 and molecular weight of 223.7 and 259.2 $g.mol^{-1}$ as the free base and the hydrochloride salt, respectively (Cohen and Trevor, 1974). The predicted octanol/buffer partition coefficient of norketamine is 166 (estimated using ALOGPS 2.1 software). Currently, norketamine is only available for research purposes but not approved for human use.

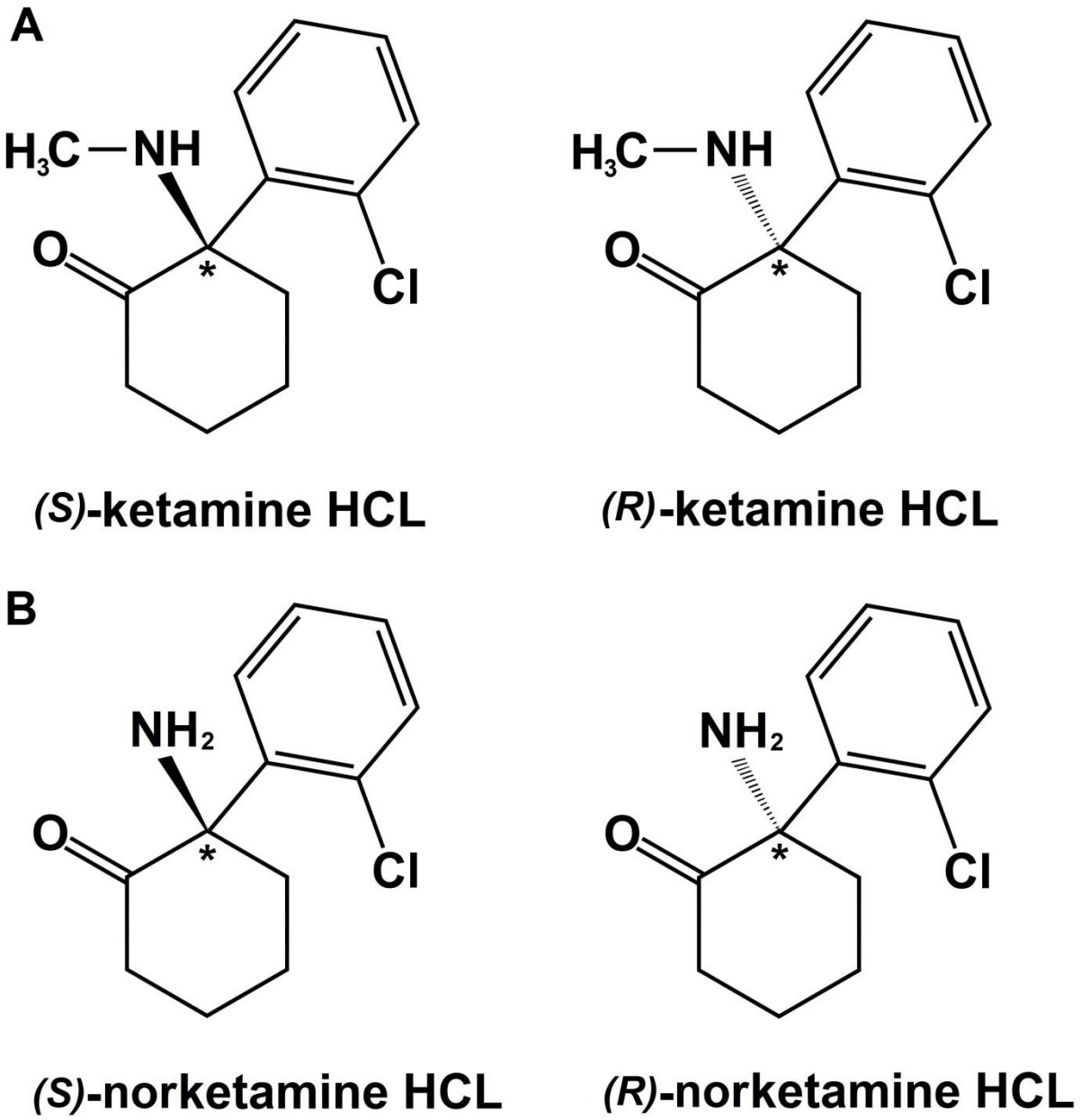

Figure 1-1. Chemical structures of enantiomers of A) ketamine and B) norketamine. Symbol * indicates the chiral centre of the molecule.

Table 1-1. Pharmacological differences between *(S)*- and *(R)*-ketamine

Activity	***(S)*/*(R)*-ketamine ratio**	**Reference**
Anaesthetic potency	3	White *et al.* (1980)
Analgesic potency	3-4	Klepstad *et al.* (1990); Mathisen *et al.* (1995)
Incidence of psychotomimetic effects at equianaesthetic doses	1/7	White *et al.* (1980)
Incidence of psychotomimetic effects at equianalgesic doses	1	Mathisen *et al.* (1995)
Affinity for PCP site in human brains	6-7	Oye *et al.* (1992)
Volume of distribution at steady state	1.1	Geisslinger *et al.* (1995)
In vivo plasma clearance rate	1.1	Geisslinger *et al.* (1995)
In vitro hepatic clearance rate#	1.2	Kharasch and Labroo (1992)

estimated using human liver microsomes.

1.3 Classical analgesic mechanisms of ketamine

Since Domino *et al.* reported the first clinical use of ketamine in 1965, hundreds of studies have investigated the mechanism(s) of action of this drug, yet the exact mechanism is still not entirely clear, as ketamine interacts with a wide range of receptors and transporters that contribute to pain processing, including *N*-Methyl-$_{D}$-Aspartate (NMDA) receptors (Anis *et al.*, 1983; Lodge *et al.*, 1982), opioid receptors (Finck and Ngai, 1982; Smith *et al.*, 1980), muscarinic and nicotinic acetylcholine receptors (Durieux, 1995; Yamakura *et al.*, 2000), serotonin receptor (Crisp *et al.*, 1991), dopamine receptor (Seeman *et al.*, 2005) and monoamine transporters (Tso *et al.*, 2004). However, the most widely accepted analgesic mechanism is non-competitive antagonism of neuronal NMDA receptors, which leads to the

inhibition of central sensitisation, a state that is characterised by the increase in responsiveness to peripheral noxious inputs in the spinal cord neurons (Woolf *et al.*, 1988). This section will briefly review the current knowledge of the role of NMDA receptors in pain development, opioid actions and ketamine analgesia.

1.3.1 Antagonism of NMDA receptors by ketamine

Orser *et al.* (1997) showed that ketamine inhibited the NMDA receptor, a ionotropic glutamate receptor (see *1.3.2 Roles of NMDA receptors in pain*, page 8-12, for more information of NMDA receptor), by a dual mechanism: firstly, it decreases the channel opening time of NMDA receptors by blocking the PCP binding site of the channel pore; and secondly, it reduces the channel opening frequency by allosterically inhibiting the hydrophobic domain of NMDA receptors. At 1 μM, racemic ketamine decreased the frequency of channel opening and the mean opening time of NMDA receptors by 56% and 32%, respectively. These inhibitions prevent the flux of ions, especially Ca^{2+}, which leads to the attenuation of glutamate-mediated nociceptive responses in dorsal horn neurons and the subsequent development of central sensitisation (see section *1.3.2 Roles of NMDA receptors in pain* for more details).

The blockade of the PCP binding site by ketamine has been found to be stereoselective in both rodents and humans (Ebert *et al.*, 1997; Hustveit *et al.*, 1995; Oye *et al.*, 1992). In rat brain homogenates, *(S)*-ketamine (K_i = 0.3 μM) was approximately 5- and 1.7-fold more potent in displacing [^{3}H]-Dizocilpine (MK-801) from the PCP binding site compared with *(R)*-ketamine (K_i = 1.4 μM) and racemic ketamine (K_i = 0.5 μM), respectively (Ebert *et al.*, 1997). Electrophysiological assays in the same study found that *(S)*-ketamine exerted approximately 3- to 4-fold more potent inhibitory effects on NMDA response than *(R)*-ketamine in rat cerebral cortex and spinal cord. In human hippocampal, frontal cortical and

occipital cortical homogenates, *(S)*-ketamine was approximately 6 to 7 times more potent than *(R)*-ketamine in displacing [^{3}H]-MK-801 binding (the estimated IC_{50} were 1.5 to 3 μM and 7 to 14 μM for *(S)*- and *(R)*-ketamine, respectively, Oye *et al.*, 1992).

As the primary metabolite, the binding of norketamine enantiomers to NMDA receptors has also been tested, but only in rodents since the compound is not available for human use. Ebert *et al.* (1997) reported that the binding affinity of *(S)*-norketamine (K_i = 1.7 μM) and *(R)*-norketamine (K_i = 13 μM) to NMDA receptors in rats were approximately 1/5th and 1/10th of their parent compound, respectively. Accordingly, the authors hypothesised that norketamine contributes to ketamine analgesic, probably via the antagonism of NMDA receptor. A later animal study using chronic constriction nerve injury model and the formalin test demonstrated that both norketamine enantiomers have anti-hyperalgesic and anti-allodynic effects but have little effect on nociceptive pain in rats, which is a common feature of NMDA-receptor antagonists (Holtman *et al.*, 2008). Interestingly, the anti-hyperalgesic effect of norketamine (about 50 to 67% that of ketamine) was relatively higher considering its low NMDA activity (10 to 20% that of ketamine), suggesting a possible involvement of other anti-hyperalgesic mechanisms (Swartjes *et al.*, 2011).

1.3.2 Roles of NMDA receptors in pain

NMDA receptors, the major glutamate-gated ion channels in mammalian nervous systems, play fundamental roles in the excitatory synaptic transmission and have been associated with synaptic plasticity, pathogenesis of pain (Petrenko *et al.*, 2003) and many neuropsychiatric disorders (Lakhan *et al.*, 2013). NMDA receptors are heteromeric proteins, and are composed of multiple subunits within a repertoire of three subtypes: GluN1, the channel-forming and glycine binding subunit; GluN2, the glutamate-binding subunit; and GluN3, the function-modulatory subunit (Kohr, 2006). Most central NMDA receptors are tetrameric assemblies of

two GluN1 and two GluN2 subunits (structure is illustrated in Figure 1-2, page 10). The physiological and pharmacological properties of the receptor are determined by the four subunits of GluN2 (namely GluN2A, 2B, 2C and 2D), which are expressed in different regions of the brain (Mori and Mishina, 1995).

The activation of NMDA receptors requires the binding of glutamate to the GluN2 subunit, the binding of glycine to the GluN1 subunit and most importantly, the removal of GABAergic signalling-controlling magnesium (Mg^{2+}) blockade of the receptor channel pore. The channel blockade by Mg^{2+} prevents the activation of NMDA receptors by the glutamate released from pre-synaptic neurons in response to weak stimuli. However, high intensity and/or persistent stimuli lead to the release of a sufficient amount of glutamate, substance P and other excitatory amino acids from hyperactive primary afferent fibres, which activate post-synaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and the metabotropic glutamate receptors (mGluRs). The activation of these receptors results in sustained membrane depolarisation of postsynaptic dorsal horn neurons that expels Mg^{2+} from the NMDA channel pore and subsequently allows the influx of calcium (Ca^{2+}) and sodium (Na^{+}) in exchange for potassium (K^{+}) through the activated NMDA receptors. The increase in intracellular Ca^{2+} level facilitates the synthesis and secretion of neurotransmitters and other pain modulators that further sustain the neuronal hyperexcitability, such as prostaglandin and nitric oxide (NO), via the activation of calmodulin dependent protein kinase II (CaMKII), protein kinase C (PKC) and NO synthase (Park *et al.*, 2000) (an illustration of this process can be found in Figure 1-3, page 12). Consequently, dorsal horn neurons decrease their threshold to noxious information carried by Aδ- and C-fibres (hyperalgesia) and responses to innocuous information carried by myelinated Aβ-fibres that normally do not contribute to pain such as touch and vibration (allodynia).

Hyperalgesia and allodynia can also be produced by numerous drugs, of which opioid-induced hyperalgesia is most common. Opioid-induced hyperalgesia and allodynia, together with opioid tolerance, physical and psychological dependence, are major problems restricting the effectiveness of chronic opioid therapy. It has been shown that cross-regulation between opioid receptors and NMDA receptors in the same postsynaptic neuron may contribute to both opioid-induced hyperalgesia and opioid tolerance (Mao *et al.*, 1995).

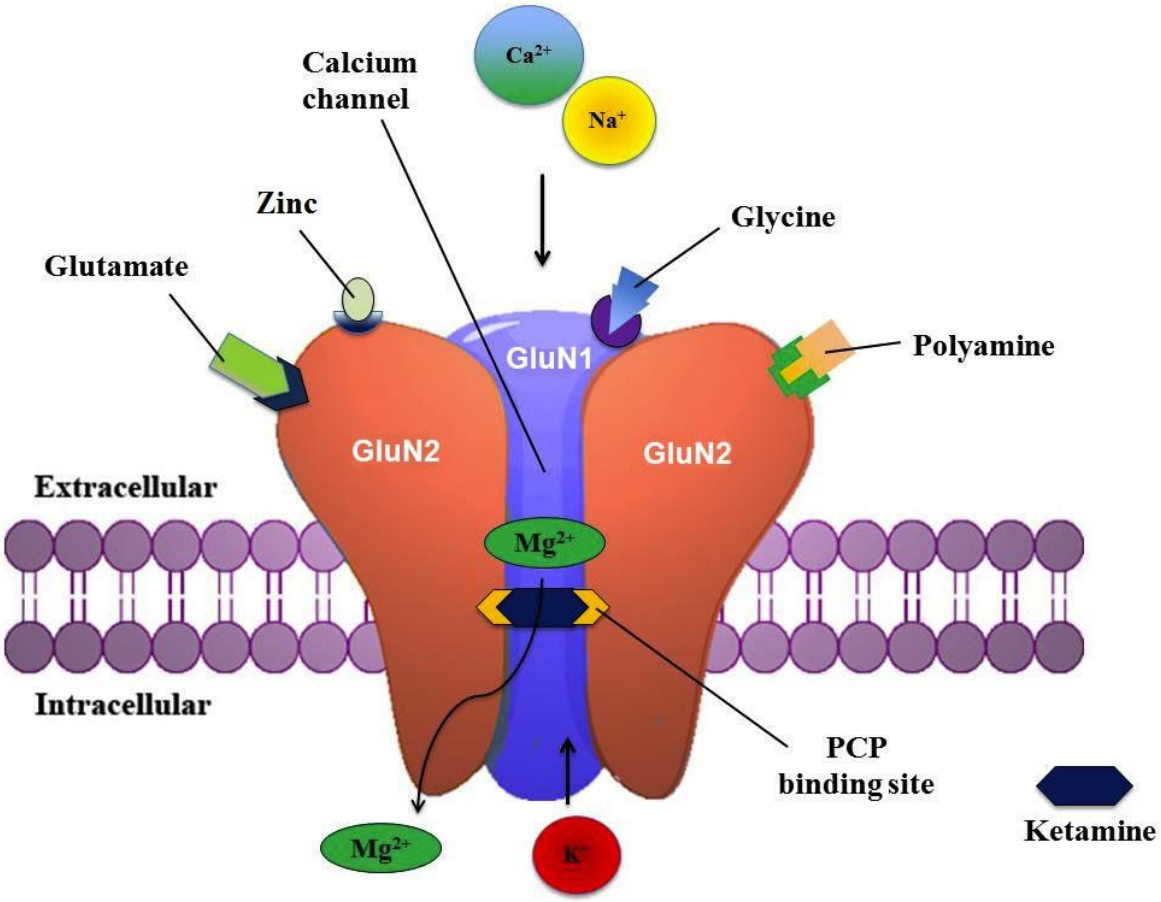

Figure 1-2. A typical tetrameric structure neuronal NMDA receptor in human central nervous system (CNS) and ligands involved in NMDA receptor activation. For clarity, only one GluN1 subunit is shown here.

Prolonged exposure to opioids facilitates the influx of Ca^{2+} by a) increasing the extracellular glutamate level through inhibition of glutamate transporters; and b) phosphorylating NMDA receptors that increase the channel opening and partly removes the Mg^{2+} blockade of channel pore (Chen and Huang, 1992). Consequently, these changes result in sensitisation of postsynaptic neurons, which leads to the development of hyperalgesia and allodynia (Mao *et al.*, 1995). In addition, the elevation of intracellular Ca^{2+} initiates a cascade of protein phosphorylation and transcriptional regulation that eventually results in the desensitisation of opioid receptors, in other words, decreased sensitivity to opioids (Chen and Huang, 1992; Garzon *et al.*, 2012; Mao *et al.*, 2002).

In summary, NMDA receptor-mediated Ca^{2+} influx plays a fundamental role in the development of neuropathic pain and opioid actions. Accordingly, ketamine, as an inhibitor of the ion channel, exhibits analgesic and opioid-sparing activities. However, there is increasing evidence that the antagonism of the NMDA receptor is unlikely to be the sole mechanism underlying ketamine's actions (see section *1.6 the non-classical analgesic mechanisms of ketamine*, page 40-48, for more details regarding other mechanisms).

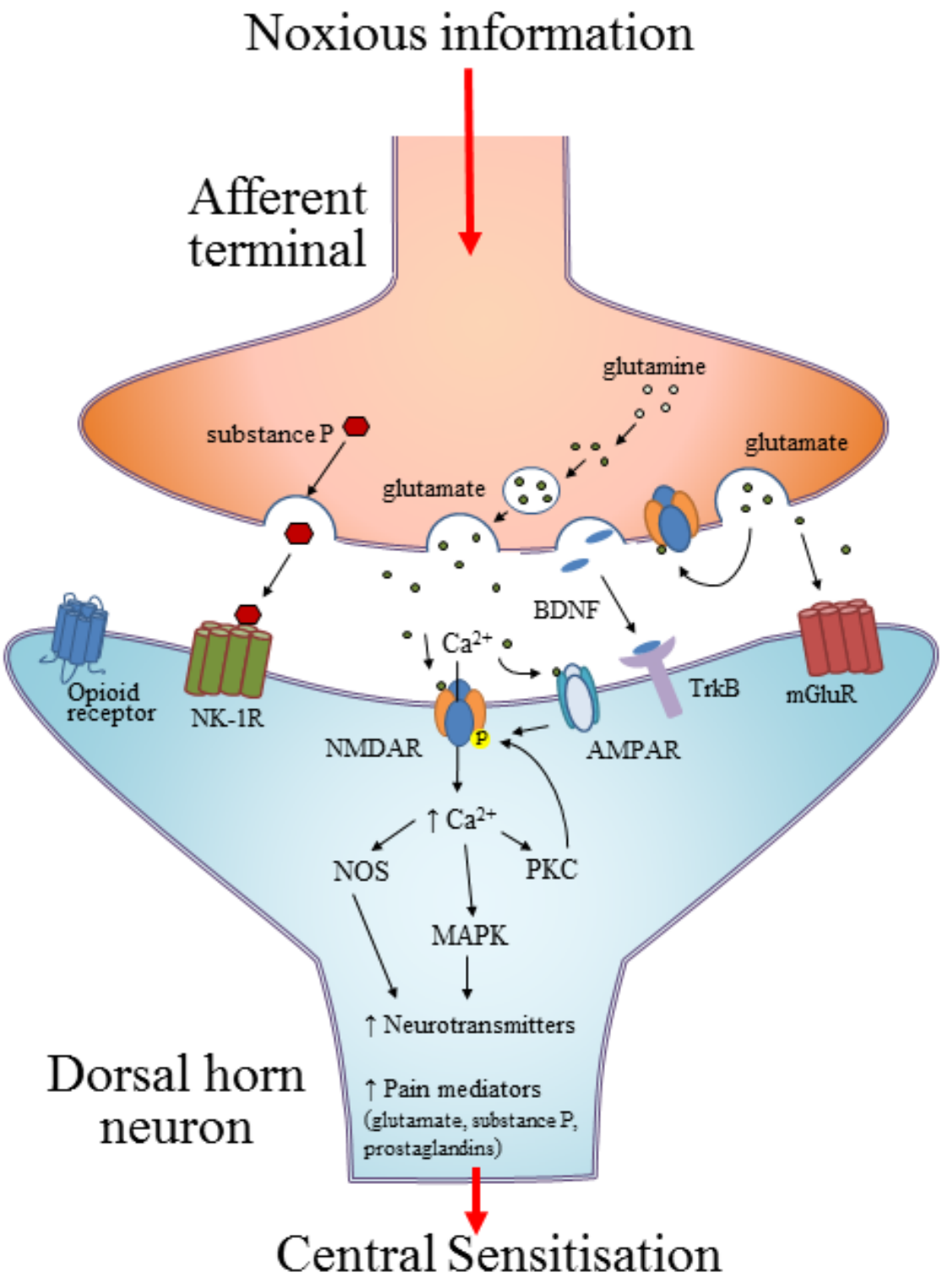

Figure 1-3. Roles of NMDA receptors in the amplification of pain signals and the development of central sensitisation. AMPAR: APMA receptor; MAPK: mitogen-activated protein kinases; NK-1R: neurokinin 1 receptor; NMDAR: NMDA receptor, TrkB: tyrosine receptor kinase B.

1.4 Pharmacokinetics of ketamine

Although ketamine at low doses produces good analgesic and opioid-sparing effects, its applications are limited by the low response rate and large interindividual variability in drug responses (Noppers *et al.*, 2010) (see section *1.5 the clinical applications of ketamine at sub-anaesthetic doses*, page 32-39, for more details). One possible cause for these limitations is the interindividual difference in pharmacokinetics, as previous clinical trials reported an up to 4-fold difference in plasma concentrations after a 40 min intravenous (i.v.) administration of 0.4 $mg.kg^{-1}$ ketamine in 10 neuropathic pain patients (Kvarnström *et al.*, 2004; Persson *et al.*, 1998). Such variability is possibly due to a difference in drug clearance. This section will review previous ketamine pharmacokinetic studies and discuss potential factors affecting ketamine clearance and plasma concentration.

1.4.1 Clearance

As a highly lipophilic drug, ketamine is extensively eliminated by hepatic metabolism, the fraction excreted unchanged of ketamine after an i.v. dose was only approximately 2 to 3% (Wieber *et al.*, 1975). The high hepatic extraction ratio (0.9) suggests that ketamine clearance is mainly determined by hepatic blood flow (Haas and Harper, 1992). The estimated plasma clearance rate of racemic ketamine following i.v. administration was approximately 70 $L.h^{-1}$ (see Table 1-2 for more details, page 15-17). The plasma and blood clearance have a same numerical value, as ketamine's blood to plasma ratio is approximately 1. The plasma clearance of *(S)*-ketamine was approximately 10-20% greater than *(R)*-ketamine (Geisslinger *et al.*, 1993; Peltoniemi *et al.*, 2012; White *et al.*, 1985; Yanagihara *et al.*, 2003), which is most likely due to the stereoselectivity in metabolism, as *in vitro* studies showed that the human liver microsomal metabolism of *(S)*-ketamine was approximately 20% higher than *(R)*-ketamine (Kharasch and Labroo, 1992; Yanagihara *et al.*, 2001).

1.4.1.1 Metabolism

Although interindividual difference in ketamine hepatic clearance may primarily be due to the difference in hepatic blood flow, additional variability in hepatic clearance may arise from its metabolism, which is mainly to norketamine by two cytochrome P450 (CYP) enzymes, CYP2B6 and CYP3A4 (Clements and Nimmo, 1981; Hijazi and Boulieu, 2002a; Kharasch and Labroo, 1992; Yanagihara *et al.*, 2001), whose expression and activity are considerably different between individuals due to the influence of disease states, medicine use, and genetic variability (Zanger and Schwab, 2013). The interindividual difference in CYP3A4 and CYP2B6 function, and its potential impact on ketamine clearance will be discussed in section *1.4.1.1.2.1 CYP2B6* (page 22-29) and *1.4.1.1.2.2 CYP3A4* (page: 29-31)

Various metabolites of ketamine have been identified in humans. Of them, the most important metabolite, both quantitatively and functionally, is believed to be norketamine, as it is a weak antagonist of NMDA receptors that produces analgesia in animal models with 1/5th to 1/3rd of the analgesic potency and half of the anti-hyperalgesic potency of ketamine (Ebert *et al.*, 1997; Holtman *et al.*, 2008; Swartjes *et al.*, 2011). Norketamine can be further hydroxylated at different positions on the cyclohexone ring to form inactive enantiomeric metabolites, 4-, 5- and 6-hydroxynorketamine, which can either be conjugated and excreted in urine, or undergo further oxidative metabolism to form dehydronorketamine (DHNK), the most prevalent metabolite in urine and second most prevalent metabolite in plasma (Chang and Glazko, 1972; Cheng *et al.*, 2007; Zhao *et al.*, 2012). Ketamine can also be directly hydroxylated into 6-hydroxyketamine, a quantitatively minor metabolite in humans (Zhao *et al.*, 2012). The scheme in Figure 1-4 (page 18) shows the pathways of the formation of ketamine metabolites in humans.

Table 1-2. Pharmacokinetic parameters of racemic ketamine and both enantiomers

Intravenous administration

Study	Subjects	Dose	Clearance ($L.h^{-1}$; 75 kg)	Volume of distribution (L; 75 kg)	Distribution half-life (min)	Terminal half-life (min)
Wieber *et al.* (1975)	N=5	RacK: 2.5 $mg.kg^{-1}$	74 ± 17	V_{ss}: 204 ± 22	11	151
Clements and Nimmo (1981)	N=5, all M, HV, 34 ± 1 yo, 3 occasions CO (> 7 days apart)	RacK: 125 µg.kg^{-1}	73 ± 6	V_{ss}: 158 ± 23	17	186
		RacK: 250 µg.kg^{-1}	86 ± 5	V_{ss}: 233 ± 30		
White *et al.* (1985)	N=5, all M, HV, 36 ± 3 yo, 3 occasions CO (7-14 days apart)	RacK: 50 $mg.min^{-1}$ (5-7 mins)	72 ± 21	V_c: 75 ± 30, $V_{d\ area}$: 218 ± 38	13 ± 9	132 ± 32
		SK: 25 $mg.min^{-1}$ (5-7 mins)	96 ± 7	V_c: 120 ± 53, $V_{d\ area}$: 353 ± 83	23 ± 15	158 ± 45
		RK: 75 $mg.min^{-1}$ (5-7 mins)	78 ± 11	V_c: 68 ± 53, $V_{d\ area}$: 293 ± 98	12 ± 9	155 ± 42
Geisslinger *et al.* (1993)	N=45, 10 F, patients undergoes surgery, 18-59 yo, group 1 (n=21) received SK, group 2 (n=24) received RacK	SK: 1 $mg.kg^{-1}$	74 ± 26	V_{ss}: 210 ± 120	NR	143 ± 76
		RacK: 2 $mg.kg^{-1}$	SK: 86 ± 32	V_{ss}: 225 ± 90		155 ± 57
			RK: 74 ± 22	V_{ss}: 248 ± 98		149 ± 47
Ihmsen *et al.* (2001)	N=10, all M, 28 ± 4 yo, 2 occasions CO (14 days apart), arterial plasma concentration measured	SK, 0.1 $mg.kg^{-1}.min^{-1}$, + 0.08 $mg.kg^{-1}.min^{-1}$, infusion	SK: 125 ± 17	V_{ss}: 216 ± 55 V_c: 24 ± 5	2.7 ± 0.3	146 ± 33
			RacK: 70 ± 8	V_{ss}: 174 ± 63 V_c: 16 ± 3	2.9 ± 0.3	196 ± 73
		RacK, 0.17 $mg.kg^{-1}.min^{-1}$ + 0.15 $mg.kg^{-1}.min^{-1}$	SK: 88 ± 4	V_{ss}: 229 ± 47 V_c: 24 ± 4	2.9 ± 0.2	190 ± 31
			RK: 65 ± 6	V_{ss}: 103 ± 55 V_c: 8 ± 3	1.5 ± 0.3	137 ± 36

Table 1-2. Pharmacokinetic parameters of racemic ketamine and both enantiomers (continued)

Intravenous administration

Study	Subjects	Dose	Clearance (L h^{-1}; 75 kg)	Volume of distribution (L; 75 kg)	Distribution half-life (min)	Terminal half-life (min)
Persson *et al.* (2002)	N=10, all M, HV, 24-62 yo, 2 occasions CO (> 3 days apart), venous and arterial plasma concentrations were measured	RK or SK: 7 mg	**Venous**:			
			SK: 100 ± 28	V_{ss}: 200 ± 44 V_c: 57 ± 31	6.7 ± 4	113 ± 40
			RK: 89 ± 28	V_{ss}: 200 ± 56 V_c: 53 ± 25	7.3 ± 3	127 ± 36
			Arterial:			
			SK: 97 ± 18	V_{ss}: 112 ± 33 V_c: 17 ± 5	3.2 ± 1	80 ± 25
			RK: 86 ± 23	V_{ss}: 121 ± 23 V_c: 17 ± 6	3.5 ± 1	102 ± 25
Yanagihara *et al.* (2003)	N=3, all M, HV, 27-40 yo, 5 occasions CO (7 days apart)	RacK: 20 mg	SK: 84 ± 7	V_{ss}: 175 ± 56	NR	117 ± 48
			RK: 77 ± 14	V_{ss}: 175 ± 42		127 ± 47
Chong *et al.* (2009)	N=6, 5 M, 33-66 yo, 3 occasions CO (1-2 days apart)	RacK: 10 mg	67 (52-67)	V_z: 370 (296-444)	NR	312 (204-384)
Peltoniemi *et al.* (2012)	N=11, HV, 20-27 yo, 4 occasions CO (4 weeks apart)	SK: 0.1 $mg.kg^{-1}$	84 ± 21	V_{ss}: 427 ± 118	12	354 ± 66

Table 1-2. Pharmacokinetic parameters of racemic ketamine and both enantiomers (continued)

Other administration routines

Study	Subjects	Route of administration	Dose	Peak plasma concentration ($ng.mL^{-1}$)	Peak time (min)	Bioavailability (%)
Grant *et al.* (1981)	N=6, HV, 32 ± 2 yo, 3 occasions CO (> 7 days apart), bitter test was masked by orange juice	Oral	RacK: 0.5 $mg.kg^{-1}$	44 ± 25	30 ± 12	17 ± 6
		i.m.	RacK: 0.5 $mg.kg^{-1}$	240 ± 121	22 ± 10	93 ± 5
Yanagihara *et al.* (2003)	N=3, all M, HV, 27-40 yo, 5 occasions CO (7 days apart)	Oral	RacK: 50 mg	SK: 40 ± 12 RK: 43 ± 13	30 ± 30 33 ± 15	18 ± 4 18 ± 5
		Sublingual	RacK: 50 mg	SK: 57 ± 23 RK: 62 ± 21	37 ± 21 40 ± 20	32 ± 7 32 ± 8
		Intranasal	RacK: 25 mg	SK: 29 ± 14 RK: 29 ± 17	18 ± 5 23 ± 10	46 ± 13 44 ± 11
		Rectal	RacK: 50 mg	SK: 39 ± 30 RK: 43 ± 31	23 ± 6 23 ± 6	31 ± 31 29 ± 19
Chong *et al.* (2009)	N=6, 5 M, neuropathic pain patients, 33-66 yo, 3 occasions CO (1-2 days apart)	Oral	RacK: 25 mg	21 (12-35)	120 (72-150)	24 (17-27)
		Sublingual	RacK: 25 mg	30 (24-32)	30 (18-48)	24 (19-49)
Peltoniemi *et al.* (2012)	N=11, HV, 20-27 yo, 4 occasions CO (4 weeks apart)	Oral	SK: 0.3 $mg.kg^{-1}$	12 ± 6	42 (18-60)	11 ± 1

M: male; F: female; HV: healthy volunteer; CO: crossover design; RacK: racemic ketamine; SK: *(S)*-ketamine; RK: *(R)*-ketamine; V_{ss}: volume of distribution at steady state; V_c: volume of distribution to central compartment; $V_{d\ area}$: volume of distribution for area under the curve; V_z: volume of distribution in the elimination phase; NR: not reported; yo, years old. Data are mean ± s.d or median (range).

