# Magnesium recurarisation differences between no reversal, neostigmine/glycopyrrolate reversal and sugammadex reversal of neuromuscular block in an *in vivo* rat model

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# Abstract

The neuromuscular junction (NMJ) is a synapse with one of the highest safety margins in the human body. The use of neuromuscular blocking agents to inhibit neuromuscular transmission is sufficient to produce skeletal muscle paralysis, a mechanism used to facilitate muscle relaxation during surgery. Residual neuromuscular block postoperatively has been found to be a major risk factor for postoperative complications. Sudden reinstatement of neuromuscular block (recurarisation), through use of magnesium, has also been observed clinically. This has led to a reluctance to use magnesium postoperatively for fear of recurarisation. Recurarisation following reversal of neuromuscular blockade with neostigmine or sugammadex has not been evaluated in a formal study, and for this reason, this study investigated recurarisation after 30 mg/kg magnesium sulphate ($MgSO_4$) following reversal of neuromuscular blockade with neostigmine, two dosages of sugammadex or when reversal was omitted. Prior to investigating recurarisation, the effects of magnesium on neuromuscular transmission in the absence of neuromuscular blocking agents was investigated, in order to determine a standard clinical dose that did not produce detectable, by Train-of-Four Ratio (TOF-R) or Twitch 1 height (%T1), neuromuscular impairment.

An *in vivo* rat model was used in which the sciatic nerve was exposed in rats anesthetised with isoflurane and the common calcaneus tendon attached to a force transducer. The response of the muscle to train-of-four (TOF) supramaximal stimulation was recorded electronically and captured to a spreadsheet. Sciatic nerve stimulation was performed as a TOF stimulus every 20 sec throughout the experiment. During a 5-minute stabilisation period baseline measurements were obtained. During recordings TOF-R and %T1 values were measured.

During the magnesium dose-effect experiments, each rat was given a bolus dose of one of: 0.9% saline (n=4), or 10 mg/kg (n=4), 30 mg/kg (n=6), 60 mg/kg (n=6) or 100 mg/kg (n=6) magnesium sulphate ($MgSO_4$) in a volume of 1 mL/kg in saline.

During the magnesium recurarisation experiments, each rat was paralysed with rocuronium 0.5 mg/kg and allowed to spontaneously reach recovery of T4 at which point four methods

of reversal of neuromuscular blockade were then adopted. The first group received no intervention at reappearance of T4 and recovered spontaneously (SPON), while in the remaining 3 groups reversal was performed after the appearance of a clearly detectable T4 twitch. Reversal was performed one of: 0.04 mg/kg neostigmine (NEO) plus glycopyrrolate, 4 mg/kg sugammadex (SUG4) and 16 mg/kg sugammadex (SUG16). After recovery of TOF-R > 0.9, magnesium sulphate (30 mg/kg) was administered and the effect recorded for 15 minutes.

In the magnesium dose-effect experiment: No neuromuscular impairment (TOF-R and %T1) was observed in the magnesium 10 and 30 mg/kg groups. In the 60 mg/kg group, a significant reduction (compared to saline) of TOF-R (0.253) of relatively brief duration was seen, but the depression of %T1 (24.0%) was not statistically significant. At 100 mg/kg, significant impairment of both %T1 (58.0%) and TOF-R (0.498) was seen that lasted beyond the duration of the recording.

In the magnesium recurarisation experiment: recurarisation was observed in all reversal groups. Recurarisation was maximal following SPON, with a maximum reduction of TOF-R of 0.451. Recurarisation after SUG4 assessed by TOF-R was depressed by 0.280 that was not significantly different from recurarisation following SUG16 (0.096) or NEO (0.174). Full recovery of TOF-R at 15 minutes was seen in all groups except for SUG4 (0.725).

It was found that at a dose of 30 mg/kg of magnesium sulphate could be used in this model to evaluate magnesium recurarisation, as it does not produce detectable (by TOF-R or %T1) neuromuscular impairment by itself.

Recurarisation was observed after magnesium was administered following all forms of reversal, but was greatest following spontaneous recovery or reversal with 4 mg/kg of sugammadex, however by increasing the dose of sugammadex (to 16 mg/kg) used for reversal the recurarisation effect was minimised.

In conclusion, the continued occurrence of magnesium recurarisation in rats reversed with moderate doses of sugammadex is most likely explained by sufficient extraction of rocuronium out of the NMJ and hence facilitated recovery from neuromuscular block. A continued presence however of residual rocuronium is able to bind the receptor when ACh release is depressed by subsequent administration of magnesium.

