Effect of novel ketamine-analogue R5 on brain activation and select behavioural parameters

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Abstract

Ketamine is a common anaesthetic that works through complex neural mechanisms, including multiple molecular and circuitry targets. Importantly, it promotes analgesia, though it also induces undesirable effects, such as agitation, disorientation, hallucinations and nausea. Thus, there is an ongoing search for novel ketamine analogues that influence a similar repertoire of brain targets as ketamine, whose beneficial effects are potentiated. The present project, utilizing rats as an animal model, focused on examining functional properties of the ester-analogue of ketamine, R5, whose potentially beneficial profile had been suggested by preliminary studies. First, brain activation patterns following R5 compared to ketamine (and another ester-analogue control compound, R1) were assessed by employing immunohistochemical detection of an immediate-early gene product, c-Fos. R5 produced a somewhat similar pattern of activity as ketamine, whereas more profound differences in c-Fos were detected after R1. It was particularly striking in areas related to pain and addiction, including the anterior insular cortex (AIC) and paraventricular nucleus (PVN). Therefore, in the subsequent set of experiments, effects of R5 on pain- and addiction-related behavioral parameters were assessed in rats injected with R5 intracerebroventricularly (ICV) or intraparenchymally. It was found that BaCl attenuated ICV R5-induced analgesia. AIC administration of R5 produced modest analgesia in the tail-flick test. Finally, PVN R5 reduced naltrexone-precipitated exercise-induced withdrawal. In sum, R5 shows an analgesic effect similar to ketamine, most likely by targeting a similar subset of brain sites, which suggests that this particular ester-analogue can be considered as a good candidate for conceptualizing future pain management strategies.

Acknowledgements

I would like to sincerely thank Dr. Pawel Olszewski, Dr. Anica Klockars, Oscar Klockars, Sarah Gartner, Erin Wood, Chris Batterton and Dr. Stuart Hameroff for their invaluable aid during the completion of this thesis. Supported by: ANZCA: Australia & New Zealand College of Anaesthetists.

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1. Introduction

Anaesthesia has been operationally defined as (1) immobility in response to noxious stimuli, and (2) amnesia. Other clinically relevant effects include analgesia, or loss of pain sensation, and hypnosis, or the loss of consciousness; but such effects do not uniquely define the anaesthetic state [5].

A comprehensive electroencephalographic analysis of anaesthetic-induced loss of consciousness was conducted by John and Prichep [6]. Using various anaesthetic drugs and techniques, they found that loss of consciousness is a fairly abrupt transition (less than 20 ms) involving interruption of γ (gamma) synchrony between frontal and posterior cortical regions. Similarly, Imas *et al.*[7] showed that volatile anaesthetics disrupt frontal–posterior cortical γ synchrony [8]. Gamma (40 Hz) synchrony is currently the best neural correlate of consciousness, and anaesthetics disrupt this synchrony by altering London dipole forces within hydrophobic regions of microtubule proteins, that allow microtubules to synchronise sensory inputs into a conscious experience.

How this occurs is becoming better understood. Anatomically, general anaesthetics have been shown to bind in, and affect various brain regions including the posterior cingulate cortex, orbitofrontal cortex, the right angular gyri, and the thalamus [9]. Different effects of anaesthesia may be anatomically differentiated. Loss of consciousness appears to be associated with the cerebral cortex [7; 10; 11], amnesia (loss of memory) with the limbic system [12-14], and immobility and analgesia (loss of movement and pain, respectively) with the spinal cord [15-18].

1.1 Mechanism of anaesthetic action

1.1.1 Site of action

Anaesthetics were found to bind with high affinity in non-polar, hydrophobic pockets in receptors for γ-aminobutyric acid type A (GABA-A), nicotinic acetylcholine, and glutamate in the brain, and glycine receptors in the spinal cord [2].

1.1.2 How anaesthetics inhibit consciousness

At the turn of the 20th century Meyer and Overton showed that potency of structurally dissimilar anaesthetic gas molecules correlated precisely over many orders of magnitude with one factor, solubility in a non-polar, 'hydrophobic' medium akin to olive oil [19; 20]. In the 1980s Franks and Lieb showed anaesthetics acted in such a medium within proteins, suggesting post-synaptic membrane receptors were the site of activity [21]. However network-level approaches fail to account for the Meyer Overton correlation, and direct anaesthetic effects on synaptic receptors are variable and inconsistent. Without synapses or networks, single cell organisms like slime mould and paramecium perform clever cognitive activities executed largely by cytoskeletal microtubules, which are inhibited by anaesthetic gases.

In the mid-19th century, the famous French physiologist Claude Bernard showed that anaesthetic gases reversibly halt purposeful cytoplasmic streaming inside slime mould, amoeboid single cell organisms. Bernard saw purposeful cytoplasmic activity as an essential feature of living systems, with anaesthesia acting in a common, unitary fashion to prevent it [22]. We now know that purposeful cytoplasmic activity in both slime mould and brain neurons depends on dynamics of cytoskeletal proteins comprising actin filaments and

microtubules [2]. Indeed slime mould movements allow the single cell creature to escape mazes, and 'solve equations' [23] by purposeful extension of cytoskeletal tendrils. Studies following Bernard later showed anaesthetic effects on cytoplasmic streaming were mediated directly in the cytoplasm, rather than via cell membrane effects [24; 25], Claude Bernard's anaesthetic action on purposeful streaming therefore occurs directly in the cytoplasm.

In the 1980's Franks and Lieb found that anaesthetics act directly within proteins, in lipid-like, non-polar 'hydrophobic pockets' [21], e.g. composed of aromatic amino acid π-electron resonance clouds.

Rod Eckenhoff's group surveyed all anaesthetic-binding proteins, measuring binding of radio-labelled halothane anaesthetic at clinically relevant concentrations in brain neurons from mice and human samples. Using chromatography they found halothane binding to 23 membrane proteins and 34 cytoplasmic proteins. Among the cytoplasmic proteins were cytoskeletal proteins including actin, and tubulin, the subunit component of microtubules [26].

1.1.3 Which proteins mediate anaesthesia?

Proteomic analysis of genetic expression following exposure to the anaesthetic halothane in animals suggests functional effects through protein networks involved in "neuronal growth, proliferation, division and communication", all microtubule-dependent functions [26].

Under normal conditions, it was shown [27-30] that London force dipoles in intra-protein hydrophobic regions coupled and oscillated coherently (gamma synchrony), and this coupling was suggested to be necessary for conscious awareness. Anaesthetic gases were suggested to bind in these non-polar, hydrophobic regions by their own London force coupling, dispersing endogenous dipoles necessary for consciousness [31]. Craddock *et al*. further showed that anaesthetic gas molecules bound in these same regions, and could act there to prevent consciousness, as shown in *Fig. 1*. This has been acknowledged as the 'quantum mobility theory' of anaesthetic action [32].

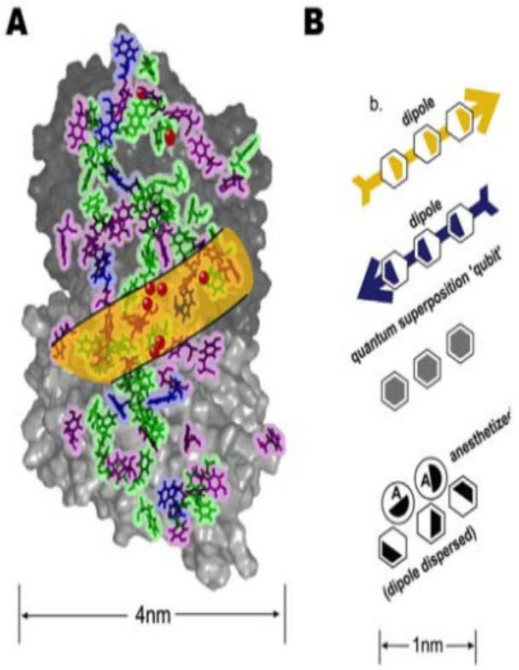


Figure 1. Tubulin, dipoles and anaesthetics. (A) Single tubulin dimer showing beta monomer (dark grey), alpha monomer (light grey), phenylalanine residues (magenta), tryptophan residues (blue), tyrosine residues (green), and persistent predicted volatile anaesthetic binding sites (red) with (in yellow) a hypothetical hydrophobic 'quantum' channel of aromatic rings. (B) Aromatic rings (e.g. phenyl and/or indole) within hydrophobic channel showing oscillating London force dipoles necessary for quantum mobility theory. Below, anaesthetics disperse dipoles, preventing consciousness [2].

Amnesia, or impairment of memory formation, is one pillar of anaesthetic action and generally ascribed to altering synaptic plasticity. However synaptic membrane proteins which mediate synaptic sensitivity are transient and re-cycled over hours to days, and yet memories can last lifetimes. Craddock *et al.* [33], and Janke & Kneussel [34] have suggested memory is encoded by post-translational modifications in microtubules in neuronal dendrites and cell bodies,

where microtubules are uniquely configured and stable. Anaesthetic effects on microtubules could thus account for amnesia, as well as loss of consciousness and prevention of purposeful behaviour, which is consistent with empirical observations.

The Meyer-Overton correlation of anaesthetic solubility in olive oil-like media to pharmacologic potency ensues from binding by van der Waals London ('dipole dispersion') forces [2], which are extremely weak quantum-level couplings. In London forces, non-polar (but polarizable) electron clouds between nearby atoms and molecules induce transient dipoles in each other, like tiny bar magnets, which then attract due to charge-separation by the resultant attractive Coulomb forces. Polar deviation from perfect non-polarity accounts for variable effects among different anaesthetic molecules [35; 36].

Soluble intravenous anaesthetics like propofol, ketamine, etomidate and barbiturates have non-polar groups attached to polar structures. As established by Meyer and Overton over a century ago, non-polar London force binding is the *sine qua non* of anaesthesia. Assuming tubulin (or some other protein) were the primary on-target binding site for anaesthetics, what is it that anaesthetics do there which prevents consciousness?

In functioning (non-anaesthetised) proteins, interactions among non-polar groups in hydrophobic pockets help determine protein conformation and function. The interactions occur by van der Waals (London dipole dispersion) forces, weak instantaneous dipole–dipole couplings between pairs of complementary electrons in molecular orbitals ('electron clouds') of adjacent non-polar amino acid side groups.

Anaesthetics alter the endogenous van der Waals London forces normally occurring in hydrophobic pockets which require electron mobility; electron pairs must be relatively free to roam among allowed orbitals. Evidence supports the idea that anaesthetics retard electron mobility—the movement of free electrons in a corona discharge is inhibited by anaesthetics (Hameroff and Watt, 1983). By forming their own van der Waals London forces in hydrophobic pockets, anaesthetics may retard electron mobility required for protein dynamics, quantum states and consciousness (*Fig. 1A*). We may then understand `non-anaesthetics' as occupying relevant pockets without altering electron mobility, and convulsants as occupying relevant pockets and forming cooperative van der Waals interactions which enhance electron mobility and protein dynamics *(Fig. 1B*). [37]

The normal function of anaesthetic-sensitive GABA-A and glycine receptors are inhibitory; anaesthetics therefore appear to (1) potentiate the activity of inhibitory proteins; and (2) inhibit the activity of excitatory receptors.

Both inhibitory and excitatory protein receptors act collectively. Supporting this notion, normal GABA-A and glycine inhibitory action depends on the integrity of cytoskeletal microtubules [38; 39].

In recent years, Eckenhoff and colleagues have found anaesthetic action in microtubules, cytoskeletal polymers of the protein tubulin that form inside brain neurons. ‘Quantum mobility’ in microtubules has been proposed to mediate consciousness. Through molecular modelling [2] have shown: (1) olive oil-like non-polar, hydrophobic quantum mobility pathways (‘quantum channels’) of tryptophan rings in tubulin, (2) binding of anaesthetic gas molecules in these channels; and (3) capabilities for π-electron resonant energy transfer. This energy transfer is due to exciton hopping among tryptophan aromatic rings in

quantum channels, a similar to photosynthesis protein quantum coherence. This mode of action describes how anaesthetic molecules can impair π-resonance energy transfer and exciton hopping in tubulin quantum channels, and thus account for selective action of anaesthetics on consciousness and memory.

The view of anaesthetic mechanism presented here may be summarized as a testable two part 'unitary quantum hypothesis': (1) Anaesthetics influence quantum states responsible for consciousness (i.e., coherent superposition of endogenous van der Waals London forces) in hydrophobic pockets of certain brain proteins. (2) Anaesthetics act by preventing stable, resonant quantum states in these hydrophobic pockets.

The Penrose–Hameroff model [40-42] shows that 'preconscious' quantum coherent superposition/quantum computing originates in neuronal microtubules and can be sustained on the order of 25 ms (i.e. coherent 40 Hz) until objective reductions ('conscious events') occur, as conscious moments occur when the quantum wave collapses and integrates all the sensory input into a moment of consciousness, accounting for the non-computable property of consciousness [37]. In summary, macroscopic quantum effects among neural proteins could account for enigmatic features of consciousness and anaesthetic action.

1.2 Ketamine

Ketamine is one anaesthetic agent commonly used in hospitals around the world, and has been shown to bind to NMDA receptors important for learning & memory, having an inhibitory effect.

Ketamine's widespread use is largely due to its ability to not suppress the respiratory system as strongly as other anaesthetics, making it useful in limited

oxygen situations, and is one of the only anaesthetics that produce analgesic, hypnotic & amnesic effects.

While ketamine is an effective anaesthetic and tool for understanding consciousness, it is reported to have side-effects of agitation/arousal, nausea, and is often associated with disorientation and hallucinations, occurring more commonly in women & children.

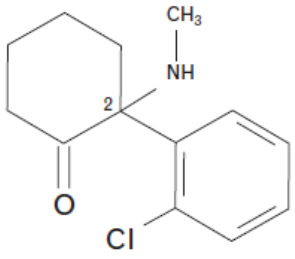
The history of ketamine begins in the 1950s at Parke-Davis and Company's laboratories in Detroit, Michigan, USA. At that time, Parke-Davis were searching among cyclohexylamines for an 'ideal' anaesthetic agent with analgesic properties.

Maddox, a chemist, discovered a process which led to the synthesis of phencyclidine or PCP [N-(1-phenyl-cyclohexyl)-piperidine] on 26 March 1956 [45; 46].

PCP caused increases in blood pressure, respiratory rate and minute volume, with conservation of corneal and laryngeal reflexes. These studies revealed genuine narcosis, with a cataleptic state, potent amnesia and analgesia produced by PCP anaesthesia [47]. EEG showed no signs of producing hypnotic or sleep-like states, and produced unacceptable reactions in many test subjects [48]. Chen [49] defined catalepsy as a 'characteristic akinetic state with a loss of orthostatic reflexes but without impairment of consciousness, in which the extremities appear to be paralysed by motor and sensory failure'[48].

Parke-Davis continued to develop related compounds, one of the agents, synthesised in 1962 by Calvin Stevens, produced excellent anaesthesia and was short-acting. It was selected for human trials as CI-581 [2-(O-chlorophenyl)-2-methyl-amino cyclohexanone] [45], and because it was a ketone together with an amine, was named ketamine (*Fig. 3*). Surprisingly, McCarthy *et al.* [50] described it in 1965 as being a compound with cataleptic, analgesic and anaesthetic action but without hypnotic properties.

Fig. 3



CI-581, Ketamine [2-(O-chloro-phenyl)-2-methyl-amino cyclohexanone].

Figure 2. Ketamine (CI-581) structure (Mion, 2017).

Ketamine was finally characterised as a dissociative anaesthetic, which was later described as the electrophysiological and functional dissociation between thalamocortical and limbic systems, characterized by complete analgesia combined with only superficial sleep [51].

After being patented by Parke-Davis for human and animal use in 1966, ketamine became available by prescription in 1969 in the form of ketamine hydrochloride, under the name of Ketalar. It was officially approved for human consumption by the United States Food and Drug Administration in 1970 and, because of its sympathomimetic properties and its wide margin of safety, was administered as a field anaesthetic to soldiers during the Vietnam war [48].

Ketamine provided potent analgesia but was less potent and of considerably shorter duration of action than PCP, particularly with regard to psychic problematic effects. The introduction of ketamine in Britain in late 1969 had been described as a ‘disaster’ from which the drug never recovered [52]. In 1970,

hallucinations were considered so unpleasant that patient acceptance was much lower than with the barbiturates [53].

In 1971, Sadove et al. [54] demonstrated that 'subdissociative' doses (0.44mg/kg) of ketamine possessed analgesic properties with moderate side effects.

1.2.1 Emergence Reactions

Ketamine [racemic (2-(2-chlorophenyl)-2-(methylamino) cyclohexanone] is a dissociative anaesthetic with profound analgesic and sympathomimetic properties. It has major advantages over opioids: minimal respiratory depression, preservation of protective airway reflexes, no hyperalgesic effects, a reduction in analgesic tolerance, and efficacy in opioid-resistant pain [55; 56]. The most important adverse effects of ketamine are its psychotomimetic properties, which occur at drug concentrations of just 100 ng/mL [57]. Because ketamine has an elimination half-life of 2 to 3 hours [56; 58]; patients may experience prolonged (20–120 minutes) psychic disturbances after anaesthesia. This limits the clinical utility of ketamine in adults [59].

"Emergence reactions" refer to a constellation of subjective effects encountered on emergence from ketamine general anaesthesia [60; 61]. Referred to as "psychomimetic", "hallucinogenic", or "psychedelic", they include intense alterations in mood, perception, thinking, body awareness and self-control. Some patients find these terrifying, whereas others do not [57].