Oral etamine undergoes extensive first-pass elimination, which results in only 17-24% bioavailability and a norketamine concentration that was 4 times that of its parent compound (Grant *et al.*, 1981). Likewise, the bioavailabilities of ketamine after sublingual, intranasal and rectal administration were low (Table 1-2, page 15-17).

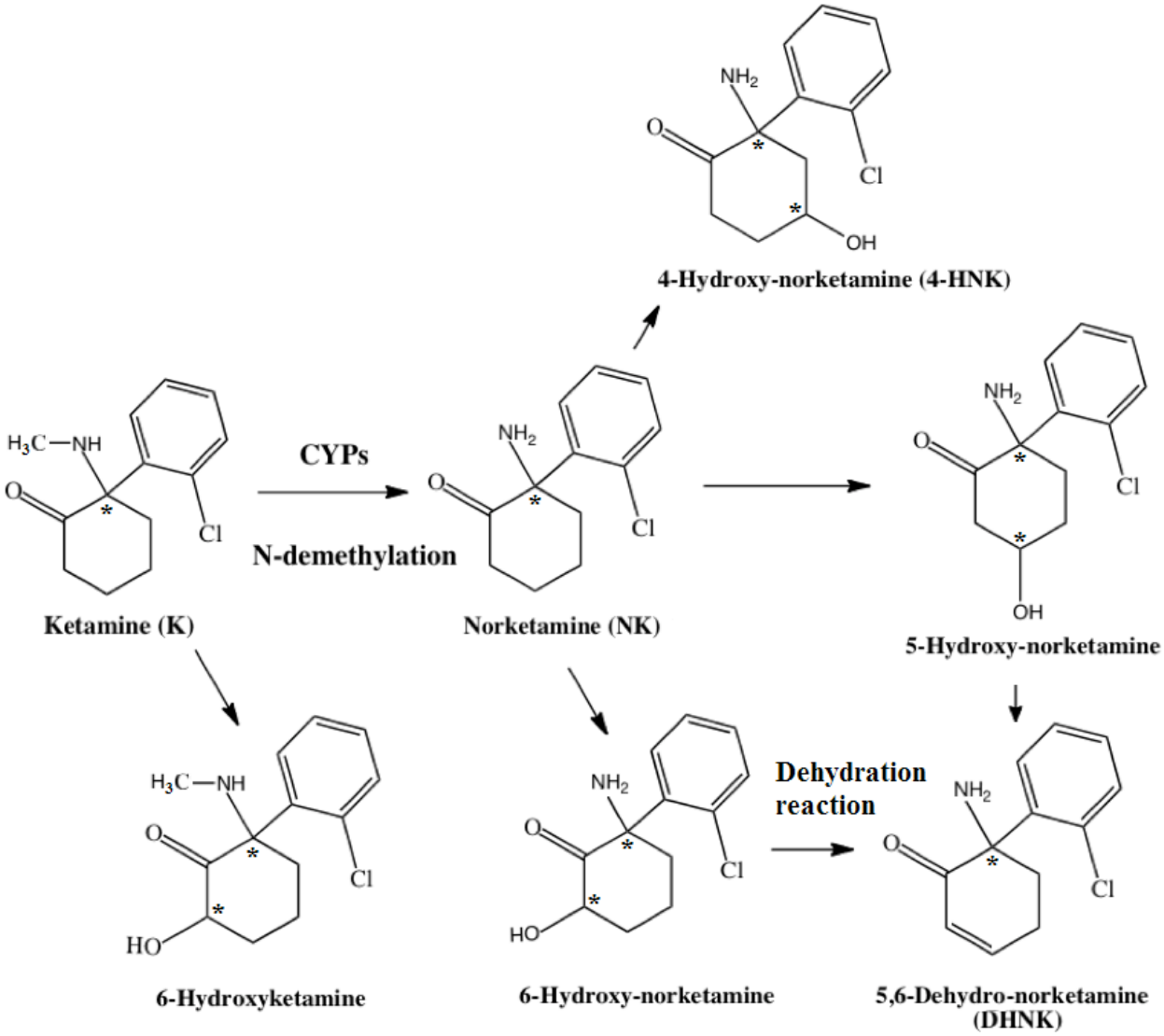

Figure 1-4. The metabolism of ketamine in humans. Symbol * indicate the chiral centre of the molecule.

1.4.1.1.1 The *N*-demethylation of ketamine to norketamine

The *in vitro* metabolism of ketamine has been investigated in several studies. Kharasch *et al.* (1992) first demonstrated that norketamine formation exhibited biphasic kinetics in human liver microsomes (HLM), indicating that the *N*-demethylation of ketamine is mediated by two enzymes. The estimated kinetic parameters (K_m and V_{max}) suggested that one enzyme has high substrate affinity (K_m: 30 to 50 μM) with low catalytic capacity (V_{max}: 2 to 6 nmol.min^{-1}.nmol P450^{-1}), and the other one has low affinity with a slightly higher capacity (K_m: 600 to 800 μM, V_{max}: 9 to 15 nmol.min^{-1}.nmol P450^{-1}). Moreover, this study showed that the *in vitro* metabolism rate of *(S)*-ketamine was approximately 20% greater than *(R)*-ketamine, which is in good agreement with *in vivo* observations.

The biphasic kinetics of microsomal ketamine *N*-demethylation and estimated values of K_m and V_{max} reported by Kharasch *et al.* were then confirmed by two independent studies in the early 2000s (Hijazi and Boulieu, 2002a; Yanagihara *et al.*, 2001). In addition, these two studies examined the contribution of each individual CYP isoform on the metabolism of *(S)*- and *(R)*-ketamine using specific CYP inhibitors and cDNA-expressed human CYP isoforms. However, there was an inconsistency in the predominant CYP isoforms responsible for the *N*-demethylation of ketamine between the two studies.

Yanagihara *et al.* (2001) showed that cDNA-expressed CYP2B6 enzyme has the highest *(S)*- and *(R)*-ketamine *N*-demethylation activities followed by CYP3A4 and CYP2C9, among the 12 tested cDNA-expressed CYP isoforms. The estimated CL_{int} values (V_{max}/K_m) of the cDNA-expressed CYP2B6 for ketamine *N*-demethylation were at least 7 and 10 times higher than those of CYP3A4 and CYP2C9, respectively. The greater catalytic activity of CYP2B6 for ketamine *N*-demethylation is likely due to its approximately 10 times higher substrate binding affinity compared to CYP2C9 and CYP3A4. Further, inhibition assays showed that

the formation of norketamine enantiomers was substantially suppressed by an inhibitory monoclonal antibody against CYP2B6 (~80%) but not by the antibodies against CYP3A4 and CYP2C9, suggesting minor contributions from these two enzymes.

In contrast, Hijazi *et al*. (2002) reported that CYP3A4 was the primary isoform responsible for the *N*-demethylation of ketamine in human livers. Although the authors also found that cDNA-expressed CYP2B6 is the enzyme with the highest ketamine catalytic activity, they argued that the contribution of CYP2B6 in HLMs may be 1/10th to half that of CYP3A4, considering the relatively low CYP2B6 expression level in human livers. However, their inhibition assays showed that orphenadrine and ketoconazole, chemical inhibitors specific for CYP2B6 and CYP3A4, respectively, had similar effects in inhibiting ketamine *N*-demethylation in pooled HLMs. The result indicates that the contribution of CYP2B6 and CYP3A4 were similar in HLMs, although the specificity of these inhibitors is debateable (see *1.4.1.1.2.1.2 inhibition and induction of CYP2B6*, page 23-25, and *1.4.1.1.2.2.1 regulation of CYP3A4 gene expression*, page 30-31, for details). As Hijazi *et al.* pointed out, the contribution of CYP2B6 and CYP3A4 to ketamine *N*-demethylation is likely dependent on their relative expression level, hence it is logical that the interindividual differences in ketamine pharmacokinetics are associated with the variability in enzyme expression and function.

1.4.1.1.2 Cytochrome P450 enzymes

In humans, cytochrome P450s are the major enzymes involved in the metabolism of xenobiotics and the degradation of endogenous compounds, catalysing approximately 75% of oxidative biotransformation of drugs (Guengerich, 2007). To date, 57 functional human CYP gene and 59 pseudogenes (a dysfunctional gene or gene that is not expressed) have been identified, which are classified into 18 families (>40% amino acid homology) and 44

subfamilies (>59% amino acid homology) on the basis of gene sequence similarity. Each enzyme is named with abbreviation CYP followed by an Arabic number representing its family, a capital letter indicating the subfamily, if there is one, and another number at the end indicating the specific isoform. For example, the cytochrome P450 enzyme family 2, subfamily D, polypeptide 6 is written as CYP2D6. The gene encoding the protein has the same name of the protein, but noted by italicised font. For example, the gene encoding CYP2D6 enzyme is written as *CYP2D6*.

Of these 57 CYP enzymes, only about a dozen enzymes from the CYP1, CYP2, and CYP3 gene families are responsible for the metabolism of xenobiotics, including 70-80% of clinical used drugs (Zanger and Schwab, 2013), while other enzymes participate more in the synthesis of hormones and the metabolism of endogenous compounds (Nebert and Russell, 2002). In general, each substrate is metabolised only by one enzyme, however, for some enzymes, overlapping of substrate specificity is not unusual, especially for CYP2B6 (Wang and Tompkins, 2008). Due to influences of both genetic and non-genetic factors (such as age, sex, disease, diet and medicine use), the expression and function of many CYP enzymes show substantial inter- and intra-individual variability (Zanger and Schwab, 2013), some of which has been shown to have substantial impact on pharmacokinetics and drug response. Among all CYP enzymes, the effects of changes in CYP3A4 and CYP2D6 enzyme expression or activity on pharmacokinetics of drugs has been relatively well established due to their wider substrate spectrum (Figure 1-5, next page). In contrast, CYP2B6 has not attracted much attention until recently because of its relatively low protein content in the liver.

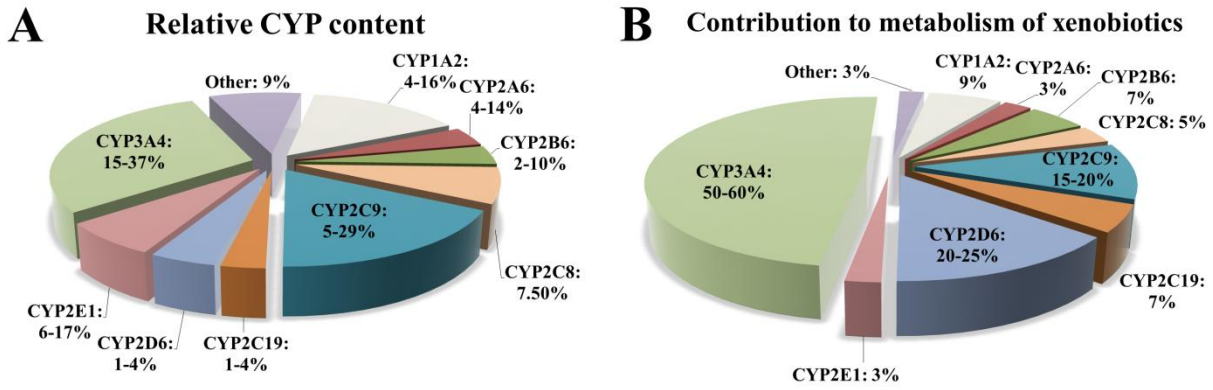

Figure 1-5. Pie charts of A) relative hepatic expression of each CYP enzymes in humans, and B) contribution of each CYP isoforms to the metabolism of xenobiotics in humans. The sum contribution of each CYP isoform to the metabolism of xenobiotics is over 100% due to overlapping in enzyme's substrate specificities. Figures were generated according to the data reported by Zanger and Schwab (2013) and PharmGKB database (Whirl-Carrillo *et al.*, 2012).

1.4.1.1.2.1 CYP2B6

Human CYP2B6 is expressed mainly in the liver, and to a lesser extent in the brain (Miksys *et al.*, 2003) and kidney (Aleksa *et al.*, 2005) but is not detectable in the small intestine (Thelen and Dressman, 2009). Initially, CYP2B6 was thought to constitute <1% of total hepatic CYP content and have a negligible contribution to drug metabolism, due to the lack of a sensitive and specific antibody for detection. More recent studies showed that CYP2B6 accounts for 2 to 10% of total hepatic CYP content (Figure 1-5A), but the inter-individual difference in expression level of hepatic enzyme can be as high as 100-fold (Wang and Tompkins, 2008; Zanger *et al.*, 2007).

1.4.1.1.2.1.1 CYP2B6 substrates

CYP2B6 has been found to be involved in the metabolism of approximately 7% of xenobiotics and 4% of the top 200 prescribed drugs in US (Rendic, 2002; Zanger *et al.*, 2008),

but often not as the predominant isoform, the major isoform responsible for the metabolism (Wang and Tompkins, 2008). The clinically important drugs that are predominantly metabolised by CYP2B6 include the atypical anti-depressant and smoking cessation agent, bupropion (Hesse *et al.*, 2000); the HIV-1 reverse transcriptase inhibitor, efavirenz (Ward *et al.*, 2003); anti-cancer agents, cyclophosphamide and ifosfamide (Chang *et al.*, 1993); analgesics, methadone and pethidine (Ramirez *et al.*, 2004; Totah *et al.*, 2008); anaesthetics, ketamine and propofol (Court *et al.*, 2001; Yanagihara *et al.*, 2001), and the anti-epileptic agent, *(S)*-mephobarbital (Kobayashi *et al.*, 1999).

1.4.1.1.2.1.2 Inhibition and induction of CYP2B6

Two of the major factors contributing to the variability in CYP2B6 enzyme expression and ketamine pharmacokinetics is enzyme inhibition and induction. Peltoniemi *et al.* (2011) reported that oral ticlopidine, a CYP2B6 inhibitor, increased the mean total area under the plasma concentration-time curve ($AUC_{0-\infty}$) of 0.2 $mg.kg^{-1}$ oral *(S)*-ketamine to 318% and decreased the norketamine to ketamine AUC ratio by approximately 50% in healthy volunteers. In addition, Noppers *et al.* (2011b) reported that oral rifampicin, an inducer of CYP2B6, CYP2C9, CYP2C19 and CYP3A4, decreased $AUC_{0-300min}$ of plasma concentrations of *(S)*-ketamine and *(S)*-norketamine following i.v. administration of 40 mg or 80 mg of *(S)*-ketamine by 10% and 50%, respectively.

A previous study by Walsky *et al.* (2006) evaluated the *in vitro* CYP2B6 inhibition with 227 commonly prescribed drugs and other xenobiotics at 30 μM and identified 30 compounds that reduced CYP2B6's bupropion hydroxylase activity by more than 50%. Among those inhibitors, two platelet aggregation inhibitors, clopidogrel and ticlopidine had greatest potency (IC_{50} = 0.02 and 0.15 μM, respectively), however, both of which are not ideal as model inhibitors for *in vitro* experiments due to their poor CYP2B6 specificity (Turpeinen

and Zanger, 2012). Other relatively potent (IC_{50} < 1 μM) or moderate inhibitors of CYP2B6 (IC_{50} < 10 μM) that are commonly prescribed include antidepressants (norfluoxetine, paroxetine, sertraline and tranylcypromine), anti-fungal agents (clotrimazole, itraconazole, ketoconazole and voriconazole), anti-asthmatic agents (mometasone and montelukast), anti-cholesterol drugs (ezetimibe and fenofibrate), anti-estrogens (raloxifene and tamoxifen), anti-hypertensive drugs (amlodipine and felodipine), Alzheimer's disease medication (memantine), anti-allergic agent (loratadine), anti-cancer agent (ThioTEPA) and antiretroviral agent (ritonavir) (Jeong *et al.*, 2009; Walsky *et al.*, 2006)**.** Notably, ketoconazole at 10 μM was previously used as an inhibitor of CYP3A in the two *in vitro* studies that reported major contribution of CYP3A4 in ketamine *N*-demethylation (Hijazi and Boulieu, 2002a; Mossner *et al.*, 2011). Since ketoconazole is also a relatively potent inhibitor of CYP2B6 (Walsky *et al.*, 2006), its inhibitory effect on liver microsomal norketamine formation may not be all attributed to the inhibition of CYP3A4. This may explain the observations of Mossner *et al*. (2011) that microsomal ketamine *N*-demethylation was substantially inhibited by ketoconazole but not by CYP3A4-specific monoclonal inhibitory antibody. Of all currently known CYP2B6 inhibitors, ThioTEPA (N,N',N''-triethylenethiophosphoramide) has the highest CYP2B6 selectivity, therefore, it is possibly a better model inhibitor for *in vitro* experiments.

It has been reported that CYP2B6 expression was elevated by a large number of CYP3A4 and CYP2C inducers, possibly via activation of nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR) (Faucette *et al.*, 2007; Ward *et al.*, 2003). Interestingly, some CYP2B6 substrates such as efavirenz and cyclophosphamide are also CYP2B6 inducers and thus may subsequently increase their own metabolism (Faucette *et al.*, 2007; Lindley *et al.*, 2002).

1.4.1.1.2.1.3 *CYP2B6* genetic polymorphisms

Another major factor contributing to the interindividual variability in CYP2B6 enzymatic expression and/or activity, and consequent differences in drug response is the *CYP2B6* genetic polymorphism. The most common form of the genetic polymorphism in human *CYP2B6* is a single nucleotide polymorphism (SNP), a single base pair alteration in the DNA sequence. According to the National Center for Biotechnology Information (NCBI) single nucleotide polymorphism database (dbSNP) (http://www.ncbi.nlm.nih.gov/projects/SNP/), over one thousand SNPs have been identified in the coding and noncoding regions of the human *CYP2B6* gene (accessed in July 2014). Some SNPs or combination of several SNPs (haplotype) that alter transcription, splicing, translation, post-transcriptional and post-translational modifications are designated with a unique allelic number (Sim and Ingelman-Sundberg, 2010). Generally, the gene sequence containing no genetic polymorphisms is designated as the wild-type (**1* allele). Although, for *CYP2B6*, the **1* allele has been detected with highest allelic frequencies (Klein *et al.* 2005), the wild-type may not always be the most prevalent allele for every *CYP* gene and ethnic group.

According to the Human Cytochrome P450 Allele Nomenclature Database (http://www.cypalleles.ki.se/cyp2b6.htm), thirty-six allelic variants have been identified in human *CYP2B6* gene to date, fifteen of which have been demonstrated to alter either the expression or function of CYP2B6 enzyme (accessed in July 2014). These expression- or function-impairing alleles are generally identified with low allelic frequencies (<1% of populations) or only in specific ethnic groups. The one exception, however, is the most prevalent and clinically important *CYP2B6* allelic variant, the *CYP2B6*6* allele, which has been detected in relatively high allelic frequencies (15 to 48%) in all major ethnic groups (Table 1-3, page 27). The *CYP2B6*6* allele is a haplotype consisting of two linked SNPs: c.516G>T (rs3745274) located on exon 4 and c.785A>G (rs2279343) located on exon 5,

which result in amino acid changes Q172H and K262R, respectively. A previous study using human cell lines transfected with cDNAs carrying *CYP2B6* variants showed that Q172H leads to an aberrant splicing of CYP2B6 pre-mRNA lacking of exons 4 to 6, which may be responsible for the substantial decrease in enzymatic expression and activity (Hofmann *et al.*, 2008). The presence of K262R substrate-dependently altered (either increased or decreased) the metabolic activities of cDNA-expressed CYP2B6 protein (Ariyoshi *et al.*, 2011). Clinically, the *CYP2B6*6* homozygous genotype (*CYP2B6*6/*6*) has been associated with greater plasma exposure (approximately 3-fold higher plasma AUC) and more severe neuropsychological toxicity of efavirenz (Rotger *et al.*, 2005). It has also been reported to affect pharmacokinetics of nevirapine (Rotger *et al.*, 2005), sibutramine (Shinde *et al.*, 2013), mirtazapine (Jaquenoud Sirot *et al.*, 2012), bupropion (Qin *et al.*, 2012) and *(S)*-methadone (Crettol *et al.*, 2006) in a substrate-dependent manner. Although CYP2B6 has been involved in ketamine metabolism *in vitro* (Hijazi and Boulieu, 2002a; Yanagihara *et al.*, 2001), the impacts of *CYP2B6*6* on ketamine metabolism and clinical response are yet to be determined.

In contrast to *CYP2B6*6*, the clinical relevance of other allelic variants of *CYP2B6* have scarcely been investigated. The *CYP2B6*5* allele (c.1459C>T, rs3211371), the second most common allelic variant in Caucasians, has been associated with lower *CYP2B6* expression and the liver microsomal hydroxylation of bupropion but not efavirenz (Desta *et al.*, 2007). Clinically, the presence of at least one *CYP2B6*5* in *CYP2C19*2* non-carriers enhanced the anti-platelet activity of clopidogrel by approximately 25% (Kassimis *et al.*, 2012). *CYP2B6*18* (c.983T>C, rs28399499) and *CYP2B6*16* (c.983T>C and c785A>G) alleles, expressed in 2-12% and 7-19%, respectively, in African populations, have been associated with high plasma efavirenz concentration and toxicity of efavirenz (Maimbo *et al.*, 2012; Wyen *et al.*, 2008). However, the key nucleotide change for these two alleles, c.983T>C, has not been found in other major populations.

Table 1-3. Allelic frequencies of expression- or function-impairing alleles of *CYP2B6* gene in Caucasian, African, Asian and Hispanic populations.

Allele	Key nucleotide change(s)	Amino acid change(s)	Functional change(s)	Allelic frequency %			
				Caucasians	Africans	Asians	Hispanics
*CYP2B6*4*	c.785A>G (rs2279343)	K262R (not in combination with other SNPs)	↑ expression & activity	4[a]	0[b]	5-12[b]	NA
*CYP2B6*5*	c.1459C>T (rs3211371)	R487C	↓ expression & activity	9-12	0-4	1-4	0-7
*CYP2B6*6*	c.516G>T (rs3745274), c.785A>G	Q172H, K262R	↓ expression & ↑ or ↓ activity (substrate dependent)	25-32	35-46	15-21	24-48
*CYP2B6*8*	c.415A>G (rs12721655)	K139E	↓ expression	0.8	0	0[b]	0
*CYP2B6*11*	c.136A>G (rs35303484)	M46V	↓ expression	0-2	0	0	0-1.1
*CYP2B6*12*	c.296G>A (rs36060847)	G99E	↓ expression	0.4-1.2	0.4-0.6	0.6	22
*CYP2B6*14*	c.419G>A (rs35773040)	R140Q	↓ activity	0.2[a]	0[b]	0[b]	NA
*CYP2B6*15*	c.1172T>A (rs35979566)	I391N	↓ expression	0-1	0	0	2.2
*CYP2B6*16*	c.785A>G c.983T>C (rs28399499)	K262R, I328T	↓ expression & activity	0[c,d]	7-19[c,d]	0[c]	NA
*CYP2B6*18*	c.983T>C	I328T	↓ expression & activity	0	2-12	0	0-1

Table 1-3. Allelic frequencies of expression- or function-impairing alleles of *CYP2B6* gene in Caucasian, African, Asian and Hispanic populations. (continued)

Allele	Key nucleotide change(s)	Amino acid change(s)	Functional change(s)	Allelic frequency %			
				Caucasians	Africans	Asians	Hispanics
CYP2B6*19	c.516G>T, c.785A>G, c.1006C>T (rs34826503)	Q172H, K262R, R336C	↓ expression	0	0-1.6[b]	0	NA
*CYP2B6*20*	c.503C>T (rs36056539) c.516G>T, c.785A>G,	T168I, Q172H, K262R	↓ expression	0	0-1.6[b]	0	NA
*CYP2B6*21*	c.1282C>A (rs35010098)	P428T	↓ expression	0	0-1.6[b]	0	NA
*CYP2B6*22*	c.-82T>C	promoter	↑ expression	2.4	0-2.5	0-2.5	2.4
*CYP2B6*26*	c.499C>G (rs3826711) c.516G>T, c.785A>G,	P167A, Q172H, K262R	↓ expression & activity	0	0	1.1	NA
*CYP2B6*27*	c.593T>C (rs36079186)	M198T	↓ expression & activity	0	0.6[e]	0	0
*CYP2B6*28*	c.1132C>T (rs36079186)	R378 termination	nonsense mutation	0	0.6[e]	0	0

Arrows indicate the functional consequence of the allele: increase (↑) or decrease (↓) on enzyme expression and activity *in vitro* (data obtained from The Human CYP Allele Nomenclature Database). Data of allelic frequencies were obtained from NCBI dbSNP database, or reported by a. Lang *et al.* (2004), b. Klein *et al.* (2005), c. Chen and Huang (1992); Solus *et al.* (2004), d.Wang *et al.* (2006), e. Rotger *et al.* (2007). NA: not available.

1.4.1.1.2.2 CYP3A4

The human CYP3A subfamily, consisting of four functional members: CYP3A4, CYP3A5, CYP3A7 and CYP3A43, is the most abundant CYP subfamily in human livers. Of the four isoforms, CYP3A4 is the quantitative dominant enzyme in most people, which accounts for approximately 85% of total liver microsomal CYP3A content and 15 to 37% of total hepatic cytochrome P450 content (Ohtsuki *et al.*, 2012). Additionally, it is also the most abundant isoform in human small intestine, representing 33 to 87% of total small intestinal CYP content (Paine *et al.*, 2006). In humans, CYP3A4 is responsible for the metabolism of approximately 50% of clinically important drugs, as it is capable of catalysing various reactions including hydroxylation, hydrolysis**,** dehydrogenation, *N*-demethylation, *N*- and *O*-dealkylation.

CYP3A5, the second most abundant isoform in CYP3A subfamily, may constitute up to 50% of the total CYP3A hepatic content and act as the predominant drug-metabolising CYP isoform in its expressers, since it has a catalytic specificity similar to CYP3A4. However, it is rarely expressed in non-African populations due to the prevalence of a defective allele variant: *CYP3A5*3* (g.6986A>G, rs776746) (Xie *et al.*, 2004). The allelic frequency of *CYP3A5*3* among Caucasians is 88-97%, Hispanics 59-85%, Asians 69-77%, African Americans 34-37% and Sub-Saharan Africans 13-18% (obtained from NCBI dbSNP, accessed at July 2014). In contrast to CYP3A4 and CYP3A5, CYP3A7 and CYP3A43 are quantitatively insignificant in adult livers and their contribution to drug metabolism is believed to be minor (Domanski *et al.*, 2001; Ohtsuki *et al.*, 2012; Westlind *et al.*, 2001).

1.4.1.1.2.2.1 Regulation of *CYP3A4* gene expression

In humans, the expression of CYP3A4 shows extremely large inter-individual variability. A previous study showed that CYP3A4 contents varied from 5 to 376 and 0.5 to 33 pmol.mg^{-1} in 60 HLMs and 31 jejunal homogenates, respectively (Lin *et al.*, 2002). Unlike CYP2B6, the variability in CYP3A4 enzyme expression is more likely due to drug- or inflammation-induced transcriptional regulation rather than genetic factors, as only 5 alleles: CYP*3A4*4* (c.352A>G, rs55951658), CYP*3A4*5* (c.653C>G, rs55901263), CYP*3A4*6* (c.830_831insA, rs4646438), *CYP3A4*18* (c.878T>C, rs28371759) and *CYP3A4*22* (g.20493C>T, rs35599367) of the 24 currently identified allelic variants of *CYP3A4* (accessed at July 2014) have been found to alter catalytic activities of enzyme *in vivo* (Hsieh *et al.*, 2001; Kang *et al.*, 2009; Wang *et al.*, 2011). Of them, the *CYP3A4*22* allele found only in Caucasians with an allelic frequency of 2-7% may explain 7 and 12% variability in the hepatic CYP3A4 mRNA and protein levels, respectively (Elens *et al.*, 2011; Wang *et al.*, 2011). A recent *in vitro* study by Wang *et al.* (2011) demonstrated that this allele was associated with approximately 40% decrease in CYP3A4 mRNA expression and approximately 60% decrease in testosterone 6β-hydroxylase activity in human livers. The *CYP3A4*4, *5, *6* and **18* allelic variants have also been associated with decreased CYP3A4 activities but were identified only in East Asian populations with allelic frequencies of 0.5 to 2% (Dai *et al.*, 2001; Hsieh *et al.*, 2001; Kang *et al.*, 2009).

The multiple active sites of CYP3A4 not only give the enzyme a broad substrate spectrum but also make CYP3A4 susceptible to inhibition and induction by a large number of drugs and xenobiotics. The induction of *CYP3A4* gene expression, similar to the induction of *CYP2B6*, is mainly mediated by the activation of nuclear receptors PXR and CAR (Zanger and Schwab, 2013). Some potent CYP3A4 inducers commonly prescribed include antibiotics (rifampicin), anti-depressants (St. John's Wort), anti-epileptic agents (carbamazepine and phenytoin) and

anti-retroviral drug (efavirenz) (FDA, Drug Development and Drug Interactions: Table of substrates, inhibitors and inducers, http://www.fda.gov/drugs/developmentapprovalprocess/developmentresources/druginteractionslabeling/ucm093664.htm). The inhibition of CYP3A4 is primarily the functional consequence of irreversible binding of compounds to CYP3A4 (Zhou *et al.*, 2005). Common CYP3A4 inhibitors include antibiotics (troleandomycin, clarithromycin and erythromycin), anti-fungal agents (itraconazole, ketoconazole, fluconazole), HIV medications (lopinavir/ritonavir and nelfinavir) and grapefruit juice (http://www.fda.gov/drugs/developmentapprovalprocess/developmentresources/druginteractionslabeling/ucm093664.htm). Notably, many of these CYP3A4 regulators such as rifampicin, efavirenz, itraconazole and ketoconazole also alter CYP2B6 content due to the fact that *CYP2B6* and *CYP3A4* share similar regulatory mechanisms. Hence co-administration of these CYP3A4 and CYP2B6 dual regulator may considerably reduce the metabolism of ketamine and aggravate the toxicity of ketamine, which should be considered before prescription.