# List of abbreviations

| | |
|---|---|
| ACh | Acetylcholine |
| AChE | Acetylcholinesterase |
| AMG | Acceleromyography |
| ANOVA | Analysis of variance |
| ATP | Adenosine triphosphate |
| $Ca^{2+}$ | Calcium ion |
| CaMKII | $Ca^{2+}$/calmodulin-dependent protein kinase II |
| ECG | Electrocardiogram |
| EMG | Electromyography |
| $H_0$ | Null hypothesis |
| $H_1$ ($H_2$… etc.) | Alternative hypothesis |
| $[Iso]_{ave}$ | Average isoflurane concentration |
| mAChR | Muscarinic acetylcholine (cholinergic) receptor |
| $Mg^{2+}$ | Magnesium ion |
| MMG | Mechanomyography |
| $Na^+$ | Sodium ion |
| nAChR | Nicotinic acetylcholine (cholinergic) receptor |
| NMB | Neuromuscular block |
| NMBA | Neuromuscular blocking agent |
| NMJ | Neuromuscular junction |
| NMT | Neuromuscular transmission |

| | |
|---|---|
| rNMB | Residual neuromuscular block |
| PACU | Post-anaesthesia care unit |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PLC | Phospholipase C |
| SD | Standard deviation |
| SEM | Standard error of the mean |
| T1 | First evoked twitch of the TOF twitches |
| $T1_{raw}$ | Raw T1 value (in mV) prior to administration of magnesium |
| $T1_{onset}$ | Time to $\%T1_{min}$ after magnesium administration |
| T2 | Second evoked twitch of the TOF twitches |
| T3 | Third evoked twitch of the TOF twitches |
| T4 | Fourth evoked twitch of the TOF twitches |
| $T_{anaesth}$ | Time spent under anaesthesia when 1$^{st}$ experimental drug administered |
| TOF | Train-of-Four |
| TOF-R | Train-of-Four Ratio |
| $TOF_{fade}$ | Train-of-Four Fade |
| $TOF_{min}$ | Minimum TOF-R reached after magnesium administration |
| $TOF_{onset}$ | Time to $TOF_{min}$ after magnesium administration |
| VGCC | Voltage-gated calcium channels |
| %T1 | Baseline corrected T1 |
| $\%T1_{min}$ | Minimum %T1 value reached after magnesium administration |
| $\Delta TOF$ | Greatest depression in TOF-R after magnesium administration |
| $\Delta T1$ | Greatest depression in %T1 after magnesium administration |

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# 1. INTRODUCTION

This thesis concerns magnesium's ability to reinstate drug-induced paralysis (known as neuromuscular block) after apparently adequate recovery, through its action on the functional contact point of the nerve and muscle (neuromuscular junction). Intravenous magnesium sulphate has many therapeutic applications in the pre-, intra- and post-operative period. Unfortunately, due its ability to potentiate neuromuscular block produced by neuromuscular blocking drug used during anaesthesia, its use is generally avoided. When magnesium interacts with these drugs, it tends to leave a degree of residual muscle weakness postoperatively, and thus its use in the perioperative period is often advised against. This is due to residual muscle relaxation after general anaesthesia being associated with respiratory complications, especially when an endotracheal tube is not supporting ventilation or protecting the airway from aspiration of vomitus.

The use of pharmacological (drug-based) methods to reverse neuromuscular block has for over 50 years involved enhancing the chemical signal from the nerve to the muscle and overpowering the neuromuscular blocking drug, with a drug known as neostigmine. A recently developed pharmacological method of reversing neuromuscular block induced specifically by rocuronium and to a lesser extent by vecuronium, involves the use of a modified cyclodextrin molecule to bind the neuromuscular blocking molecule irreversibly in the blood stream, often likened to a molecular 'vacuum cleaner' specific to the drug.

Studies (animal or clinical) comparing magnesium-induced reinstatement of neuromuscular block (recurarisation) between the different pharmacological regimes of reversing neuromuscular block are lacking in the literature and magnesium has such potential for use during and after general anaesthesia, that it was important to investigate whether recurarisation could be prevented or the effect size reduced by using a particular method of reversal.

This text presents a study investigating the mechanism of the phenomenon of magnesium recurarisation and how it differs between different neuromuscular block reversal strategies.