Subjects have often described altered physical sensations or body image:

"Floating, very care-free feelings", "complete annihilation of idea of self" "The experience seems to be a mystical experience, an incomprehensible

comprehension of the universe. There seemed to be no past, present or future, just existence, life and death at the same time." [57]

Emergence delirium or excitement were common and these increased somewhat in severity with dosage but the pattern of changes was not clear cut. A small (2.5-5.0 mg) dose of diazepam intravenously rapidly controlled these symptoms, but larger doses re-induced anaesthesia and caused respiratory depression. Except with the smallest induction dose, ketamine was followed by more vomiting and/or nausea than methohexitone. The difference was particularly marked 1-6 hours after anaesthesia where the incidence with any dose of ketamine was significantly higher ($P<0.05$) than with methohexitone [53]. Patient acceptance was lower than any alternative drug, therefore there is still a need for analogues with much shorter half-lives to avoid the concomitant use of sedatives/ hypnotics [53].

Ketamine analogues with very rapid clearance might limit the psychomimetic period to only a few minutes [59].

1.2.2 Modified ketamine: Strategy to improve clinical use of ketamine

In developing a novel analogue to overcome these limitations, the limited structure–activity relationships of analogues of ketamine have shown that its anaesthetic effects are related closely to its physicochemical properties, with its (more polar) secondary 6-hydroxy metabolite having no anaesthetic properties [62]. This suggested to Harvey et al., [59] that an ester with similar lipophicity to ketamine might retain desirable anaesthetic properties, but would be rapidly hydrolysed by serum esterases to the corresponding very polar and thus non-anaesthetic ionised acid. SN 35210 (R1) was the first ketamine ester-analogue

designed for rapid offset via esterase-mediated hydrolysis. The compounds of interest were synthesized from norketamine *Fig. 4(1*), prepared from commercially available (2-chlorophenyl)(cyclopentyl) methanone. 13 Esters were then prepared by treatment of norketamine with the appropriate alkyl halides Br(CH2)nCO2(CH2)R, and converted to the hydrochloride salts with HCl gas *(Fig. 4).* Purity was determined by high-performance liquid chromatography (HPLC) monitoring at 272 nm and was ≥95% for all compounds [59].

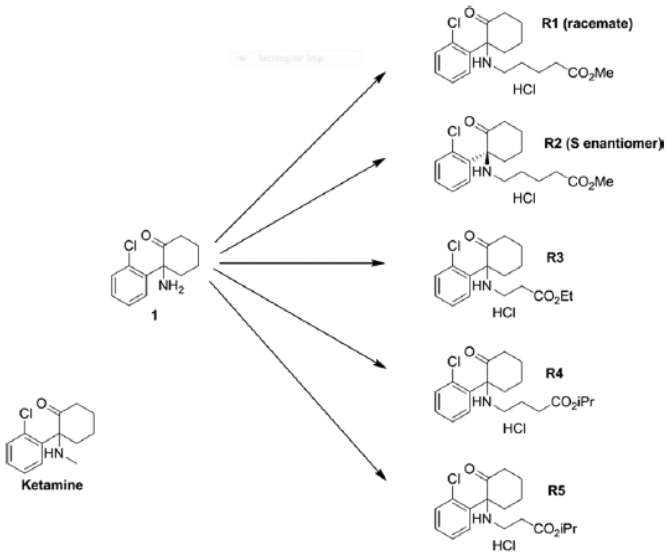


Figure 3. Synthesis of study drugs (R1–R5) from norketamine (1). Structure of ketamine provided for comparison. All analogues are racemic save R2, which is the S-enantiomer of R1. Me = methyl; Et = ethyl; iPr = isopropyl. (Harvey et al., 2015).

1.3 Associated brain regions

Ketamine anaesthesia produces changes in neuronal activity within a variety of networks, particularly those associated with nociception, stress and reward circuitry, and even addiction-related pathways. By understanding the role each of these brain regions play, brain activation may be compared after different treatment drugs to help select behavioural studies that can further investigate these findings.

1.3.1 Rostral agranular insular cortex: Nociception pathway

The connectivity of the rostral agranular insular cortex (RAIC) suggests it is involved in multiple aspects of pain behaviour (*Fig. 5*). Projections to the RAIC from medial thalamic nuclei are associated with motivational/affective components of pain. RAIC projections to mesolimbic/mesocortical ventral forebrain circuits are likely to participate in the sensorimotor integration of nociceptive processing, while its brainstem projections are most likely to contribute to descending pain inhibitory control [63].

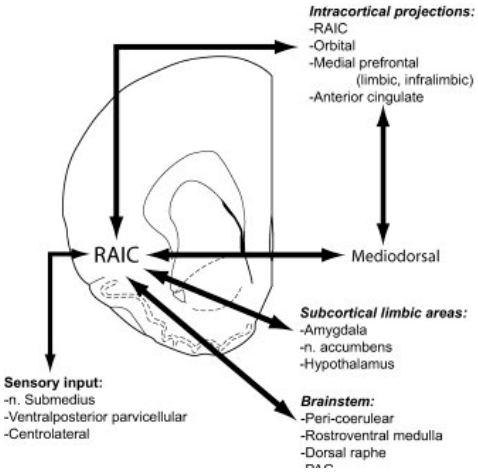


Figure 4. RAIC pain pathways (Jasmin et al., 2004).

1.3.2 Area postrema: Nausea

This region is associated with nausea, therefore decreased neuronal activity here may imply a reduction in symptoms of nausea (*Fig. 6*). One study on anaesthesia with nitrous oxide found that the euphoric properties of nitrous oxide may be a reflection of the neuronal activity of mesocortical dopaminergic projection, whereas the increased incidence of post-exposure emesis (nausea) may be a reflection of the neuronal activity of the medullary dopaminergic system [64].

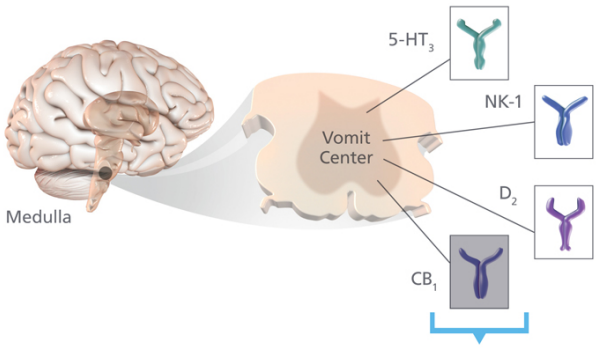


Figure 5. Medulla nausea pathway (Murakawa et al., 1994)

1.3.3 Nucleus of the solitary tract: Peripheral sensory relay center

The nucleus of the solitary tract (NTS) plays a pivotal role in neural cardiovascular regulation and integration [65]. Integrating nociceptive and cardiorespiratory afferents, the NTS plays a major role in mediating the reflex tachycardia evoked by somatic noxious stimulation [66].

The nucleus of the solitary tract (NTS) is a key gateway for meal-related signals entering the brain from the periphery. One study found that increased activity correlated with reduced appetite and body weight [67].

Satiety signals arising from the gastrointestinal (GI) tract and related digestive organs during food ingestion and digestion are conveyed by vagal sensory afferents to the hindbrain nucleus of the solitary tract (NTS) [68]. Chronic stress causes hypothalamo–pituitary–adrenal (HPA) axis hyperactivity and cardiovascular dyshomeostasis.

Noradrenergic (NA) neurons in the nucleus of the solitary tract are considered to play a role in these changes. Studies have suggested that corticotropin-releasing hormone (CRH) originating within the hypothalamic paraventricular nucleus (PVN) acts in the nucleus of the solitary tract (NTS) to regulate sympathetic and cardiovascular functions [69].

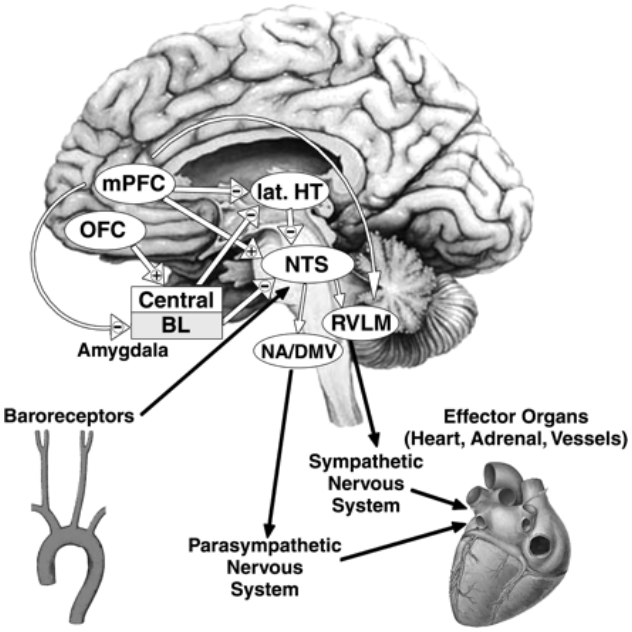


Figure 6. Characterization of cortical and subcortical influence on baroreflex function. Nucleus tractus solitarius (NTS), nucleus ambiguus (NA), and dorsal motor vagal nucleus (DMV) are important structures involved in efferent and afferent vagal activity. The lateral hypothalamus (lat HT) and the amygdala (basolateral nucleus, BL) generate inhibitory control over parasympathetic structures in the brain stem during stressful events and in anxiety. In contrast, the medial prefrontal cortex (mPFC) and the orbitofrontal cortex (OFC) facilitate vagal output and inhibit the rostral ventrolateral medulla (RVLM). –, mainly inhibitory influence; +, facilitatory influence (Bär et al., 2007).

1.3.4 Bed nuclei of the stria terminalis: Anxiety, addiction and stress response

Emerging evidence suggests the bed nucleus of the stria terminalis (BNST) mediates both anxiety and addiction through connections with other brain regions, including the amygdala and nucleus accumbens [70]. Rodent studies provide compelling evidence that the BNST plays a central role in sustained threat

monitoring, a form of adaptive anxiety, and in the withdrawal and relapse stages of addiction [71].

The BNST is ideally situated to instigate allostatic changes in the brain through its dense connections with the paraventricular nucleus (PVN) of the hypothalamus, the node of the hypothalamic–pituitary–adrenal (HPA) axis that initiates cortisol responses. Indeed, BNST lesions alter stress-related cortisol release [72], suggesting that the BNST may play an important role in disorders triggered by stress response, including anxiety and addiction (*Fig. 8*) [71].

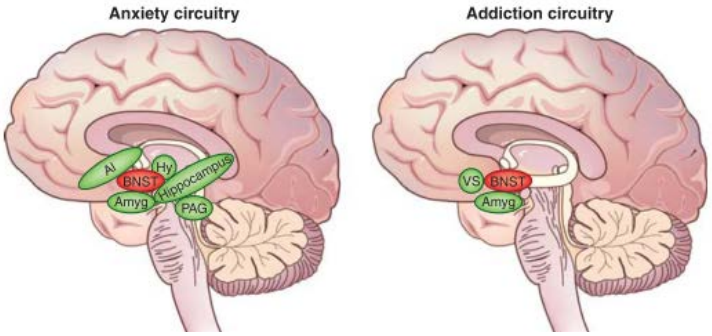


Figure 7. Human anxiety and addiction circuits. The BNST is a central node in both anxiety and addiction neurocircuitry. (left) The BNST is centrally located to influence human anxiety responses, with connections to multiple limbic and brainstem regions that mediate defensive response to threat, including the amygdala, anterior insula, hippocampus, hypothalamus, and periaqueductal gray. (right) The BNST is engaged during the negative emotional stage of withdrawal and interacts with the amygdala and ventral striatum, including the shell of the nucleus accumbens and ventral tegmental area, to mediate negative reinforcement.

AI, anterior insula; Amyg, amygdala; BNST, bed nucleus of the stria terminalis; Hy, hypothalamus; PAG, periaqueductal gray; VS, ventral striatum (S. Avery et al., 2016).

1.3.5 Amygdala: Emotion and memory

The amygdala is a heterogeneous structure that has been implicated in a wide variety of emotion-related functions, most notably in fear conditioning, and is made up of distinct regions including the basolateral amygdala (BLA) and central nucleus of the amygdala (CeA). The BLA mediates associations between predictive stimuli and the sensory properties of biologically significant events, and the CeA mediates the association of predictive stimuli with the affective or emotional properties of those events. Whereas the BLA mediates the emotional significance of specific rewards, the CeA seems to establish the general affective response that underlies the nonspecific reinforcing effect of those events [73].

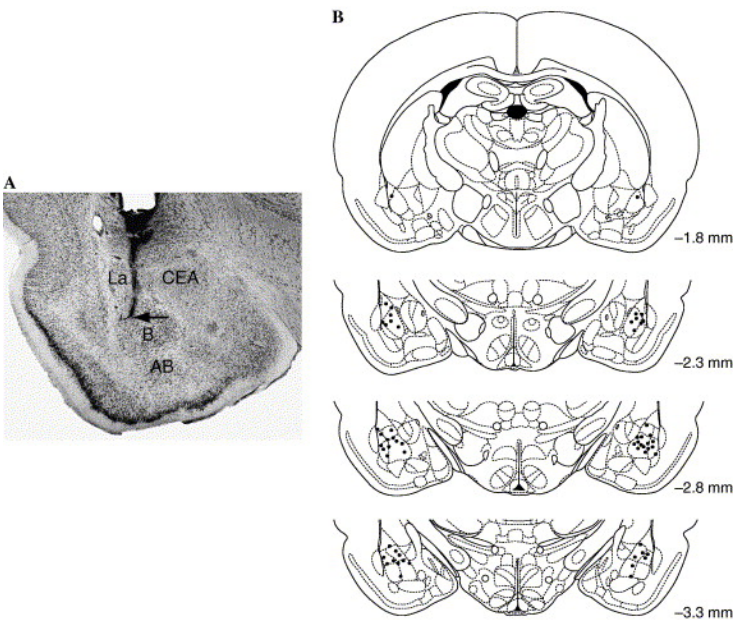


Figure 8. Cannula placement in the BLA. (A) Representative photomicrograph illustrating the placement of a cannula and needle tip in the BLA. Arrow points to the needle tip. B, basal; AB, accessory basal; CEA, central amygdala; La, lateral amygdala. (B) Infusion needle tips in the BLA of 30 randomly selected rats included in the experiment. (Roozendaal et al., 2006).

1.3.5.1 Basolateral amygdala: Fear response

The basolateral amygdala has been implicated in unconscious processing of threat-response [74] and acquisition of conditioning/reward-seeking behaviour [75]. Extensive work using pavlovian fear conditioning procedures revealed a role for the basolateral amygdala (BLA) in both the acquisition and expression of

conditional fear [76]. Lesions of the BLA have been shown to inhibit the acquisition of fear response and conditional learning, implicating the integrity of this region as important for conditional learning. The suppression of fear behaviour by intra-BLA muscimol (inhibition) was associated with increased c-Fos expression in the central nucleus of the amygdala (CeA) [77].

1.3.5.2 Central nucleus of the amygdala: Stimulating emotional response

The CeA has been found to play a role in mediating fear- and anxiety-related behavioural and endocrine responses. Ablation studies show it is responsible for freezing behaviour upon perceiving a threat, and production of corticotrophin-releasing factor (CRF), suggesting the CeA is involved in mediating fear- and anxiety-related behavioural and pituitary-adrenal responses, as well as in modulating brain CRF activity [78]. Similarly, the suppression of fear behaviour by intra-BLA muscimol (inhibition) was associated with increased c-Fos expression in the central nucleus of the amygdala (CeA) [77].

1.3.5.3 Caudate Putamen: Memory-retrieval, habit-formation and learned response

Modulated by the amygdala, this region is important for memory and learning. In studies, neuronal activity of the dorsolateral CPU attenuates cocaine-seeking behaviour after abstinence and inhibits explicit conditioned-stimuli- and context-induced reinstatement after extinction training, implicating the CPu in habit learning [79]. The dorsolateral caudate putamen (dlCPu), a mediator of stimulus–response associations [80], exerts more fundamental influence over cocaine seeking after abstinence than other brain regions. Dopaminergic neuron activity in the dlCPu has been associated with presentation of reward-paired stimuli [81]. Stimulation studies show that the caudate putamen is required for the expression

and consolidation of memory, while inhibition results in attenuated memory consolidation and retrieval [82], and has been implicated in hallucinations, mediating prefrontal behaviours and in the conceptual integration of memories [83].

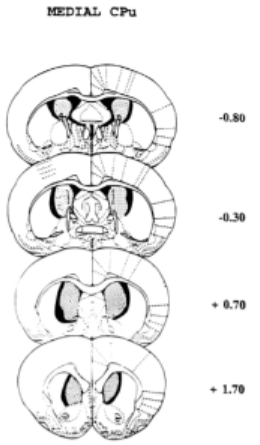


Figure 9. A schematic drawing of the largest and smallest lesions of the medial caudate nucleus (medial CPu). The lesioned areas are represented by the shaded areas. (Packard & Teather, 1998)

1.3.6 Paraventricular nucleus of the thalamus: Arousal

The paraventricular nucleus of the thalamus (PVT) is part of a group of midline, and intralaminar thalamic nuclei which are hypothesized to play a role in arousal [84-86]. While the function of the PVT remains poorly understood, experiments looking at the expression of Fos protein as a measure of neuronal activity have consistently shown that neurons in the PVT are activated during periods of arousal [86]. The PVT has also been implicated in the regulation of food intake and hypothalamic–pituitary–adrenal activity during chronic stress [87]. Based on the evidence that the PVT is involved in arousal and receives a substantial orexin innervation, it is reasonable to suggest that the PVT may play a part in the arousal functions of orexins.