In addition to drug-mediated enzyme inhibition, CYP3A4 content is substantially regulated by sex (higher mean CYP3A4 content in female) and pathological states, particularly those that result in proinflammatory responses (e.g. cancers) (Aitken *et al.*, 2006; Diczfalusy *et al.*, 2008; Hunt *et al.*, 1992; Morgan, 1997; Schmidt *et al.*, 2001). An *in vitro* study using cultured human hepatocytes showed that proinflammatory cytokines including IL-1, IL-6, tumour necrosis factors (TNF), transforming growth factor, and interferon reduce the expression of CYP3A4 mRNA and protein by up to 95% and 90%, respectively (Aitken and Morgan, 2007).

1.4.1.2 Excretion

Following a single i.v. ketamine administration, ketamine and norketamine were detectable in the first hour and up to 23 hours and dehydronorketamine was detectable in urine for up to 60

hours after injection. Only approximately 2 to 3% of ketamine can be recovered as unchanged drug from urine after a single i.v. injection. The recovered norketamine and dehydronorketamine in urine was approximately 1.6% and 16% of the administrated dose respectively. The remaining injected dose of ketamine was renally excreted as glucuronide conjugates of metabolites and intermediates (Wieber *et al.*, 1975).

1.4.2 Distribution

As a drug with a high lipid solubility, ketamine is rapidly distributed to highly perfused tissues (such as the brain, heart and liver) and subsequently redistributed to less perfused tissues (White *et al.*, 1976). The distribution half-life of racemic ketamine after i.v. injection was approximately 10 to 20 min (Table 1-2, page 15). A less than 10 min distribution half-life when administered over a relatively long infusion duration (30 min) was also reported, however, which may be due to the difficulty in measuring distribution during infusion (Persson *et al*,. 2002). The volume of distribution of racemic ketamine at steady-state (V_{ss}) was about 2 to 6 $L.kg^{-1}$, which is approximately 60- to 150-fold greater than the plasma volume, indicating an extensive distribution of ketamine in humans (Geisslinger *et al.*, 1993; Ihmsen *et al.*, 2001; Persson *et al.*, 2002; Yanagihara *et al.*, 2003). No significant stereoselective difference in the values of V_{ss} was observed by these pharmacokinetic studies.

1.4.3 Plasma protein binding

The percentage of ketamine and norketamine bound to human serum proteins is 47 to 64% and 50 to 54%, respectively (Dayton *et al.*, 1983; Hijazi and Boulieu, 2002b). Dayton *et al.* (1983) showed that the bound fraction of tritium-labelled racemic ketamine to human serum albumin, α_1-acid glycoprotein (AAG) and γ-globulin at 37 ℃ were 15% to 30%, 10 to 48% and 3 to 8%, respectively, dependent on the pH and serum protein concentrations. These

values were comparable with those reported by Hijazi *et al.* (2002). Since the concentration of plasma proteins is largely determined by diseases, such as cancers (Phillips *et al.*, 1989), the disease-induced alteration in plasma protein binding may partially explain some variations in ketamine apparent clearance of total drug. However, as mentioned earlier, the high hepatic extraction ratio suggests that the influence of plasma protein binding on ketamine clearance is relatively small compared with the change in liver blood flow.

1.4.4 Summary

Clinical trials have reported several-fold interindividual difference in ketamine plasma concentrations (50 to 350 $ng.mL^{-1}$, after a 40 min infusion of 0.4 $mg.kg^{-1}$) in pain patients (Kvarnström *et al.*, 2004). Previous studies reported that the minimal plasma concentration of ketamine required for analgesia was 150 $ng.mL^{-1}$ (Clement *et al.*, 1981). Hence the variability in plasma concentrations may be a factor influencing ketamine analgesic response. Since ketamine is extensively cleared in the liver with a high extraction ratio (Haas and Harper, 1992), the variation in plasma concentration may potentially reflect the difference in ketamine clearance. In humans, ketamine is rapidly distributed and mainly cleared by hepatic CYP-mediated *N*-demethylation. CYP2B6 and CYP3A4 have been related to ketamine *N*-demethylation *in vitro* but the relative contribution of each enzyme remains controversial (Hijazi and Boulieu, 2002a; Yanagihara *et al.*, 2001). The expression and function of human hepatic CYP2B6 and CYP3A4 are highly affected by genetic (particularly for CYP2B6) and non-genetic factors (Zanger and Schwab, 2013), hence the difference in enzyme function may explain the interindividual variation in plasma concentration.

However, previous *in vivo* pharmacokinetic studies found little variation in ketamine plasma concentrations and clearance rates (Clements and Nimmo, 1981; White *et al.*, 1985). A possible reason for such a discrepancy is that the pharmacokinetic parameters were almost

exclusively assessed in young healthy male volunteers, which may not reflect the influence of age, disease, or medication use on the pharmacokinetics of ketamine that commonly occur in chronic pain patients. Moreover, the population sizes of these pharmacokinetic studies were too small to determine the effects of genetic variants of *CYP2B6* on ketamine clearance. Accordingly, the purpose of the current project was to investigate ketamine metabolism *in vitro* and plasma clearance of ketamine at steady-state in chronic pain patients in an attempt to reveal the impact of *CYP2B6* genetic variability and non-genetic factors on ketamine pharmacokinetics.

1.5 The applications of sub-anaesthetic ketamine in pain management

In the past two decades, a large number of randomised controlled clinical trials (RCT) have been conducted to examine the efficacy and safety of sub-anaesthetic doses of ketamine in the treatment of different types of pain. A sub-anaesthetic ketamine dose is defined as an intramuscular (i.m.) bolus dose of < 2 $mg.kg^{-1}$ or an i.v. injection of < 1 $mg.kg^{-1}$ or a continuous i.v. infusion rate of < 1.2 $mg.kg^{-1}.h^{-1}$ (Schmid *et al.*, 1999). At such doses, although ketamine still results in psychotomimetic adverse effects, the incidence of adverse effects was relatively low (< 8%) compared with anaesthetic application (2 to 60%, average 20-30%) and these effects were generally mild and well-tolerated by patients (Krestow, 1974; Laskowski *et al.*, 2011; Oduntan and Gool, 1970; White *et al.*, 1982).

The most frequently investigated uses of sub-anaesthetic ketamine were: a) supplements to opioid-based postoperative acute pain treatments; and b) treatment of chronic non-malignant pain. In addition, the use of ketamine in the treatment of cancer pain, especially opioid refractory cancer pain, has been assessed in many trials, most of which, however, lack sufficient quality due to their small participant number or inappropriate research design. This

section will summarise the findings of the literature on the therapeutic effectiveness and limitations of ketamine analgesia or co-analgesia in humans.

1.5.1 Ketamine for postoperative opioid analgesia

The effectiveness of sub-anaesthetic doses of ketamine as an adjuvant to postoperative opioid analgesia has been examined in three systematic reviews (Bell *et al.*, 2006; Laskowski *et al.*, 2011; Subramaniam *et al.*, 2004), all of which concluded that the addition of low doses of ketamine to opioid analgesia was useful to improve pain relief and/or reduce the postoperative 24 h opioid requirement. However, these beneficial effects of ketamine were only observed in approximately 60-70% of all clinical trials. Laskowski *et al.* (2011) found that ketamine is likely to be two times more effective in improving opioid analgesic effects in surgical procedures that were associated with high pain severity state (pain score $\geq$ 7 on a 0-10 visual analogue scale [VAS]) than in those associated with moderate (VAS score 4 to 7) or low pain severity state (VAS score $\leq$ 4). In addition to pain severity, ketamine co-analgesia was affected by its route of administration. Subramaniam *et al*. (2004) showed that ketamine had the greatest possibility to improve opioid analgesia when given by continuous i.v. infusion compared with other routes of administration, whilst the combination of ketamine and morphine i.v. patient controlled analgesia (PCA) treatment failed to improve analgesia and reduce morphine consumption. This finding suggested that maintenance of steady-state plasma concentration is possibly important for ketamine's analgesia. Further, ketamine's effects on opioid consumption and time to first analgesic effect appeared to be greater when at doses of > 1 $mg.kg^{-1}$ or $\leq$ 0.5 $mg.kg^{-1}$ compared with that at doses in-between (Laskowski *et al.*, 2011). This result suggests a possibility that ketamine may exert its analgesic effects via different mechanisms at low and high plasma concentrations.

1.5.2 Ketamine for the treatment of chronic pain

1.5.2.1 Ketamine for the treatment of chronic non-cancer pain

The effectiveness of ketamine for acute relief of chronic non-cancer pain has been reported (Bell, 2009; Noppers *et al.*, 2010). I.V. infusion was the most frequently employed and probably the most effective route of administration in randomised controlled trials (RCTs), as approximately 95% (19 of 20) trials employing i.v. infusion regimens reported > 50% pain relief by ketamine (see *Appendix II dosing regimens employed in published RCTs investigating ketamine for pain management*, page 158-161, for more details). In contrast, other routes of administration, particularly oral administration, have been associated with low analgesic efficacy (Noppers *et al.*, 2010). However, it should be noted that the analgesic effects of i.v. ketamine were observed in only 40-50% of patients (Graven-Nielsen *et al.*, 2000; Kvarnström *et al.*, 2003; Kvarnström *et al.*, 2004; Lemming *et al.*, 2005; Sörensen *et al.*, 1997), and that there were considerable differences in the magnitude and duration of pain relief between ketamine responders. As mentioned earlier in section *1.4 Pharmacokinetics of ketamine* (page 13-32), one possible explanation for such a problem is the variability in plasma concentrations. Another possible explanation is the heterogeneous nature of pain (Felsby *et al.*, 1996). Many patients suffer from one or more forms of chronic pain that may involve both nociceptive and neuropathic components; and ketamine is expected to be more effective against the neuropathic component, since its primary mechanism of action is hypothesised to be suppression of central sensitisation.

Due to the fact that plasma concentration of ketamine rapidly decline after the termination of infusion (half-life = 2-3 h), only a few studies followed patients for more than 48 h after a short-term i.v. ketamine infusions (Backonja *et al.*, 1994; Mitchell and Fallon, 2002; Sörensen *et al.*, 1997). Surprisingly, Mitchell *et al.* (2002) and Sörensen *et al.* (1997) reported that the analgesic effects of ketamine persisted for 5 days after a single infusion in some patients. This

persistent pain relief by ketamine was more common and long-lasting after multiple day long-term i.v. infusion regimens (Amr, 2010; Schwartzman *et al.*, 2009; Sigtermans *et al.*, 2009). A semi-quantitative analysis by Noppers *et al.* (2010) examined the effect of infusion duration on the magnitude and length of treatment effect and found that the analgesic effect of ketamine generally dissipated over 48 h following infusion after short infusion scheme (< 2 h). In contrast, infusions over 10 h have approximately 95% probability of providing an analgesia lasting over 48 h (Noppers *et al.*, 2010). For example, Sigtermans *et al.* (2009) reported that a 4.2-day i.v. infusion of *(S)*-ketamine (1.2 to 7.2 $\mu g.kg^{-1}.min^{-1}$) produced significant pain relief for 12 weeks in patients with complex regional pain syndrome (CRPS). The authors speculated that the persistent pain relief by ketamine is due to long-term desensitisation of the central NMDA receptors, however, this hypothesis has yet to be tested.

Although ketamine analgesia is associated with lower response rate when the drug was administered via alternative routes (Noppers *et al.*, 2010), it is worth noting that sufficient analgesia was achieved at a low plasma ketamine concentration in some patients. For example, it has been shown that topical ketamine produced acute reduction of local allodynia and mechanical hyperalgesia in CRPS patients, with plasma concentrations lower than the lower limit of quantification (1 $ng.mL^{-1}$) (Finch *et al.*, 2009). This finding indicates that ketamine analgesia is possibly not only due to inhibition of central sensitisation, but also involves peripheral mechanisms, although the exact mechanism(s) remains unclear. Apart from the antagonism of NMDA receptors in the peripheral neurons (Petrenko *et al.*, 2003), other logical speculation involves the suppression of peripheral pro-inflammation, since animal studies demonstrated that ketamine at sub-anaesthetic doses attenuated peripheral proinflammatory responses in rats (Sun *et al.*, 2004; Yu *et al.*, 2007) (see section *1.6 anti-inflammatory mechanism of ketamine*, page 40-48, for more details).

1.5.2.2 Ketamine for the treatment of chronic cancer pain

Chronic pain is one of the most frequent complications of cancer. Approximately 30 to 50% of patients under chronic treatment for solid tumours suffer from chronic pain and the prevalence among those patients with advanced disease is 70 to 90% (Portenoy and Lesage, 1999). At present, treatment of chronic cancer pain is still mostly dependent on opioid-based therapy, which, unfortunately, is often associated with opioid-tolerance, dependence, hyperalgesia and allodynia (Portenoy, 2011). Accordingly, adjuvant analgesics are added in an attempt to increase the therapeutic index of opioids by reducing opioid dose or adverse effects. The concept of using adjuvant ketamine with chronic opioids for cancer pain management has been deduced from the positive effects of ketamine on acute opioid analgesia in postoperative settings.

Despite increasingly being employed, the evidence that supports the use of adjuvant ketamine to opioid-based chronic cancer pain treatment was mostly provided by open label studies, uncontrolled trials and case series, but not RCTs with sufficient population size (Bell *et al.*, 2012). A systematic review published in 2003 (Bell *et al.*, 2003) and its updated revision in 2012 (Bell *et al.*, 2003; Bell *et al.*, 2012) identified only two RCTs with appropriate experimental design and more than 10 participants in each treatment group. Although the two included studies demonstrated positive effects of ketamine in improving analgesic effects (Mercadante *et al.*, 2000) or reducing opioid consumption (Yang *et al.*, 1996), the data are insufficient to reach any evidence-based conclusions due to the small population of participants (a total of 30 patients were involved in these two RCTs).

During the time of my PhD candidature, a large, double-blinded phase III RCT investigated the effects of subcutaneous ketamine in 181 patients with cancer pain (Hardy *et al.*, 2012). Patients were randomised to receive 24 h continuous s.c. infusion of ketamine (n = 93) or

placebo saline control (n = 92) at a rate of 100 mg / 24 h, and escalating doses of 300 and 500 mg / 24 h if there was no improvement in pain relief. No significant differences in pain score and breakthrough analgesic doses between ketamine (analgesic response rate = 31%) and placebo group (analgesic response rate = 27%) were observed, but the incidence of adverse effects in the ketamine group was approximately two times greater than the placebo group. Based on these findings, Hardy *et al.* concluded that there is no net clinical benefit of ketamine when used as a co-analgesic to standard treatments of cancer pain. Admittedly, this study has a high quality of study design and the largest sample size among all the published trials, but more RCTs with high quality are needed to confirm their results, since these observations are inconsistent with the findings of a large number of non-RCTs. However, in my opinion, the problems of lack of standardisation in dosing regimens and knowledge of pharmacokinetics of ketamine in chronic cancer patients have to be addressed before the initiation of further trials.

1.5.3 Summary

Sub-anaesthetic ketamine is probably effective in improving postoperative opioid analgesia in surgeries that induce severe acute pain and useful for the treatment of various chronic non-cancer pain, but its effectiveness in the treatment of chronic cancer pain needs to be confirmed by more high quality RCTs. The major problem that limited the use of sub-anaesthetic ketamine in pain treatment was the low efficacy and the considerable interindividual variability in drug response. One possible explanation for such an interindividual difference is the variability in ketamine clearance and therefore its plasma concentrations. In addition, ketamine, particularly when it was administered via off-label routes (oral and topical administration), produced sufficient analgesic effects at a plasma concentration lower than the minimal requirement for analgesia (150 $ng.mL^{-1}$, Clement *et al.*, (1981)). This suggests that ketamine analgesia is mediated by distinct mechanisms that are

involved in a concentration-dependent manner. To minimise these variabilities and guide dosing decisions, more fundamental research is required to investigate the causes of the differences in ketamine pharmacokinetics and the precise analgesic mechanism(s) of ketamine over a range of concentrations.

1.6 The non-classical analgesic mechanisms of ketamine

Although it is well accepted that the attenuation of central sensitisation via the non-competitive antagonism of NMDA receptors is the primary mechanism responsible for ketamine's actions, ketamine has also been found to interact with several other neuronal targets *in vitro,* including opioid receptors (Finck and Ngai, 1982; Hirota *et al.*, 1999; Hustveit *et al.*, 1995; Sarton *et al.*, 2001; Smith *et al.*, 1987), cholinergic receptors (Durieux, 1995; O'Neill *et al.*, 2013; Yamakura *et al.*, 2000), and monoaminergic receptors and transporters (Bannister *et al.*, 2009; Kapur and Seeman, 2002; Seeman *et al.*, 2009). However, the inhibition constant (K_i) values of both ketamine enantiomers for most of these targets were relatively low (over 10 μM in humans, compared with 2 to 4 μM for NMDA receptors). These low ligand-receptor affinities (see Appendix II for more information, page 158-161) suggest that these targets may be clinically relevant only at anaesthetic ketamine doses, whilst their contributions to ketamine analgesia at sub-anaesthetic doses are likely to be minor. Since the mechanism of ketamine anaesthesia is beyond the scope of this thesis, the involvement of these targets in ketamine's actions will not be discussed in detail here.

In addition to the interactions with these neuronal targets, several recent *in vitro* experiments demonstrated that ketamine reduces the synthesis of several proinflammatory cytokines, namely IL-1β, IL-6 and TNF-α, in both peripheral and central immune cells (Chang *et al.*, 2009; Welters *et al.*, 2010; Wu *et al.*, 2008; Wu *et al.*, 2012). Since there is solid evidence that indicates the important roles of proinflammatory cytokines in the pathogenesis of

neuropathic pain, this inhibition of proinflammatory responses may be a potential non-neuronal contributor to ketamine analgesia (Clark *et al.*, 2013). The section will briefly discuss the role of proinflammatory cytokines in the development of neuropathic pain and the analgesic activities of ketamine.

1.6.1 Anti-inflammatory mechanism of ketamine analgesia

Clinically, the attenuation of surgery-induced systematic proinflammatory responses in patients who received perioperative ketamine anaesthesia has been frequently reported. At the early stage of this PhD, our group conducted a meta-analysis that showed a mean preoperative-postoperative IL-6 concentration difference (95% confidence interval) of -71 (-101 to -41) $pg.mL^{-1}$ in 331 patients received perioperative ketamine (Dale *et al.* (2012), see Appendix III, 161-173). Given the role of proinflammatory cytokines in the pathogenesis of neuropathic pain, this inhibition of early postoperative IL-6 inflammatory response by ketamine indicates a potential contribution of anti-proinflammatory activities to ketamine analgesia. In addition to clinical observations, the inhibitory effect of ketamine on proinflammatory cytokine production has also been reported by *in vitro* studies using both peripheral and central immune cells.

1.6.1.1 Ketamine and peripheral immune response

Ketamine has been reported to inhibit the activities of macrophages and neutrophils. This inhibition of peripheral innate immune cells may contribute to ketamine's anti-hyperalgesic and anti-allodynic effects, and may explain ketamine analgesia at plasma concentrations lower than the limit of detection (1 $ng.mL^{-1}$, Lynch *et al.* (2003)).

Macrophages, which are differentiated monocytes stimulated by infection or tissue damage, play important roles in both innate and adaptive immunity. They phagocytose pathogens and instruct other immune cells by expressing and releasing a wide range of substances including proinflammatory cytokines and NO (Nathan, 1987), which contribute to neuropathic pain by directly sensitising nociceptive fibres and/or stimulating the production of other pain mediators such as bradykinin and prostaglandins (Sommer and Kress, 2004). Ketamine suppresses not only the phagocytic and oxidative ability of macrophages (Chang *et al.*, 2005), but also the production of proinflammatory cytokines. Several *in vitro* experiments have shown that racemic ketamine at 10 to 1000 μM reduced the synthesis of IL-1β, IL-6 and TNF-α mRNA in lipopolysaccharide (LPS)-stimulated isolated murine macrophage or macrophage-like cells, but has no effect in the unstimulated cells (Chang *et al.*, 2005; Chang *et al.*, 2009; Wu *et al.*, 2008). These findings suggest that ketamine may exert anti-inflammatory activities through the inhibition of Toll-Like Receptor (TLR)-4 signalling pathway, since LPS mainly acts on the TLR4/myeloid differentiation protein (MD)-2 protein complex (Shimazu *et al.*, 1999; Viriyakosol *et al.*, 2001). The binding of LPS to TLR4/MD-2 complex activates a mitogen-activated protein kinase signalling (MAPK) cascade (also known as the Ras/Raf/MEK/ERK/IKK signalling cascade), which leads to the activation and translocation of several transcriptional factors that can induce gene expression of proinflammatory cytokines, namely nuclear factor kappa B (NF-κB), interferon regulatory factors (IRFs), activator protein (AP)-1 and cAMP response element-binding protein (CREB) (O'Neill *et al.*, 2013). It has been shown that ketamine inhibits the activation of transcriptional factors possibly via the suppression of the phosphorylation of several essential kinases in the MAPK signalling cascade, including Ras, Raf, MAPK, extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNKs) and IκB kinase (Chang *et al.*, 2009; Wu *et al.*, 2008), (Figure 1-6, page 45). In addition, ketamine reduced the DNA binding activity of NF-κB and AP-1 (Welters *et al.*, 2010; Wu *et al.*, 2008). However, whether this downregulation of the

MAPK signalling cascade by ketamine is due to the direct antagonism of TLR4 receptors is not entirely clear. Chang *et al.* (2009) reported that ketamine disrupted the binding of LPS to the LPS-binding protein (LBP), a cofactor presenting LPS to the LPS-binding pocket of the TLR4 receptor. However, the binding of ketamine to TLR4 receptors has not been examined.

Neutrophils are one of the major cell types in the innate immune system. It is the first immune cell type to arrive at the site of infection during inflammation and the predominant immune cell type involved during the acute inflammatory stage (Witko-Sarsat *et al.*, 2000). Neutrophils can eliminate pathogens via three mechanisms: direct phagocytosis, degranulation (a process that releases microbicidal molecules from granules) and release of a neutrophil extracellular trap that binds and kill bacteria (Brinkmann *et al.*, 2004). Being the first responder, another major role of neutrophils is recruiting and directing other immune cells, such as macrophages and lymphocytes, via the secretion of various cytokines, chemokines and growth factors (Witko-Sarsat *et al.*, 2000). This chemoattraction may be important during the early stages of neuropathic pain development, since both macrophage and lymphocytes have been associated with the pathogenesis of neuropathic pain (Moalem and Tracey, 2006; Scapini *et al.*, 2000). Ketamine has been shown to inhibit IL-8 and superoxide production in the activated neutrophils likely via downregulation of the p38 MAPK-mediated pathway (Lu *et al.*, 2010; Zu *et al.*, 1998), and the migration of neutrophils to the site of inflammation (Hofbauer *et al.*, 1998; Zahler *et al.*, 1999) possibly via modulation of the expression of adhesion molecules: cluster of differentiation (CD)-11b, CD16, CD18 and CD62L (Welters *et al.*, 2010; Westlind *et al.*, 2001).

1.6.1.2 Ketamine and central immune cells

Anti-inflammatory effects of ketamine have also been observed in two major innate immune cells in the CNS: microglia and astrocytes (Chang *et al.*, 2009; Wu *et al.*, 2012), both of

which are glial cells, the non-neuronal components of the nervous system. Microglia, account for 5-10% of total glial cells in the CNS, and function as the resident macrophages of the human CNS. Under normal physiological conditions, microglia exist in the CNS in a ramified morphology and constantly elongate through the surrounding areas. When injury or immunological stimuli are detected, ramified microglia are rapidly activated and transformed into an amoeboid morphology, which allows activated microglia to phagocytose and communicate with other immune cells. Astrocytes are the most abundant cell type in the human brain, constituting approximately 85% of glial cells (Chao *et al.*, 1996). In addition to providing metabolic and trophic support to neighbouring neurons (Volterra and Meldolesi, 2005), one important role of astrocytes is regulation of glutamate homeostasis via the excitatory amino-acid transporters (EAATs). Astrocytes remove approximately 80 to 90% of extracellular glutamate in the human brain, which is converted into glutamine by glutamine synthase (Coulter and Eid, 2012; Martinez-Hernandez *et al.*, 1977). Glutamine is then transported back to the presynaptic neurons and converted back to glutamate in excitatory synapses or GABA in inhibitory synapses (Coulter and Eid, 2012).

The roles of microglia and astrocytes in pain processing are well-documented (Watkins *et al.*, 2001). Both cells contribute to pain processing by releasing pain modulators that amplify pain signalling rather than direct involvement in signal transmission (Figure 1-7, page 48). Briefly, microglia and astrocytes are activated by a variety of spinal neurotransmitters and neuromodulators that are released in response to peripheral nerve injury or inflammation. Upon activation, both cells also release additional neurotransmitters or neuromodulators, some of which, such as NO, bradykinin or prostaglandins are directly involved in nociceptive processing (Milligan and Watkins, 2009); others such as glutamate, D-serine and proinflammatory cytokines can result in hyperexcitability of neurons by means of direct

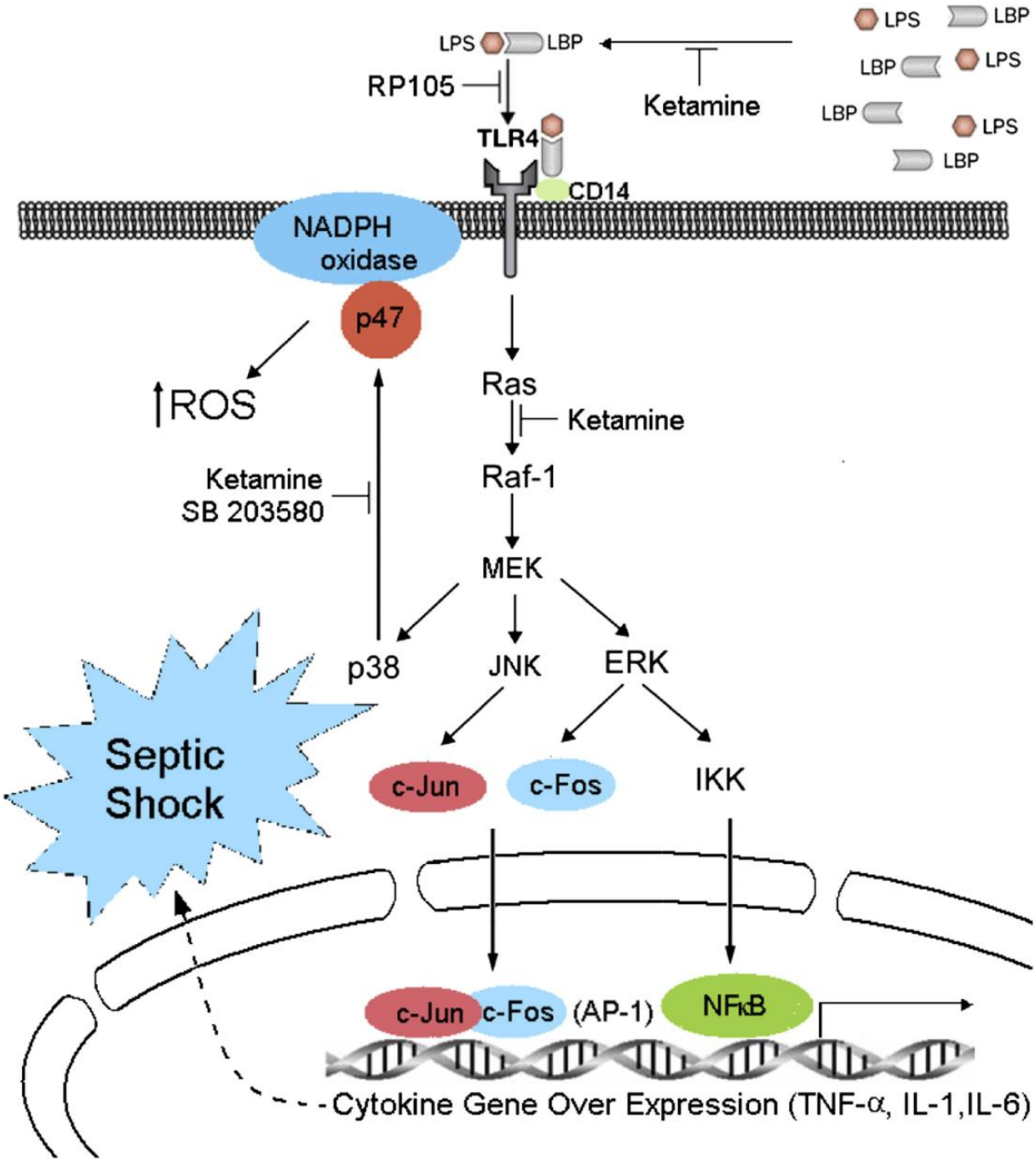

Figure 1-6. Potential molecular mechanisms underlying the inhibitory effect of ketamine on TLR4 signalling-mediated production of proinflammatory cytokines. Ketamine inhibits: a) the recognition of LPS by TLR4 receptor via disruption of LPS-LBP binding; and b) the translocation and activation of nuclear factors via suppression of Ras/Raf/MEK/ERK/IKK signalling cascade. Figure obtained from Liu *et al.* (2012), used with author's permission.

activation of NMDA receptors or upregulation of NMDA receptor activities (Petrenko *et al.*, 2003; Viviani *et al.*, 2003; Wolosker *et al.*, 1999; Zhang *et al.*, 2008), which ultimately leads to central sensitisation and the development of neuropathic pain. In addition to the secretion of pain modulators, the glutamate-glutamine cycle between astrocytes and neurons has been associated with central sensitisation (Chiang *et al.*, 2007).

TLR4 signalling-mediated inflammatory responses have also been implicated in opioid action. A growing body of evidence suggests that chronic administration of opioids stimulates TLR4 signalling and proinflammatory cytokine expression in glia. A recent study demonstrated that 5 days intrathecal morphine infusion increased the expression of TNFα, IL-1β and IL-6 mRNA in rat dorsal spinal cord by 2.5-, 13- and 111-fold, respectively (Shen *et al.*, 2011). Moreover, opioid tolerance, opioid-induced hyperalgesia and allodynia can be attenuated by pharmacological and genetic blockade of TLR4 receptors or inhibition of proinflammatory cytokines (Hutchinson *et al.*, 2007; Hutchinson *et al.*, 2008; Hutchinson *et al.*, 2010; Raghavendra *et al.*, 2002; Shavit *et al.*, 2005). Therefore, it is logical to speculate that the attenuation of opioid-induced central immune responses may, partly, contribute to the opioid-sparing effects of ketamine.

Racemic ketamine has been found to inhibit the production of NO and IL-1β in LPS-activated microglia cells and the production of IL-1β, IL-6 and TNFα in LPS-activated astrocytes at high anaesthetic concentrations (Chang *et al.*, 2009; Wu *et al.*, 2012). Similar to the inhibitory mechanism on peripheral immune cells, the mechanism underlying these effects has been proposed to be the suppression of TLR4 signalling. In microglia, racemic ketamine at 100 μM decreased the LPS-induced phosphorylation of ERK1/2 by approximately 15% but had no effect on the induced JNK1/2 and p38 MAPK phosphorylations (Chang *et al.*, 2009). In astrocytes isolated from infant rat spinal cord, racemic ketamine at 100 μM reduced the LPS-

induced TLR4 protein expression and phosphorylation of NF-κB by approximately 40 and 50%, respectively (Wu *et al.*, 2012). Interestingly, it has been shown that glutamate evoked only small Ca^{2+} elevations in embryonic and postnatal rat spinal cord astrocytes (Ahmed *et al.*, 1990; Ziak *et al.*, 1998), suggesting this inhibition of NF-κB phosphorylation by ketamine may not be due to the decrease in NMDA receptors-mediated Ca^{2+} influx. induced TLR4 protein expression and phosphorylation of NF-κB by approximately 40 and 50%, respectively (Wu *et al.*, 2012). Interestingly, it has been shown that glutamate evoked only small Ca^{2+} elevations in embryonic and postnatal rat spinal cord astrocytes (Ahmed *et al.*, 1990; Ziak *et al.*, 1998), suggesting this inhibition of NF-κB phosphorylation by ketamine may not be due to the decrease in NMDA receptors-mediated Ca^{2+} influx. Taken together with the observations that ketamine inhibits TLR4 signalling in peripheral immune cells, these findings suggest the potential involvement of TLR4 signalling in ketamine's anti-inflammatory and analgesic mechanisms.

In comparison with ketamine, the effects of norketamine on proinflammatory responses have yet to be determined. As mentioned earlier in section *1.3.1 Antagonism of NMDA receptors by ketamine* (page 7) and *1.4.1.1 metabolism* (page 1314), norketamine has 10-20% of the NMDA receptor affinity but approximately half of the anti-hyperalgesic potency of ketamine, indicating a potential contribution of non-NMDA mechanisms to norketamine anti-hyperalgesia. Hence, it is possible that norketamine exhibits some anti-proinflammatory activities that may play a role in the attenuation of hyperalgesia. In addition, the effects of each individual enantiomer of ketamine and norketamine on TLR4 signalling need to be investigated. This may be important in explaining the discrepancy between stereoselectivity in NMDA receptor binding affinity (6- to 7-fold difference in humans) and in the analgesic potency (3- to 4-fold difference) of ketamine, since none of other currently purposed targets

show a stereoselective preference on *(R)*-ketamine (lower K_i values for *(R)*-ketamine see Appendix II: page 158-161).

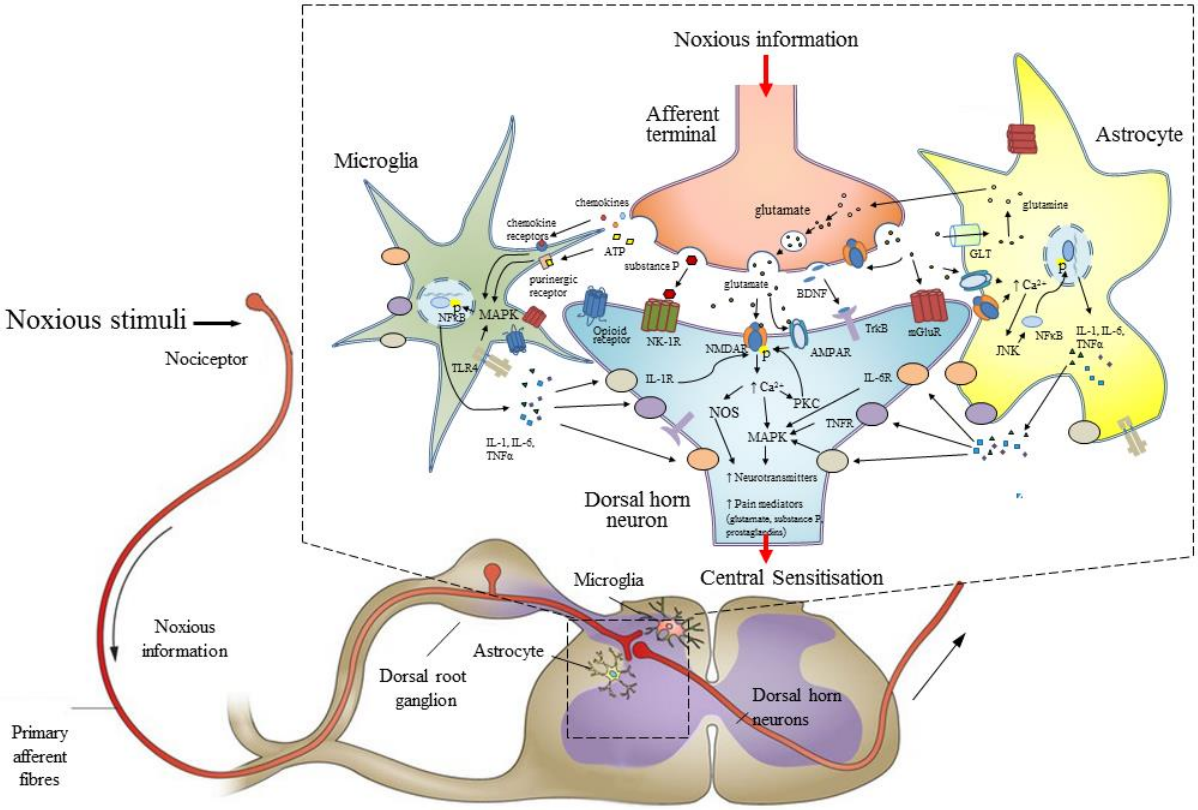

Figure 1-7. The role of glia-neuronal cross-regulation in the amplification of pain signals and the development of central sensitisation. The diagram of dorsal horn and primary sensory neurons were adapted from (Malfait and Schnitzer, 2013).

1.7 Summary, aims and hypotheses

Ketamine at sub-anaesthetic doses, when administrated i.v., produces some improvement in acute and chronic opioid analgesia without severe psychotomimetic adverse effects. However, its low analgesic efficacy and the substantial interindividual variability in drug response restrict its therapeutic applications.

One potential factor contributing to this variability is ketamine plasma concentrations, since clinical studies showed a several-fold interindividual difference in plasma concentrations in neuropathic pain patients who received ketamine via i.v. infusion. This variation in plasma concentration is possibly due to the difference in ketamine clearance, which is a consequence of the change in hepatic blood flow and CYP enzyme activities. Unlike clinical trials, the variation in ketamine plasma concentration was not observed in previous pharmacokinetic studies in humans, probably because these studies were almost exclusively conducted in young healthy volunteers and in small scales (less than 10 subjects), which may not be able to reflect genetic and non-genetic influences on ketamine's pharmacokinetics. Several *in vitro* studies demonstrated that ketamine is mainly metabolised by CYP2B6- and CYP3A4-mediated *N*-demethylation to norketamine, however, the predominant isoform remains controversial. Identification of the relative contribution of these two CYP enzymes may help clinicians to minimise the influence of drug-induced changes in ketamine clearance, as the expression and activity of both CYP2B6 and CYP3A4 are vulnerable to influences of disease and drugs. In addition, at least for *CYP2B6*, the impact of genetic variants on catalytic activities is of particular important. Some variants, such as the most common *CYP2B6*6* allelic variant, have been implicated with clinically relevant changes in drug responses in a substrate-dependent manner. Thus, it is possible that these genetic variants are associated with the interindividual variability in ketamine plasma clearance and therefore plasma concentrations, which potentially has an impact on analgesic efficacy.

Another factor that possibly affects ketamine analgesia is the heterogeneous nature of pain. In patients who received ketamine orally or topically, adequate analgesic and opioid-sparing effects were frequently observed at plasma ketamine concentrations that are substantially lower than the minimum requirement for analgesia following i.v. administration (150 $ng.mL^{-1}$). This observation may be a consequence of two possibilities. Firstly, there is a contribution

of norketamine to the analgesic effects of ketamine, as norketamine has been shown to be a weak NMDA receptor antagonist and its plasma concentrations were up to 4 times of ketamine's when ketamine was given orally. Secondly, there may be an alternative analgesic mechanism of ketamine at low concentration ranges. Although the primary analgesic mechanism of ketamine is believed to be the inhibition of NMDA receptor-mediated hyperexcitability in dorsal horn neurons and the subsequent development of central sensitisation, there is a discrepancy between the stereoselectivity in ketamine's NMDA receptor inhibitory potency and analgesic potency. Recent findings showed that ketamine exerts immunomodulatory effects on peripheral and central innate immune cells possibly via inhibiting TLR4 signalling. As there is increasing evidence that TLR4 signalling-mediated inflammatory responses in these innate immune cells play important roles in the development of neuropathic pain and depression disorders, anti-inflammation may be an alternative mechanism that is involved in ketamine's analgesic and opioid-sparing activities. However, the effects and stereoselectivity of ketamine and norketamine on TLR4-mediated inflammatory response at low concentrations had not been determined. Moreover, the molecular mechanism underlying this inhibition of TLR4 signalling is unclear.

Therefore the hypotheses and aims of this PhD project were:

Hypothesis 1: CYP2B6 is the predominant CYP enzyme for ketamine *N*-demethylation at clinically relevant concentrations in both HLMs and expressed proteins. The HLMs carrying the *CYP2B6*6* allelic variant or expressed *CYP2B6*6* variant protein will show a lower ketamine metabolic activity. *(S)*-ketamine will exhibit a greater metabolism rate than *(R)*-ketamine.

Aim 1: To evaluate the relative contribution of CYP2B6 and 3A4 to the *N*-demethylation of ketamine enantiomers and assess the impact of the *CYP2B6*6* allelic variant on ketamine *N*-demethylation using HLMs and expressed human CYP2B6 and 3A4 enzymes and protein variants.

Hypothesis 2: There will be large interindividual variability in steady-state ketamine plasma clearance, ketamine plasma concentration, and ketamine to norketamine concentration ratios in chronic pain patients. The variability in plasma concentrations can be explained by the *CYP2B6*6* allelic variant, as ketamine plasma clearance rates will be substantially reduced in *CYP2B6*6* allele carriers. The steady-state ketamine plasma concentrations will be associated with the difference in ketamine's analgesic and adverse effects.

Aim 2: To investigate the plasma clearance of ketamine in patients with chronic opioid-refractory pain in order to identify: a) the impact of the *CYP2B6*6* allelic variant on steady-state ketamine plasma clearance and ketamine to norketamine concentration ratio; and b) the relationship between the plasma ketamine and norketamine concentrations and analgesic and adverse responses of ketamine.

Hypothesis 3: Both ketamine and norketamine stereoselectively interact with TLR4/MD-2 heterodimer complex and inhibit the *in vitro* LPS-stimulated IL-6 production at low concentration.

Aim 3: To examine the innate immune pharmacology of ketamine and norketamine enantiomers by determining: a) the binding characteristics of ketamine and norketamine to TLR4/MD-2 using an *in silico* docking simulation assay; and b) the effects of the enantiomers

of ketamine and norketamine at different concentrations on the LPS-stimulated IL-6 production in HEK293 cells stably expressing human TLR4 and co-signalling molecules.

Chapter 2. The CYP2B6*6 allele significantly alters the N-demethylation of ketamine enantiomers in vitro.

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As discussed earlier, the interindividual variability in ketamine plasma concentrations is likely due to differences in clearance. Since ketamine is mainly cleared to norketamine by hepatic CYP2B6 and CYP3A4, whose expression and activities show large inter- and intra-individual variability (Zanger and Schwab, 2013), ketamine plasma concentration are possibly affected by factors that alter the function of these two CYP enzymes. Although both CYP2B6 and CYP3A4 have been associated with ketamine metabolism, there is a contradiction in previous *in vitro* pharmacokinetic studies on ketamine as to which is the predominant enzyme. In my opinion, the relative contribution of each enzyme to ketamine metabolism needed to be clarified before investigating the relationship between the variability in plasma ketamine clearance and the factors altering the functions of these two enzymes (Hijazi and Boulieu, 2002a; Yanagihara *et al.*, 2001). In addition, an *in vitro* study investigating the degree of influence of the *CYP2B6* genetic polymorphism on ketamine metabolism would provide preliminary data to allow a more appropriate design of the population pharmacokinetic analysis. I was particularly interested in the impact of the *CYP2B6*6* allele, the most common allelic variant of *CYP2B6* gene, since it has been associated with the decrease in both the expression and function of CYP2B6 (Hofmann *et al.*, 2008). Thus, the aim of this study was to: a) evaluate the relative contribution of CYP2B6 and 3A4 to the N-demethylation of ketamine enantiomers; and b) assess the impact of the *CYP2B6*6* allelic variant on ketamine N-demethylation using HLMs, and expressed human CYP2B6 and 3A4 enzymes and protein variants.

In this study, the relative contribution of CYP2B6 and CYP3A4 to ketamine metabolism was determined using inhibitors with a higher specificity to the target CYP enzyme. The results showed a predominant role of CYP2B6 not CYP3A4 in the *N*-demethylation of ketamine to norketamine at clinically relevant concentrations, and a marked reduction of ketamine

metabolism by the presence of the *CYP2B6*6* allele in both HLMs and cDNA-expressed enzymes.

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Chapter 3. CYP2B6*6 allele and age substantially reduce steady-state ketamine clearance in chronic pain patients: an influence on adverse effects.

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Statement of Authorship

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Author Contributions

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Signature		Date	06/08/2014

The first study (Chapter 2, page 53-67) identified a large impact of the *CYP2B6*6* allelic variant on ketamine *N*-demethylation *in vitro*, suggesting a likely influence of the *CYP2B6*6* allele on the plasma clearance of ketamine. Thus, a clinical pharmacokinetic study was then conducted to test whether this *in vitro CYP2B6*6* allele-induced reduction of ketamine metabolism is applicable to *in vivo*. According to magnitude of impact on the *CYP2B6*6* genotype on ketamine metabolism determined in the first study (at least 40% reduction) and the allelic frequency of the *CYP2B6*6* in Caucasian populations (Table 1-3, page 27), I estimated that a study with a population size of 50 will give approximately 80% power to identify an effect of *CYP2B6*6* allele on ketamine plasma clearance. The effects of other *CYP2B6* genetic variants, if they were detected, would also be investigated but not be the focus of the study due to their low prevalence in Caucasians. In addition to the *CYP2B6* genetic variability, other factors modulating CYP activities such as sex, age, disease and medicines may affect ketamine clearance. However, the influence these factors has not been well investigated previously, as ketamine pharmacokinetics have been studied almost exclusively in young healthy males (Clements and Nimmo, 1981; White *et al.*, 1985; Yanagihara *et al.*, 2003). Hence, the aims of this study were to investigate the plasma clearance of racemic ketamine in patients with chronic opioid-refractory pain in order to identify: a) the impact of the *CYP2B6*6* allelic variant on steady-state ketamine plasma clearance and ketamine to norketamine concentration ratio on steady-state ketamine plasma clearance and ketamine to norketamine concentration ratio; and b) the relationship between the plasma ketamine and norketamine concentrations and analgesic and adverse responses of ketamine. Influences of other CYP2B6 genetic polymorphisms and several other non-genetic

factors modulating CYP activities including sex, age, disease and medicines on ketamine plasma clearance were also investigated.

Racemic ketamine pharmacokinetics and responses (both analgesic and adverse) were examined in two study populations. The first study population consisted of 14 subjects who were part of a group of ketamine recipients in a hypothesis-driven research comparing the analgesic response of ketamine with placebo treatment (Hardy *et al.*, 2013). The second study population consisted of 35 subjects from an open-labelled study without a placebo control, and which use was more similar to how ketamine is used in chronic opioid-refractory pain patients. Plasma concentrations of racemic compounds not each enantiomer were quantified as no significant stereoselective difference in pharmacokinetics were observed *in vivo* (Yanagihara *et al.*, 2003). The steady-state plasma profiles of ketamine and norketamine were analysed in a combined group of the two populations, since ketamine dosing protocols were almost identical in the two studies. Plasma concentrations of ketamine and norketamine at steady-state conditions showed greater than 5- and 7-fold interindividual variability, respectively. A substantially lower ketamine plasma clearance was found in patients with the *CYP2B6*6* allele and in older patients. The age-related reduction in ketamine clearance is likely due to the decrease in hepatic blood flow with age. The *CYP2B6*6* genotype, the age of the patient, and their combined impact explained approximately 40%, 30% and 60% of variability in ketamine plasma concentrations at steady-state, respectively, while sex and medication had no significant effects on ketamine pharmacokinetics. The reduction in ketamine clearance in *CYP2B6*6* carriers supports the findings of my *in vitro* study and

provides more evidence for the impact of the *CYP2B6*6* allele on ketamine pharmacokinetics. This study also linked ketamine plasma clearance with its adverse effects, as the steady-state plasma clearance of ketamine in patients who experienced adverse effects was approximately 15% lower (45.6 vs 52.6 L.h-1, P = 0.04) in those who did not.

Ideally the relationship between plasma ketamine concentrations and drug efficacy should be examined in a combined population to increase its statistical power. Unfortunately, due to the fundamental differences in study designs and pain assessment tools between the two study populations, it is inappropriate to analyse analgesic results as a combined group. Therefore, these results were not included in the manuscript and further assessment in one large group is required. Instead the influences of *CYP2B6*6* genetic variant and plasma concentrations on ketamine analgesic efficacy were evaluated within each population and reported in section *3.1 relationship between ketamine PK and analgesic response*, page 96-99). These preliminary data found no relationship between plasma concentrations and analgesic responses. The result may possibly be due to the largely heterogeneous nature of pain, as the analgesic efficacy was appeared to be higher in patients who suffered from neuropathic pain, however, each population had limited size to provide sufficient statistical power for the detection of a clinically meaningful difference. Thus, further investigation on the influences of pain variability on ketamine analgesia is required.

Summary

Aim: Ketamine analgesia is limited by low intrinsic efficacy compounded by large interindividual variability in drug responses, possibly due to the heterogeneity in drug concentration. The *CYP2B6*6* allele is associated with substantially reduced ketamine metabolism *in vitro*, and therefore, may affect ketamine clearance. Our aims were to examine the impact of the *CYP2B6*6* allele on ketamine plasma clearance, and on adverse effects in chronic pain patients.

Methods: *CYP2B6* genotypes were identified in 49 chronic pain patients who received 24 h continuous subcutaneous infusions of ketamine. Steady-state plasma concentrations of ketamine ($C_{ss,k}$) and norketamine ($C_{ss,nk}$) were determined using HPLC.

Results: The median plasma clearance of ketamine after 100 mg / 24 h dose was significantly lower in patients with the *CYP2B6*6/*6* (21.6 $L.h^{-1}$) and *CYP2B6*1/*6* (40.6 $L.h^{-1}$) genotypes compared with patients with the *CYP2B6*1/*1* genotype (68.1 $L.h^{-1}$, $P < 0.001$). Patients who experienced adverse effects had lower plasma clearance (45.6 $L.h^{-1}$) than those who did not (52.6 $L.h^{-1}$, $P = 0.04$). The *CYP2B6*6* genotype and age, and their combined impact explained 40%, 30% and 60% of the variation in $C_{ss,k}$, respectively. Similar results were observed after higher doses.

Conclusions: The *CYP2B6*6* allele is associated with a substantial decrease in steady-state ketamine plasma clearance in chronic pain patients. The decreased clearance and resultant higher plasma concentrations may be associated with a higher incidence of ketamine adverse effects.

Introduction

Ketamine, an *N*-methyl-D-aspartate (NMDA) receptor antagonist, has been used as an anaesthetic for over 40 years. The drug is being increasingly used at sub-anaesthetic doses in the management of acute postoperative pain and chronic refractory pain, either solely or in combination with opioids (Bell *et al.*, 2012; Laskowski *et al.*, 2011; Noppers *et al.*, 2010). Although sub-anaesthetic ketamine is said to produce some degree of analgesic and opioid-sparing effects in chronic pain patients, the analgesia, both when it is given alone or combined with an opioid, is limited by low efficacy and large interindividual variability in analgesic responses and by adverse effects warranting drug cessation (Hardy *et al*., 2012; Kvarnström *et al.*, 2004; Laskowski *et al.*, 2011; Noppers *et al.*, 2010; Sörensen *et al.*, 1997).

One potential causal factor of this variability is the interindividual differences in ketamine pharmacokinetics (PK); a previous clinical trial reported an up to 4-fold variation in plasma concentrations among neuropathic pain patients receiving a single 40 min intravenous infusion of 0.4 $mg.kg^{-1}$ ketamine (Kvarnström *et al.*, 2004). Ketamine is a high hepatic clearance drug with an estimated high hepatic extraction ratio (0.9) indicating that its clearance is sensitive to changes in hepatic blood flow (Haas *et al.*, 1992). Additional variability in hepatic clearance may arise from its metabolism which is primarily to norketamine by two cytochrome P450 (CYP) enzymes, CYP2B6 and CYP3A4, whose expression and catalytic activities show large variability in humans (Yanagihara *et al.*, 2001; Zanger *et al.*, 2013). Moreover, a previous randomised placebo-controlled trial reported a

negative contribution of *(S)*-norketamine to the analgesic effects of *(S)*-ketamine on heat pain in healthy volunteers, indicating another potential pharmacokinetic (PK) influence of ketamine on analgesia (Olofsen *et al.*, 2012). Although large interindividual differences in the clearance of sub-anaesthetic ketamine were not observed in previous PK studies (Clements *et al.*, 1981; White *et al.*, 1985; Yanagihara *et al.*, 2003), these studies were conducted in young healthy male volunteers, which may not accurately represent clinical populations whose liver function and CYP enzyme activities show large variability due the differences in age, disease states and medication use (Zanger *et al.*, 2013). In addition, the population size of these PK studies (Clements *et al.*, 1981; White *et al.*, 1985; Yanagihara *et al.*, 2003) was too small ($n \leq 10$) to systematically detect any impact of genetic variations, particularly the *CYP2B6*6* allele, the most prevalent defective allelic variant of the *CYP2B6* gene, on ketamine metabolism and potentially on response (Turpeinen *et al.*, 2012). Our recent *in vitro* study identified that the intrinsic clearance of ketamine was decreased by up to 89% in human liver microsomes and 55% in cDNA-expressed CYP2B6 protein by the presence of the *CYP2B6*6* allele (Li *et al.*, 2013). However, the clinical impact of the *CYP2B6*6* allele on *in vivo* ketamine clearance and adverse responses are yet to be determined.

The primary aim of the present study was to examine: the impact of the *CYP2B6*6* allelic variant on the steady-state plasma clearance of racemic ketamine and plasma concentrations of ketamine and norketamine in patients with chronic opioid-refractory pain. The secondary aim was to investigate the association between adverse effects and the plasma clearance of ketamine.

Methods

Subjects

Two study populations with a total number of 49 patients were included in this study. Characteristics of the 49 patients were as follows: 1) 23 of 49 were male; 2) median age was 64 years (range: 35 to 87); and 3) all were Caucasians except 2 patients with Sri Lankan ethnic origin. Blood samples were obtained from all patients and clinical profiles (including, when available, information about disease states, pain types, comorbidities, opioid consumption and other concurrent non-opioid medications) were obtained from 45 patients. Medications that may induce or inhibit the function of CYP2B6 or CYP3A4 were identified according to a cytochrome P450 drug interaction table (Flockhart, 2007).

The first study population consisted of 14 Caucasian subjects (7 males and 7 females, median age of 67.5 years [range: 45 to 86]) who were part of a group of ketamine recipients in a large multicentre Australian-wide double-blinded randomised placebo-controlled trial investigating the clinical benefits of co-analgesic ketamine in cancer pain (Hardy *et al.*, 2012). These 14 subjects agreed to have blood taken for DNA analysis. The eligible criteria and the dosing procedures were previously described (Hardy *et al.*, 2012). The study was registered on the Australian and New Zealand Clinical Trials Registry (ANZCTR with trial ID: ACTRN12607000501448). Ethical approval was obtained from ethics committees at all sites (Human Research Ethics Committees (HREC) including Repatriation General Hospital HREC [reference no. 69/07], Calvary Health Care Sydney HREC [approval no. 2008.12.04],

South Western Sydney Area Health Service HREC [reference no. 07/RPAH/193], Mater Health Services HREC [reference no. 1171A], Peter MacCallum Cancer Centre Ethics Committee [reference no. 07/56], and St Vincent's Hospital HREC [reference no. 07/RPAH/193]). All participants provided written informed consent.

The second study population consisted of 35 chronic opioid-refractory pain patients from an open-label study (16 males and 19 females) with a median age of 64 years (range: 35 to 87). Patients with poor venous access, previously received ketamine, and in whom significant hypertension or tachycardia would be potentially dangerous were excluded. Patients were recruited at Supportive and Palliative Care Unit and Pain Unit at Monash Medical Centre, Victoria, Australia. The study was registered on the ANZCTR (ACTRN12613000327785). Ethical approval was obtained from the Southern Health HREC, Victoria (reference no. 12159L) and the University of Adelaide HREC (reference no. H-2013-002). All participants provided written informed consent.

Ketamine Dosing protocol

In both populations, racemic ketamine hydrochloride (Ketalar®) was administrated via 24 h continuous subcutaneous infusion (CSCI) with a three level dose-escalation method that has been previously described (Jackson *et al.*, 2001). Briefly, patients received ketamine at an initial dose level of 100 mg / 24 h. Pain scores were assessed using a 0-10 Brief Pain Inventory (BPI) in the first study, and a 0–10 patient reported Numeric Rating Scale (NRS) in the second study. In both scale, 0 indicated no pain and 10 indicated worst possible pain.

Adverse effects of ketamine were assessed using Clinician-Administered Dissociative States Scale in the first study and National Cancer Institute Common toxicity Criteria in both studies, and recorded on a 0-4 scale, (0-patient alert, 4-patient unarousable). Pain score and adverse effects were assessed before, twice daily (for the first study population) or every four hours (for the second study population) during the infusion, and at the end of the 24 h infusion. Neuropathic pain were assessed using Leeds Assessment of Neuropathic Pain Scale. The dose was then increased to 300 mg / 24 h and finally 500 mg / 24 h, if the reduction in pain score by < 2 from baseline on the 0 to 10 pain scale and no intolerable adverse effects (requiring cessation of ketamine) occurred. Venous whole blood samples were collected at the end of 24 h infusion. One to two millilitres of blood samples were immediately stored at -20˚C and used for DNA extraction. Plasma samples were separated from the rest of the whole blood samples by centrifugation at 2000×g at room temperature and then stored at -20˚C until the quantification of drug.

DNA extraction and CYP2B6 genotyping

Genomic DNA was isolated from whole blood using Maxwell® 16 instrument with Maxwell® 16 Blood DNA purification kit (Promega Co. Sydney, NSW, Australia) according to the manufacturer's protocol. SNPs related to the *CYP2B6*4* (c.785A>G, rs2279343), *CYP2B6*6* (c.516G>T, rs3745274 and c.785A>G), *CYP2B6*8* (c.415A>G, rs12721655) and *CYP2B6*13* (c.415A>G, c.516G>T and c.785A>G) alleles were screened by previously described PCR – restriction fragment length polymorphism assays (Li *et al.*, 2013).

Quantification of ketamine and norketamine plasma concentrations

Plasma concentrations of ketamine and norketamine were quantified by a high performance liquid chromatography assay with UV detection. Quantification of ketamine and norketamine as the free base was performed with standard curves consisting of 8 standards that ranged from 20 to 800 $ng.mL^{-1}$. Ketamine (Sigma-Aldrich, Castle Hill, NSW, Australia) and norketamine (Toronto Research Chemicals, distributed by Sapphire Bioscience Pty. Ltd., Waterloo NSW 2017, Australia) standards were prepared by diluting aqueous stocks (10 $\mu g.mL^{-1}$) with drug-free human plasma. Plasma samples and standards at a volume of 1 mL were spiked with 150 ng of internal standard (ephedrine, purchased from Faulding Co. Ltd. Torrensville, SA, Australia), and then alkalised with 500 µl of saturated sodium carbonate before being extracted into 6 mL of 70:30 v/v hexane:ether (Chem-Supply Pty Ltd., Gillman, SA, Australia). Drugs were then back-extracted into 60 µl of 0.1 M hydrochloric acid after centrifugation at 2000×g at room temperature for 10 min. Fifty microliter aliquots were injected into the HPLC system and analysed using a previously described method (Li *et al.*, 2013). Inter- (n = 6) and intra-assay (n = 6) variabilities for both ketamine and norketamine were determined by the analysis of duplicates of quality control samples (QC) at low (50 $ng.mL^{-1}$), medium (150 $ng.mL^{-1}$) and high (350 $ng.mL^{-1}$) concentrations. The inter- and intra-assay imprecision and inaccuracy of all QC samples were less than 10%. Extraction efficiency was determined by comparing the peak areas of each QC sample and the internal standard from extracted samples with those from non-extracted aqueous solutions. The median (range) extraction efficiency for ketamine, norketamine and the internal standard were 90% (88 to

93%), 88% (86 to 91%) and 81% (79 to 83%), respectively. The lower limit of quantification for both compounds was 10 ng.mL^{-1}.

Data analysis

The deviation of genotype frequencies for each variant allele from Hardy-Weinberg equilibrium was tested by the chi-squared test. Plasma clearance of ketamine at steady-state conditions (CL_{ss}) was calculated as $K_0/C_{ss,k,}$ where K_0 was the infusion rate and $C_{ss,k}$ was ketamine steady-state plasma concentration. The differences in $C_{ss,k}$, the steady-state norketamine plasma concentration ($C_{ss,nk}$), CL_{ss} and plasma ketamine to norketamine metabolic ratio (KET/NK MR) among patients with different *CYP2B6*6* genotypes were examined by Jonkeheere-Terpstra tests using SPSS Statistics 20 (IBM, Armonk, NY, USA). The differences in CL_{ss} and KET/NK MR between three doses were analysed using Friedman test. The difference in CL_{ss} at 100 mg / 24 h dose between patients who experienced adverse effects and who did not was analysed using Mann-Whitney tests. The relative impact of *CYP2B6*6* allele, sex, age and potential inhibitors/inducers of CYP2B6 or CYP3A4 on the variation in ketamine PK were analysed using linear regressions in R software with the package "relaimpo" (Grömping, 2006; R Core Team, 2013). Due to a fundamental difference in pain assessment tools between the two studies, the influences of ketamine PK on the ketamine analgesic responses was not analysed as a combined group (Breivik et al., 2008). Results were considered statistically significant when $P<0.05$. Data are presented as median (range) unless specified.