In turn, the PVT could integrate information from a number of arousal centers and relay this information to the nucleus accumbens, amygdala, and prefrontal cortex to place these forebrain structures in a state of arousal or readiness necessary for behavioural responding. For example, the nucleus accumbens is connected with

behavioural motor control systems and has been implicated in defensive behaviours and feeding [88].

Thus, with afferents from brainstem and hypothalamic cell groups and efferents to limbic centers, the PVT is in a position to integrate visceral and circadian information, and modulate limbic functions. In addition to HPA activity, chronic stress also modifies activity in systems that regulate core temperature and energy balance [87].

1.3.7 Hypothalamus

1.3.7.1 Paraventricular nucleus of the hypothalamus: Stimulating stress response

The paraventricular nucleus of the hypothalamus is an integration centre between the central and peripheral autonomic nervous systems. In line with this role, it is characterized by a very complex architecture and is involved in numerous functions, from stress to the

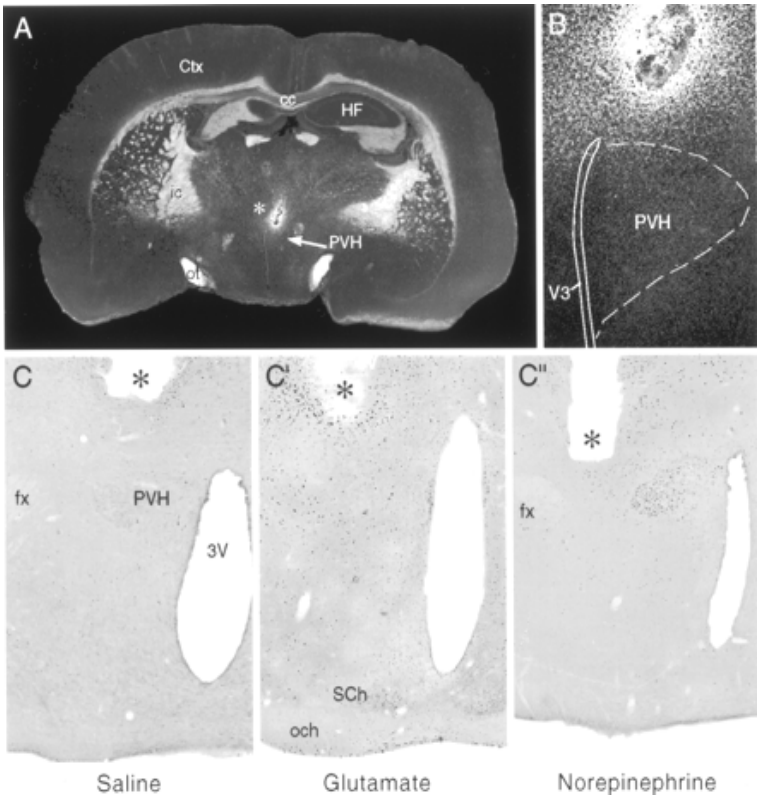


Figure 10. The extent and placement of microinjections into the PVH. A, Dark-field photomicrograph showing the location and extent of a microinjection into the region just dorsal to the PVH (arrow). B, Enlarged view of injection site showing that the gradient of diffused injectate spreads to involve the PVH. C, Bright-field photomicrographs of immunoperoxidase preparations (for Fos-IR) to show representative microinjection cannula placements in rats injected with saline (C), glutamate (C') or norepinephrine (C"). Magnifications: A, 8.5×; B, 70×; C, 25×. cc, Corpus callosum; Ctx, cerebral cortex; fx, fornix; HF, hippocampal formation; ic, internal capsule; och, optic chiasm; ot, optic tract; SCh, suprachiasmatic nucleus (Cole & Sawchenko, 2002).

control of feeding, body energy balance, blood pressure, heart rate and sexual activity [89].

Physical stress and cytokines have been shown to increase the secretion of corticotropin-releasing factor (CRF) by the paraventricular nucleus of the hypothalamus (PVN) [90].

The paraventricular hypothalamic nucleus is a key site for marshalling integrated and adaptive responses to stress. The PVN houses (1) parvocellular neurosecretory neurons expressing corticotropin-releasing factor (CRF) for the initiation of the endocrine (pituitary–adrenal) arm of the generalized stress response, (2) neurons projecting to central autonomic cell groups, including sympathetic preganglionics that constitute the neural arm of the stress response, and (3) magnocellular neurosecretory cells that release oxytocin and arginine vasopressin (AVP) from the posterior pituitary in response to more specific physiological [91].

1.3.7.2 Dorsomedial hypothalamus: Cardiovascular response to emotional fear

Shown to play a key role in the cardiovascular changes associated with emotional or exteroceptive stress, the DMH may represent a higher order hypothalamic center responsible for integrating autonomic, endocrine and even behavioural responses to emotional stress [92].

Muscimol is an agent found to be inhibitory to virtually all mammalian neurons by virtue of its GABA-a receptor agonist properties. Microinjection of muscimol has come to be a standard technique to achieve acute reversible suppression of neuronal activity in a discrete region of the brain. Treatment in the DMH with muscimol was found to dramatically reduce the number of Fos positive neurons in both subregions of the PVN [92]. Inhibition of DMH was found to result in inhibition of PVN (stress response). Stress response behaviour observed by

stimulation of the DMH included increase of respiratory rate, heart rate and arterial pressure [92].

1.3.7.3 Ventromedial hypothalamus: Regulation of circulatory system and food intake

Neural activity in the VMH has been found to be associated with suppression of the circulatory system in rats. Blood pressure and heart-rate were found to correlate negatively with activity of the VMH during anaesthesia [93].

Stimulation studies show that electrical stimulation of the ventromedial hypothalamus (VMH) causes a reduction in food intake, whereas electrolytic or chemical lesions in this area result in hyperphagia and obesity in the rat [94]. The ventromedial hypothalamic nucleus (VMH) is also necessary for the integrated hormonal response to hypoglycemia, as the VMH is sensitive to hypoglycemia for full neuronal activity of catecholamine and glucagon secretion and is a key glucose sensor for hypoglycemic counter-regulation [95].

1.3.8 Nucleus accumbens: Learning, fear and motivation

Several lines of evidence suggest that the nucleus accumbens (NAcc) may play an important role in relating reward processes to the action–outcome associations that underlie instrumental learning [96; 97] [98]. The nucleus accumbens, an area primarily involved in motivation also forms an integral part of the fear-processing circuit, as it receives projections from both the anterior cingulate and the basolateral amygdala (BLA). Pietersen et al. found sustained anxiety responses that persist beyond the immediate threat are mediated, at least in part, by structures such as the nucleus accumbens [99].

The NAcc itself is a heterogeneous structure and can be further divided into anatomically distinct core and shell subregions [100].

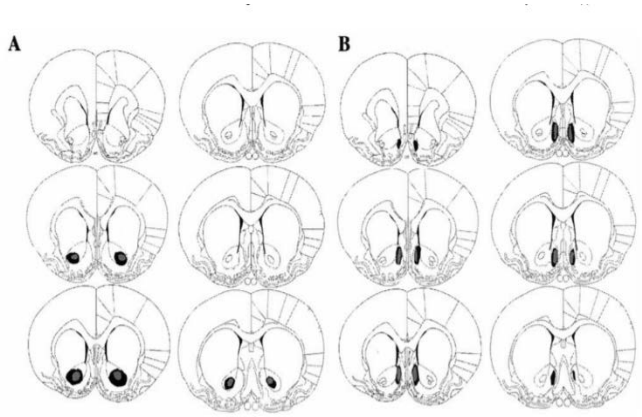


Figure 11. Schematic representation of excitotoxic lesions of the NAC core (A) and shell (B) (Corbit et al., 2001).

1.3.8.1 Accumbens core: Learning, fear and directing action toward reward

The core region plays a key role in selecting or initiating actions based on the reward value of their consequences. Core-lesioned animals show reduced rates of acquisition learning and response. The core appears to be involved in mediating the impact of evaluative processes via which animals encode the incentive value of the instrumental outcome on the performance of goal-directed actions [98]. Ketamine anaesthesia was shown to lead to suppression of c-Fos expression associated with drug reward, eliminating perception of reinforcing effects [101].

1.3.8.2 Accumbens shell: Anticipation of reward/fear and motivation

The second network involving the accumbens shell, appears to be involved in mediating the excitatory effects of stimuli that anticipate reward on goal-directed performance [98]. Lesions of the shell have been found to attenuate lever pressing for stimuli paired previously with reward [102]. The shell of the accumbens is a central structure through which feedback from cues associated with reward help to activate and guide actions that are instrumental to gaining access to basic commodities.

The shell region may mediate the impact of stimulus–reward associations on instrumental performance, and be responsive to signals for reward [98].

1.3.8.3 Ventral tegmental area: Reward/fear expression and startle response

The ventral tegmental area is a group of dopaminergic and glutaminergic neurons that work with the accumbens, stimulated during reinforcement or fear learning and reward-seeking. Studies show evidence of reinforcing effects produced by microinjection of morphine into the VTA [103], suggesting the reinforcing property of opiates may be mediated by neural pathways originating in the VTA [104].

Nicotinic acetylcholine receptors (nAChRs), especially those located in the ventral tegmental area, are important in mediating the stimulatory, dopamine enhancing and reinforcing effects of ethanol [105].

Lesions also produced suppressed fear expression during conditioned startle response experiments, suggesting the role of the VTA in regulating levels of aversive emotional arousal [106].

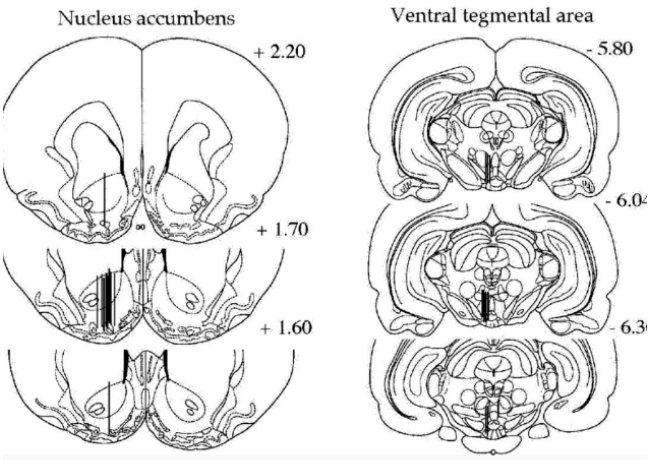


Figure 12. Coronal rat brain sections showing the probe placements (illustrated by vertical lines) in the nucleus accumbens and the ventral tegmental area of rats. The numbers in each brain section indicate millimetres from bregma (Larsson et al., 2005).

2. Aim

As ketamine promotes effective analgesia but also induces undesirable effects, there is an ongoing search for novel analogues whose beneficial effects are potentiated. To examine the functional properties of a novel ester-analogue of ketamine, R5, that has a potentially beneficial profile as indicated by preliminary studies, brain activation and select behavioural parameters in rat animal models are investigated.

2.1 Specific aims:

1. Investigate brain activation patterns following administration of ketamine and ester-analogues, by employing immunohistochemical detection of an immediate-early gene product, c-Fos.
2. c-Fos immunoreactivity showed significant changes in neuronal activity of brain regions associated with nociception and addiction, including the AIC and PVN. R5 produced a somewhat similar pattern of activity as ketamine, whereas more profound differences in c-Fos were detected after R1.

Therefore, in the subsequent set of experiments, effects of R5 on pain- and addiction-related behavioural parameters were investigated.

3. Methodology

3.1 Identify which areas of the animal brain show significant differences in immunoreactivity patterns between treatment groups

c-Fos staining has been used as a reliable method of quantifying neuronal activity [107]. c-Fos is a proto-oncogene that is expressed within dopaminergic neurons following depolarization. The protein product, c-Fos protein, can be identified by immunohistochemical techniques. Therefore, c-Fos expression can be used as a marker for neuronal activity throughout the neuraxis following peripheral stimulation. A thorough review found this procedure as an effective method of quantifying recent neuronal activity in dopaminergic neurons [108]. Therefore, by analysing the brain tissue of rats infused intravenously with different anaesthetics, the pathways underlying the effects of those anaesthetics may be better understood. Also, the neuroendocrine bases of adverse side-effects can be better understood, and anaesthetic chemicals can be refined to avoid post-anaesthesia adverse reactions.

In this study, we intravenously infused ketamine as well as two new anaesthetics, dubbed R1 and R5, into the tail vein of rats over the course of 45 minutes and subsequently analysed the brain tissue of these rats for the presence of the immediate early gene product, c-Fos.

All experiments were undertaken at the University of Waikato Animal Facility (E2.26). Ethical approval was obtained from the University of Waikato Animals Ethics Committee for all experiments:

965: Effects of novel intravenous anaesthetics on rat brains, 2015.

#1020: Exercise addiction withdrawal scoring, 2017.

3.1.1 Animal Preparation

Twenty-two adult female Sprague Dawley rats of 4 months old and weighing 267.8 +/- 28.8g were studied. Rats were single-housed in standard rat cages with fresh wood-shaving bedding and ad libitum access to standard chow (Diet 86, Sharpes Stockfeed Ltd) and water unless stated otherwise.

On the experimental day, rats were atraumatically restrained in perspex tube with tail port. Venous cannulation of the marginal tail vein was undertaken and secured before animals were returned to their home cage. Rats were randomly assigned to one of four treatment groups: 1) control (saline) 2) ketamine, 3) R1 (SN 35210), and 4) R5 (SN 35563).

Ketamine (Hospira Australia Pty Ltd, Australia) was administered at 10mg/ml diluted in 0.9% saline solution. R1 and R5 were synthesised by the Auckland Cancer Society Research Centre, Auckland, New Zealand, and solubilized in 0.9% saline at 10mg/ml prior to administration.

3.1.2 Drug administration

Mini-bore intravenous tubing was attached to the venous cannula of the tail vein for drug administration. For the purposes of this study loss of righting reflex (the ability of the animal to spontaneously right from a position of dorsal recumbancy to a position of sternal recumbancy - LORR) served as the key measure of sedation.

Control animals received an intravenous infusion of 0.9% saline solution at 0.4 ml/kg/min for 45 minutes.

Ketamine group animals received intravenous ketamine (10mg/ml) infusions initially at 20mg/kg/min until LORR, and thereafter at 2mg/kg/min to 45 minutes.

R1 group animals received intravenous R1 (10mg/ml) initially at 20mg/kg/min until LORR, and thereafter at 4mg/kg/min to 45 minutes.

R5 group animals received intravenous R5 (10mg/ml) infusions initially at 20mg/kg/min until LORR, and thereafter at 4mg/kg/min to 45 minutes.

Infusions of ketamine, R1, and R5 were titrated in an up-and-down fashion to maintain a consistent level of sedation (defined by maintained LORR) for the duration of drug infusions.

Infusion rates in ketamine treated animals trended downwards from the initial 20mg/kg/min due to developing respiratory depression. Conversely infusion rates in the R1 and R5 treated groups were increased from the initial 4mg/kg/min due to lightening of sedative effect.

Following completion of drug infusions all animals were maintained in their home cages for a period of 15 to 30 minutes. Time to return of righting reflex (the time from cessation of drug infusion to first spontaneous righting) was recorded. At 60 to 75 minutes following commencement of study drug injection animals underwent perfusion and brain dissection.

3.1.3 Perfusion/brain dissection

An hour after drug administration, animals were anesthetised with urethane (35% dissolved in 0.9% saline, 3mL, intraperitoneally (i.p.) administered), and perfused rapidly through the aorta with 50 ml of saline followed by 500 ml of 4% paraformaldehyde in 0.1 phosphate buffer (pH 7.4) . Brains were removed and post-fixed overnight in the same fixative at 4°C. 60µm thick coronal vibratome sections of the brain were processed as free-floating sections for standard single antigen immunostaining of c-Fos. Sections were rinsed in 50 nM TBS (pH 7.4–7.6), and then pre-treated for 10 min in 3% H_2O_2, 10% methanol (diluted in TBS). After rinsing in TBS they were further incubated overnight at 4°C in the primary rabbit-anti-Fos antibody (diluted 1:40 000; Synaptic Systems, Australia) washed in TBS, and subsequently incubated for 1h at room temperature in the secondary

goat-anti-rabbit antibody (1:400; Vector Laboratories). Following four washes in TBS, sections were incubated for 1h with avidine–biotin peroxidase complex (1:800; Elite Kit, Vector Laboratories). The vehicle for all incubations was a solution of 0.25% gelatin and 0.5% Triton X-100 in TBS. The peroxidase in the tissue was visualized with 0.05% diaminobenzidine (DAB), 0.01% hydrogen peroxide and 0.3% nickel sulfate (12-min incubation). Sections were washed in TBS to stop the reaction, mounted onto gelatin-coated slides, air-dried, dehydrated in ascending concentrations of ethanol, soaked in xylene (Merck KGaA, Germany) and embedded in Entellan (Merck KGaA, Germany). The number of Fos-positive nuclei was counted bilaterally for each neuroanatomical region of interest using ImageJ Software and manual counting, with boundaries defined according to the atlas [110], on 2–4 sections per animal. Images provided by a CCD camera attached to a Nikon Eclipse 400 microscope were analysed using Nikon NIS Elements image software. Densities of Fos-immunoreactive nuclear profiles (per 1 mm) were averaged per animal, and then per experimental group. Statistical analysis of data was performed using ANOVA followed by Bonferroni's post-hoc test and Fisher analysis, and values were considered significantly different when $p<0.05$ [111].