Results

Genotyping detected the *CYP2B6*1* (wild-type), *CYP2B6*5*, *CYP2B6*6* and *CYP2B6*7* alleles, with allelic frequencies of 71%, 3%, 24% and 1%, respectively. No *CYP2B6*4*, *CYP2B6*8* and *CYP2B6*13* variant alleles were detected in any of the participants. The frequencies of all genotypes were in Hardy-Weinberg equilibrium. Thirty-five patients (72%) received the dose-escalation regimen, 25 of whom received the highest 500 mg / 24 h dose. The other 14 patients received only one dose (either 100 mg or 300 mg / 24 h). The median values of CL_{ss} and KET/NK MR were not substantially different between the three doses (Table 1, P = 0.13 and 0.31 for CL_{ss} and KET/NK MR, respectively).

The steady-state plasma concentration profiles of ketamine in patients with various *CYP2B6* genotypes are listed in Table 1. At the end of the 100 mg / 24 h CSCI of ketamine, the median values of $C_{ss,k}$ and KET/NK MR were significantly higher in patients with the *CYP2B6*6/*6* genotype than those with the *CYP2B6*1/*6* and the *CYP2B6*1/*1* genotypes ($P < 0.001$, Figure 1A & 1B). The estimated CL_{ss} in patients with the *CYP2B6*6/*6* genotype was approximately 40% and 59% of that in patients with the *CYP2B6*1/*1* and the *CYP2B6*1/*6* genotypes, respectively ($P < 0.001$, Figure 1C). Similar gene-dose effects were observed after the 300 and 500 mg / 24 h CSCIs (Figure 2). Linear regression analyses showed that the *CYP2B6*6* allele explained 40%, 43% and 41% of the variation in $C_{ss,k}$; 30%, 43% and 52% of the variation in CL_{ss}; and 42%, 39% and 48% of the variation in KET/NK MR after 24 h CSCI of 100, 300 and 500 mg ketamine, respectively ($P < 0.001$ for all regressions).

In addition to the *CYP2B6*6* genotype, the age of the patients significantly affected ketamine PK at 100 and 300 mg / 24 h doses (Figure 3), explaining 28% of $C_{ss,k}$ (P = 0.0005), 31% of CL_{ss} (P = 0.0002) and 12% of KET/NK MR (P = 0.02) variability at the 100 mg / 24 h dose; and 22%, 22% and 11% of $C_{ss,k}$ (P = 0.006), CL_{ss} (P = 0.006) and KET/NK MR (P = 0.05) variability, respectively, at the 300 mg / 24 h dose. Linear two-factor regressions showed that together the *CYP2B6*6* allele and age explained approximately 62% and 61% of variability in ketamine $C_{ss,k}$ after the 100 mg ($F_{(3,37)}$ = 23.9, P < 0.001) and 300 mg / 24 h doses ($F_{(3,29)}$ = 54.2, P < 0.001), respectively (Figure 4).

No significant sex differences in ketamine plasma concentration were observed (P = 0.16, 95% CI = -10 to 35; P = 0.15, 95% CI = -13 to 96; and P = 0.41, 95% CI = -11 to 205 at the 100, 300 and 500 mg / 24 h doses, respectively). Three patients received a medication that may inhibit the function of CYP2B6 (clopidogrel) and one patient received an inducer of CYP2B6 and CYP3A4 (carbamazepine). The PK parameters for these patients at the 100 mg / 24 h dose were highlighted in Figure 1. No patient received any inhibitor of CYP3A4. Neither inclusion of sex nor other drugs or both in the linear two-factor (*CYP2B6* genotype and age) regressions improved the model in predicting ketamine steady-state plasma concentrations. Exclusion of the data of the two patients of Sri Lankan ethnic origin did not alter the results of data analysis.

Adverse effects of ketamine were reported by 18 patients, including 11 patients with the *CYP2B6*1/*1* genotype (which was approximately 42% of all patients with the *CYP2B6*1/*1*

genotype), 3 patients with *CYP2B6*1/*6* genotype (20% of all *CYP2B6*1/*6* carriers), all 3 patients with the *CYP2B6*/*6* genotype and one patient with the *CYP2B6*5/*6* genotype. The adverse response rate was 36% and 42% in the first and second study population, respectively. When the two study populations were analysed together, the CL_{ss} at 100 mg / 24 h dose in patients who experienced adverse effects of ketamine (45.6 [20.0-77.6] $L.h^{-1}$) was significantly lower than that for those who did not (52.6 [22.8-121] $L.h^{-1}$, P = 0.04, 95% CI = 0.1 to 23.3), no statistically significant difference in CL_{ss} was found in each individual study (CL_{ss} in patients who reported adverse effect and who did not was 30.4 [21.0-32.9] $L.h^{-1}$ and 42.0 [22.8-65.5] $L.h^{-1}$, respectively, for the first population, P = 0.12, 95% CI = -6.4 to 32.6; and 47.9 [20.0-72.6] and 60.8 [21.6-121] $L.h^{-1}$ respectively, for the second population, P = 0.09, 95% CI = -3.9 to 25.3). Drowsiness, which was reported by 10 patients, was the most common adverse effect, followed by hallucination (n = 8), dizziness (n = 4) and confusion (n = 3). No patient required cessation of ketamine due to adverse effects.

Discussion

The present study provides the first evidence that the *in vivo* steady-state metabolic clearance of ketamine to norketamine during CSCI is substantially decreased by the number of *CYP2B6*6* variant alleles present and is also impacted by increasing age. To the best of our knowledge, this is also the first study reporting the steady-state plasma concentration profiles of ketamine and norketamine following a subcutaneous administration with a dose-escalation regimen.

The estimated median steady-state plasma clearance of ketamine was approximately 50 $L.h^{-1}$, which is expected to be similar to the blood clearance rate, as the blood/plasma ratio of ketamine is approximately 1 (Hijazi *et al.*, 2001). This clearance rate was relatively lower compared with the estimates of a previous study by Goldberg et al (Goldberg *et al.*, 2010), who reported that the mean clearance of ketamine was approximately 60 $L.h^{-1}$ in 16 patients with complex regional pain syndrome who received a 5-day continuous intravenous infusion of racemic ketamine. The discrepancy is possibly due to the older population in our study (mean ± SD age: 67 ± 12 years) compared to that of Goldberg et al study (mean ± SD age: 33 ± 10 years). Linear regression analyses of our study showed that age explained 22 to 31 % of interindividual variation in clearance rates. The reduction in ketamine clearance in elderly subjects is possibly a consequence of substantial age-related decreases in liver volume, hepatic blood flow, and total P450 content, and possibly the enzymatic function of CYP2B6 and CYP3A4 (Cotreau *et al.*, 2005; Parkinson *et al.*, 2004; Sotaniemi *et al.*, 1997; Wynne *et al.*, 1989).

Additionally, the results of linear regression analyses suggested that the *CYP2B6*6* allele is likely to be the major factor influencing ketamine steady-state plasma concentrations, which by itself explained approximately 40% of the over 5-fold interindividual variability. Such an effect of the *CYP2B6*6* allele on ketamine plasma concentrations is most likely due to its impact on metabolic clearance to norketamine, as median CL_{ss} in patients carrying the *CYP2B6*1/*6* and *CYP2B6*6/*6* genotypes were 60% to 70% and 40%, respectively, of those in patients with the *CYP2B6*1/*1* genotype. According to the equation: $CL_H = Q_H \times$

($f_{ub}CL_{int}$)/(Q_H+$f_{ub}CL_{int}$), where CL_H is hepatic clearance, Q_H is hepatic blood flow, f_{ub} is the unbound fraction of drug in plasma, and CL_{int} is intrinsic clearance (Rowland and Tozer, 2011), and given that ketamine's fraction unbound was approximately 53% and the average hepatic blood flow in people aged 60-70 was approximately 72 $L.h^{-1}$, the estimated intrinsic clearance of ketamine was decreased by at least 64% and 86% in patients carrying the *CYP2B6*1/*6* and *CYP2B6*6/*6* genotypes, respectively (Dayton *et al.*, 1983; Wynne *et al.*, 1989). Such a finding is almost identical to the result of our recent *in vitro* study, in which the intrinsic clearance of ketamine to norketamine was decreased by at least 62% and 84% in human liver microsomes with the *CYP2B6*1/*6* and *CYP2B6*6/*6* genotypes, respectively (Li *et al.*, 2013). In addition to the most common *CYP2B6*6* allele, the *CYP2B6*5* and *CYP2B6*7* alleles were also identified in our patients. Although the extremely low genotype frequencies prevent a feasible analysis, it is interesting to note that the ketamine clearance in the patient with *CYP2B6*6/*7* genotype was almost identical to those in patients with *CYP2B6*6/*6* genotype. This may reflect a possible functional impairment in carriers of the *CYP2B6*7* allele. The *CYP2B6*5* allele may also have a minor effect on ketamine clearance, as the ketamine to norketamine metabolic ratios in the two patients with the *CYP2B6*1/*5* genotype were at least 1.4-fold higher than that in the carriers of the *CYP2B6*1/*1* genotype. However, further *in vivo* and *in vitro* studies are required to examine these individual effects of the *CYP2B6*5* and *CYP2B6*7* alleles on ketamine clearance.

In contrast to the *CYP2B6*6* genotype and age, sex and potential inhibitors/inducer of *CYP2B6* had no significant effect on ketamine clearance and hence were not included in our

model of prediction of steady-state ketamine plasma concentrations following CSCI. However, the influence of inhibitors/inducers may need to be taken into consideration when predicting ketamine concentration after oral administration, as previous studies reported large effects of a CYP2B6 inhibitor (ticlopidine) and CYP3A4 inducers (rifampicin and St. John's Wort) on the exposure to oral ketamine in healthy volunteers (Noppers *et al.*, 2011; Peltoniemi *et al.*, 2012; Peltoniemi *et al.*, 2011).

The variability in plasma clearance and subsequently influence on plasma concentrations may impact on the incidence of ketamine adverse effects, as the plasma concentrations of ketamine in patients who experienced adverse effects were approximately 15% lower than those who did not. Notably, the adverse effects of ketamine were reported by all three patients with the *CYP2B6*6/*6* genotype, indicating a possibility that these patients may be more vulnerable to the adverse effects of ketamine. However this finding needs further assessment with a large number of *CYP2B6*6* carriers. Because two individual studies were not initially designed to investigate the concentration-adverse effect relationship, each population had limited size to provide enough statistical power to reveal a 15% difference in ketamine clearance (power were 45% and 60% for the first and second population respectively).

Since one of the main limitations for use of ketamine as an analgesic is its adverse effect profile, particularly in relation to neurocognitive adverse effects, given the influence of the *CYP2B6*6/*6* allele on plasma concentration and incidence of adverse effects of ketamine described in this study, it is possible that testing patients for the allele may allow for better

patient selection and improve number needed to treat and number needed to harm rates in clinical practice. Future studies are needed to inform the potential clinical utility of this finding.

In conclusion, I showed that the *CYP2B6*6* allele, the most common genetic variant of the *CYP2B6* gene, was associated with substantial decreases in ketamine steady-state plasma clearance in chronic pain patients. In addition, clearance was substantially reduced in the elderly. The combined impact of the *CYP2B6*6* allele and age explained approximately 60% of interindividual variation in steady-state ketamine plasma concentrations. This variability in ketamine concentration may contribute to the incidence of adverse effects.

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Tables

Table 1. Ketamine steady-state plasma concentrations (C_{ss},k), ketamine to norketamine metabolic ratio (KET/NK MR) and ketamine steady-state clearance rates (CL_{ss}) in patients with the *CYP2B6*1/*5*, *CYP2B6*5/*6* and *CYP2B6*6/*7* genotypes in comparison with median (range) values in patients with the *CYP2B6*1/*1*, *CYP2B6*1/*6*, and *CYP2B6*6/*6* genotypes.

CYP2B6 **genotype**	**C_{ss} (ng.mL^{-1})**			**KET/NK MR**			**CL_{ss} (L h^{-1})**		
	100 mg/24 h	300 mg/24 h	500 mg/24 h	100 mg/24 h	300 mg/24 h	500 mg/24 h	100 mg/24 h	300 mg/24 h	500 mg/24 h
*CYP2B6*1/*1*	66 (30-158) (n=23)	159 (121-317) (n=17)	275 (174-483) (n=10)	0.9 (0.5-1.8) (n=23)	0.9 (0.6-2.1) (n=17)	0.8 (0.6-1.6) (n=10)	54.5 (22.8-120.9) (n=23)	68.1 (34.2-89.3) (n=17)	65.8 (37.4-103.8) (n=10)
*CYP2B6*1/*6*	98 (51-136) (n=15)	267 (141-366) (n=13)	394 (283-687) (n=12)	1.5 (0.7-3.7) (n=15)	1.6 (0.8-4.9) (n=13)	1.7 (0.8-3.1) (n=12)	37.8 (26.5-70.8) (n=15)	40.6 (29.6-76.6) (n=13)	45.9 (26.3-63.9) (n=12)
*CYP2B6*6/*6*	168 (154-181) (n=3)	395 (298-407) (n=3)	598 (570-626) (n=2)	2.5 (2.1-3.1) (n=3)	2.5 (2.1-3.2) (n=3)	2.6 (2.2-2.9) (n=2)	21.6 (19.9-23.5) (n=3)	27.4 (26.6-36.3) (n=3)	30.3 (28.9-31.7) (n=2)
*CYP2B6*1/*5*	87			2.2			41.5		
*CYP2B6*1/*5*	69	230	466	1.6	1.4	1.7	52.6	47.1	38.8
*CYP2B6*5/*6*	71	214	365	1.7	1.8	1.9	50.9	50.6	49.5
*CYP2B6*6/*7*	172			4.6			21.0		
Overall	76 (30-181) (n=45)	214 (121-407) (n=35)	366 (174-687) (n=26)	1.3 (0.5-4.6) (n=45)	1.3 (0.6-4.9) (n=35)	1.4 (0.6-3.1) (n=26)	47.6 (19.9-120.9) (n=45)	50.6 (26.6-89.3) (n=35)	49.3 (26.3-103.8) (n=26)

Figures

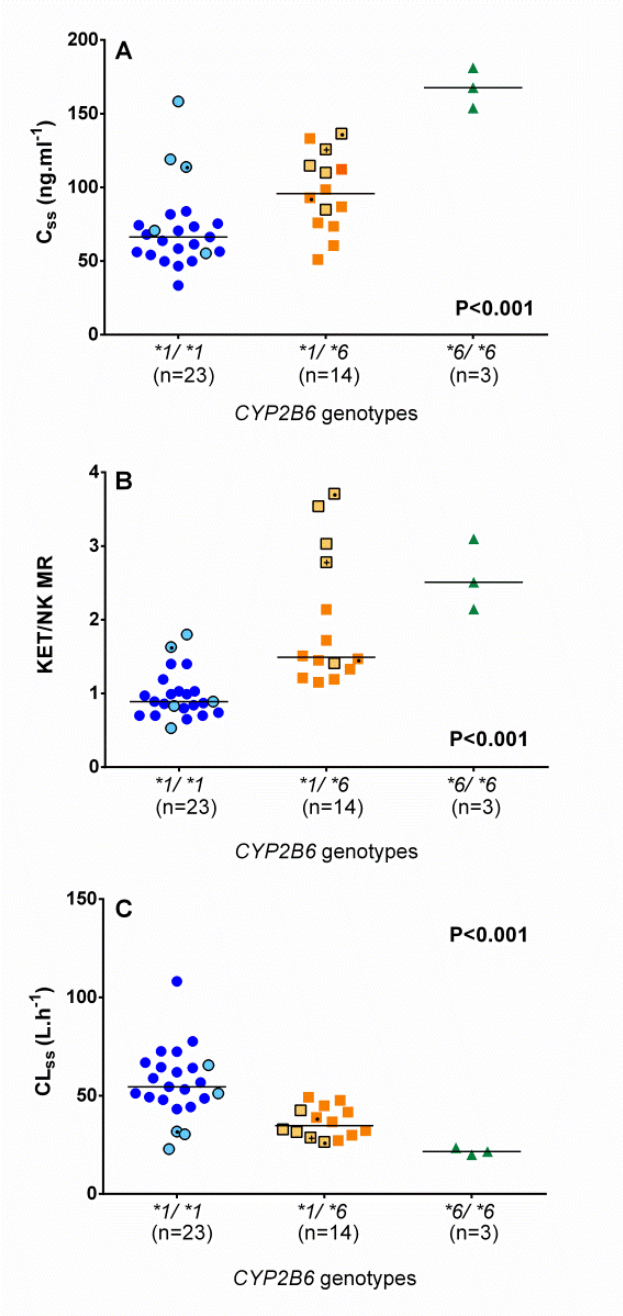

Figure 1. Influence of the *CYP2B6*6* allelic variant on: A) ketamine steady-state plasma concentration ($C_{ss,k}$); B) ketamine to norketamine metabolic ratio (KET/NK MR); and C) ketamine steady-state plasma clearance (CL_{ss}) in chronic pain patients who received a 100 mg / 24 h CSCI. Symbols with lighter filled colour and black border represent patients from the first population. Symbols with a dot (●) and plus (+) sign represent patients who received clopidogrel and carbamazepine, respectively. Lines indicate the median values for each genotype group. All P values were obtained from Jonckheere-Terpstra tests.

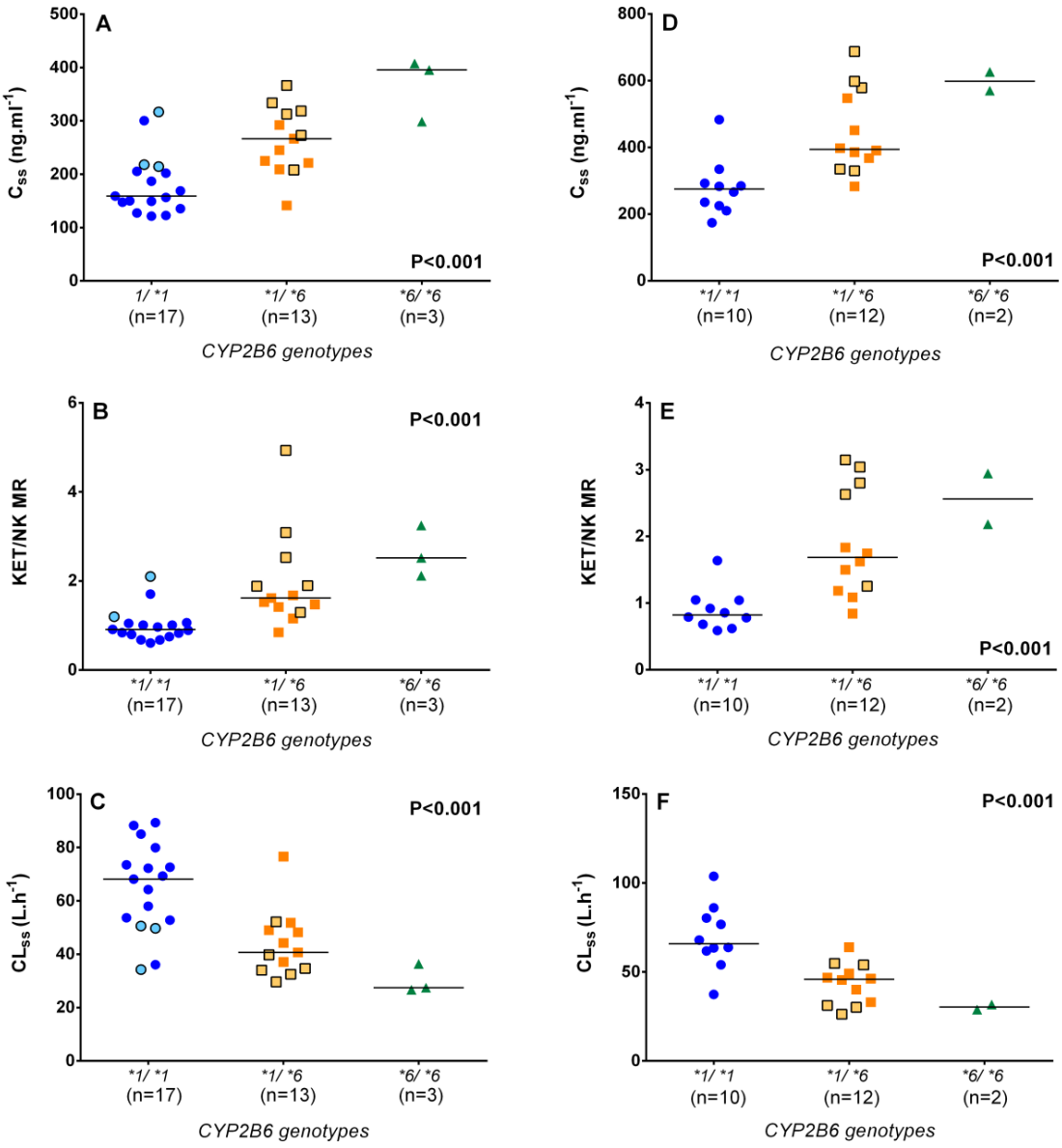

Figure 2. The impact of the *CYP2B6*6* allelic variant on: A, D) ketamine steady-state plasma concentration ($C_{ss,k}$); B, E) ketamine to norketamine metabolic ratio (KET/NK MR); and C, F) ketamine steady-state plasma clearance (CL_{ss}) in chronic pain patients who received a 300 mg / 24 h (A, B, C) and 500 mg / 24 h (D, E, F) CSCI, respectively. Symbols with lighter filled colour and black border represent patients from the first population. Lines indicate the median values for each genotype group. All P values were obtained from Jonckheere-Terpstra tests.

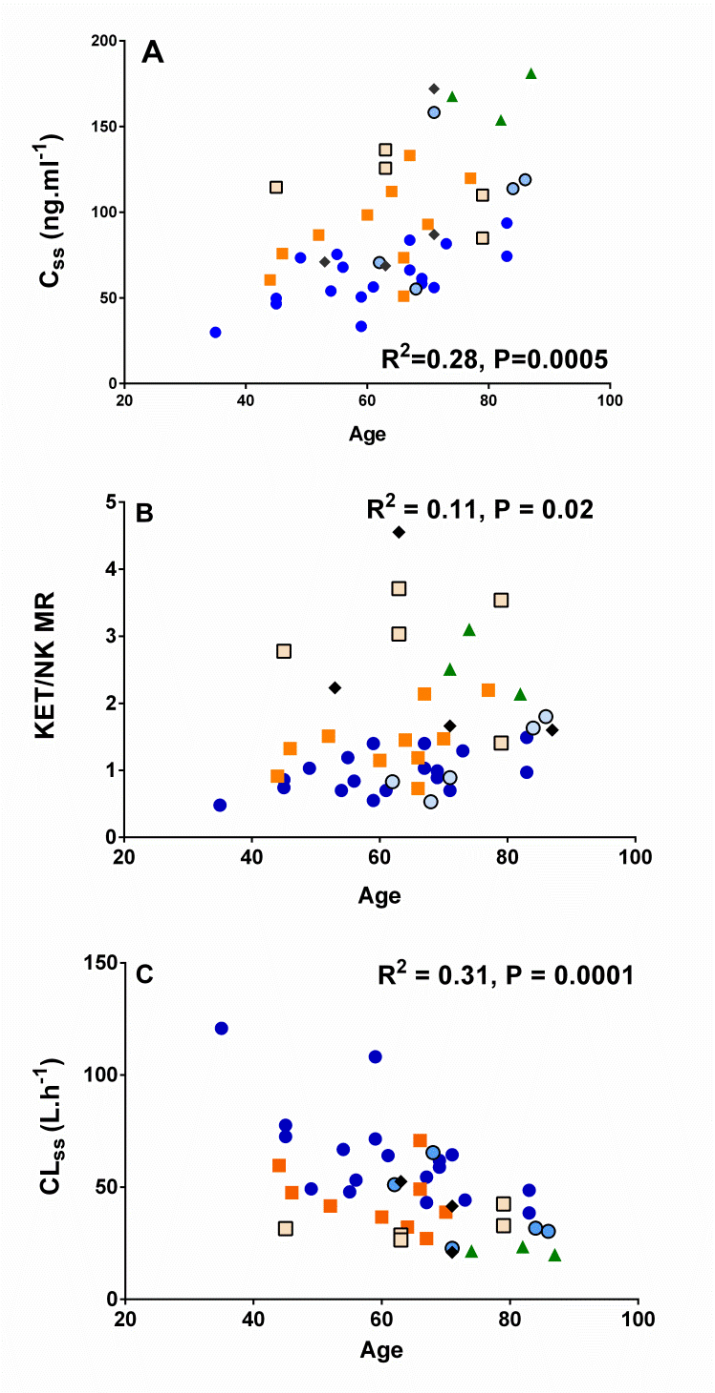

Figure 3. Linear regression between the age of the patients and: A) ketamine steady-state plasma concentration ($C_{ss,k}$); B) ketamine to norketamine metabolic ratio (KET/NK MR); and C) ketamine steady-state plasma clearance (CL_{ss}) in 45 patients who received a 100 mg / 24 h CSCI. Blue circles (●), orange square (■), green triangle (▲) and black rhombus (◆) represent patients with the *CYP2B6*1/*1*, *CYP2B6*1/*6*, *CYP2B6*6/*6* and other *CYP2B6* genotypes, respectively. Symbols with lighter filled colour and black border represent patients from the first population.

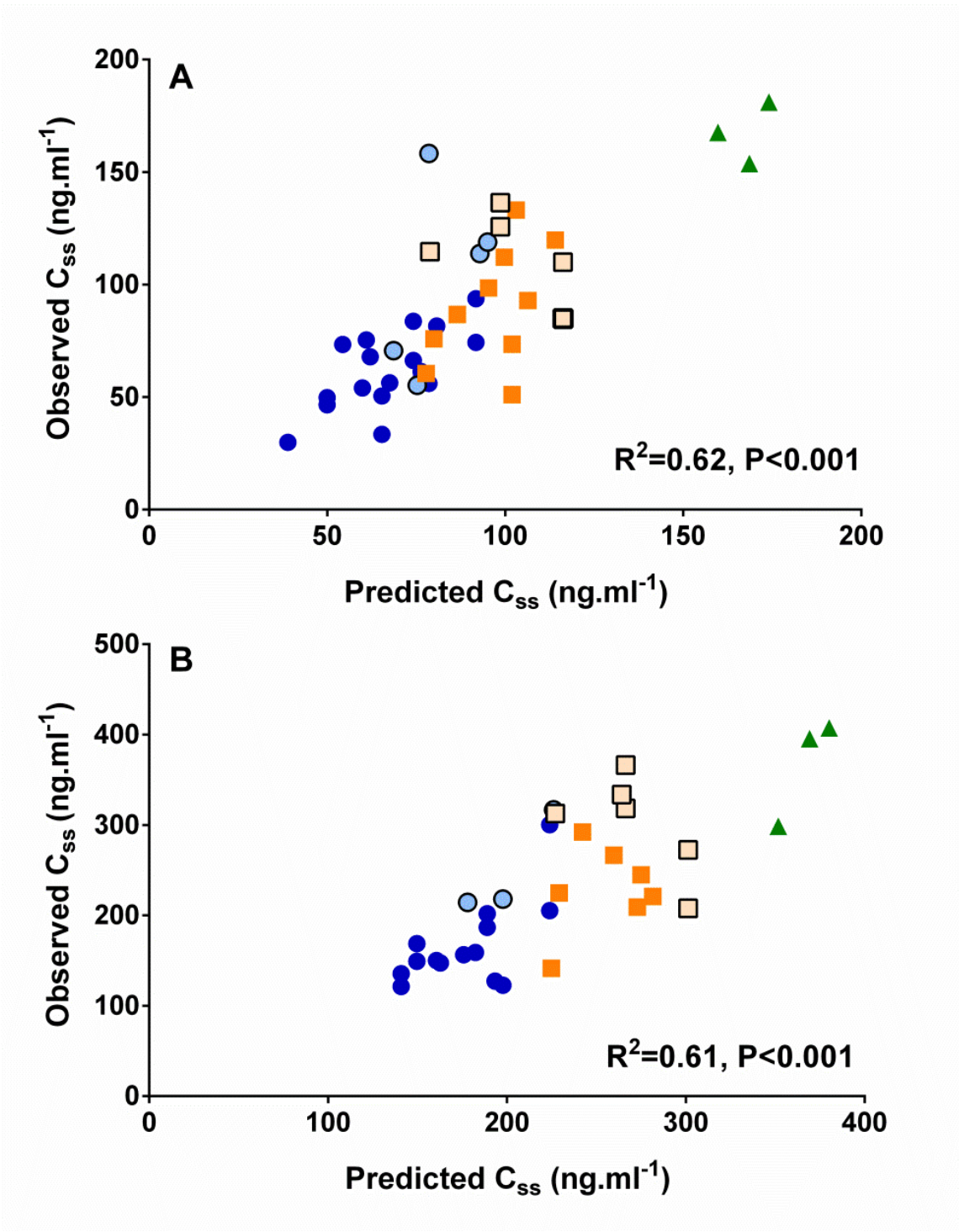

Figure 4. Linear two-factor regressions (*CYP2B6*6* allele and age) between the model-predicted and observed ketamine steady-state plasma concentrations (Css,k) at: A) a 100 mg / 24 h; and B) 300 mg / 24 h doses. Blue circles (●), orange square (■) and green triangle (▲) represent patients with the *CYP2B6*1/*1*, *CYP2B6*1/*6* and *CYP2B6*6/*6* genotypes, respectively. Symbols with lighter filled colour and black border represent patients from the first population.

3.1 Relationship between ketamine PK and analgesic response

The population of the current study consisted of subjects from two studies that were fundamentally different in their study designs. The first study was a hypothesis-driven research comparing the analgesic response of ketamine with placebo treatment (Hardy *et al.*, 2013), whiles the second study was an open-labelled study without a placebo control, and which use was more similar to how ketamine is used in such populations. Additional to the study design, there was a difference in the pain assessment tools between the two studies: BPI, which was employed in the first study, is designed to assess chronic pain intensity and the degree of pain interference with life. Pain severities at 'its worst', 'least, 'average' over the last 24 h, and at the time of assessment were recorded; In contrast, NRS, which was employed in the second study, is designed to assess the intensity of acute pain but not to capture pain-related interference with life (Breivik *et al.*, 2008). Therefore, the sensitivity and effectiveness of two pain assessment tools for identifying pain-induced suffering are likely to be different (Krebs *et al.*, 2007). As such, the influence of ketamine PK variability on analgesic response was analysed separately in two study populations.

3.1.1 First study population

The 14 patients of the first study population were from a group of ketamine recipients in Hardy's study who consented to participate in the pharmacogenetics substudy (Hardy *et al.*, 2013). Five of the 14 patients (36%) suffered from neuropathic pain (assessed using Leeds Assessment of Neuropathic Pain Scale), a proportion that was not substantially different from that of the cohort reported in the entire Hardy's study (approximately 30%). Approximately 43% patients (6 of 14) reported an improvement in pain after ketamine. Of these 6 responders, 3 responded at the 100 mg / 24 h dose, 1 responded at the 300 mg / 24 h dose, and 2 responded only at the highest dose, 500 mg / 24 h. However, the median values of $C_{ss,k}$, $C_{ss,nk,}$

CL_{ss} and KET/NK MR in those patients were not significantly different fro77m those of non-responders at the same dose (Table 3-1). Although the analgesic response rate was slightly higher in neuropathic pain patients (60%, 3 of 5) than in nociceptive pain patients (33%, 3 of 9) in this subgroup of 14 subjects, the original study reported that response was independent of pain type in a population of 93 subjects (Hardy *et al.,* 2013). Since the first population has a small number of subjects, and importantly, no patient carrying the *CYP2B6*6/*6* genotype, substantial variabilities in ketamine clearance and plasma concentrations were not observed in this population. Accordingly, the population has a limited ability to examine the influence of the variability in ketamine PK on analgesic efficacy, and hence the concentration-analgesic response relationship was primarily analysed on the basis of the data obtained from the second study population, whose population size was much larger.

Table 3-1. Median (range) values of $C_{ss,k}$, $C_{ss,nk}$, KET/NK MR and CL_{ss} for ketamine responders and non-responders in the first study population. Median values are highlighted.

	Dose/ 24 h	**Responder**	**Non-responder**	Difference between medians
$C_{ss,k}$ ($ng.mL^{-1}$)	100 mg	**87** (55-172) (n=3)	**114** (69-158) (n=10)	P = 0.71
	300 mg	**317**	**273** (208-366) (n=9)	
	500 mg	**400** (335-466) (n=2)	**588** (330-687) (n=4)	P = 0.40
$C_{ss,nk}$ ($ng.mL^{-1}$)	100 mg	**39** (38-104) (n=3)	**54** (24-178) (n=10)	P = 0.71
	300 mg	**151**	**168** (67-322) (n=9)	
	500 mg	**192** (107-278) (n=2)	**227** (207-263) (n=4)	P = 0.99
KET/NK MR	100 mg	**2.2** (0.5-4.6) (n=3)	**1.7** (0.8-3.7) (n=10)	P = 0.93
	300 mg	**2.1**	**1.9** (0.7-4.9) (n=9)	
	500 mg	**2.4** (1.7-3.2) (n=2)	**2.7** (1.3-3.0) (n=4)	P = 0.66
CL_{ss} ($L.h^{-1}$)	100 mg	**41.5** (21.0-65.5) (n=3)	**31.6** (22.8-52.6) (n=10)	P = 0.81
	300 mg	**34.2**	**39.8** (29.6-52.1) (n=9)	
	500 mg	**46.3** (38.8-53.9) (n=2)	**30.7** (26.3-54.8) (n=4)	P = 0.53

3.1.2 Second study population

Approximately 61% patients (19 of 31) from the second study population reported an improvement in pain after ketamine. Of these 19 responders, 5 responded at the 100 mg / 24 h dose, 4 responded at the 300 mg / 24 h dose, and 10 responded only at the highest dose, 500 mg / 24 h. Similar to the result for the first study population, no significant differences in median values of $C_{ss,k}$, $C_{ss,nk}$, CL_{ss} and KET/NK MR between ketamine responders and non-responders were identified in this second study population (Table 3-2, next page). The analgesic response rate was approximately 77% (10 of 13) in neuropathic pain patients and 50% (9 of 18) in non-neuropathic pain patients (P = 0.16, odds ratio = 3.3 [95% CI: 0.7 to 16]). The result suggested a potentially greater analgesic effect of ketamine against neuropathic pain than other pain types, although further research with a much larger size is required to confirm this finding. Given that pain variability may be a determinant of ketamine analgesia, there is a possibility that the impact of clearance variability on analgesia, if any, was overwhelmed by the heterogeneous nature of pain. Moreover, the higher analgesic efficacy against neuropathic pain reflects the fact that the analgesic activity of adjuvant ketamine is more attributable to the attenuation of pain hypersensitivity and opioid tolerance rather than the direct suppression of nociceptive transmission (Visser *et al.,* 2006).

Interestingly, 11 of 13 patients (85%) who experienced adverse effects responded to ketamine with a median CL_{ss} value of 48.6 $L.h^{-1}$, the two patients who had adverse effects but not responded to ketamine were both *CYP2B6*6/*6* carriers (CL_{ss} were 23.5 and 20.0 $L.h^{-1}$, respectively). In contrast, only 8 of 18 patients (44%) who did not experience adverse effects reported an improvement in pain. The median CL_{ss} in these 8 patients was 53.1 $L.h^{-1}$, whilst that in 10 patients who had no adverse effect nor analgesic response was 59.7 $L.h^{-1}$. This result suggests a possible analgesic response-adverse effect relationship, which need further investigations.

Table 3-2. Median (range) values of $C_{ss,k}$, $C_{ss,nk}$, KET/NK MR and CL_{ss} for ketamine responders and non-responders in the second study population. Median values are highlighted.

	Dose/ 24 h	**Responder**	**Non-responder**	Difference between medians
$C_{ss,k}$ ($ng.mL^{-1}$)	100 mg	**61** (33-120) (n=5)	**74** (47-181) (n=22)	P = 0.42
	300 mg	**150** (148-205) (n=4)	**209** (121-407) (n=19)	P = 0.27
	500 mg	**210** (174-570) (n=10)	**266** (225-626) (n=9)	P = 0.51
$C_{ss,nk}$ ($ng.mL^{-1}$)	100 mg	**62** (24-91) (n=5)	**65** (43-86) (n=22)	P = 0.42
	300 mg	**182** (123-258) (n=4)	**126** (119-258) (n=19)	P = 0.56
	500 mg	**266** (196-415) (n=10)	**254** (213-485) (n=9)	P = 0.46
KET/NK MR	100 mg	**1.3** (0.6-2.2) (n=5)	**1.2** (0.7-3.1) (n=22)	P = 0.99
	300 mg	**0.9** (0.8-1.2) (n=4)	**1.0** (0.6-3.3) (n=19)	P = 0.29
	500 mg	**0.8** (0.7-2.2) (n=10)	**1.1** (0.6-2.9) (n=9)	P = 0.29
CL_{ss} ($L.h^{-1}$)	100 mg	**58.9** (30.2-108.2) (n=5)	**49.2** (20.0-77.6) (n=22)	P = 0.49
	300 mg	**72.4** (52.8-73.5) (n=4)	**88.3** (26.7-89.3) (n=19)	P = 0.15
	500 mg	**86.0** (31.7-103.8) (n=10)	**67.9** (28.9-80.3) (n=9)	P = 0.51

Chapter 4. Ketamine and norketamine stereoselectively inhibit LPS-induced IL-6 synthesis in a time-dependent manner: potential involvement of multiple pathways

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Statement of Authorship

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Contribution to the Paper	Participated in research design Conducted experiments Performed data analysis Wrote the writing of the manuscript		
Signature		Date	14/04/2014

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Contribution to the Paper	Participated in research design Performed data analysis Contributed the writing of the manuscript		
Signature		Date	14/04/14

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Contribution to the Paper	Contributed to data analysis and the writing of the manuscript		
Signature		Date	22/04/2014

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Contribution to the Paper	Participated in research design Performed data analysis Contributed the writing of the manuscript		
Signature		Date	14/04/2014

It has been proposed that ketamine analgesia is potentially a consequence of the attenuation of pain hypersensitivity (hyperalgesia and allodynia) rather than the direct suppression of nociceptive transmission, due to its inhibitory effects on central sensitisation (Joly *et al.*, 2005; Stubhaug *et al.*, 1997). Thus the drug is expected to be more effective in treating neuropathic pain than other pain types. This greater analgesic efficacy of ketamine against neuropathic pain was found in the second study (77% vs 50%), although further confirmation is required, as the difference observed in chapter 3 did not reach statistical significance (P = 0.16), perhaps due to the small sample size.

Although the antagonism of NMDA receptor plays a significant role in the attenuation of central sensitisation, it may not be the only molecular mechanism that contributes to such process. However, most currently proposed non-classical analgesic mechanisms are involved only at high ketamine concentrations, and have limited ability to explain the discrepancy between the stereoselectivity in analgesic potency and that in NMDA affinity. As discussed earlier, the stereoselective difference in analgesic potency between *(S)*- and *(R)*-ketamine (3- to 4-fold) was relatively minor considering a slightly larger difference in their NMDA receptors binding affinities (6- to 7-fold) in humans (*1.6.1.2 ketamine and central immune cells*, page 43-48). However, similar to the NMDA receptor, almost all of these non-NMDA analgesic targets such as opioid, dopamine and monoaminergic systems show either no stereoselective preference or a preference on *(S)*-ketamine (see *Appendix II: Binding affinity of racemic ketamine and enantiomers to various targets* for more information, page 158-161).

Previous *in vitro* studies showed that high concentrations of ketamine attenuated endotoxin-stimulated production of proinflammatory cytokines in murine macrophage cells, possibly via suppression of the LPS/TLR4 signalling pathway, although the exact molecular target required further investigation (Chang *et al.*, 2009; Wu *et al.*, 2008). Clinically, a meta-

analysis by our group revealed that intraoperative ketamine attenuates postoperative concentrations of IL-6, one of the biomarkers of proinflammatory response (Dale *et al.*, 2012). Since there is a growing body of evidence suggesting that proinflammatory cytokines induce or facilitate neuropathic pain (Sommer and Kress, 2004), it was speculated that ketamine-induced inhibition of proinflammatory response contributes to ketamine's analgesic activity. Therefore, the level of proinflammatory response may partly influence ketamine analgesic responses, and hence may contribute to the inter- and intra-variability in ketamine response.

Unfortunately, another clinical trial investigating the association between the proinflammatory response and the clinical outcome of ketamine analgesia in chronic pain patients was not planned at the commencement of the candidature, due to the time and funding constraints. Additionally, although there are some indications that ketamine attenuates proinflammatory responses *in vitro*, the inhibition was only observed in murine cell lines and at the higher concentrations correlated with anaesthetic applications. The suppressive effects of ketamine on proinflammatory response at analgesically relevant concentrations needed further examination using human cells. Moreover, data on the stereoselectivity in ketamine's anti-proinflammatory activity was lacking at present. Therefore, I aimed to examine the innate immune pharmacology of ketamine and norketamine enantiomers by determining: a) the binding characteristics of ketamine and norketamine to TLR4 and MD-2, an important co-factor of TLR4 signalling, and their heterodimer complex (TLR4/MD-2) using an *in silico* docking simulation assay; and b) the effects of the enantiomers of ketamine and norketamine at different concentrations on the LPS-stimulated IL-6 production in HEK293 cells stably expressing human TLR4 and co-signalling molecules.

The *in silico* simulation results showed that ketamine enantiomers bind to amino acid residues that are required for the interaction between LPS and TLR4/MD-2 heterodimer complex, suggesting that the direct blockade of TLR4 by ketamine may contribute to the regulation of TLR4 signalling. The *in vitro* results demonstrated that *(R)*-ketamine may be more potent, at least acutely, in attenuating IL-6 production than *(S)*-ketamine. This finding may partly explain the discrepancy between ketamine's stereoselectivity in analgesic potency and the NMDA receptor binding affinity. In addition, the time-dependent inhibition results suggested a possible mechanistically-based difference between acute and chronic effects. Although the details of these mechanisms require further investigation, the present study provided some evidence that soluble co-factors of TLR4 signalling are required for the acute effect, whereas the chronic effect may be independent of direct drug-TLR4 interaction.

Structured Summary

Background and Purpose. Racemic ketamine inhibits the interleukin-6 (IL-6) inflammatory response. This study examined the contribution of enantiomers of ketamine and its active metabolite, norketamine, to the inhibition of lipopolysaccharide (LPS)-induced IL-6 production in HEK293 cells expressing human toll-like receptor 4 (TLR4) and co-signalling molecules. The binding of ketamine and norketamine to TLR4 were also explored *in silico*.

Experimental Approach. LPS-induced IL-6 production in cells that were pre-incubated with ketamine and norketamine enantiomers at different concentrations and exposure times was quantified using ELISA. The mechanistic characterisation of the drug responses were examined using *in silico* docking simulations and *in vitro* by removing these drugs from the supernatant prior to LPS stimulation.

Key Results. The IL-6 inhibition by the *(S)*-enantiomers but not the *(R)*-enantiomers of ketamine and norketamine was enhanced by the length of pre-incubation. *(R)*-ketamine caused 2-fold greater inhibition than *(S)*-ketamine after 20 min pre-incubation but similar inhibition after 4 h or longer exposure. *(S)*-norketamine reduced IL-6 production by approximately 30% after 8 h exposure but had no acute effect. IL-6 production was inhibited by both ketamine enantiomers when drug was removed from the supernatant after 4 h but not after 20 min pre-incubation. *In silico* docking simulations showed that ketamine binds to the LPS-binding site of myeloid differentiation protein 2 (MD-2), a TLR4 co-signalling factor.

Conclusions and Implications. *(R)*-ketamine is more potent, at least acutely, in attenuating IL-6 production than *(S)*-ketamine. The time-dependent inhibition by *(S)*-ketamine and *(S)*-norketamine may reflect a mechanistically-based difference between acute and chronic effects. The acute effect possibly requires co-factors of TLR4 signalling, whereas the chronic effect appears to be independent of direct drug-TLR4 interaction.

Keywords: ketamine; norketamine; ketamine enantiomers; LPS; TLR4; MD2; inflammation; IL-6

List of non-standard abbreviations:

CD14, cluster of differentiation 14; DMEM, Dulbecco's Modified Eagles Medium; DXO, dextrorphan; FBS, fetal bovine serum; HEK293, human embryonic kidney 293 cell line; IL-6, interleukin-6; LPS, lipopolysaccharide; LBP, LPS binding protein; MD-2, myeloid differentiation protein 2; PBS, phosphate buffered saline; RPMI medium, Roswell Park Memorial Institute 1640 medium; TLR4, toll-like receptor 4.

Ketamine is an analgesic/co-analgesic for acute and chronic pain due to its anti-hyperalgesic and opioid-sparing effects (Laskowski *et al.*, 2011; Noppers *et al.*, 2010), primarily via suppressing central sensitisation through non-competitive antagonism of the NMDA receptor (Joly *et al.*, 2005; Stubhaug *et al.*, 1997). However, other neuronal targets may be involved including opioid and monoaminergic systems (De Kock *et al.*, 2007; Persson, 2010). Further, ketamine inhibits the early postoperative interleukin-6 (IL-6) inflammatory response in humans (Dale *et al.*, 2012), and *in vitro* endotoxin-stimulated IL-6 production in a time-dependent manner (Wu *et al.*, 2008). This action is likely via suppression of toll-like receptor 4 (TLR4)/myeloid differentiation protein 2 (MD-2) signalling (Chang *et al.*, 2009; Chen *et al.*, 2009; Wu *et al.*, 2008; Wu *et al.*, 2012), suggesting ketamine-induced control of innate immune signalling may contribute to ketamine's complex analgesic action. This agrees with the role of TLR4-mediated pro-inflammatory activity in the pathogenesis of pain (Nicotra *et al.*, 2012) and the initiation and maintenance of opioid tolerance, hyperalgesia and allodynia (Ji *et al.*, 2014; Thomas *et al.*, 2012).

Ketamine also has rapid anti-depressant effects (Katalinic *et al.*, 2013) at similar doses to its analgesic effects (Ibrahim *et al.*, 2012; Zarate Jr *et al.*, 2012). Although unclear, the cellular and molecular mechanisms underlying this therapeutic effect are complex and may involve a link between the neuronal NMDA receptor blockade and the innate immune attenuation.

The pharmacology of ketamine is complicated by its stereochemistry and the formation of an active metabolite, norketamine. In general, ketamine is clinically used as a 1:1 racemic mixture of *(S)*- and *(R)*-ketamine, with *(S)*-ketamine having 4 times greater analgesic potency compared with *(R)*-ketamine (Klepstad *et al.*, 1990), most likely due to its 5-fold higher binding affinity to the NMDA receptor (Oye *et al.*, 1992). *(S)*- and *(R)*-norketamine have $1/10^{th}$ to $1/5^{th}$ NMDA affinity of the parent drug enantiomers, respectively (Ebert *et al.*,

1997), and attenuated mechanical and thermal hyperalgesia and allodynia in rats (Holtman *et al.*, 2008). However, the inhibition of the innate immune response by norketamine, and ketamine enantiomers have yet to be determined.

The present study aimed to examine the innate immune pharmacology of ketamine and norketamine enantiomers by determining: a) the *in silico* binding characteristics to TLR4/MD-2; and b) the *in vitro* inhibition of LPS-induced IL-6 expression following acute- and long-term exposure (LPS is the classical ligand for TLR4).

Methods

Materials

(+)-Naloxone (a TLR4 antagonist that has no affinity for opioid receptors) was a kind gift from Dr. Kenner Rice (Chemical Biology Research Branch, NIDA and NIAAA, NIH, Rockville, MD, USA). Amitriptyline hydrochloride, Dulbecco's Modified Eagles Medium (DMEM, with 1000 mg L^{-1} glucose, L-glutamine and sodium bicarbonate), fetal bovine serum (FBS), LPS from *Escherichia coli* (strain: 0111:B4), penicillin-streptomycin, phosphate buffered saline (PBS), (*S*)-ketamine hydrochloride, trypan blue, trypsin-EDTA solution and synthetic triacylated lipoprotein Pam3CSK4 were obtained from Sigma–Aldrich (Castle Hill, NSW, Australia). Roswell Park Memorial Institute 1640 (RPMI) medium, (+)-MK801 maleate, dextrorphan (DXO) and proguanil were purchased from Life Technologies (Mulgrave, VIC, Australia), Abcam Biochemicals (distributed by Sapphire Bioscience, Waterloo, NSW, Australia), Roche (Sydney, NSW, Australia) and AkzoNobel (Macclesfield, Cheshire, UK), respectively. *(R)*-norketamine, *(S)*-norketamine and *(R)*-ketamine were prepared as previously described (Li *et al.*, 2013). Human IL-6 enzyme-linked

immunosorbent assay (ELISA) kits were from eBioscience (distributed by Jomar Bioscience, Kensington, SA, Australia). Cell culture flasks (75 cm²) and Nunc™ MicroWell™ 96-well microplates were obtained from BD Falcon™ (North Ryde, NSW, Australia) and Thermo Fisher Scientific (Scoresby, VIC, Australia), respectively.

Experiment 1 - In silico docking simulations

The pdb (protein database) files for the crystal structure of the human TLR4 and MD-2 complex (PDBID: 3FXI, resolution 3.10Å) and the crystal structure of human MD-2 (PDBID: 2E56, resolution 2.00Å) were obtained from Research Collaboratory for Structural Bioinformatics protein databank (http://www.rcsb.org). These pdb files were modified by removing ligands, exogenous water molecules and artifacts from crystallisation using Molergro Molecular Viewer (2012.2.5). The modified structures were then added with polar hydrogen atoms using AutoDockTools (version 1.5.6) and loaded in PyRx virtual screening tools (Pyrx – Python Prescription 0.8, http://pyrx.sourceforge.net) as macromolecules. Structures of *(S)*- and *(R)*-ketamine hydrochloride in (structure data file) format were obtained from PubChem compound database. Sdf files were then converted to pdb format using Molergro Molecular Viewer. The three-dimensional structures of *(S)*- and *(R)*-norketamine hydrochloride in pdb files were generated using Corina online demo (http://www.molecular-networks.com/online_demos/corina_demo) based on the structure of *(S)*- and *(R)*-ketamine hydrochloride that was obtained from PubChem. Pdb files for the four compounds were loaded in PyRx virtual screening tools as ligands. The docking of ligands to the TLR4/MD-2 dimer complex or single MD-2 molecules was conducted using AutoDock Vina program in Pyrx virtual screening tools (Wolf, 2009). Vina search space was set with default maximised parameters (for TLR4/MD-2 dimer complex: centre X: 11.2472, Y: -5.2793, Z: -5.1027; dimensions (Angstrom) X: 79.3335, Y: 105.4492, Z: 139.7796; for single MD-2: centre X:

0.4226, Y: 23.7980, Z: 13.1096; dimensions (Angstrom) X: 47.2670, Y: 45.1448, Z: 35.6681). Exhaustiveness was set at 8. For each ligand, the predicted binding sites for all conformations were visualised using AutoDockTools to analyse the optimal conformation with the lowest free binding energy and greatest frequency, and the important protein residues that were involved in the interaction between the macromolecule and the ligand.

Experiment 2 - Effects of 20 min pre-incubation with ketamine and norketamine enantiomers on LPS-induced IL-6 production

Cell cultivation

A human embryonic kidney 293 cell line (HEK293) stably expressing human TLR4 and co-signalling molecules (MD-2 and cluster of differentiation 14 (CD14), hereafter referred to HEK293-hTLR4/MD-2 cells) was kindly donated by Dr. Ashley Mansell (Centre for Innate Immunity and Infectious Diseases, Monash Institute of Medical Research, VIC, Australia). Cells were seeded in culture flasks containing 20 mL DMEM media supplemented with 10% FBS and 1% penicillin/streptomycin at a density of 2×10^6 cells per flask, maintained at 37 ℃ with 95% air/5% CO_2 and split every 4-5 days using 1×trypsin-EDTA in PBS solution. Viable cell counts were performed using trypan blue staining. Cells were plated in 96-well plates at a seeding density of 5×10^5 cells per well and maintained in RPMI-1640 media supplemented with 10% FBS (200 µl per well) for 20 h prior to experiments. Cell cultivation and experiments were performed under sterile conditions in a laminar flow hood.

LPS stimulation of cells

Cells were stimulated with LPS (0, 0.1, 1, 100 and 1000 $ng.mL^{-1}$) for 20 h. The incubation time and LPS concentration were optimised with regard to IL-6 production after incubation with LPS at a range of concentrations (14 concentrations ranging from 1 $pg.mL^{-1}$ to 10

μg.mL^{-1}) and different exposure times (20 and 30 min, and 1, 2, 4, 8, 16 and 20 h). The IL-6 concentration in the supernatant at the end of stimulation was quantified using an ELISA method according to the manufacturer's protocol. The absorbance values at a wavelength of 450 nm minus absorbance at 570 nm were measured using a microplate reader (POLARstar, BMG Labtech, Mornington, VIC, Australia). The IL-6 production after 20 h stimulation with 1 μg.mL^{-1} of the TLR1/TLR2 agonist Pam3CSK4 was also examined to ensure that the LPS response was not mediated by TLR1/TLR2 signalling. All incubations were performed in triplicate at 37 ℃.

Ketamine and norketamine treatments

Cells were pre-incubated with 1, 10, 100 μM of *(S)*-ketamine, *(R)*-ketamine, *(S)*-norketamine or *(R)*-norketamine (all dissolved in RPMI media), or the same volume of vehicle control (RPMI media) in 96-well plates for 20 min prior to stimulation with LPS for 20 h. Cell-free supernatants were collected from each well at the end of incubation for IL-6 quantification.

Experiment 3 - Time course effects of ketamine and norketamine enantiomers in comparison to NMDA and TLR4 antagonists on LPS-induced IL-6 production

The inhibitory effects of ketamine and norketamine enantiomers on LPS-induced IL-6 production after short and long durations of pre-incubation were determined. These effects were compared with known NMDA receptor antagonists (DXO and (+)-MK801), TLR4 signalling inhibitors (amitriptyline and (+)-naloxone) and a negative control with no known NMDA receptor or TLR4 activity (proguanil). Cells were pre-incubated with 10 μM of the individual testing compounds or vehicle control (PBS) for 20 and 30 min, and 1, 2, 4 and 8 h before or 20 min after 20 h LPS stimulation at 100 ng.mL^{-1}. The concentration of LPS and

drugs were selected with regard to the responses of ketamine and norketamine enantiomers in experiment 2 and the water solubility of drugs.

Experiment 4 – Cell culture supernatant replacement experiments – acute- vs long-term exposure

Cell culture supernatant replacement experiments were conducted to determine the long-term effect of ketamine and norketamine on LPS-induced IL-6 production after acute exposure. As in experiment 2 and 3, cells were initially maintained in the standard media for 20 h before treated with 10 μM of *(S)*-ketamine, *(R)*-ketamine, *(S)*-norketamine, *(R)*-norketamine and (+)-naloxone for 20 min or 4 h. At the end of these pre-incubation times, supernatants were removed and replaced with either a) fresh RPMI standard media (fresh media replacement) or b) supernatant from cell cultures that were incubated with standard media for 20 h without drug exposure or LPS stimulation (conditioned media replacement). Cells were then treated with either 100 $ng.mL^{-1}$ LPS alone or 100 $ng.mL^{-1}$ LPS plus 10 μM of the corresponding drug as a positive control (Figure 1).

Data analysis

Differences in IL-6 concentrations between treatments and corresponding controls were analysed by generalised linear mixed-effect models, which were then further analysed using analysis of variance to determine the influence of each fixed-effects term (drugs, drug concentrations and LPS concentrations for experiment 2; drugs and pre-incubation time for experiment 3; and drug treatment [drug and re-addition of drug after wash], pre-incubation time and culture media for experiment 4) and their interactions on the model and Tukey's HSD post-hoc test to determine the differences between individual treatments. Inter-plate

variability was considered as a random effect. These analyses were performed using R software with packages MASS, lme4 and multcomp (Bates *et al.*, 2012; Hothorn *et al.*, 2008; R Core Team, 2013; Venables *et al.*, 2002). Three-dimensional surface plots were generated using the graphical interface: RStudio v0.97 with package lattice (Sarkar, 2008). F-ratios and degree of numerator ($d.f_1.$) instead of P-values are reported to indicate the level of statistical significance, as the validity of estimating P-values for linear mixed-effects is debatable due to the uncertainty of denominator degrees of freedom (Bates, 2006). Results were considered statistically significant when F-values were greater than 4 for linear mixed-effects models or $P<0.05$ for Tukey's HSD tests. Treatments that reduced IL-6 production to lower than 80% of its corresponding vehicle were considered as biologically relevant. Data are present as mean ± s.d..

Results

Experiment 1 - In silico docking simulations

The majority of the docking conformations for all of the four tested ligands on the TLR4/MD-2 dimer complex were clustered in the same binding pocket of MD-2. The estimated binding energies for the optimal conformation for *(S)*-ketamine, *(R)*-ketamine, *(S)*-norketamine and *(R)*-norketamine were -6.3, -6.3, -6.7 and -6.6 kcal/mol, respectively. Due to ketamine and norketamine enantiomers having docking preference to MD-2, which is independent of TLR4 interaction, all four ligands were docked in a human MD-2 monomer with greater resolution in an attempt to improve the prediction of binding sites and the estimation of binding energy. Visualisation showed that the optimal binding sites for ketamine and norketamine enantiomers were located at different positions in the same binding pocket of MD-2. The binding energies for the optimal conformation for *(S)*-ketamine, *(R)*-ketamine, *(S)*-

norketamine and *(R)*-norketamine were -6.5, -6.6, -7.9 and -7.3 kcal/mol, respectively. The amino acid residues of the MD-2 protein that each ligand interacted with are listed in Table 1.

Experiment 2 - Effects of 20 min pre-incubation with ketamine and norketamine enantiomers on LPS-induced IL-6 production

Basal IL-6 concentrations were not different across assays ($P > 0.98$) and unaffected by the treatment with any drug alone ($P = 1.00$ for all four drugs). The stimulated IL-6 production was dependent on: a) LPS concentration ($F = 553$, $d.f_1.=4$) with a rank order of 1 $\mu g.mL^{-1}$ > 100 $ng.mL^{-1}$ > 1 $ng.mL^{-1}$ > 100 $pg.mL^{-1}$; and b) ketamine concentration ($F= 24.2$ and 53.2, $d.f_1.=3$, for *(S)*- and *(R)*-ketamine, respectively) with a rank order of 100 μM < 10 μM ≈ 1 μM for *(S)*-ketamine, and 100 μM ≈ 10 μM < 1 μM for *(R)*-ketamine (Figure 2). Although *(R)*-ketamine produced greater inhibitory effects on LPS-induced IL-6 production (range: 33 to 65% inhibition) compared with equimolar *(S)*-ketamine (range: 7 to 37% inhibition), the difference did not reach statistical significance ($F = 3.42$, $d.f_1.=1$). In contrast, norketamine enantiomers did not significantly inhibit LPS-induced IL-6 production, except *(R)*-norketamine at 100 μM where 25 to 30% inhibition at various LPS concentrations was found ($P < 0.05$).

Experiment 3 - Time course effects of ketamine and norketamine enantiomers in comparison to NMDA and TLR4 antagonists on LPS-induced IL-6 production

Basal IL-6 production was unaffected by the pre-incubation with any drug alone ($P > 0.9$). All tested drugs at 10 μM except proguanil significantly reduced LPS-induced IL-6 production, the rank order of the maximum inhibitory effects of these drugs (% inhibition versus vehicle) were: (+)-MK801 (78) ≈ (+)-naloxone (75) > *(R)*-ketamine (73) > racemic ketamine (67) =

(S)-ketamine (67) > amitriptyline (42) > *(S)*-norketamine (31) > dextrorphan (26) > racemic norketamine (16) ≈ *(R)*-norketamine (15). In contrast to pre-incubation, LPS-induced IL-6 production was not significantly inhibited by 20 min incubation with any tested compounds after LPS stimulation.

The inhibition of IL-6 production by *(S)*-ketamine and *(S)*-norketamine was dependent on pre-incubation time ($F = 10.6$ and 17.3, $d.f_1 = 5$ for *(S)*-ketamine and *(S)*-norketamine, respectively, Figure 3) whereas the IL-6 inhibition by other drugs was not (F-values ranged from 0.5 to 3.5). Tukey's HSD test showed that the stimulated IL-6 concentrations in cells that were pre-incubated with *(S)*-ketamine or *(S)*-norketamine for 4 or 8 h were significantly lower than when pre-incubated with these two drugs for less than 4 h ($P < 0.001$ and 0.01 for *(S)*-ketamine and *(S)*-norketamine, respectively). The inhibitory effect of *(S)*-ketamine and *(S)*-norketamine after 4 h pre-incubation, as average percent inhibition in IL-6 response, was approximately 1.6- and 2-fold higher, respectively, than that after 20 min pre-incubation.

The stimulated IL-6 concentration in cells pre-incubated with *(S)*-ketamine for 20 min was approximately 2-fold higher than when pre-incubated with *(R)*-ketamine ($p<0.01$). By contrast, no significant stereoselective difference in the stimulated IL-6 production was found after 4 and 8 h pre-incubations ($P = 0.99$).

Experiment 4 – Cell culture supernatant replacement experiments

Basal LPS-induced IL-6 production in cells receiving fresh media replacement was 20 ± 4% of those receiving conditioned media replacement. Moreover, no drug significantly affected stimulated IL-6 production in cells receiving fresh media replacement, except 4 h pre-incubation with *(S)*-ketamine, which reduced the stimulated IL-6 production by approximately

65% in the presence of drug (P = 0.02) and 56% without the presence of drug during stimulation (P = 0.03).