Based on the differences in immunoreactivity patterns, we were then able to conduct select behavioural studies during direct administration to regions showing significant changes to further examine these effects. This included testing of analgesic effects and co-administering the drug with an ion-channel blocker to investigate the mechanism of action for effective analgesia.

3.2 Investigate the behavioural effects of ketamine-analogues directly administered in pain and addiction pathways.

In the investigation into the action of drugs on specific brain regions, the method of direct injection into brain sites of interest is a widely used approach. This allows site-specific direct administration of a chemical to the region hypothesised to mediate a behaviour, allowing confirmation and measurement of the underlying role of a region. Using stereotaxic equipment, this can be performed quickly and humanely under anaesthetic surgery, and allows repeated administration for months after [109].

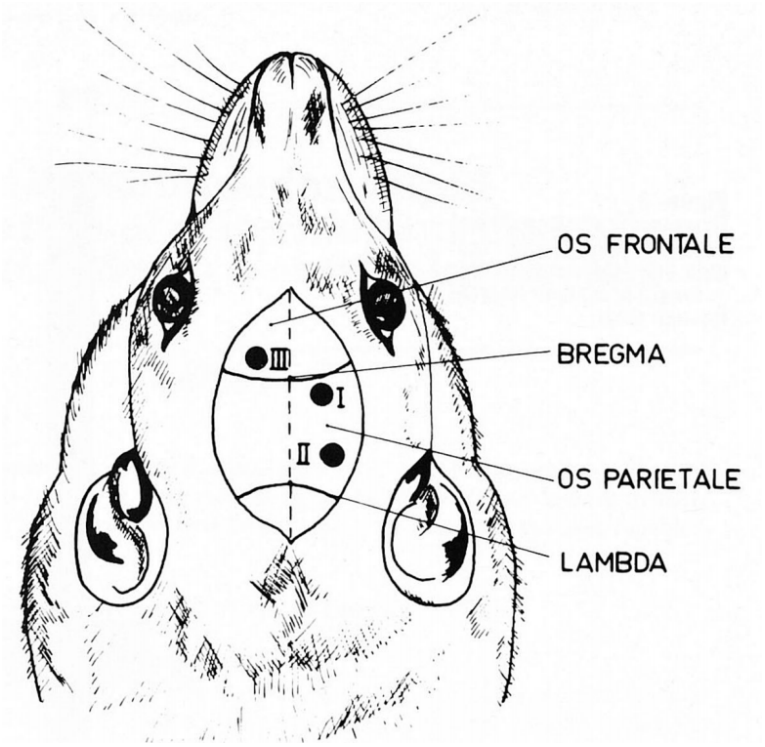


Figure 13. Schematic representation of cannula implantation (I) and anchoring screws (II, III), on rat skull (Gispen et al., 1979).

Approximately 12-week old, female Sprague Dawley rats received site-specific intracranial injections aimed at one of 3 sites of interest using coordinates from The Rat Brain Atlas [110]:

Table 1. . Coordinates for cannulation of the different brain sites used in this study. Coordinates measured in relation to bregma. AP: anterior-posterior; L: lateral; DV: dorsal-ventral

Area:	AP:	L:	DV:
PVN	-2.5	0.0	-8.1
AIC	3.0	4.0	-5.5
ICV	-1	1.5	-3.5

3.2 A. Description of surgical procedure

1. Anaesthesia:

Ketamine (90 mg/kg) and xylazine (10 mg/kg) were administered i.p., with supplements of approximately 1/3 of this dose if depth of anaesthesia was insufficient. Prior to the initiation of the surgery, anaesthetic depth was confirmed by absence of toe-pinch and tail-pinch reflex. This procedure was repeated approximately every 10 – 15 min throughout the surgery. In case of an observed toe-pinch reflex prior to the beginning of surgery, an additional 5 – 10 min waiting time was given to allow the anaesthetic to produce its full effect. A supplemental anaesthetic injection was given if the reflex was still present 15 min after the initial injection. If a visible response was observed once the procedure began, the procedure was stopped, and a supplemental injection of ketamine/xylazine was given (see above). The procedure was resumed when the toe-pinch reflex was no longer observable.

2. Intracranial cannula implantation:

Under ketamine/xylazine anaesthesia, rats were mounted into a stereotaxic instrument. The scalp was shaved, scrubbed with betadine and alcohol, and dried. Using aseptic technique, a small midline incision was made to expose the skull surface. The surface was cleaned of blood and allowed to dry (to make surface landmarks more visible). Skull landmarks (bregma and lambda) were noted and coordinates for cannula placement sites measured (based on the rat brain atlas of [110]).

Three to 4 small holes were drilled in the skull and the surface was again cleaned of blood, after which a 22 gauge stainless steel unilateral or bilateral guide cannula (Plastics One, Inc.) was lowered into the appropriate hole to the pre-determined

depth; stainless steel self-tapping screws were placed into the other holes to act as anchors. Acrylic dental cement was applied to the screws and cannulas to bind all together after drying. Sutures were placed at each end of the incision, and the animal was removed from the instrument. The cannula placement surgery itself was generally completed within 20-25 minutes.

3. Aseptic technique:

Instruments were sterilized via autoclave at least one day before a surgery day. Each pack was marked with autoclave indicator tape. Because multiple rats typically undergo surgery on any given surgery day, the tips of the instruments were re-sterilized between rats with the following sequence: immersion of the tips into hydrogen peroxide, followed by immersion in 70% isopropyl alcohol. This typically removes any blood or other debris on the tips and sterilizes them. The scalp was shaved, scrubbed with betadine and alcohol, and dried. Using aseptic technique, a small midline incision was made to expose the skull surface, and the cannula was implanted as described above. Lab coats, sterile gloves and a mask were used during surgery.

4. Surgery recovery and post-op care

After surgery, bacitracin ointment was applied to the external wound; carprofen was given (5mg/kg) as a post-operative analgesic. Carprofen was also administered on the day following surgery (same dose). The animal was kept warm and observed frequently until arousal and self-righting movement was regained (usually approximately 60 minutes after removal from stereotaxic frame). The animal was returned to its cage and maintained on ad lib food and water for 7 - 10 days before any further manipulations were done (except handling). Rats

were weighed and inspected daily for at least 3 days for any signs of illness, including weight loss, improper wound healing, sluggishness and poor grooming. Rats were allowed to recover for one week while housed in standard cages in the animal facility prior to testing.

3.2 B. Manipulations

The first behavioural study tested the analgesic effect of R5 administered directly to the nociception area of the brain, compared to saline.

1. Anterior insula cortex: Tail flick evaluation

Animals:

Twelve bilaterally cannulated adult female Sprague Dawley rats (age 147 +/- 9days; weight 239 +/-31g) were studied.

Intracranial injections:

Bilateral cannulation with two 26-gauge cannula into the anterior insular cortex with coordinates as described in Table 1.

Drug administration:

For intracranial injections, solutions were loaded into 30-cm lengths of PE-50 tubing attached to 10-µl Hamilton syringes. Drug/saline injection was delivered by 1.0 µl bolus over 30 seconds. Control animals (n=6) received 0.9% saline solution at 1µl, and test animals (n=6), R5 (SN 35563) concentration 10mg/ml at 1 µL.

Tail Flick evaluation:

A tail flick analgesia meter (Colombus Instruments, Colombus, Ohio) was used to determine pain sensitivity in the control and drug treated animals. Radiant heat was applied using a shutter-controlled lamp as a heat source focused on a spot located 6-8cm from the tip of the tail. The intensity of the beam was set at a level

producing basal latency times between 3 and 4.5 seconds. To prevent thermal tissue injury the cut off time as set at 10 seconds. A digital response time indicator with a resolution of 0.1s measured the time from initiation of stimulus until tail withdrawal (tail flick). This measurement was repeated with all animals every 5 minutes for 60 minutes in total.

The tail-flick latency (TFL) response following infusion of control and study drugs was calculated as a percentage of the maximum possible effect (MPE) such that:

$$\%MPE = [TFL\ (post\text{-}drug) - TFL\ (pre\text{-}drug) / 10\ sec - TFL\ (pre\text{-}drug)] \times 100\%$$

3. Intracerebroventricle: Tail flick evaluation

In an attempt to further investigate the mechanism of action by which R5 produces analgesic effects, a second behavioural study administered R5 directly to the HPA-axis by ICV injection, preceded by barium chloride administration. This aimed to test the hypothesis that barium blocks two-pore potassium (K2P) ion channels in neuronal membranes required for analgesic effects of anaesthetics.

Animals:

Nine unilaterally cannulated adult female Sprague Dawley rats (age 147 +/- 9days; weight 239 +/-31g) were studied.

Intracranial injections:

Implanted unilaterally (right) with one 26-gauge cannula into the anterior horn of the lateral ventricle with coordinates as described in Table 1.

Cannulas were held in place with dental cement, and patency maintained with occlusive stylets.

Drug administration:

For intraventricular injections, solutions were loaded into 30-cm lengths of PE-50 tubing attached to a 10-µL Hamilton syringe prefilled with test agents. All injections were delivered by 1.0 µL bolus over 30 seconds. Stock solutions of NaCl 0.9% (control), BaCl2 10mg/mL (test), and R5 (SN 35563) 10mg/mL were prepared.

Tail Flick evaluation:

A tail flick analgesia response was measured as above.

Test algorithm:

Following assessment of baseline tail flick latency (time 0) rats underwent interventricular injection of 1µL saline (control [n=4]) or 1 µL barium chloride

(test [n=5]) at time equals 2 minutes. TFL was reassessed at 3 minutes. Further 1µL injections of saline or barium solutions were undertaken at 5 minutes, and followed immediately by interventricular injection of R5 (SN 35563) at 1µL. TFL evaluation was undertaken at 1 minute intervals from time 6 minutes to 10 minutes, then at 5 minute intervals thereafter to 60 minutes.

Statistical analysis:

Statistical analysis of all variables was undertaken using RMANOVA.

3. Paraventricular nucleus of the hypothalamus: Exercise withdrawal

Finally, as the nociception system is also involved in addiction and withdrawal behaviour, the effect of R5 in reducing addictive-like, exercise-induced withdrawal symptoms was investigated. This was achieved by direct administration of R5 to the PVN after addictive-like, exercise-induced withdrawal, before behavioural effects were scored and compared.

Animals:

After recovery from cannulation, 16 rats were housed in running wheel chambers for one week to habituate, then assigned to one of two groups for one week:

- Food restricted to 1hr per day (n=8)
- Free (ad libitum) access to food (n=8)

During this period, food intake and weight was monitored daily. After this period, rats were administered 1.0 mg/kg naltrexone via i.p. (opioid blocker) to induce opioid withdrawal effects, in the morning at the time that they are normally given food access, followed by the site-specific administration of an anaesthetic agent via cannula.

The following anaesthetics and doses were used:

1. R5 (SN35563): 10µg/µL, 1µL

2. Saline (control): 0.9% solution, 1µL

Intracranial injections:

The stylet (or dummy cannula) was removed and replaced by an injection cannula with a 1 mm protrusion beyond the tip of the guide cannula. The injection cannula was connected via PE tubing to of 10-µl Hamilton syringes prefilled with either R5 or saline, and a volume of 0.5 microliters/side was injected over a period of approximately 60 seconds. The injector was left in place for another 30 seconds to allow the injected substance to diffuse into the tissue. To minimize stress, the animal was placed in a small cage during this 2 minute period. The injector was then removed, the stylet replaced, and the rat was then placed in a transparent observation chamber.

Behavioural Scoring:

Withdrawal symptoms were then recorded by behavioural scoring over a one hour period, before being returned to running wheel cages in the animal facility. Withdrawal behaviours observed were scored in accordance with [112] in 2 ways:

1) body weight loss in 1 hr (1 for every 1% of weight loss), wet dog shakes (1-2 shakes = 2; 3 or 4 shakes = 3; 4 or more shakes = 4), escape attempts (2-4 attempts = 1, 5 to 9 attempts = 2, and 10 or more attempts = 3)

2) Abnormal posture/writhing, teeth chattering, ptosis (drooping eyelids), diarrhoea, profuse salivation, swallowing movements, abnormal postures, and chromodacyorrhea (red tears) were scored for their presence

The total withdrawal scores were calculated as the sum of all of the individual withdrawal scores.

Following each injection, the animals received 2 days of ad libitum access to food, followed by 2 days of restricted access to food for those animals that were in the food restricted group, which means that animals received one naltrexone and one site-specific treatment injection every 4 days. In total each animal received 2 treatments and naltrexone administrations over 6 weeks.

4. Results

4.1 Identify which areas of the animal brain show significant differences in immunoreactivity patterns between treatment groups.

All animals receiving sedative drug infusion displayed rapid onset sedation characterised by loss of righting reflex. Return of righting reflex time was significantly greater in the ketamine group when compared with the R1 (SN 35210) group (p=0.0079), and the R5 (SN35563) treated group (p=0.0079). One animal from the ketamine and R1 (SN 35210) group died on induction of anaesthesia following inadvertent drug overdose.

Administered drug doses in conjunction with behavioural metrics are presented in Table 2.

Table 2. Anaesthetic drug dosing and behavioural metrics.

	Control (n=5)	**Ketamine (n=5)**	**SN 35210 (n=5)**	**SN 35563 (n=5)**
Time to LORR (sec)	NA	63.0 (56-72.5)	66.0 (49.5 – 69.5)	70.0 (55.5 – 87.5)
Dose to LORR (mg/kg)	NA	21.0 (18.7 – 24.2)	22.0 (19.8 – 23.2)	23.3 (18.5 – 29.2)
Total dose (mg/kg)	NA	95.0 (92.5 – 103)	221 (201 – 237)	203 (198 – 209)
Time to RORR (sec)	NA	613 (593 – 776)	45.0 (29.5 – 56.0)	70.0 (55.5 – 87.5)

All data median (IQR)
LORR: loss of righting reflex
RORR: return of righting reflex

c-Fos Analysis

c-Fos immunoreactivity showed differences in patterns of neuronal activity between ketamine and novel analogues. Novel ketamine-analogues produced

somewhat different patterns of activity than ketamine, with R5 producing more profound differences than R1.

4.1.1 Anterior insula cortex:

In the AIC, c-Fos immunoreactivity following both R1 and R5 infusion was significantly inhibited compared to saline but not ketamine treatment, while ketamine infusion did not show a significant effect on c-Fos immunoreactivity (Figure 14).

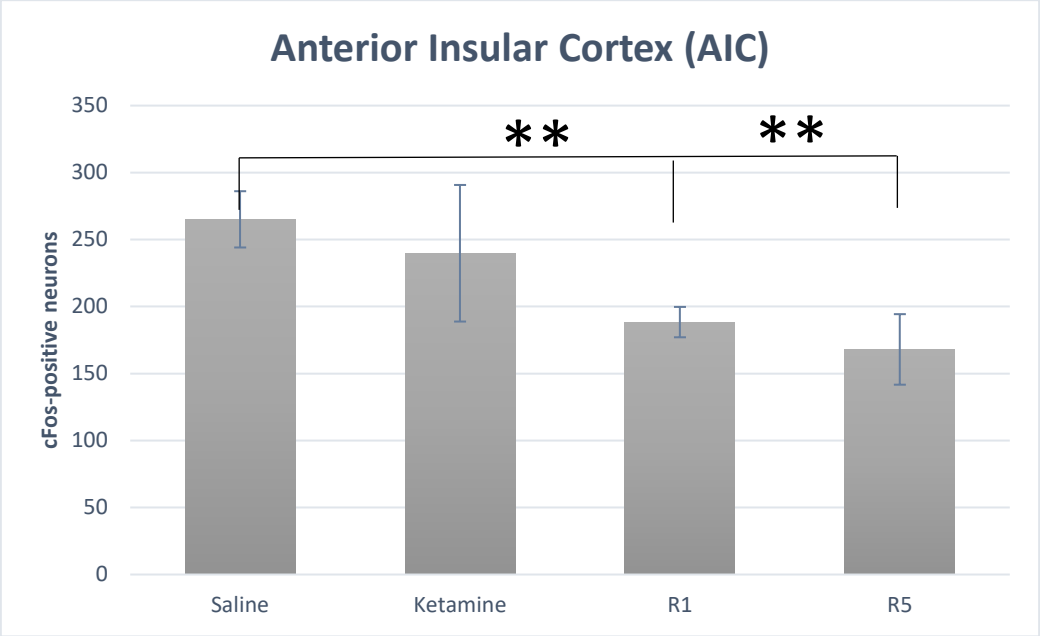


*Figure 14. Both R1 and R5-treated animals show significantly (**) reduced neuronal activity in the anterior insular cortex when compared to saline. ** = p< 0.01*

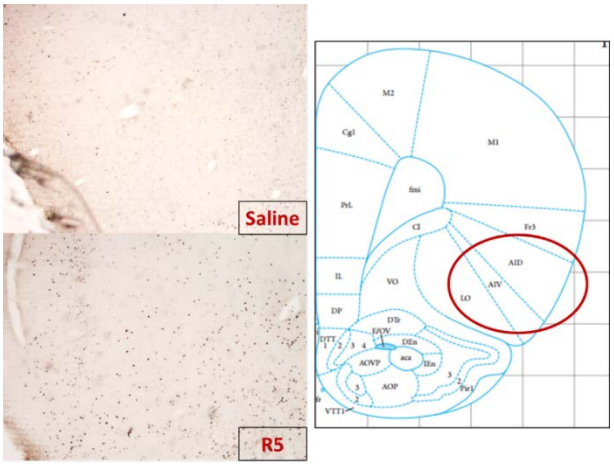


Figure 15. c-Fos immunoreactivity in the anterior insular cortex of animals treated with saline and R5.