In cells receiving conditioned media, *(S)*-ketamine, *(R)*-ketamine and (+)-naloxone significantly attenuated LPS-induced IL-6 production when the drug was present in the supernatant during LPS stimulation (Figure 1, test plate 2a). However, removal of drugs after 20 min pre-incubation (Figure 1, test plate 2b) substantially diminished the inhibitory effects of these drugs ($p<0.001$, Figure 4A). Pre-incubation with norketamine enantiomers for 20 min produced no significant effects on IL-6 production regardless of their presence during LPS stimulation (P = 1.0 and 0.91, for *(S)*- and *(R)*-norketamine, respectively when present, and P = 0.39 and 0.41, for *(S)*- and *(R)*-norketamine, respectively when absent, Figure 4A). In contrast to 20 min pre-incubation, inhibition by both ketamine enantiomers was not altered by the removal of drug after 4 h pre-incubation, whereas (+)-naloxone lost its inhibitory effect if it was not present during LPS stimulation (P < 0.001, Figure 4B). *(S)*-norketamine significantly reduced the stimulated IL-6 production regardless of its removal after 4 h pre-incubation (P < 0.001), while *(R)*-norketamine showed no significant effects on IL-6 (P = 0.99 and 0.15 while present and absent, respectively, Figure 4B).

Discussion

I show here that pre-incubation with ketamine and norketamine enantiomers stereoselectively attenuated LPS-induced IL-6 production in HEK293-hTLR4/MD-2 cells in a concentration- and time-dependent manner. The finding that 20 min pre-incubation with *(R)*-ketamine but not *(S)*-ketamine inhibited the stimulated IL-6 response at 1 μM, a clinically relevant sub-anaesthetic plasma concentration (Zarate Jr *et al.*, 2012), indicates that *(R)*-ketamine may have a greater potency than *(S)*-ketamine, at an equimolar sub-anaesthetic dose, against LPS-

triggered innate immune responses after acute exposure. Moreover, the inhibitory effect of *(S)*-ketamine at 10 and 100 μM, corresponding to anaesthetic plasma concentrations (Domino *et al.*, 1982; Idvall *et al.*, 1979), was approximately 59% and 83% that of *(R)*-ketamine, respectively. These more potent inhibitory effects of *(R)*-ketamine may indicate that the antagonism of NMDA receptors may not be an important mechanism underlying ketamine's inhibitory effects on IL-6 response, since *(R)*-ketamine has a much lower affinity to the NMDA receptors compared with *(S)*-ketamine. In contrast to ketamine, the negligible effects of norketamine enantiomers on induced IL-6 production after 20 min pre-incubation suggest a minor (if any) contribution of norketamine to the inhibition of IL-6 inflammatory response after acute exposure. Although *(R)*-norketamine inhibited IL-6 response at 100 μM, the concentration is approximately 10 times the highest achievable plasma norketamine concentration in humans after IV dosing (Domino *et al.*, 1982). A surprising observation of this series of experiments was that ketamine enantiomers only inhibited IL-6 production by high concentrations of LPS. Although the possibility of experimental errors cannot be precluded, this result may reflect the existence of distinct pathways of LPS-induced IL-6 production at different concentrations. However, this hypothesis needs to be tested in future studies.

Previous studies have reported that the inhibition of pro-inflammatory cytokine synthesis by racemic ketamine was dependent on incubation time in LPS-stimulated macrophage-like cells (Chen *et al.*, 2009; Wu *et al.*, 2008). However, this observation may be due to the time course of LPS stimulation rather than the effect of ketamine itself given that ketamine was co-administered with LPS stimulation. In our study, the effect of ketamine and norketamine at 10 μM on LPS-induced IL-6 production was investigated at 6 pre-incubation times ranging from 20 min to 8 h before the 20 h LPS stimulation in an attempt to differentiate the acute and long-term effects of ketamine and norketamine exposure. I found that the inhibitory effects of

both *(S)*-ketamine and *(S)*-norketamine were remarkably enhanced after 4 and 8 h pre-incubation compared to 20 min and may reflect a mechanistically-based difference between the acute and long-term effects of these compounds. The inhibition by *(S)*-norketamine after long-term exposure is particularly noteworthy, as acute *(S)*-norketamine exposure failed to produce biologically significant inhibition (defined as production lower than 80% the corresponding vehicle). In addition, the degree of inhibition by *(S)*-norketamine after 8 h pre-incubation (average 31% reduction in stimulated IL-6 production) was approximately half that by *(S)*-ketamine (average 63% reduction) and *(R)*-ketamine (average 66% reduction), indicating that *(S)*-norketamine may play a role in the inhibition of pro-inflammatory response to chronic ketamine exposure.

Contrary to the *(S)*-enantiomers, no time-course effects were observed for inhibition by *(R)*-ketamine and *(R)*-norketamine. Therefore, due to the increase in the *(S)*-ketamine effect, substantial differences in inhibition between *(S)*- and *(R)*-ketamine were not observed after long-term exposure. This finding agrees with the results of a previous study using human whole blood that found an equal suppressive effect of *(S)*- and *(R)*-ketamine on enterotoxin-induced tumor necrosis factor-α and IL-6 production after 6 h drug exposure (Kawasaki *et al.*, 2001). The time-independent effect of *(R)*-ketamine and the change in stereoselectivity in inhibition over drug pre-incubation time suggest that the two ketamine enantiomers may exert their inhibitory effects via distinct mechanisms that have different time courses. The observation that neither (+)-naloxone or amitriptyline inhibited LPS-induced IL-6 production in a time-dependent manner suggests that the mechanism underlying this time-dependent inhibitory effects of *(S)*-ketamine and *(S)*-norketamine was the antagonism of TLR4.

The substantial decreases in LPS-stimulated IL-6 production and the loss of inhibitory effects of *(S)*-ketamine after 20 min pre-incubation, *(R)*-ketamine and (+)-naloxone after both 20 min

and 4 h pre-incubation in cells receiving fresh media replacement but not in cells receiving conditioned media suggest important roles of cell-released soluble co-factors in the LPS elicitation and chemical inhibition of IL-6 response in HEK293-hTLR4/MD-2 cells; this is likely due to the conditioned media containing various secreted extracellular proteins that are not present in fresh media (Hutchinson *et al.*, 2010). A previous study using HEK293 cells expressing human TLRs, MD-2 and CD14 showed that the recognition of LPS by TLR4 required at least three co-factors; LPS binding protein (LBP), CD14 and MD-2. LBP is a soluble protein that delivers and accelerates the binding of LPS to membrane-bound CD14 before the LPS-CD14 complex is recognised by the TLR4/MD-2 heterodimer on the surface membrane (da Silva Correia *et al.*, 2001). Chen *et al*. (2009) showed that ketamine disrupts the binding of LPS to LBP in macrophage-like cells, which may lead to the subsequent antagonism of mitogen-activated protein kinases signalling pathway and the suppression of nuclear factor-κB activities with resultant decrease in pro-inflammatory cytokine production. In addition to LBP, the secreted MD-2 (sMD-2) has been implicated in LPS responses (Kennedy *et al.*, 2004). sMD-2 is secreted in excess amounts by HEK293-hTLR4/MD-2 cells and can form a stable complex with LPS that can directly trigger TLR4 signal transduction without the presence of CD14 and free LPS (Kennedy *et al.*, 2004; Visintin *et al.*, 2001).

The *in silico* docking simulations in the present study identified a likely interaction between human MD-2 and both ketamine and norketamine enantiomers. More importantly, several amino acid residues of MD-2 that ketamine interacts with, including leucine 54 (for *(R)*-ketamine), tyrosine 131 (for *(S)*-ketamine) and isoleucine 124 (for both *(R)*- and *(S)*-ketamine), form a loop of extensive hydrogen bonds with the hydrophobic R2 lipid chains of LPS at the core of TLR4-MD-2 main dimerisation interface (Park *et al.*, 2009). This suggests a potential impact of ketamine enantiomers on the recognition of LPS by antagonising MD-2, which may explain the decrease in IL-6 production in response to LPS and the lack of drug

inhibition in cells receiving fresh media replacement. However, the *in silico* data cannot explain the stereoselectivity in ketamine's inhibition, which is possibly due to a limitation of the AutoDock Vina program that is incapable of predicting global conformational changes upon ligand binding and their functional consequences on the efficacy of inhibition. In contrast to ketamine enantiomers, the residues of MD-2 that norketamine enantiomers interact with have not been related to LPS recognition, although the binding affinities for these interactions are relatively high. This result suggests that interaction between norketamine enantiomers and MD-2 may not interfere with the binding of LPS to TLR4, which may explain the less potent effect of norketamine on LPS-induced IL-6 production versus ketamine.

In cells receiving conditioned media replacement, the inhibitory effect of *(S)*-ketamine and *(S)*-norketamine after 4 h pre-incubation, unlike the inhibition by (+)-naloxone, was not significantly impaired by the removal of drug before LPS stimulation, indicating that the presence of drug during stimulation may not be required for such inhibition. Interestingly, the inhibition by *(S)*-ketamine after 4 h pre-incubation was also unaffected by the fresh media replacement. These results suggest that the inhibition of LPS-stimulated IL-6 production by *(S)*-ketamine and *(S)*-norketamine after long-term exposure possibly does not involve a direct interaction between these drugs and TLR4 or co-receptors, but rather is due to long-term changes in signalling cascades that lead to the downregulation of cytokine synthesis. These changes in signalling cascades may also be associated with the inhibition by *(R)*-ketamine after long-term exposure, although the direct drug-receptor interaction may also be involved, as its removal before LPS stimulation significantly but not drastically reduced the inhibition.

The changes in signalling cascades and cytokine synthesis over long-term *(S)*-ketamine and *(S)*-norketamine exposure could potentially explain a finding of recent clinical studies, that is,

the duration of ketamine analgesic effect is associated with the duration of infusion (Noppers *et al.*, 2011; Sigtermans *et al.*, 2009). Sigtermans *et al.* (2009) reported that long-term *(S)*-ketamine infusion (20 to 30 $mg.h^{-1}$ for 100 h) produced pain relief sustaining over 12 weeks following treatment in patients with complex regional pain syndrome type 1. This duration of action is much longer compared with ketamine and norketamine's less than 6 h half-life (Hijazi *et al.*, 2003). In contrast, Noppers et al showed that analgesic effects of *(S)*-ketamine did not persist beyond the 3 h after short-term infusion ketamine (0.5 $mg.kg^{-1}$, 30 min). I speculate that long-term *(S)*-ketamine exposure may cause long-term changes in pro-inflammatory cytokine synthesis that possibly contribute to the sustained ketamine analgesia. However, further experiments are required to examine this hypothesis.

In conclusion, the results of the present study suggest that *(R)*-ketamine is more potent in attenuating IL-6 production compared with equimolar *(S)*-ketamine after acute drug exposure. However, the effects of *(S)*-ketamine are substantially enhanced and may be similar to that of *(R)*-ketamine after chronic exposure. *(S)*-norketamine may also contribute to the anti-inflammatory effects of ketamine chronically but not acutely. These results indicate that long-term infusion may potentially improve ketamine analgesic and anti-depressant activities. In addition, our findings provide preliminary evidence for a mechanistically-based difference in the acute and long-term effects of both ketamine enantiomers and *(S)*-norketamine on stimulated IL-6 production, although the details of these mechanisms, especially the mechanism underlying the long-term effect of ketamine, need to be investigated in further studies.

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Author Contributions

Y Li: participated in research design; conducted experiments; performed data analysis, contributed to the writing of the manuscript

M R Hutchinson: participated in research design; performed data analysis, contributed to the writing of the manuscript

J K Coller: performed data analysis; contributed to the writing of the manuscript

A A Somogyi: participated in research design; performed data analysis, contributed to the writing of the manuscript

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Table:

Table 1. Ketamine and norketamine enantiomer interactions with amino acid residues of human MD-2 monomer derived RCSB protein databank.

Ligands	**Residues**
(S)-ketamine	I52, I80, I124, Y131, C133, I153
(R)-ketamine	I52, L54, I80, F121, I124, C133, I153
(S)-norketamine	L61, I63, Y65, L71, L74, F76, I94, F104, V113, I117, L146, F147
(R)-norketamine	I63, Y65, L74, F76, F104, F147

Residues are presented as 1-letter amino acid abbreviation plus the position on MD-2 molecule. (C: cysteine; F: phenylalanine; I: isoleucine; L: leucine; V: valine; Y: tyrosine).

Figures:

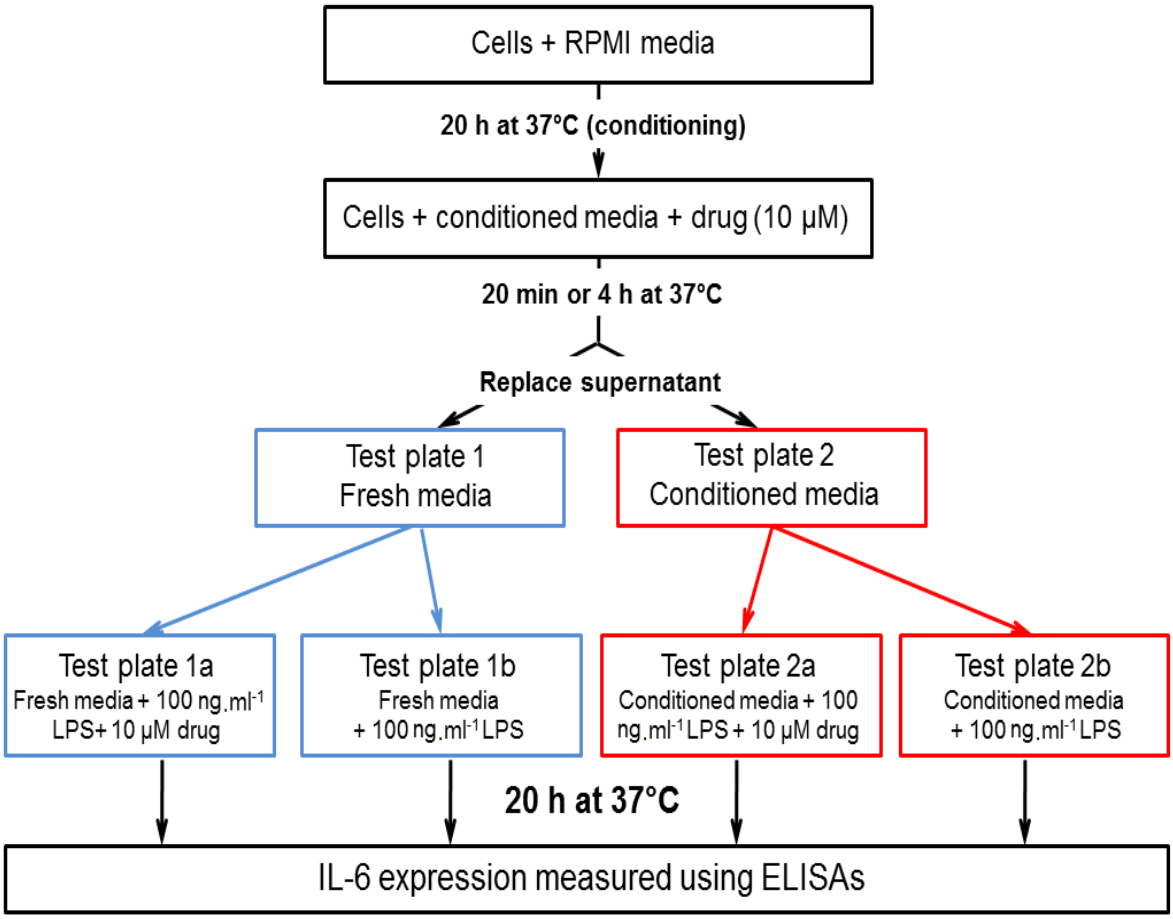

Figure 1. Design of cell culture supernatant replacement experiments. Conditioned media comprised the supernatant from cell cultures that were incubated with standard RPMI media for 20 h with no drug exposure or LPS stimulation. Drugs were *(S)*-ketamine, *(R)*-ketamine, *(S)*-norketamine, *(R)*-norketamine, (+)-naloxone and vehicle (PBS) control.

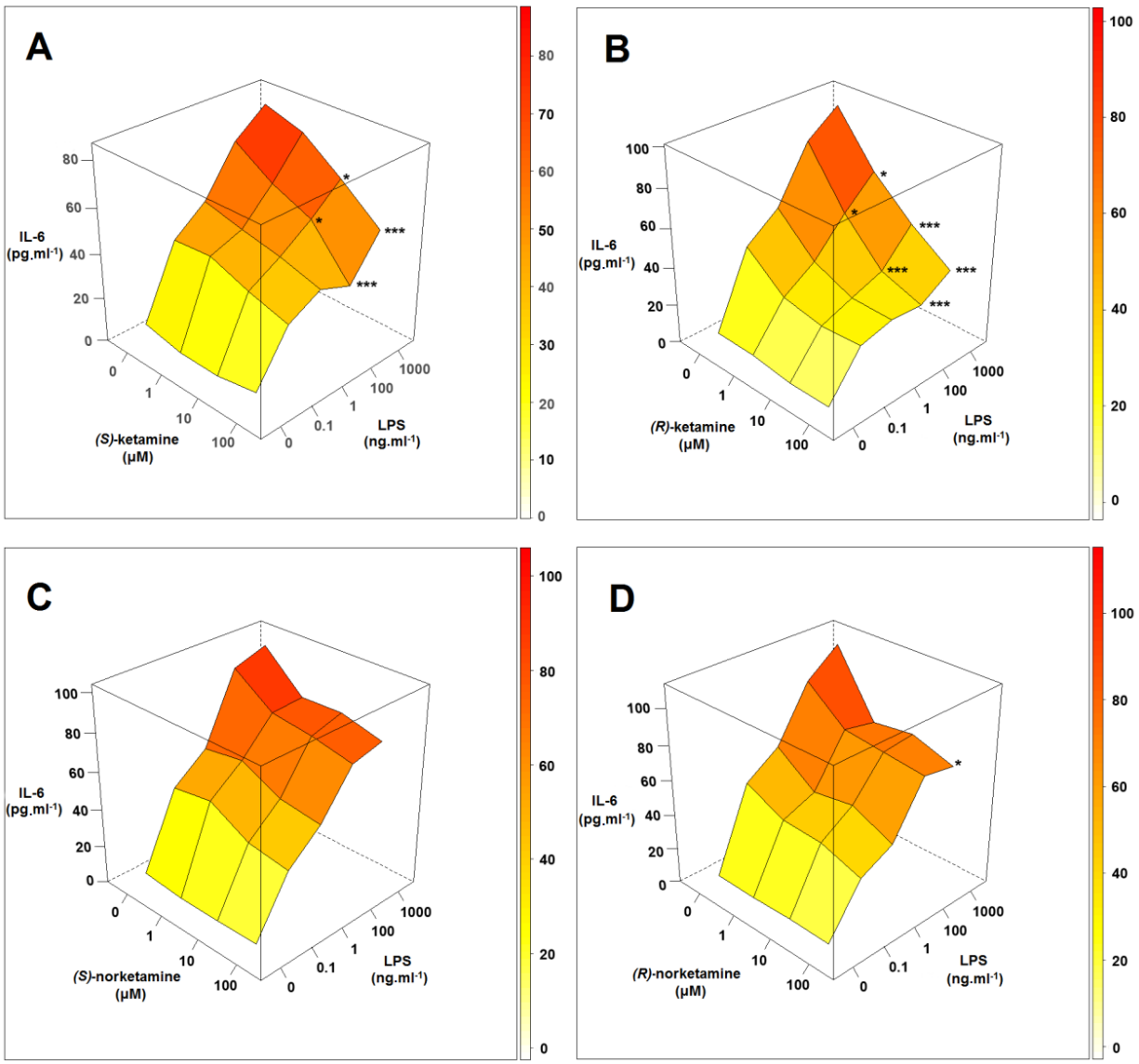

Figure 2. Effects of 20 min pre-incubation with: A) *(S)*-ketamine; B) *(R)*-ketamine; C) *(S)*-norketamine; and D) *(R)*-norketamine at 1, 10 and 100 μM on 20 h LPS-induced IL-6 production at different LPS concentrations (100 pg mL^{-1}, 1 $ng.mL^{-1}$, 100 $ng.mL^{-1}$ and 1 μg mL^{-1}). Each value represents the mean of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, LPS-induced IL-6 concentrations were significantly different from and less than 80% of that of corresponding vehicle control.

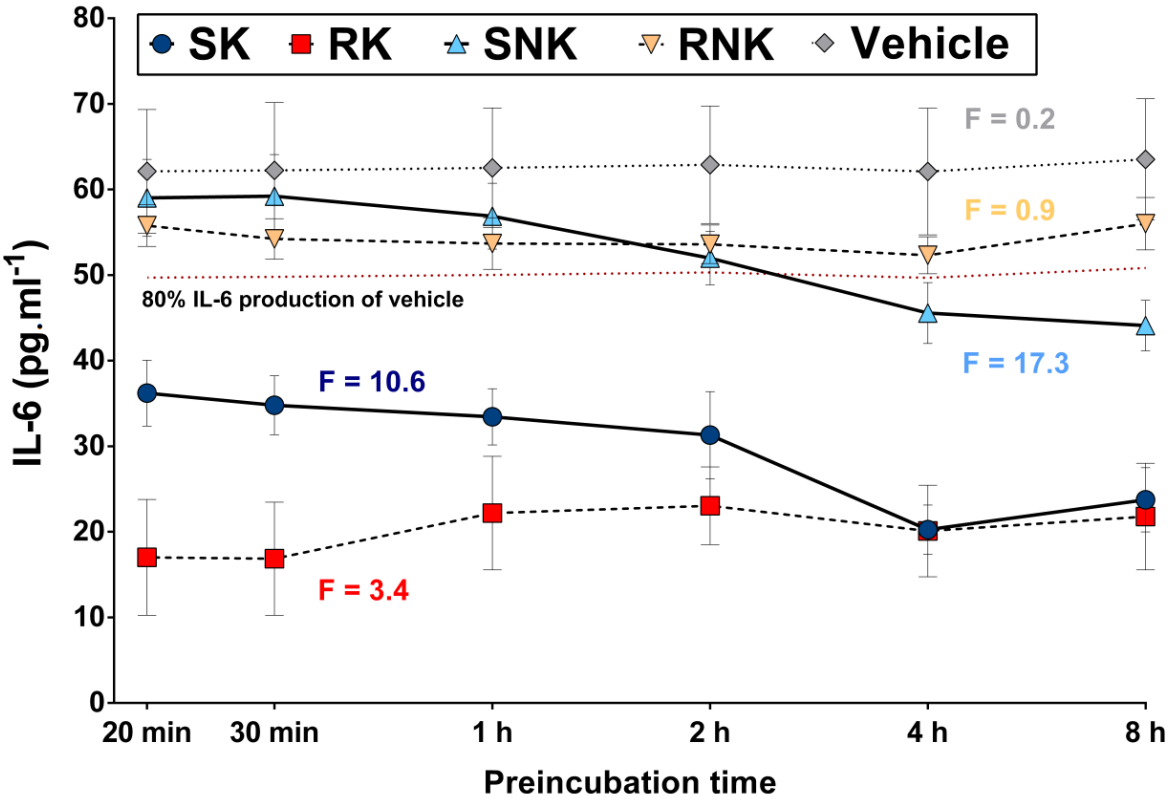

Figure 3. *(S)*-ketamine and *(S)*-norketamine but not *(R)*-ketamine and *(R)*-norketamine inhibited LPS-induced IL-6 production in a pre-incubation time-dependent manner. F-values ($d.f_1=5$) > 9 (P = 0.05, according to F-table) indicate a significant influence of pre-incubation time on IL-6 production. Data are mean ± SD of six independent experiments.

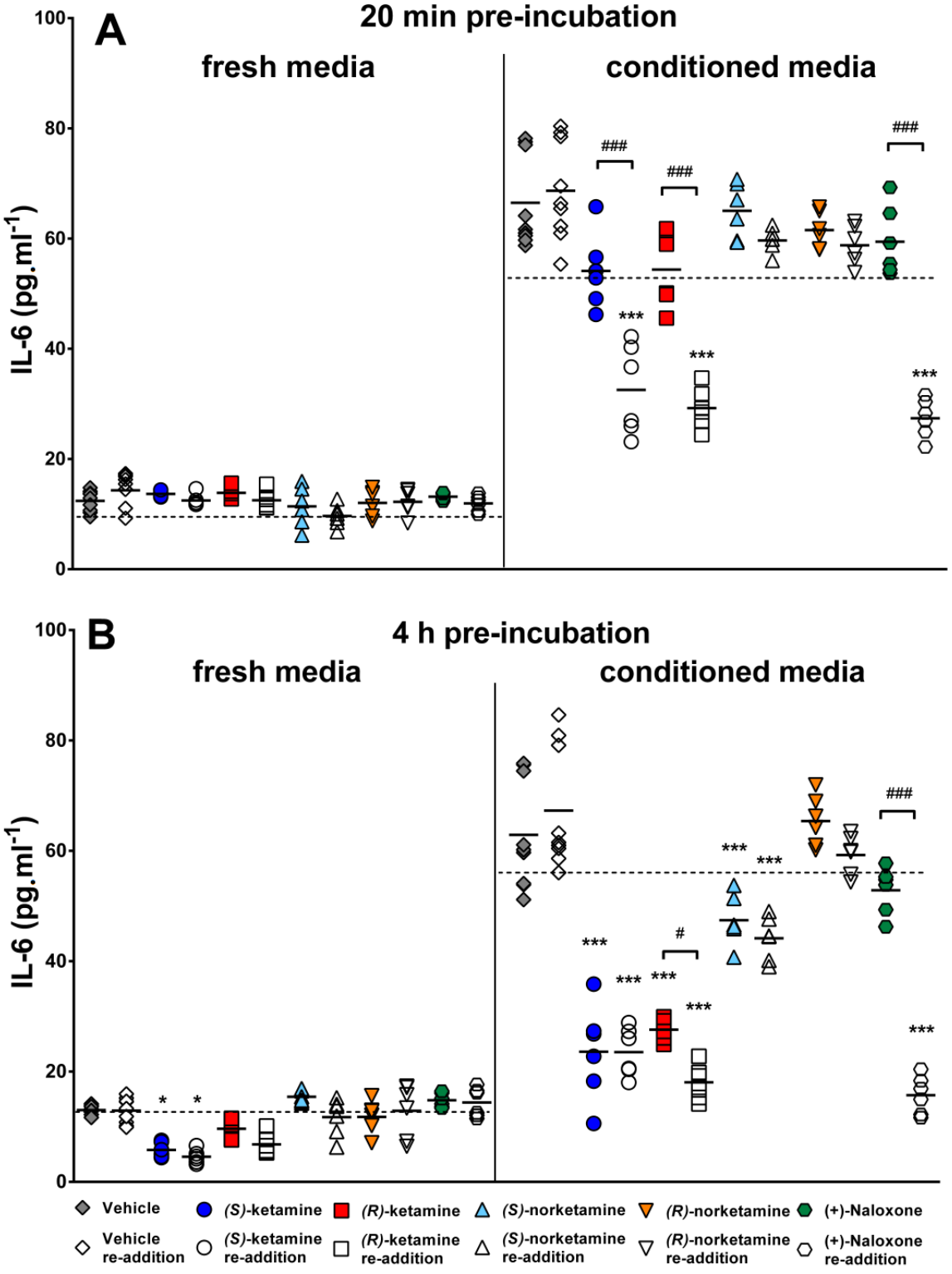

Figure 4. Basal LPS-induced IL-6 production in cells that received conditioned media replacement were substantially higher than those that received fresh media replacement. No treatment except 4 h pre-incubation with *(S)*-ketamine caused significant inhibition in cells receiving fresh media replacement. In cells receiving conditioned media replacement, (+)-

naloxone produced inhibition on LPS-induced IL-6 production only when it was re-added to the supernatant after being removed at the end of A) 20 min and B) 4 h pre-incubations. In contrast, the inhibitory effects of *(S)*-ketamine, *(R)*-ketamine and *(S)*-norketamine were substantially decreased when they were removed from the supernatant after the 20 min but not after the 4 h pre-incubation. Filled symbols represent the re-addition treatments where drugs were re-added into culture media after supernatant replacement. Dashed lines represent the mean value of 80% IL-6 production of vehicle controls. Bars show the mean value of six experiments. * $p < 0.05$, *** $p < 0.001$ significant difference in IL-6 production compared with the corresponding vehicle. # $p < 0.05$ and ### $p < 0.001$, significant difference in IL-6 production between cells that were stimulated in the presence of drugs (filled symbols) in supernatant and those that were stimulated without the drugs (open symbols).

Chapter 5. Discussion

The effectiveness of ketamine analgesia and co-analgesia in chronic pain management is limited by its low efficacy and marked interindividual variability in response. In my view, one possible cause of such limitations is the large difference in ketamine plasma concentration due to the variability in clearance. Ketamine is mainly cleared by hepatic CYP2B6 and CYP3A4-mediated metabolism to norketamine (Hijazi and Boulieu, 2002a; Yanagihara *et al.*, 2001), however, the predominant enzyme remained controversial at the start of this PhD. Identification of the relative contribution of each enzyme to ketamine metabolism may provide valuable information that help clinicians avoid potential drug-drug interactions. In addition, given that the expression and activity of CYP2B6 are largely affected by genetic factors (Zanger et al., 2007), I hypothesised that genetic variability in the *CYP2B6* gene, especially the most prevalent defective allelic variant of *CYP2B6* gene, *CYP2B6*6*, could contribute to the interindividual variability in ketamine clearance, and therefore, response. Moreover, since there was limited data on ketamine clearance in chronic pain patients, a clinical pharmacokinetic study investigating the influences of non-genetic factors, such as sex, age, disease states and medications on ketamine clearance may help clinicians make a safe and appropriate dosing decision. As such, the primary aims of my thesis were to investigate the impact of the *CYP2B6*6* allele on ketamine metabolism *in vitro*, and the influences of the *CYP2B6*6* allele and other non-genetic factors on *in vivo* ketamine clearance and its subsequent effects on analgesic response and adverse effects in chronic pain patients.

In addition to the variability in clearance, another possible cause of the interindividual variability in response is the heterogeneous nature of pain. Given that the primary analgesic effects of ketamine are the attenuation of hyperalgesia and allodynia (major symptoms of neuropathic pain), the drug is expected to be more effective against neuropathic pain. Although these analgesic effects are primarily due to the antagonism of NMDA receptor,

there is a discrepancy between the stereoselectivity in ketamine's NMDA affinity and analgesic potency (Table 1-1, page 6), which indicates a possible involvement of other analgesic mechanisms. However, all current known mechanisms have failed to explain such discrepancy, as none of which show a stereoselective preference towards *(R)*-ketamine.