4.1.2 Area postrema:

No significant changes in c-Fos immunoreactivity in the AP were observed between saline and any of the administered anaesthetics (*Figure 16.*)

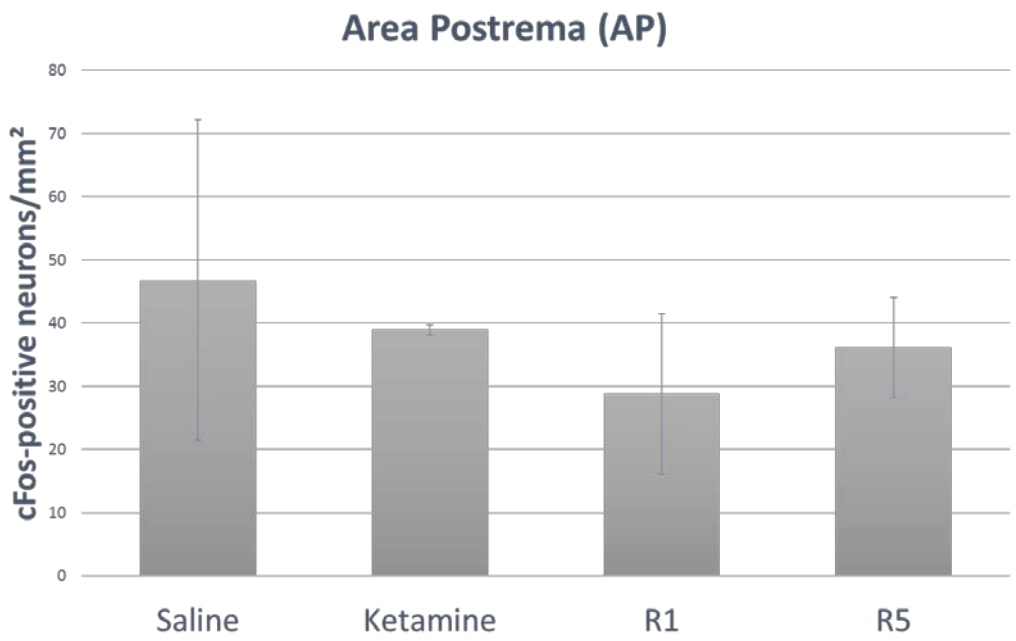


Figure 16. Neuronal activity did not differ significantly in animals treated with the different drugs in the AP.

4.1.3 Nucleus of the solitary tract:

R1-treated animals show significantly (**) increased c-Fos immunoreactivity compared to saline-treated animals in the NTS, and R5-treated animals show significantly (*) increased c-Fos immunoreactivity in this region compared to saline-treated animals. Ketamine infusion did not show a significant effect on c-Fos immunoreactivity (*Figure 17*).

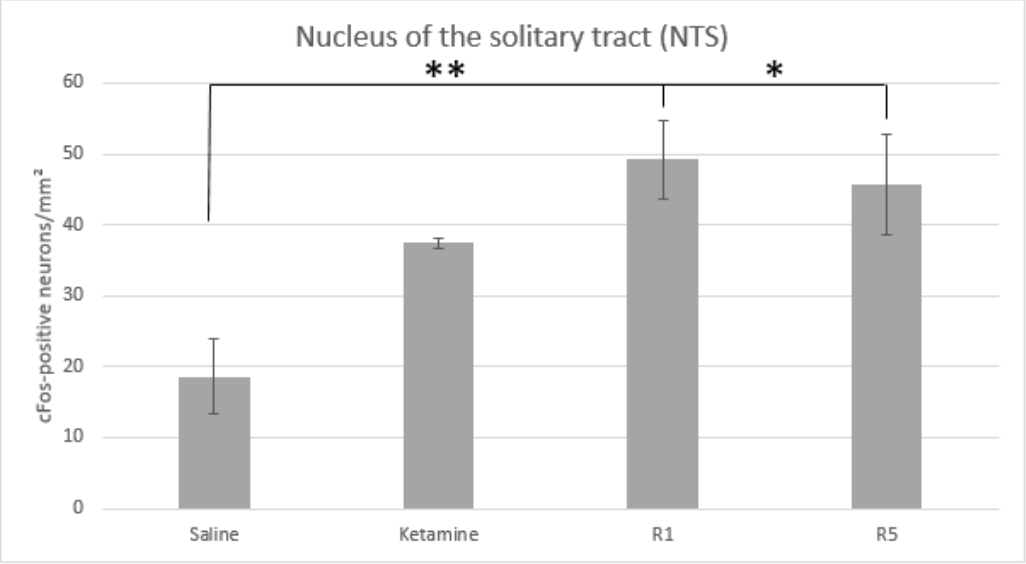


*Figure 17. R1-treated animals show significantly (**) increased neuronal activity compared to saline-treated animals in the NTS, and R5-treated animals show significantly (*) increased neuronal activity compared to saline-treated animals. * = p<0.05, ** = p<0.01*

4.1.4 Bed nuclei of the stria terminalis:

No significant changes in c-Fos immunoreactivity were observed in the BNST compared to saline- or ketamine-treated animals (*Figure 18).*

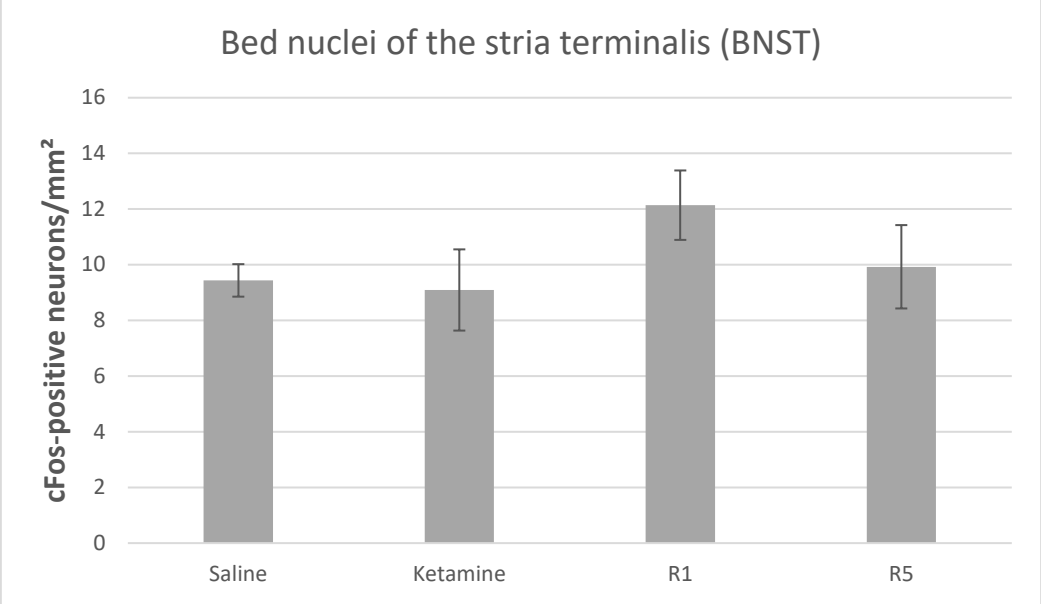


Figure 18. No significant changes were observed in neuronal activity in the BNST compared to saline-treated animals.

4.1.5 Basolateral amygdala:

R1-treated animals show significantly reduced c-Fos immunoreactivity in the BLA compared to both saline-treated (***), and ketamine-treated (**) animals *(Figure 19)*.

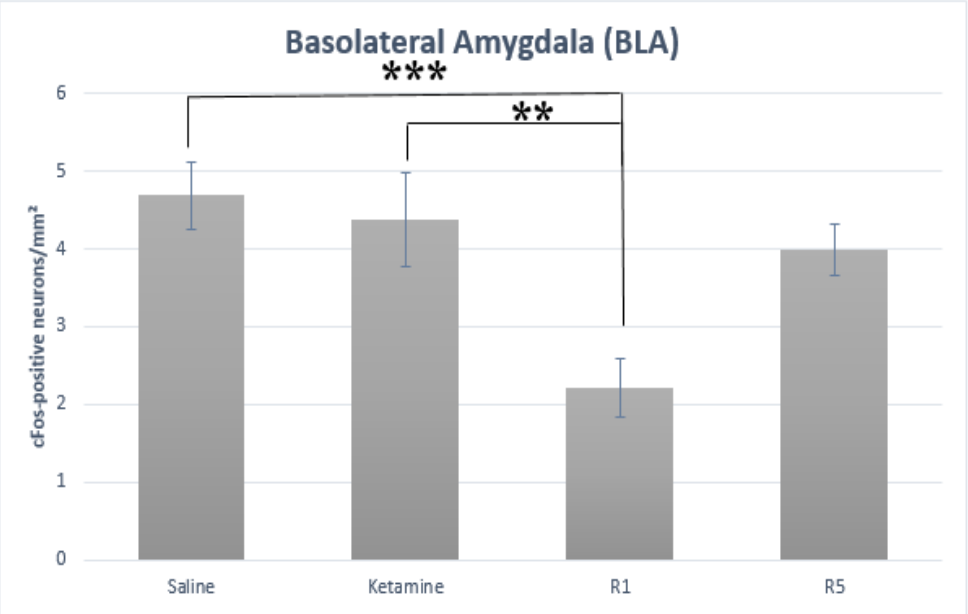


*Figure 19. R1-treated animals show significantly reduced neuronal activity in the BLA compared to both saline-treated (***), and ketamine-treated (**) animals. **=p<0.01, ***=p<0.001*

4.1.6 Central nucleus of the amygdala:

R1-treated animals show significantly (*) increased c-Fos immunoreactivity in the CeA compared to saline-treated animals, and R5-treatement significantly (**) increased c-Fos immunoreactivity in this region compared to saline administration *(Figure 20).*

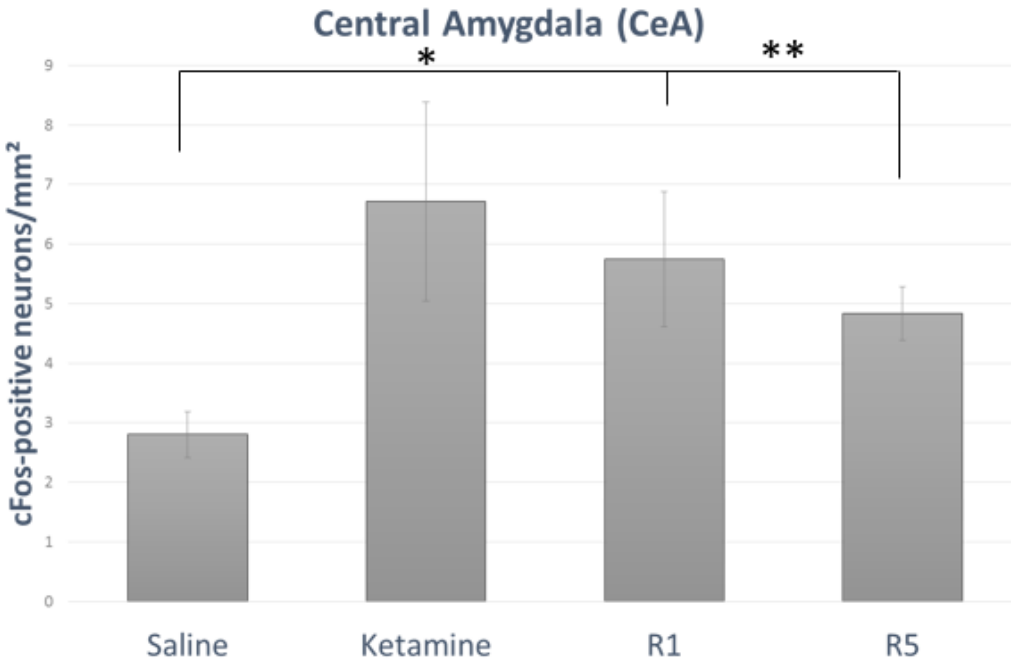


*Figure 20. R1-treated animals show significantly (*) increased neuronal activity compared to saline-treated animals in the CeA, and R5-treatement significantly (**) increased neuronal activity in this region when compared to saline administration. * = p<0.05, ** = p<0.01*

4.1.7 Caudate putamen:

Both ketamine- and R1-treated animals show significantly (*) increased c-Fos immunoreactivity in the CPu compared to saline treatment, while R5-treated animals show significantly (*) reduced c-Fos immunoreactivity in this region compared to ketamine treatment *(Figure 21)*.

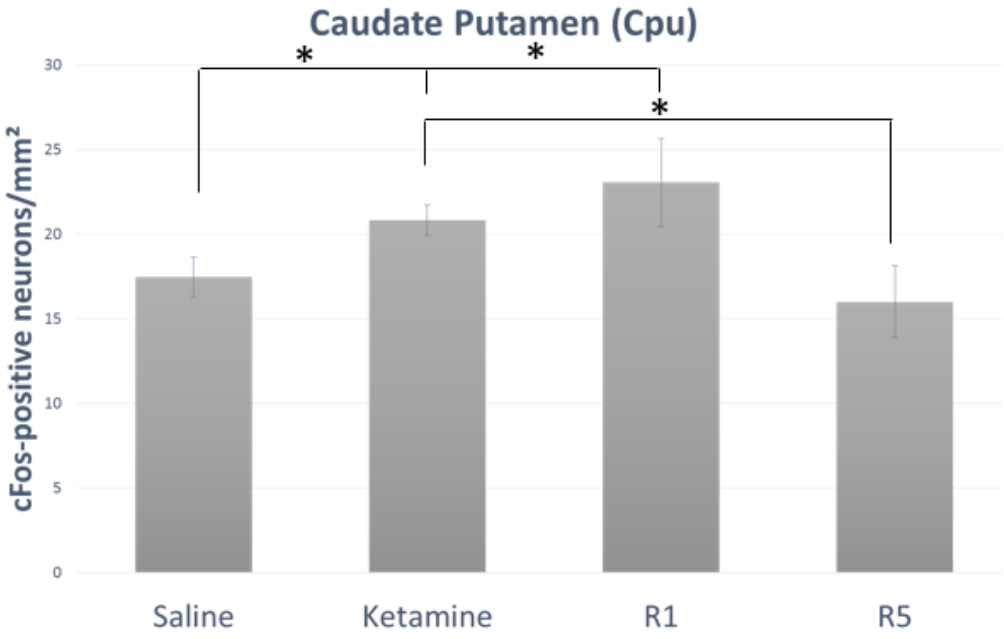


*Figure 21. Both ketamine- and R1-treated animals show significantly (*) increased neuronal activity in the CPu compared to saline treatment. R5-treated animals show significantly (*) reduced neuronal activity in this region when compared to ketamine treatment. * = p<0.05*

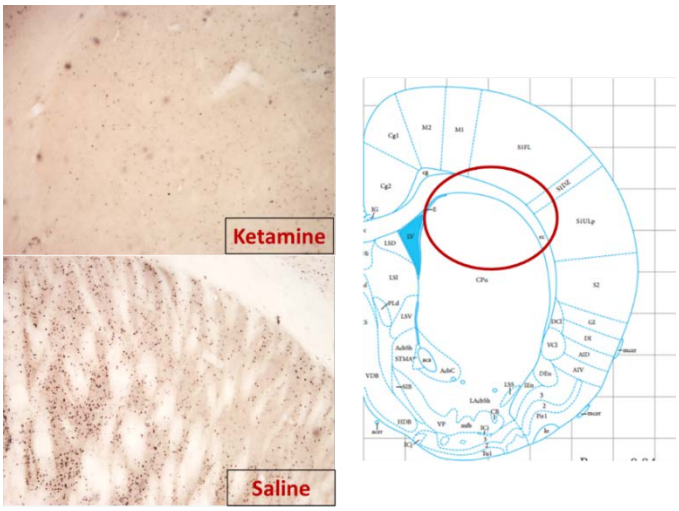


Figure 22. c-Fos immunoreactivity in the caudate putamen of animals treated with saline and ketamine.

4.1.8 Paraventricular nucleus of the thalamus:

Ketamine administration resulted in significantly (***) increased c-Fos immunoreactivity in the PVT compared to saline administration. Saline-, R1- and R5-treated animals all show significantly (***) reduced c-Fos immunoreactivity of this region when compared to ketamine (*Figure 23)*.

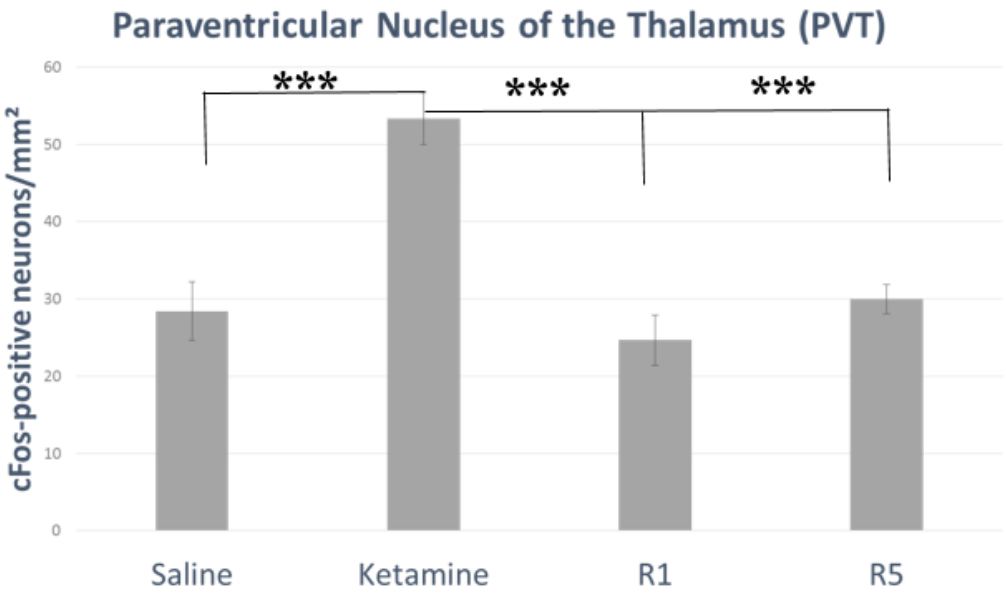


*Figure 23. Ketamine administration resulted in significantly (***) increased neuronal activity in the PVT compared to saline administration. Saline-, R1- and R5-treated animals all show significantly (***) reduced neuronal activity of this region when compared to ketamine. * = p<0.05, ** = p<0.01, *** = p<0.001*

4.1.9 Paraventricular nucleus of the hypothalamus:

R5-treated animals show significantly (***) reduced c-Fos immunoreactivity in the PVN, and ketamine-treated animals show significantly (**) reduced c-Fos immunoreactivity in this region when compared to saline. R1-treated animals show significantly (***) increased c-Fos immunoreactivity, and R5-treated animals show significantly (**) increased c-Fos immunoreactivity in this region compared to ketamine-treated animals (*Figure 24).*

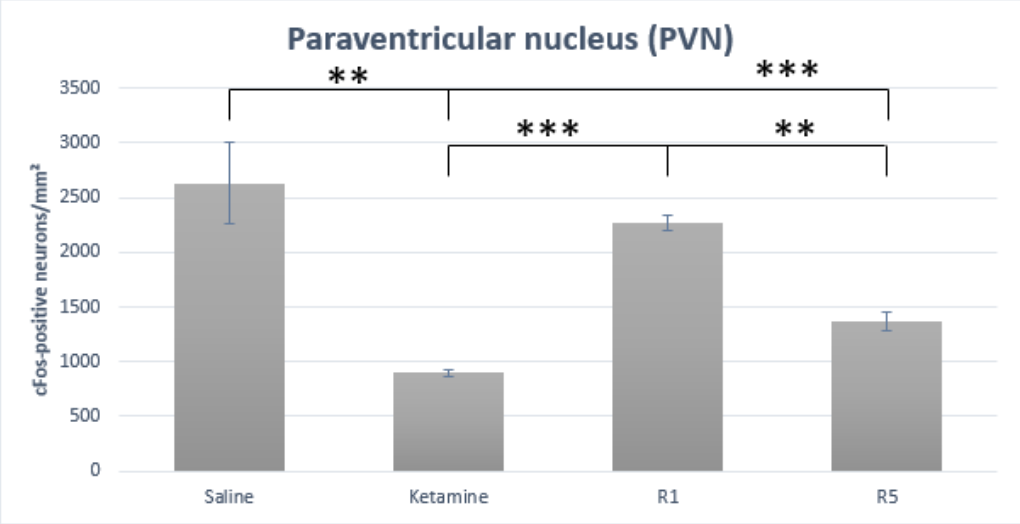


*Figure 24. R5-treated animals show significantly (***) reduced neuronal activity in the PVN, and ketamine-treated animals show significantly (**) reduced neuronal activity in this region when compared to saline. R1-treated animals show significantly (***) increased neuronal activity, and R5-treated animals show significantly (**) increased neuronal activity in this region compared to ketamine-treated animals. * = p<0.05, ** = p<0.01, *** = p<0.001*

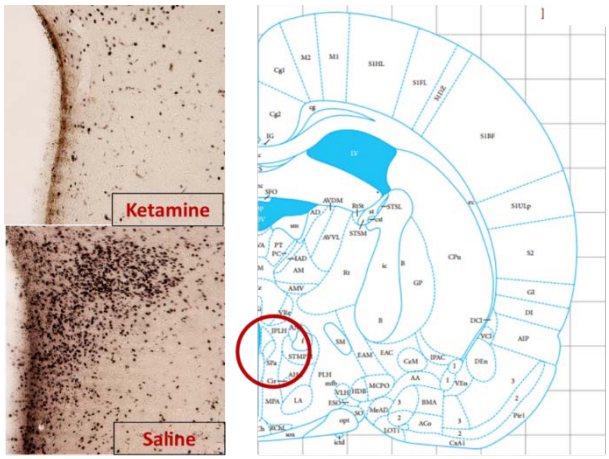


Figure 25. c-Fos immunoreactivity in the paraventricular nucleus of animals treated with saline and ketamine.

4.1.10 Dorsomedial nucleus of the hypothalamus:

Both ketamine- and R5-treated animals show significantly (***) increased c-Fos immunoreactivity in the DMH, and R1-treated animals show significantly (*) increased c-Fos immunoreactivity in this region when compared to saline treatment. R1-treated animals show significantly (***) reduced c-Fos immunoreactivity in this region when compared to ketamine treatment *(Figure 26)*.

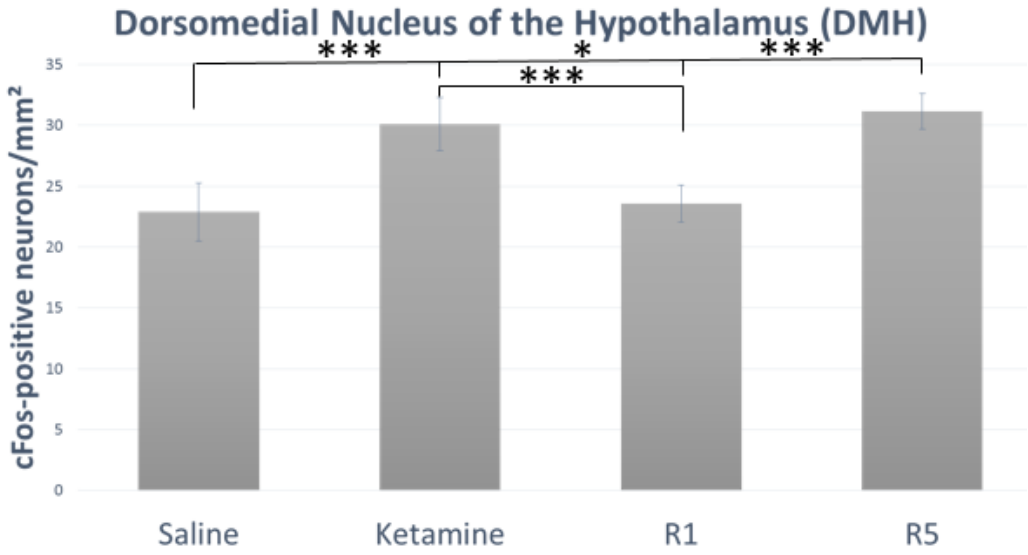


*Figure 26. Both ketamine- and R5-treated animals show significantly (***) increased neuronal activity in the DMH, and R1-treated animals show significantly (*) increased neuronal activity in this region when compared to saline treatment. R1-treated animals show significantly (***) reduced neuronal activity in this region when compared to ketamine treatment. * = p<0.05, ** = p<0.01, *** = p<0.001*

4.1.11 Ventromedial hypothalamus:

Saline-, R1- and R5-treated animals all show significantly (***) reduced c-Fos immunoreactivity in the VMH compared to ketamine treatment. R5-treated animals show significantly (***) reduced c-Fos immunoreactivity in this region when compared to saline treatment *(Figure 27).*

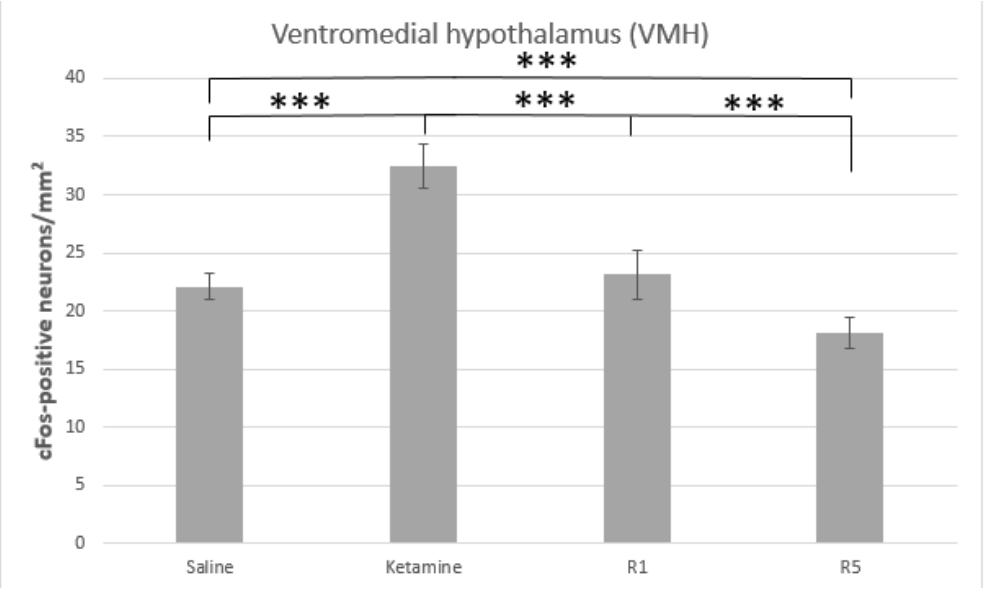


*Figure 27. Saline-, R1- and R5-treated animals all show significantly (***) reduced neuronal activity in the VMH compared to ketamine treatment. R5-treated animals show significantly (***) reduced neuronal activity in this region when compared to saline treatment. *** = p<0.001*

4.1.12 Nucleus accumbens core:

R5-treated animals show significantly (***) increased c-Fos immunoreactivity in the AcbC, and ketamine-treated animals show significantly (**) increased c-Fos immunoreactivity in this region compared to saline treatment. R1-treated animals show significantly (*) reduced c-Fos immunoreactivity compared to ketamine (*Figure 28)*.

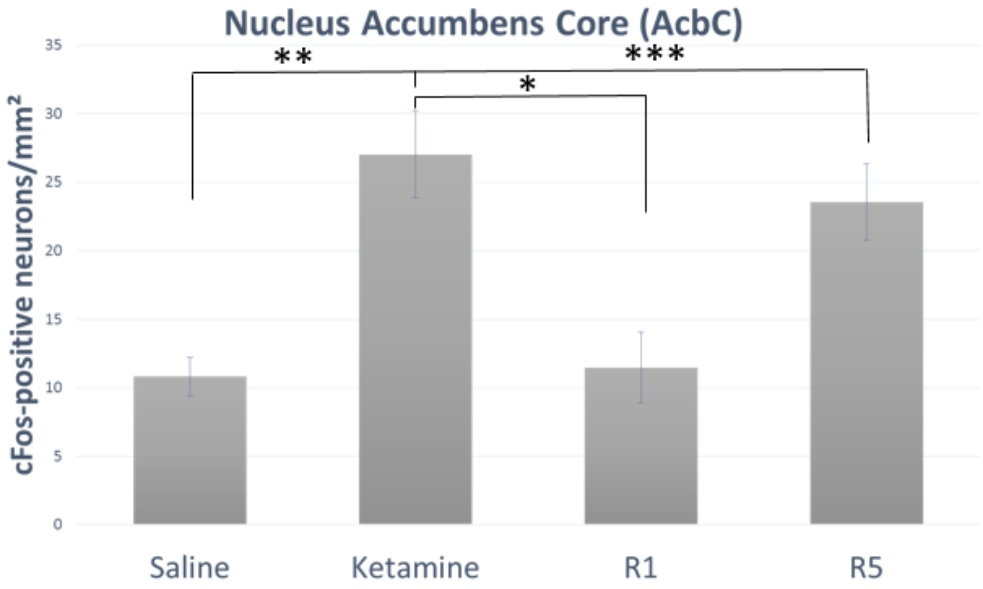


*Figure 28. R5-treated animals show significantly (***) increased neuronal activity in the AcbC, and ketamine-treated animals show significantly (**) increased neuronal activity in this region compared to saline treatment.R1-treated animals show significantly (*) reduced neuronal activity compared to ketamine. * = p<0.05, ** = p<0.01, *** = p<0.001*

4.1.13 Nucleus accumbens shell:

Ketamine-treated animals show significantly (**) increased c-Fos immunoreactivity in the AcbSh compared to saline treatment. Both R1- and R5-treated animals show significantly (***) reduced c-Fos immunoreactivity compared to ketamine treatment (*Figure 29)*.

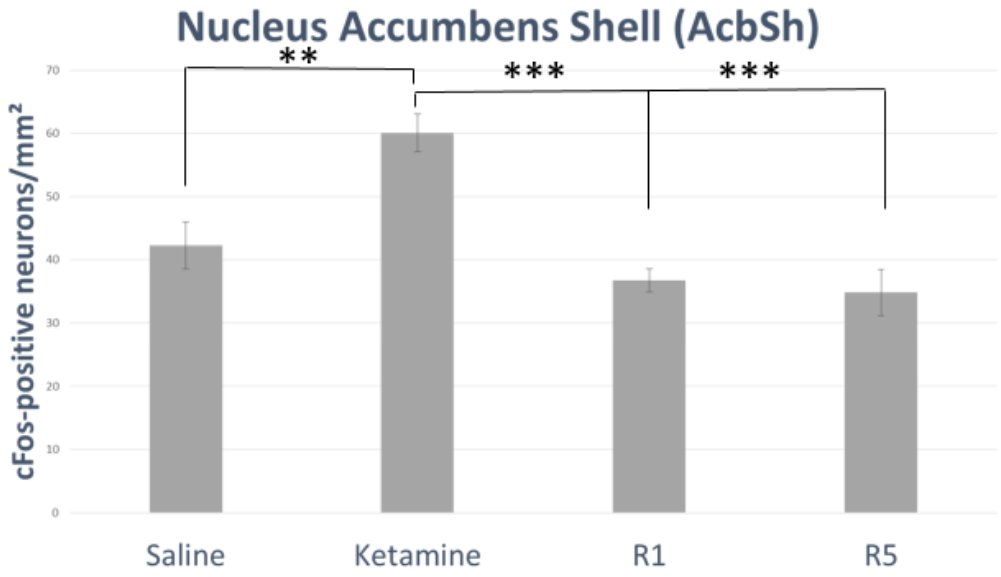


*Figure 29. Ketamine-treated animals show significantly (**) increased neuronal activity in the AcbSh compared to saline treatment. Both R1- and R5-treated animals show significantly (***) reduced neuronal activity compared to ketamine treatment. ** = p<0.01, *** = p<0.001*

4.1.14 Ventral tegmental area:

Both ketamine- and R1-treated animals show significantly (***) increased c-Fos immunoreactivity in the VTA, and R5-treated animals show significantly (**) increased c-Fos immunoreactivity in this region when compared to saline treatment. Saline-treated animals show significantly (***) reduced c-Fos immunoreactivity in the VTA, and R5-treated animals show significantly (*) reduced immunoreactivity in this region when compared to ketamine treatment (*Figure 30).*

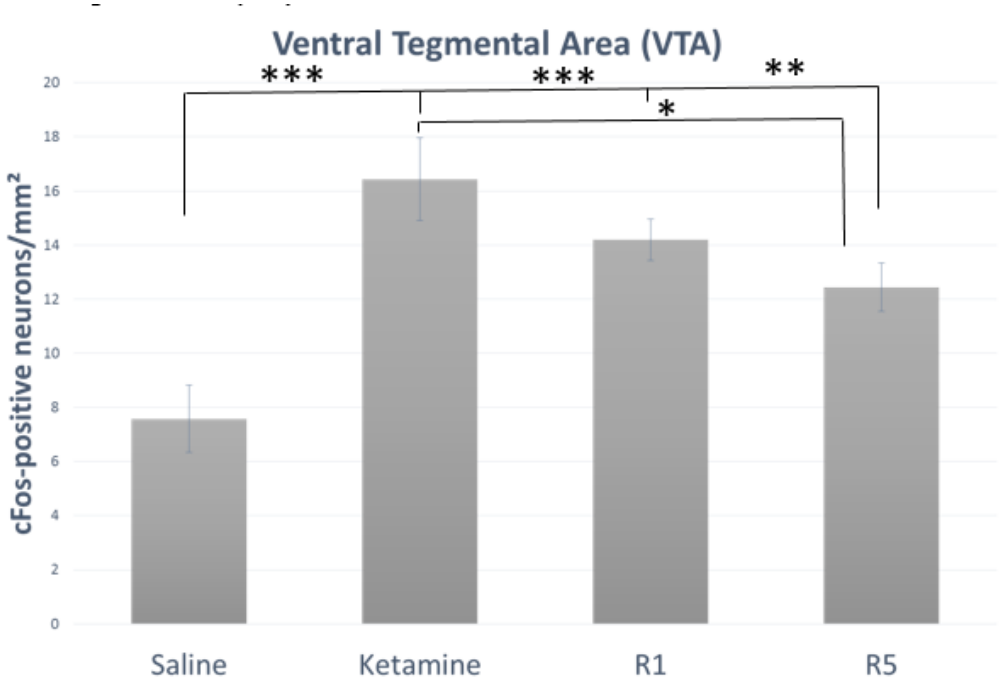


*Figure 30. Both ketamine- and R1-treated animals show significantly (***) increased neuronal activity in the VTA, and R5-treated animals show significantly (**) increased neuronal activity in this region when compared to saline treatment. Saline-treated animals show significantly (***) reduced neuronal activity in the VTA, and R5-treated animals show significantly (*) reduced neuronal activity in this region when compared to ketamine treatment. * = p<0.05, ** = p<0.01, *** = p<0.001*

4.2 Investigate the behavioural effects of ketamine-analogues directly administered in pain and addiction pathways.

c-Fos immunoreactivity showed significant changes in neuronal activity of brain regions associated with nociception and addiction. Novel ketamine-analogues produced somewhat different patterns of activity than ketamine, with R5 producing more profound differences than R1.