Recent *in vitro* findings of a suppressive effect of ketamine on proinflammatory cytokine production suggested that a possible anti-proinflammatory mechanism may underly ketamine analgesia (Dale *et al.*, 2012; Liu *et al.*, 2012). However, the stereoselectivity of such effects was unclear. In addition, the anti-proinflammatory effects of ketamine were only observed at the highest achievable anaesthetic concentrations and biologically irrelevant concentrations in animal cells. Additionally, although there was some indication that inhibition of the TLR4/MD2 pathway was involved in the inhibition of proinflammatory response by ketamine, the exact target was yet to be identified. Therefore, the secondary aim of my thesis was to explore the innate immune pharmacology of ketamine and norketamine enantiomers at anaesthetically and analgesically relevant concentrations in TLR4/MD2 transfected human cells using LPS-stimulated IL-6 production as a biomarker for proinflammatory response. The attenuation of proinflammatory cytokine may also contribute.

5.1 The impact of *CYP2B6*6* genetic variability on ketamine clearance

5.1.1 A new HPLC method for quantification of ketamine and norketamine

For the quantification of ketamine and norketamine in biological samples, a new HPLC-UV method was successfully developed and validated. The method, consisting of a two-step liquid-liquid extraction procedure and a UV detection procedure, has the capacity to assay up to 60 samples per day per HPLC system with more than 90% inter- and intra-assay accuracy and precision, and clinically relevant limit of detection (lower limit of detection is 10 ng.mL^{-}

[1]), using a standard HPLC equipment set-up. This method is relatively time-saving (3 h for the extraction and 15 h [overnight] for the UV detection per 60 samples) compared with a method previously developed in our laboratory (unpublished), whose detection procedure took approximately 30 h per 60 samples; and a method described by Cheng *et al.* (2007), whose extraction procedure took approximately 8 h per 60 samples using standard equipment.

5.1.2 The *CYP2B6*6* genetic polymorphism reduced ketamine metabolic clearance

The major finding of this thesis was the substantial impact of the *CYP2B6*6* allele on the metabolic clearance of ketamine. The *in vitro* study presented in chapter 2 demonstrated that CYP2B6 and not CYP3A4 is the predominant CYP isoform responsible for ketamine metabolism to norketamine (*N*-demethylation) in HLMs at clinically relevant concentrations. As such, the impact of the *CYP2B6*6* genotype on ketamine metabolism was subsequently examined. The results showed that intrinsic clearance for both ketamine enantiomers in HLMs was reduced by approximately 60% and 85% in the presence of one and two *CYP2B6*6* alleles, respectively. Such a reduction in intrinsic clearance is due not only to the reduction of CYP2B6 expression but also the impairment in function including the enzyme-substrate binding (decrease in K_m) and catalytic activity (decrease in V_{max}). These observations agree with the finding of Hofmann *et al.* (2008) that the *CYP2B6*6* genetic mutation induces aberrant splicing that results in a severe reduction in the expression and activity of functional mRNA and protein. The marked impact of the *CYP2B6*6* allele on microsomal metabolism of ketamine was confirmed by subsequent enzyme kinetic assays using cDNA-expressed CYP2B6 protein variants and inhibition assays in HLMs. Taken together, this study provided the first evidence of the major impact of the *CYP2B6*6* genetic variant on ketamine *N*-demethylation to norketamine *in vitro*, and indicated potential effects of the *CYP2B6*6* allele

on ketamine plasma concentration, and possibly on analgesic efficacy. Thus, the clinical importance of this *in vitro* observation was subsequently investigated.

The clinical pharmacokinetics study presented in chapter 3 confirmed the significant impact of the *CYP2B6*6* allele on ketamine steady-state plasma clearance and plasma concentrations in chronic pain patients. To the best of my knowledge, this is the first human evidence of the genetic impact of *CYP2B6* on ketamine plasma concentrations. The ketamine steady-state plasma clearance rate in patients with the *CYP2B6*1/*6* and the *CYP2B6*6/*6* genotypes were 60 to 70% and 40% of that in patients with the *CYP2B6*1/*1* genotype. Accordingly the estimated intrinsic clearance of ketamine was decreased by at least 64% and 86% in patients carrying the *CYP2B6*1/*6* and *CYP2B6*6/*6* genotypes, an effect that is almost identical to the reduced clearance observed *in vitro* with HLMs. This effect of the *CYP2B6*6* allele explained approximately 40% of the interindividual variability in ketamine steady-state plasma concentrations.

The large interindividual variability in plasma concentrations of ketamine (over 5-fold) and norketamine (over 7-fold) had not been observed in previous PK studies conducted in young male healthy volunteers (Clements and Nimmo, 1981; Geisslinger *et al.*, 1995; White *et al.*, 1985; Yanagihara *et al.*, 2003). One possible reason is that the population size of these studies ($n \leq 5$) might have been too small to identify any *CYP2B6*6* carrier, especially a *CYP2B6*6/*6* carrier (genotype frequency is approximately 6% in Caucasians). Another possible reason is that the contribution of the age-related change in hepatic function to the variability in ketamine clearance was minor, since the participants of these PK studies were young (< 40 years) and similar in their age (see Table 1-2, page 15-17). The effect of age in ketamine hepatic clearance will be discussed in the following section.

5.1.3 Reduction of ketamine clearance in elderly patients

In addition to the *CYP2B6* genetic impact, the population PK study presented in chapter 3 identified an association between ketamine plasma concentrations and the age of the patient. The ketamine plasma clearances in patients aged 40 years or less were at least twice those in patients aged 80 years or over. This age-related change in ketamine clearance explained approximately 20-30% of variability in steady-state plasma concentrations. Since the hepatic clearance of ketamine is subject to the change in liver blood flow, and it is generally accepted that there are substantial decrease in both liver blood flow and liver volume in elderly subjects (over 65 years), estimated to be approximately 30-40% and 20-30%, respectively, compared with those in subjects less than 40 years (Wynne *et al.*, 1989), the reduction in ketamine clearance is likely due to the age-related decreases in liver blood flow. Although some early studies indicated an age-related reduction in CYP3A4 activity (midazolam hydroxylase) *in vivo* (Greenblatt *et al.*, 1984; Harper *et al.*, 1985; Platten *et al.*, 1998), the finding was not confirmed by subsequent *in vitro* studies and lacked of support from *in vitro* observations (Albrecht *et al.*, 1999; Gorski *et al.*, 2003; Parkinson *et al.*, 2004). Therefore, it is less likely that the age-induced reduction in ketamine plasma clearance is due to an impairment of CYP3A4 activity.

In contrast to age, sex and CYP2B6 inhibitors/inducers showed no or minor influences on ketamine plasma clearance and concentration in our study cohort. The lack of sex difference may reflect a minor involvement of CYP3A4 enzyme in the hepatic clearance of ketamine at the tested doses, as hepatic CYP3A4 expression level has been found to be 2-fold higher in females; and many CYP3A4 substrates are metabolised faster in females than males (Wolbold *et al.*, 2003). Nevertheless, considering a previous report on the influences of CYP3A inducers on oral ketamine exposure (Noppers *et al.*, 2011b), the potential influence of CYP3A4 inducers on ketamine metabolism should not be ignored, especially after oral

dosing. The lack of influences of CYP2B6 inhibitors/inducers is possibly because ketamine was administered by continuous infusion, for which the hepatic blood flow is the major determinant of drug clearance. However, similar to CYP3A4 inducers, these drugs may still have a marked influence on ketamine PK after non-parenteral routes of administration, which requires further investigation.

5.1.4 Lower plasma clearance in patients who experienced ketamine adverse effects

The other major finding of the population PK study was an association between ketamine clearance and the incidence of adverse effects. The data showed that the steady-state plasma clearance of ketamine in patients who experienced adverse effects (median 45.6 $L.h^{-1}$) was approximately 15% lower than those who reported no adverse effects (median 52.6 $L.h^{-1}$, P = 0.04). Of particular note were the three *CYP2B6*6/*6* carriers all experienced adverse effects, indicating these patients may be more vulnerable to ketamine. These findings indicated that although the severity of ketamine adverse effects at sub-anaesthetic doses is generally mild, the incidence may be increased in patients whose CYP2B6 function is substantially impaired. However, due to the small group size, the presence of three *CYP2B6*6/*6* carriers may have unduly influenced the decrease in median plasma clearance in the "adverse effects" group. As such, there is a possibility that the large difference in plasma clearance of ketamine was exaggerated (false positive result). Therefore, the association between plasma clearance on ketamine adverse effects and the influence of the *CYP2B6*6/*6* genotype require further assessment with a larger population size. It is possible that a prior *CYP2B6*6* genotyping may help patient selection to improve "number needed to treat" and "number needed to harm rates" in clinical practice.

5.1.5 No relationship between plasma ketamine and norketamine concentrations and ketamine analgesic efficacy

In contrast with the relationship between ketamine clearance and adverse effects, no significant difference in plasma concentrations of ketamine and norketamine between responders and non-responders was identified. Additionally, the minimum dose of ketamine required for adequate analgesia was different among ketamine responders. Such results suggest that ketamine analgesic response may be independent of plasma concentrations of ketamine and norketamine. Although the driving factor(s) for this lack of concentration-analgesic efficacy is not entirely clear, considering ketamine's relatively greater efficacy against neuropathic pain, I speculated that this concentration-response relationship is obscured by the large effect of the pain variability. However, due to the small population size for each pain type group in our study ($n<15$), and that response was determined by different outcomes in both studies, it is difficult to further investigate the relationship during different pain conditions. Another potential confounding factor is opioid-resistance, since a number of patients in our study may have relied heavily on chronic opioid analgesia prior to ketamine treatment, in which opioid tolerance and opioid-induced hyperalgesia were more likely to be developed. But unfortunately, an accurate assessment of opioid tolerance and opioid-induced hyperalgesia in pain patients is constrained by difficulties (some phenomena produce similar symptoms of increasing pain but not due to opioid-resistance, such as ongoing tissue damage), which prevented the investigation of the influence of opioid-resistance on ketamine analgesia in the current study. In addition to the variability in pain and opioid-resistance, interindividual differences in the level of innate immune response may possibly contribute to the variability in ketamine analgesia, although ketamine's anti-proinflammatory ability at sub-anaesthetic concentrations requires further confirmation.

Based on the results of chapter 2 and 3, it appears that the *CYP2B6*6* allele has a large impact on ketamine metabolic clearance to norketamine, and may be related to the adverse effects of ketamine at sub-anaesthetic doses. However, the relationship between the *CYP2B6*6* allele and ketamine adverse effects and clinical meanings of this relationship require further assessment in a larger population. If this relationship was identified, prior *CYP2B6*6* genotype screening may help predict ketamine plasma concentrations and the risk of adverse effects, which would allow for a more accurate initial dose selection. However, due to the overwhelming influence of confounding factors on ketamine analgesic response, such as the heterogeneous nature of pain, the clinical impact of the *CYP2B6*6* allele on ketamine analgesic efficacy remains uncertain.

5.2 Inhibition of LPS-stimulated IL-6 production by ketamine and norketamine

As discussed earlier, ketamine is more effective against neuropathic pain than other pain types, probably because the analgesic mechanism is via the attenuation of hyperalgesia and allodynia (major symptoms of neuropathic pain) rather than a direct suppression of nociceptive transmission. Although the anti-hyperalgesic and anti-allodynic effects of ketamine have been primarily associated with the antagonism of NMDA receptor, there is a discrepancy between the stereoselectivity in NMDA affinity and analgesic potency (Table 1-1, page 6), which indicates a possible involvement of another analgesic mechanism. However, all current known mechanisms have failed to explain such discrepancy, as they have either no stereoselectivity or a stereoselective preference towards *(S)*-ketamine.

Recent *in vitro* findings of the suppressive effect of ketamine on proinflammatory cytokine production suggested that a possible anti-proinflammatory mechanism may underly ketamine analgesia (Dale *et al.*, 2012; Liu *et al.*, 2012). However, the stereoselectivity of such effects

was unclear. In addition, the anti-proinflammatory effects of ketamine were only observed at the highest achievable anaesthetic concentrations and biologically irrelevant concentrations in animal cells. Additionally, although there was some indication that inhibition of the TLR4/MD2 pathway was involved in the inhibition of proinflammatory response by ketamine, the exact target was yet to be identified. Therefore, the secondary aim of thesis was to explore the innate immune pharmacology of ketamine and norketamine enantiomers at anaesthetically and analgesically relevant concentrations in TLR4/MD2 transfected human cells using LPS-stimulated IL-6 production as a biomarker for proinflammatory response.

5.2.1 Ketamine and norketamine stereoselectively inhibits stimulated IL-6 production in a concentration- and time-dependent manner

The study present in chapter 4 showed that *(R)*-ketamine, at least acutely, was approximately 1- or 2-fold more potent in attenuating IL-6 production than equimolar *(S)*-ketamine. Additionally, only *(R)*-ketamine showed significant inhibition at an analgesically relevant concentration. To my knowledge, this is the first report of ketamine's anti-proinflammatory effect at low concentrations. The greater inhibitory effect of *(R)*-ketamine may give some explanation for the relatively minor stereoselective difference in ketamine's analgesic activity (*(S)*-ketamine is 3-4 times of *(R)*-ketamine) compared with the difference in NMDA binding affinity (*(S)*-ketamine is 6-7 times of *(R)*-ketamine). Moreover, it may be related to the greater anti-depressant potency of *(R)*-ketamine compared with *(S)*-ketamine in rodent models (Zhang *et al.*, 2014), as proinflammatory cytokines have been involved in the development of depression (Dantzer *et al.*, 2008; Dowlati *et al.*, 2010). Interestingly, it appears that the inhibitory effects of *(S)*-ketamine but not *(R)*-ketamine are significantly enhanced after chronic exposure. The chronic effects of *(S)*-ketamine and *(S)*-norketamine may explain a clinical observation that long-term infusion improves *(S)*-ketamine analgesia (Noppers *et al.*,

2011a). However, the connection between these two observations needs to be further investigated.

Unlike ketamine enantiomers, *(S)*-norketamine but not *(R)*-norketamine showed an inhibitory effect on IL-6 production, which, although was approximately 50% that of *(S)*-ketamine, only observed after long-term exposure. Such results suggest that norketamine may contribute to the inhibition of proinflammatory response with chronic ketamine treatment.

5.2.2 Potential anti-proinflammatory mechanisms of ketamine

The time-dependent inhibition of IL-6 by *(S)*-ketamine and *(S)*-norketamine may reflect a potential difference in the anti-proinflammatory mechanism between acute and chronic ketamine exposure. Therefore, the mechanisms underlying the inhibition of IL-6 production after different exposure times were explored. Although my experiments were unable to identify the precise molecular mechanisms, the results indicated that the soluble co-signalling factors for LPS-TLR4 binding, presumably MD-2 and CD14, played a crucial role in the acute inhibitory effect of ketamine. This finding is supported by the *in silico* docking simulation results, which showed likely interactions between human MD-2 and both ketamine enantiomers at the core of TLR4-MD-2 main dimerisation interface. Although norketamine enantiomers also showed docking preference on MD-2, the binding site was irrelevant to LPS-TLR4/MD-2 interaction, which may be the reason for the lack of inhibition by norketamine after acute exposure.

On the contrary, the chronic inhibitory effect of ketamine seems not dependent on a direct drug-receptor interaction, but rather to be a functional consequence as yet unidentified long-term changes in signalling cascades, which require further investigation. On the basis of these findings, I speculated that ketamine inhibits LPS-stimulated IL-6 by two mechanisms: an

acute mechanism that disrupts the recognition of LPS by TLR4 by antagonising MD-2; and a chronic mechanism causing long-term modulations of the TLR4 signalling pathway that leads to the suppression of IL-6 production. This chronic mechanism may be of particular clinical relevance as it may explain the persistent pain relief after termination of long-term *(S)*-ketamine infusion that have been observed clinically.

5.3 Conclusion

In conclusion, the findings of this thesis provide the first *in vitro* and clinical evidence that the presence of the *CYP2B6*6* allele variant substantially reduces ketamine metabolism to norketamine. This genetic impact on metabolism has a large contribution to the marked decrease in steady-state ketamine plasma clearance in patients carrying the variant, which explains to a great extent the large interindividual variability in ketamine plasma concentrations. In addition, it may be associated with a higher risk of experiencing ketamine adverse effects. Therefore, the *CYP2B6*6* genotype may be an important factor that should be taken into consideration when optimising ketamine doses for a target therapeutic plasma concentration in individual patients. However, the analgesic efficacy of ketamine cannot be simply predicted based on the *CYP2B6*6* genotype or even the plasma ketamine concentration due to the considerable influence of the heterogeneous nature of pain; the degree of opioid-resistance; and probably the level of proinflammatory response between patients.

In addition, the immunopharmacology study reported in this thesis has extended the current knowledge of the anti-proinflammatory actions of ketamine and norketamine. The data demonstrated a greater potency of *(R)*-ketamine in inhibiting stimulated proinflammatory cytokine production. This finding not only provides some explanation for the discrepancy between the stereoselectivity of ketamine in analgesic potency and the NMDA affinity, but

also indicates a possible clinical usefulness of *(R)*-ketamine in the attenuation of proinflammatory response. Furthermore, the identification of the time-dependent inhibition of IL-6 by *(S)*-ketamine and *(S)*-norketamine for the first time links a possible mechanism to the observed persistent analgesia after long-term ketamine infusion and may lead to a new path in the research of ketamine analgesia.

Chapter 6. References

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Appendices

Appendix I: Dosing regimens employed in published RCTs investigating ketamine for pain management

Study	Doses	Route(s)/ Frequency	Subjects (n, pain type)	Duration	Study type	Analgesic effects
Intravenous						
Eide *et al.* (1994)	0.15 $mg.kg^{-1}$	Single bolus	8, neuralgia	10 min	R, DB, CO	50% pain relief by KET, but not by morphine
Backonja *et al.* (1994)	0.25 $mg.kg^{-1}$		6, chronic neuropathic pain	5 min	PC, DB	Pain relief in 5/6 patients, analgesia last for 2 weeks in one patient
Persson *et al.* (1998)	0.15, 0.3, 0.45 $mg.kg^{-1}$		8, pain in the lower extremity	5 min	R, DB,CO	Pain relief by KET at 0.3 mg.kg-1 in 7/8 patients, at 0.45 $mg.kg^{-1}$ in 8/8 patients
Eide *et al.* (1995)	0.06 $mg.kg^{-1}$ + 0.036 $mg.kg^{-1}.h^{-1}$	Bolus + infusion	9, central dysesthesia pain	1 min bolus + 17-20 min infusion	R, DB	KET reduced continuous pain in 7/9 patients, median analgesic effect: 40%
Jorum *et al.* (2003)			12, neuropathic pain		R, PC, DB, CO	KET reduced the hyperalgesia to cold pain
Nikolajsen *et al.* (1996)	0.1 $mg.kg^{-1}$ + 0.049 $mg.kg^{-1}.h^{-1}$		11, phantom limb pain	1 min bolus + < 45 min infusion	R, PC, DB, CO	Complete relief of stump pain and 70-100% relief of phantom pain by KET
Felsby *et al.* (1996)	0.2 $mg.kg^{-1}$ + 0.3 $mg.kg^{-1}.h^{-1}$		10, peripheral neuropathic pain	10 min bolus + 1 h infusion	R, PC, DB	KET produced 57% reduction of spontaneous pain and 33% the area of allodynia
Baad-Hansen *et al.* (2007)	0.05 + 0.07 $mg.kg^{-1}$ [*(S)*-ketamine]	Infusion	10, atypical odontalgia	10 + 20 min	R, PC, CO	KET produced no relief of atypical odontalgia and capsaicin-evoked pain
Gottrup *et al.* (2006)	0.24 $mg.kg^{-1}$		20, nerve injury pain	30 min	R, PC, DB, CO	>33% reduction in spontaneous pain by KET in 8/19 patients and by PBO in 1/19 patients
Mercadante *et al.* (2000)	0.25, 0.5 $mg.kg^{-1}$		10, chronic cancer pain	30 min	R, DB, CO	80% and 96% pain relief by KET at 0.25 and 0.5 $mg.kg^{-1}$, respectively

Study	Doses	Route(s)/ Frequency	Subjects (n, pain type)	Duration	Study type	Analgesic effects
Intravenous						
Sörensen *et al.* (1997)	0.25, 0.5 mg.kg^{-1}	Infusion	11, fibromyalgia		PC, DB	Pain relief in 8/11 patients, 2-7 days analgesic effects in 6/8 responders
Graven-Nielsen *et al.* (2000)	0.3 mg.kg^{-1}		29, fibromyalgia	30 min	R, PC, DB	>50% pain relief in 17/29 patients
Browne and Lucki (2013)	0.3 mg.kg^{-1}		30, whiplash associated pain		R, PC, DB, CO	>57% pain relief in 14/28 patients
Kvarnström *et al.* (2003)	0.4 mg.kg^{-1}		12, peripheral neuropathic pain	40 min	R, PC, DB, CO	Pain relief in 7/12 patients, the mean value for maximal pain reduction by KET was 55%
Mitchell and Fallon (2002)	0.6 mg.kg^{-1}		18, critical limb ischemia	4 h	R, DB	KET improved opioid analgesia by 15% and 19% at 1 day and 5 days post-infusion, respectively
Max *et al.* (1995)	0.75 mg.kg^{-1}.h^{-1}		8, chronic post-traumatic pain	2 h	R, PC, DB, CO	15-100% pain relief during infusion, 20-100% reduction in mechanical allodynia
Schwartzman *et al.* (2009)	0.35 mg.kg^{-1}	Long-term infusion	9, CRPS	4 h / day for 10 days	R, PC, DB,	20% reduction in pain score for 4 weeks after infusion
Salas *et al.* (2012)	0.5, 1 mg.kg^{1} daily		11, cancer pain	2 days	R, PC, DB,	No improvement of opioid analgesia by the addition of ketamine
Amr (2010)	16 mg h^{-1}		8, neuropathic pain	1 week	R, DB	KET improved gabapentin analgesia for 2 week after infusion
Sigtermans *et al.* (2009)	5-30 mg h^{-1} [*(S)*-ketamine]		30, CRPS	4.2 days	R, PC, DB	KET reduced NRS pain score from 6.9 to 2.7 during the infusion week; significant greater pain relief by KET than PBO for week 11
Oral						
Haines and Gaines (1999)	20-100 mg	Daily	21, neuropathic pain	1 week	R, CO / OL	Pain relief in 3/21 patients, 17/21 patients experienced adverse effects of KET

Study	Doses	Route(s)/ Frequency	Subjects (n, pain type)	Duration	Study type	Analgesic effects
Oral						
Lauretti *et al.* (1999)	0.5 $mg.kg^{-1}$	Twice a day	15, chronic cancer pain	1 month	PC, DB	No significant improvement of opioid analgesia, KET reduced opioid consumption on day 20 and 30
Furuhashi-Yonaha *et al.* (2002)	0.5 $mg.kg^{-1}$	Four times a day	8, chronic neuropathic pain	1 week	R, PC, DB, CO	Mean VAS pain score reduced from 78 to 49 mm after in 8 patients received KET
Rabben *et al.* (1999)	4 $mg.kg^{-1}$	Daily	26, trigeminal neuropathic pain	3 days	R, PC, DB, CO	Nearly complete pain relief during oral KET administration in 5/26 patients
Ishizuka *et al.* (2007)	10 mg [*(S)*-ketamine]	Three times a day	15, chronic cancer pain	4 weeks	R, DB	KET improved opioid analgesia by 15% and 19% at 1 day and 5 days post-infusion, respectively
Subcutaneous						
Nicolodi and Sicuteri (1995)	0.08 $mg.kg^{-1}$	Single bolus or TID	34, migraine	3 weeks	R, DB, CO	50-100% pain relief by KET
Hardy *et al.* (2012)	100, 300, 500 mg daily	24 h continuous infusion	181, chronic cancer pain	3-5 days	R, PC, DB	No significant difference in pain score between KET (response rate = 31%) and PBS (response rate = 27%)
Topical						
Lynch *et al.* (2003)	5 mL of 0.5% ketamine	Four times a day	20, neuropathic pain	2 days	R, PC, DB, CO / OL	No significant pain improvement by KET
Finch *et al.* (2009)	0.5 mL of 10% ketamine	Once	20, CRPS	Once	R, PC, DB, CO	Approximately 30% of allodynia by KET, but no reduction of pain score

Study	Doses	Route(s)/ Frequency	Subjects (n, pain type)	Duration	Study type	Analgesic effects
Other						
Carr *et al.* (2004)	10-50 mg	Intranasal, 10 mL / spray; 1-5 sprays	22, breakthrough pain	Once	R, PC, DB, CO	14/20 (70%) patients achieved a NPIS score of ≤ 4 following KET
Ayesh *et al.* (2008)	0.55 mg	Intra-articular	18, temporomandibul-ar joint arthralgia	Once	PC, CO, DB	No significant reduction in pain score
Yang *et al.* (1996)	1 mg	i.t., twice a day	20, chronic cancer pain	3-36 months	PC, CO, DB	KET enhanced analgesic effects of intrathecal morphine
Rabben *et al.* (1999)	0.4 $mg.kg^{-1}$	i.m.	26, trigeminal neuropathic pain	One injection	PC, CO, DB	No improvement in 9/26 patients; 1 h analgesic effect in 9/26 patients; >12 h analgesic effect in 8/26 patients

CO, crossover; DB, double blinded; KET, ketamine; NPIS, numeric pain intensity scale; NRS, numeric rating scale; OL, open-label study; PBO, placebo; PC, placebo controlled; R, randomised

Appendix II: Binding affinity of racemic ketamine and enantiomers to various targets

Type of receptor	Species	Experimental design	Drug concentration	Binding affinity/inhibition constant	Stereoselectivity	Reference
NMDA receptor	Guinea pig	Displacement of [^{3}H]-TCP	Not reported	K_i: 1.1 μM (SK), 3.2 μM (RK)	SK has 2.9-fold affinity of RK	(Hustveit *et al.*, 1995)
	Rat	Displacement of [^{3}H]-MK801	SK & RK (0.1-500 μM)	K_i: 0.3 μM (SK), 1.4 μM (RK)	SK has 5-fold affinity of RK	(Ebert *et al.*, 1997)
	Human	Displacement of [^{3}H]-dizocilpine	SK & RK (10 nM-500 μM)	IC_{50}: 1.5-2.8 μM (SK), 7.2-14 μM (RK)	SK has 6 to 7-fold affinity of RK	(Oye *et al.*, 1992)
Dopamine D_2 receptor	Human	Radio-labelled ligand assay	RacK (1-10,000 nM)	K_i: 0.06 ± 0.01 μM	Not reported	(Seeman *et al.*, 2005)
	Human		SK & RacK (0.1 to 300 μM)	K_i: 0.7 ± 0.3 μM (SK), 2.3 ± 0.3 μM (RacK)	SK has 3-fold affinity of RacK	(Kapur and Seeman, 2002)
	Rat	Radio-labelled ligand assay	RacK (0.1 to 300 μM)	K_i: 1.0 ± 0.2 μM	Not reported	(Kapur and Seeman, 2002)
μ-opioid receptor	Human	Displacement of [^{3}H]-DAMGO	RacK (0.5 μM to 5 mM)	K_i: 12 ± 0.8 μM	Not reported	(Hirota *et al.*, 1999)
	Chinese hamster	Displacement of [^{3}H]-DPN	RacK (0.3 μM to 10 mM) SK & RK (3 μM to 1mM)	pK_i: 4.4 ± 0.02 μM (RacK), 4.5 ± 0.01 μM (SK), 4.1 ± 0.05 μM (RK)	No significant difference	(Hirota *et al.*, 1999)

Type of receptor	Species	Experimental design	Drug concentration	Binding affinity/inhibition constant	Stereoselectivity	Reference
μ-opioid receptor	Guinea-pig	Displacement of [^{3}H]-DPN	Not reported	K_i: 28 μM (RK), 11 μM (SK)	SK has 2.5-fold affinity of RK	(Hustveit *et al.*, 1995)
κ-opioid receptor	Chinese hamster	Displacement of [^{3}H]-DPN	RacK (0.3 μM to 10 mM) SK & RK (3 μM to 1mM)	pK_i: 4.6 ± 0.04 μM (RacK), 4.6 ± 0.03 μM (SK), 4.2 ± 0.03 μM (RK)	No significant difference	(Hirota *et al.*, 1999)
	Guinea-pig	Displacement of [^{3}H]-U69593	Not reported	K_i: 24 μM (RK), 100 μM (SK)	SK has 4.2-fold affinity of RK	(Hustveit *et al.*, 1995)
δ-opioid receptor	Chinese hamster	Displacement of [^{3}H]-DPN	RacK (0.3 μM to 10 mM) SK & RK (3 μM to 1mM)	pK_i: 4.6 ± 0.04 μM (RacK), 4.6 ± 0.03 μM (SK), 4.2 ± 0.03 μM (RK)	No significant difference	(Hirota *et al.*, 1999)
	Guinea-pig	Displacement of [^{3}H]-DPDPE	Not reported	K_i: 130 μM (RK), 130 μM (SK)	No significant difference	(Hustveit *et al.*, 1995)
σ receptor	Guinea-pig	Displacement of [^{3}H]+SKF10047	Not reported	K_i: 131 μM (SK), 19 μM (RK)	RK has 6.7-fold affinity of SK	(Hustveit *et al.*, 1995)
Muscarinic receptor	Guinea-pig	Displacement of [^{3}H]-DPDPE	Not reported	K_i: 20 μM (SK), 37 μM (RK)	SK has 1.8-fold affinity of RK	(Hustveit *et al.*, 1995)
	Rat	Currents flow across the membrane	RacK (0.1 μM to 1 mM)	IC_{50}: 5.7 μM	Not reported	(Durieux, 1995)

Type of receptor	Species	Experimental design	Drug concentration	Binding affinity/inhibition constant	Stereoselectivity	Reference
5-HT_3 receptor	Human	Currents flow across the membrane	RacK (1-100 μM)	IC_{50}: 910 ± 30 μM	Not reported	(Yamakura *et al.*, 2000)
Noradenaline transporter	Human	Radioligand binding assay	RacK (10 μM-100 mM)	K_i: 67 ± 26 μM	Not reported	(Nishimura *et al.*, 1998)
Serotonin transporter	Rat	Radioligand binding assay	RacK (10 μM-100 mM)	K_i: 162 ± 28 μM	Not reported	(Nishimura *et al.*, 1998)
Dopamine transporter	Rat	Radioligand binding assay	RacK	K_i: 46.9 μM (SK), 390 μM (RK)	SK has 8-fold affinity of RK	(Nishimura and Sato, 1999)

Data is present as mean or mean ± SD; SK: *(S)*-ketamine; RK: *(R)*-ketamine; RacK: racemic ketamine; K_i: inhibitor constant; IC_{50}: half maximal inhibitory concentration; pK_i: $-\log K_i$

Appendix III:

Does intraoperative ketamine attenuate inflammatory reactivity following surgery? A systematic review and meta-analysis.

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