Based on these differences in immunoreactivity patterns, select behavioural studies were conducted, first testing the analgesic effect of R5 administered directly to the nociception area of the brain, compared to saline.

4.2.1 AIC - Tail flick

Direct bilateral injection of R5 into the insular cortex resulted in modest attenuated pain response in rats.

Raw and %MPE tail flick responses are presented graphically in figures 34 & 35 respectively.

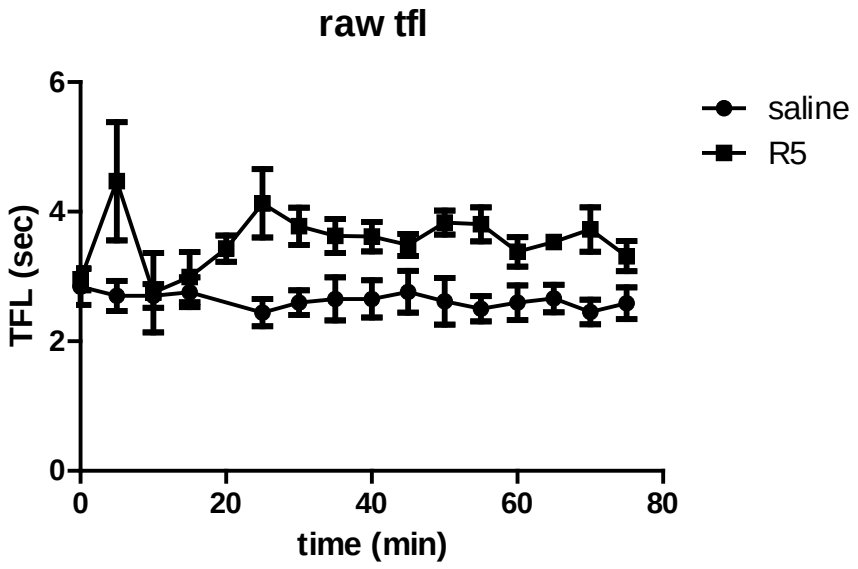


Figure 31. AIC raw tail flick response data. Direct bilateral injection into the insular cortex of R5-treated animals show modest attenuated tail flick response compared to saline-treated animals.

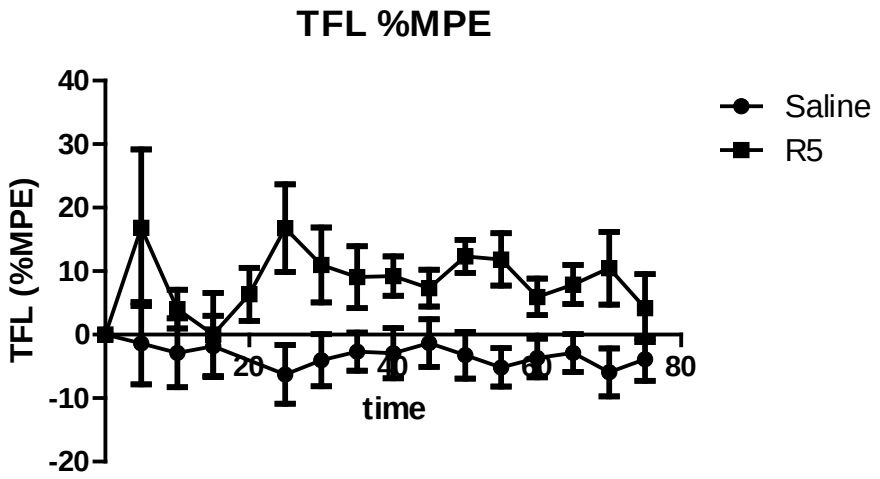


Figure 32. AIC tail flick % MPE data. Direct bilateral injection into the insular cortex of R5-treated animals show modest attenuated tail flick response compared to saline-treated animals

4.2.2 Intracerebroventricle (ICV) - Tail flick

In an attempt to further investigate the mechanism of action by which R5 produces analgesic effects, a second behavioural study administered R5 directly to the HPA-axis by ICV injection, preceded by barium chloride administration. This aimed to test the hypothesis that barium blocks two-pore potassium (K2P) ion channels in neuronal membranes required for analgesic effects of anaesthetics.

Interventricular injections of saline and R5 (SN 35563) were well tolerated.

Interventricular injection of barium chloride resulted in mild irritation in all test animals.

Barium chloride (BaCl) injection into the ICV preceding R5 anaesthetic administration into the same site, is shown to inhibit the analgesic effect of R5, resulting in reduced tail flick latency responses, when compared to sodium chloride (NaCl)-treated animals.

The results show a marked decrease in tail flick response time (6s to 3s), up to 8mins after administration of barium chloride and R5, when compared to saline

and R5. After the 8min mark, barium chloride appears to be metabolized and begins to show similar behavioural responses between groups.

Raw TFL responses and TFL as %MPE are presented in figures 36 & 37 below.

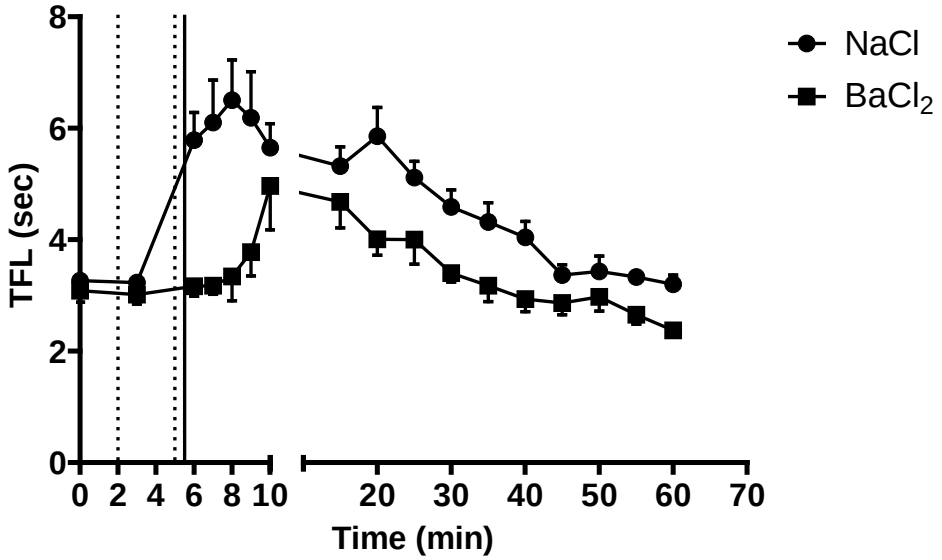


Figure 33. ICV raw tail flick responses according to group. BaCl-treated animals show clearly reduced response times when compared to saline-treated animals. Data: mean (SEM). Dotted lines represent saline/barium injection. Solid line represents SN 35563 injection. 2-way RMANOVA p=0.0006; max difference at 6,7,8,9 minutes p<0.001 all.

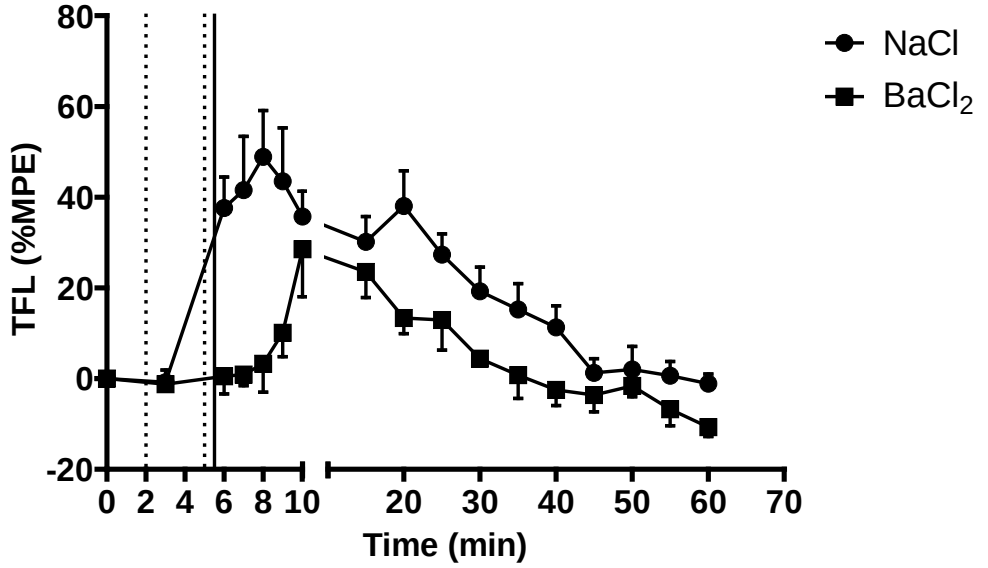


Figure 34. ICV tail flick responses %MPE according to group. BaCl-treated animals show clearly reduced response times when compared to saline-treated animals. Data: mean (SEM). Dotted lines represent saline/barium injection. Solid line represents SN 35563 injection. 2-way RMANOVA p=0.005; max difference at 6,7,8,9 minutes p<0.001 all.

4.2.3 PVN - Exercise withdrawal

Finally, as the nociception system is also involved in addiction and withdrawal behaviours, the effect of R5 in reducing addictive-like, exercise-induced withdrawal symptoms was investigated. This was achieved by direct administration of R5 to the PVN after addictive-like, exercise-induced withdrawal, before behavioural effects were examined.

Direct injection of R5 administered to the PVN shows a strong trend (p=0.0592) of inhibiting exercise withdrawal behaviour compared to saline-treated animals (*Figure 35)*.

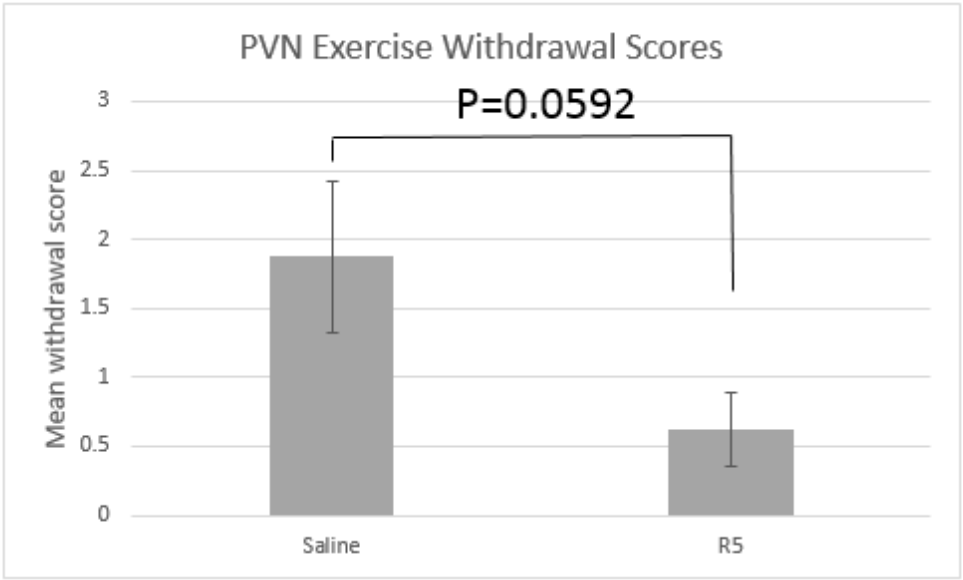


Figure 35. PVN exercise withdrawal results show a strong trend of R5-treated animals exhibiting reduced withdrawal behaviour compared to saline treatment. p=0.0592

5. Discussion

5.1 Identify which areas of the animal brain show significant changes in activity between treatment groups.

The present study used novel molecular analogues of ketamine to investigate the comparative neuronal activity when administered in rat animal models, with an aim to identify novel drugs that influence a similar repertoire of brain targets as ketamine, while attenuating the unpleasant side-effects of ketamine use.

c-Fos immunoreactivity showed significant changes in neuronal activity of brain regions associated with these symptoms. Novel ketamine-analogues produced somewhat different patterns of activity than ketamine, with R5 producing more profound differences than R1.

5.1.1 Emetic pathway

AP - Nausea

No significant changes in neuronal activity of the area postrema, commonly associated with emetic (nausea) responses, were observed between saline and any of the administered anaesthetics.

I would encourage repeating this study with a larger sample, as error bars are large and the small sample shows some indication of all anaesthetics inhibiting nausea activity slightly when compared to saline, with R1 showing inhibition more so than the other anaesthetics; a result that would be advantageous in refining the common side-effect of nausea after ketamine anaesthesia. The small sample sizes seen here are a result of the method of sample collection and the anatomical location of this brain region. During vibratome sectioning of the fixed brain, the brain is first cut in half and affixed to the slide to fit under

the blade, but in this process this small part of the brainstem is often damaged. Other methods of sectioning could be utilized in future to overcome this issue.

5.1.2 Nociception pathway

AIC- Nociception

Both R1 & R5 show significant inhibition of pain pathways in the agranular insula cortex when compared to saline treatment, indicating active analgesia. Interestingly however, regular ketamine did not show significant reduction in activity of this region when compared to saline, even though we would assume analgesia was induced. When compared to ketamine, both R1 and R5 showed slightly reduced activity of this region, which may indicate a stronger analgesic effect than ketamine.

Nociception-pathway neuronal activity studies have associated activity in the AIC with the subjective evaluation of heat/pain, whereas the posterior insula cortex is activated when painful stimulus is applied, regardless of subjective evaluation [113]. This may imply that our observation of significantly reduced activity in the AIC after administration of R1 and R5, correlates with significantly reduced subjective experience of pain.

5.1.3 Stress Pathway

NTS - Peripheral sensory relay center/ cardiovascular response to stress

R1 is shown to produce a significant increase in activity of this region associated with regulating sympathetic and cardiovascular systems in response to stress, when compared to saline. R5 also produces a significant increase in activity when compared to saline, but to a lesser extent than R1.

This region is associated with control of physiological response, such as food intake and stress response. Release of CRH from the PVN during stress, has been shown to activate the NTS to regulate sympathetic and cardiovascular functions [69].

Results showing that increased activity in this region when compared to saline, after any anaesthetic administration, may signify more activity of the stress response circuit than when compared to saline, especially as this correlates with results in the PVN and associated areas. This is not overly surprising as one would expect any induction of anaesthesia to be slightly more stressful than no treatment.

When comparing the anaesthetics, our results show that the novel ketamine analogues R1 and R5 may produce slightly increased activity of this region associated with stress response, when compared to regular ketamine, but any differences are not significant.

R1 produced the highest levels of activity in regions associated with response to stress, including the PVN.

PVN - Stimulating stress response

Significant inhibition of this region was produced by ketamine and R5, when compared to saline. R1 produced significantly ($p<0.0001$) increased, and R5 produced significantly ($p=0.003$) increased neuronal activity of this region associated with stimulating stress responses (PVN), when compared to ketamine. This may imply that anaesthetic agents inhibit the activity in this region associated with stress response, however our data showed that normal ketamine inhibits activity in this region more significantly than the novel analogues (R1 and R5).

R1 was the least effective anaesthetic agent at inhibiting this region, and perhaps the stress response.

c-Fos analysis showed that R1 also produced the most neuronal activity in the NTS region, an associated area implicated in stimulating the stress response. Studies have shown that stress factors can influence CRH release in PVN that in turn stimulates the NTS to regulate sympathetic and cardiovascular responses to stress [69]. This supports the suggestion that R1 may stimulate the stress response more than regular ketamine anaesthesia.

BNST - Anxiety, addiction & stress response

No significant differences in activity between ketamine and the novel analogues were observed, demonstrating that these novel analogues do not significantly increase, or reduce activity of the BNST, an anxiety and stress response associated region, compared to the currently used ketamine anaesthetic. R1 produced slightly more neuronal activity of this region than any other treatment, though differences were not deemed significant.

VMH - Regulation of circulatory system & food intake

R1, R5 and saline all showed significantly reduced neuronal activity of this region associated with reduced circulatory system response compared to ketamine.

Neuronal activity in the VMH has been found to be associated with suppression of the circulatory system in rats. Blood pressure and heart-rate were found to correlate negatively with activity of the VMH during anaesthesia [93].

Neuronal activity levels produced by R1 are similar to that of saline, while R5 produced significant inhibition of this region when compared to saline, therefore implying that R5 may trigger an increase in heart-rate and blood pressure.

Ketamine seems to produce much greater stimulation of this region, which may result in more reduced circulatory responses during ketamine anaesthesia. R5 however produced significant inhibition of this region, which may imply an increase in blood pressure and heart-rate.

This result correlates with increased activity produced in the NTS by R5 that is associated with increased heart-rate in response to stress.

5.1.4 Arousal pathway

PVT- Arousal

Ketamine produced very significantly increased neuronal activity of the arousal-associated area (PVT), when compared to saline.

However, both R1 and R5 produced very significantly reduced neuronal activity of the arousal-related region (PVT), when compared to ketamine. This would imply that the new ketamine analogues (both R1 and R5) may produce reduced arousal after anaesthesia, a common emergence reaction upon recovering from ketamine anaesthesia.

5.1.5 Fear pathway

BLA - Conditional fear responses

R1 showed very significantly inhibited neuronal activity in the BLA region, responsible for acquisition of fear responses, when compared to saline. R1 also shows very significantly reduced neuronal activity of this area, when compared to ketamine.

R1 produced significantly reduced activity of the BLA, which has previously been associated with fear behaviour. [77]. This evidence may imply that R1 produces reduced fear behaviour, when compared to ketamine. The suppression of fear behaviour by intra-BLA muscimol (inhibition) has been associated with increased c-Fos expression in the central nucleus of the amygdala (CeA). This correlates with the observed significant increase in neuronal activity in the CeA after administration of R1 also.

This may illustrate that the novel anaesthetic R1, significantly reduces some aspects of fear responses after anaesthetic administration, compared to ketamine anaesthesia. This would be advantageous in eliminating the disturbing emergence reactions that follow ketamine anaesthesia, often associated with fearful responses.

CeA - Stimulating emotional response

All anaesthetics showed an increase in activity of this region, responsible for emotional responses, when compared to saline.

Increased activity of the CeA has been associated with suppression of fear response [77]. This may imply that all anaesthetics agents tested in the present study reduce emotional response, although R5 produced the most significant

increase in activity when compared to saline, implying that R5 may suppress emotional response significantly.

When compared to ketamine, both novel analogues appear to produce less activity of this region, though any difference was not significant.

AcbC – Learning, fear and directing action toward reward.

Both ketamine and R5 produce significantly (P=0.0011 and P=0.0009 respectively) increased neuronal activity in the AcbC, an area associated with motivation and fear, when compared to saline.

However, R1 produces significantly reduced neuronal activity of this region when compared to ketamine, similar to that of saline.

This may imply that as ketamine and R5 significantly stimulate this region associated with fear and motivation, and R1 does not produce this effect, that R1 does not produce increased fear responses observed during ketamine anaesthesia.

The nucleus accumbens, an area primarily involved in motivation also forms an integral part of the fear-processing circuit, as it receives projections from both the anterior cingulate and the basolateral amygdala (BLA). This correlates with the lack of neuronal activity produced by R1 in the BLA, where both ketamine and R5 also significantly increased activity.

This reinforces the view that R1 does not produce significantly increased fear responses observed after administration produced by the other anaesthetics.

AcbSh - Anticipation of reward/fear and motivation

Ketamine produces very significantly increased neuronal activity of the brain region associated with the anticipation of reward or fear, when compared to saline.

However, both R1 and R5 produce significantly less neuronal activity of this region, when compared to ketamine, similar to that of saline. This reinforces the notion that R1 produces significantly reduced fear-related behaviour than ketamine does.

VTA – Reward/fear expression, startle response

While all anaesthetics significantly increase stimulation of this region associated with fear expression, R5 produced significantly reduced activity of this region when compared to ketamine. Lesions of the VTA produced suppressed fear expression during conditioned startle response experiments, suggesting the role of the VTA in regulating levels of aversive emotional arousal [106].

This may imply that R5 produces significantly reduced fear-expression behaviour than ketamine anaesthesia.

DMH - Cardiovascular response to emotional fear

Both ketamine and R5 produced significantly increased neuronal activity in the brain region associated with controlling cardiovascular responses to fear (DMH) when compared to saline, however R1 showed significantly reduced neuronal activity in this region, when compared to ketamine. Studies show symptoms of stress response behaviour observed by stimulation of DMH included increase of respiratory rate, heart rate and arterial pressure [92].This may imply that R1 produces a significant reduction in stimulation of the cardiovascular responses to fear than regular ketamine does. R1 was again shown to influence this region the least compared to saline, as seen in the PVN; however increased neuronal activity of the DMH by ketamine and R5 correlated with inhibition of activity in the PVN.

5.1.6 Amnesia/memory retrieval and hallucination pathway

CPu - Memory-retrieval, habit-formation and learned response

Ketamine and R1 both produced significantly increased immunoreactivity in the region associated with habit and memory (CPu) when compared to saline. However, R5 did not produce significant changes compared to saline, implying that memory-retrieval regions, often associated with hallucinations, may not be activated in the way ketamine and R1 do. Stimulation studies show that the caudate putamen is required for the expression and consolidation of memory, while inhibition results in attenuated memory consolidation and retrieval [82], and has been implicated in hallucinations, mediating prefrontal behaviours and in the conceptual integration of memories [83]. This may imply that more significant amnesia and reduced hallucinations are produced during R5 administration, than ketamine which often produces emergence reactions associated with hallucinatory behaviour. R5 also produced significantly reduced activity of this region when compared to ketamine.

5.2 Investigate the behavioural effects of ketamine-analogues directly administered in pain and addiction pathways.

By then focussing on nociception and addiction areas that showed significant changes, we were able to conduct behavioural studies during direct administration to the sites of interest, to further examine these effects. This included testing of analgesic effects and co-administering the drug with an ion-channel blocker to investigate the mechanism of action for effective analgesia.

5.2.1 AIC: Tail flick study

c-Fos results of intravenous administration showed that all anaesthetic agents produced inhibition of neuronal activity in this region associated with perception of pain, with the novel ketamine analogues producing the most significant effect.

Therefore, R5 was administered directly to this region to examine if this results in effective analgesia measured by behavioural responses.

Direct injection:

- Direct bilateral injection of R5 into the insular cortex resulted in modest attenuated pain response in rats (but significantly less than systemic administration).

The observed result that direct injection into the AIC resulted in significantly less inhibited pain response than systemic administration implies that regions other than the AIC are responsible for the full analgesic effect produced by systemic administration.

5.2.2 ICV: Tail flick study

In an attempt to further investigate the mechanism of action by which R5 produces analgesic effects, a second behavioural study administered R5 directly to the HPA-axis by ICV injection, preceded by barium chloride administration. This aimed to test the hypothesis that barium blocks two-pore potassium (K2P) ion channels in neuronal membranes required for analgesic effects of anaesthetics.

By directly administering into the ventricle, diffusion into the entire hypothalamic region can be attained without damage to any specific area [114]. To investigate the hypothesis that analgesic effects of these drugs require functional two-pore potassium (K2P) ion channels in neuronal membranes, direct administration of R5 into the ventricle preceded by either barium chloride or saline was conducted. Barium has been shown to block (K2P) two-pore potassium ion channels in neuronal membranes, blocking anaesthetic agents from reaching the hydrophobic pockets of microtubules to initiate anaesthetic effects [115; 116].

The results show that R5 is an effective analgesic when administered in this region, and that barium chloride produced a marked decrease in tail flick response time (6s to 3s), up to 8mins after administration of barium chloride and R5, when compared to saline and R5. After the 8min mark, barium chloride appears to be metabolised and begins to show more similar responses between groups.

These results imply that barium chloride produced inhibition of the analgesic effect of R5 early on, before being metabolized after around 8 minutes, after which the analgesic effect was similar between groups.

The result of the administration of BaCl preceding anaesthetic administration described in the present study reinforced the hypothesis of barium K2P blockade, as the group receiving BaCl showed markedly decreased tail-flick response times when compared to the saline control group.

Therefore, functioning K2P ion channels seem to be required for anaesthetic agents to produce effective analgesia.

5.2.3 PVN: Exercise withdrawal study

Finally, as the nociception system is also involved in addiction and withdrawal behaviours, the effect of R5 in reducing addictive-like, exercise-induced withdrawal symptoms was investigated. This was achieved by direct administration of R5 to the PVN after addictive-like, exercise-induced withdrawal, before behavioural effects were examined.

The results of the exercise withdrawal study showed a strong trend ($p=0.0592$) of R5 producing reduced opioid-withdrawal behaviour symptoms, when compared to saline.

This opioid-withdrawal behaviour included escape attempts, teeth chattering and wet-dog shakes, associated with increased stress and arousal.

c-Fos studies of R5 intravenously show that the drug produces significantly inhibited immunoreactivity in the PVN, associated with reduced stress response. This reinforces the behavioural findings that R5 has inhibited stress responses associated with opioid withdrawal.

6. Conclusions and perspectives

6.1 Conclusions

The present project, utilizing rats as an animal model, focused on examining functional properties of the ester-analogue of ketamine, R5, whose potentially beneficial profile had been suggested by preliminary studies. First, brain activation patterns following R5 compared to ketamine (and another ester-analogue control compound, R1) were assessed by employing immunohistochemical detection of an immediate-early gene product, c-Fos.

- R5 produced a somewhat similar pattern of activity as ketamine, whereas more profound differences in c-Fos were detected after R1. It was particularly striking in areas related to pain and addiction, including the anterior insular cortex (AIC) and paraventricular nucleus (PVN).

In response to these neurological markers, behavioural studies were shown to reinforce these findings, with R5 producing analgesic effects when administered directly to associated regions (AIC), and reduce stress-associated withdrawal behaviour when administered to the PVN.

- AIC administration of R5 produced modest analgesia in the tail-flick test.
- PVN R5 reduced naltrexone-precipitated exercise-induced withdrawal.
- BaCl attenuated ICV R5-induced analgesia.

In summary, R5 shows an analgesic effect similar to ketamine, most likely by targeting a similar subset of brain sites, which suggests that this particular ester-analogue can be considered as a good candidate for conceptualizing future pain management strategies.

6.2 Perspectives

Ketamine has long been shown to produce disturbing emergence reactions that have severely reduced patient acceptance, even though ketamine is one of the safest anaesthetic agents available. These reactions often include severely increased arousal and fear responses upon waking up, nausea and even hallucinations.

The present study intended to refine the effects of ketamine anaesthesia by testing novel ketamine analogues in their comparative effects. c-Fos expression shows significant changes in activity of brain regions associated with these symptoms.

Specifically, R1 produced increased analgesia, decreased fear response and arousal, however indicates an increase in some aspects of stress response, compared to ketamine.

R5 also produced increased analgesia, and possibly amnesia, as memory retrieval regions were inhibited, perhaps contributing to reduced hallucinations. Decreased arousal, stress and fear -associated activity was also observed, however some measures of cardiovascular stress regions were shown to increase in activity (*Figure 36*).

Significant changes compared to ketamine:

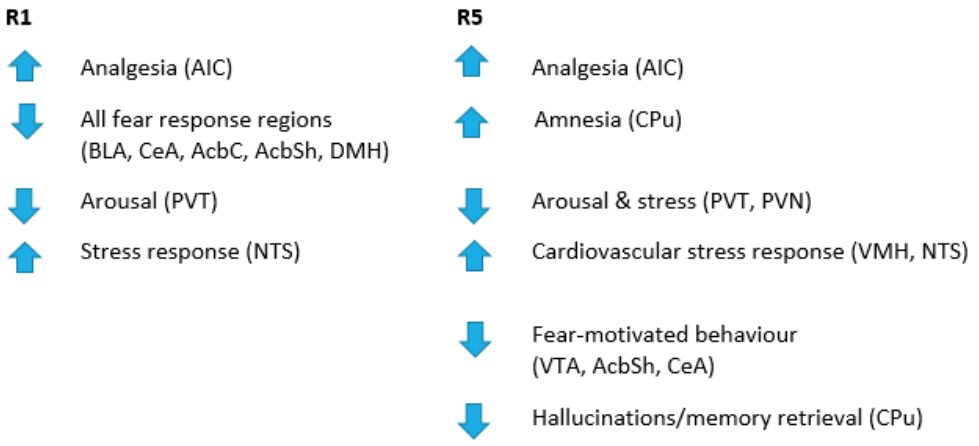


Figure 36. Summary of function-related c-Fos results for each novel anaesthetic

In response to these neurological markers, behavioural studies were shown to reinforce these findings, with R5 producing analgesic effects when administered directly to associated regions (AIC), and reduce stress-associated behaviour when administered to PVN.

The present study therefore shows that novel ketamine-analogues produce similar effects to ketamine, however R5 in particular elicits significant changes in patterns of neuronal activity relating to pain pathways, and therefore may be an especially eligible candidate for further research to bring this drug to clinical use, as it appears to reduce signs of troubling side-effects often reported with ketamine anaesthesia.

Future research could utilise rt-PCR techniques to better understand the comparative changes occurring at a gene-expression level contributing to reduced side-effects during anaesthesia. Findings such as the fact that R5 produced significantly reduced neuronal activity of the caudate putamen, a region associated with memory-recall and hallucinations, would be interesting to follow up with behavioural studies perhaps in humans. By examining the

difference in subjective experiences under anaesthesia, the hypothesis that R5 may produce reduced hallucinations, one of the most troubling emergence reactions and a reason it is abused recreationally, can be investigated, leading to a safer clinical drug. Finally, to better address the common side-effect of nausea after ketamine anaesthesia, utilising an alternative method of slicing the brain before measuring immunoreactivity of the area postrema would increase the sample size and better allow observation of any significant effect in this area.

7. Supplementary Material

Table 2. AIC c-Fos p-values

		p-value	
ketamine	r1	0.2939	
	r5	0.1834	
	saline	0.6115	

		p-value	
saline	r1	0.0059	**
	r5	0.0085	**
	ketamine	0.6115	

Table 3. AP c-Fos p-values

		p-value
ketamine	r1	0.6512
	r5	0.8527
	saline	0.7875

		p-value
Saline	r1	0.5054
	r5	0.5927
	ketamine	0.7875

Table 4. NTS c-Fos p-values

		p-value	
ketamine	r1	0.2268	
	r5	0.5529	
	saline	0.0781	trend

		p-value	
saline	r1	0.0071	**
	r5	0.0253	*
	ketamine	0.0781	trend

Table 5. BNST cFos p-values

		p-value
ketamine	r1	0.1433
	r5	0.6947
	saline	0.8748

		p-value
saline	r1	0.1329
	r5	0.8268
	ketamine	0.8748

Table 6. BLA cFos p-values

		p-value	
ketamine	r1	0.0079	**
	r5	0.57	
	saline	0.6684	

		p-value	
saline	r1	0.0007	***
	r5	0.256	
	ketamine	0.6684	

Table 7. CeA cFos p-values

		p-value	
ketamine	r1	0.6866	
	r5	0.3747	
	saline	0.0577	Strong trend

		p-value	
saline	r1	0.0177	*
	r5	0.0045	**
	ketamine	0.0577	Strong trend

Table 8. CPu cFos p-values

		p-value	
ketamine	r1	0.304	
	r5	0.017	*
	saline	0.025	*

		p-value	
saline	r1	0.0332	*
	r5	0.5147	
	ketamine	0.025	*

Table 9. PVT cFos p-values

		p-value	
ketamine	r1	<0.0001	***
	r5	<0.0001	***
	saline	<0.0001	***

		p-value	
saline	r1	0.4584	
	r5	0.6961	
	ketamine	<0.0001	***

Table 10. PVN cFos p-values

		p-value	
ketamine	r1	<0.0001	***
	r5	0.003	**
	saline	0.0011	**

		p-value	
saline	r1	0.3577	
	r5	0.0002	***
	ketamine	0.0011	**

Table 11. DMH cFos p-values

		p-value	
ketamine	r1	<0.0001	***
	r5	0.0775	trend
	saline	<0.0001	***

		p-value	
saline	r1	0.0332	*
	r5	<0.0001	***
	ketamine	<0.0001	***

Table 12. VMH cFos p-values

		p-value	
ketamine	r1	<0.0001	***
	r5	<0.0001	***
	saline	<0.0001	***

		p-value	
saline	r1	0.1396	
	r5	<0.0001	***
	ketamine	<0.0001	***

Table 13. AcbC cFos p-values

		p-value	
ketamine	r1	0.0201	*
	r5	0.573	
	saline	0.0011	**

		p-value	
saline	r1	0.8053	
	r5	0.0009	***
	ketamine	0.0011	**

Table 14. AcbSh cFos p-values

		p-value	
ketamine	r1	<0.0001	***
	r5	<0.0001	***
	saline	0.0019	**

		p-value	
saline	r1	0.2054	
	r5	0.1771	
	ketamine	0.0019	**

Table 15. VTA cFos p-values

		p-value	
ketamine	r1	0.16	
	r5	0.0297	*
	saline	0.0009	***

		p-value	
saline	r1	0.0001	***
	r5	0.0053	**
	ketamine	0.0009	***

Table 16. PVN exercise withdrawal p-values

		p-value	
saline	R5	0.0592	Strong trend

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