## 1.1. The Neuromuscular junction

The neuromuscular junction (NMJ) is point of contact of the nervous system to a skeletal muscle fibre. Here, chemical transmission of the neural signal across a synapse is necessary to produce an action potential on the muscle cell membrane (sarcolemma), which downstream initiates the process of muscle contraction.

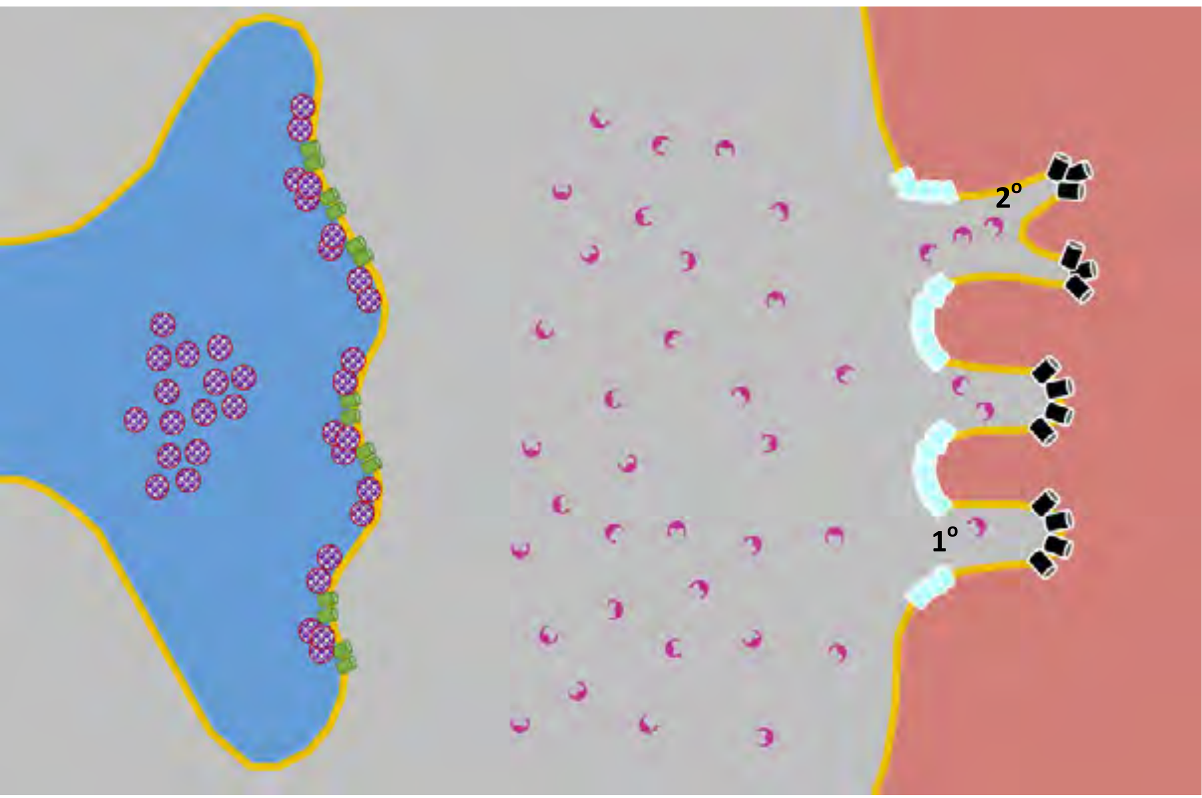



**Figure 1:** Schematic of the neuromuscular junction. The prejunctional division is made up of nerve terminal containing the ACh-filled synaptic vesicles ( ) located at the active zones adjacent to voltage-gated calcium channels ( ). The neuromuscular cleft is made up of a basement membrane (not depicted), extracellular matrix and the acetylcholinesterase enzyme ( ). The postjunctional division is made up of primary (**1°**) and secondary (**2°**) folds of the postjunctional (muscle) membrane, nicotinic cholinergic receptors ( ) and voltage-gated sodium channels ( ). (Adapted from Martyn et al. 2015)[1]

### 1.1.1. Morphology

The neuromuscular junction is composed of the axon terminal of the motor neuron (presynaptic component), the synaptic cleft (synaptic component) and the skeletal muscle sarcolemma. (Figure 1)

The motor neuron is a large myelinated axon originating from the spinal cord. As the axon approaches the muscle it branches in order to innervate multiple skeletal muscle fibres, which are then collectively known as a motor unit. Where the axon terminal reaches the muscle membrane it branches again and loses its myelin sheath. The Schwann cells then associate with the axon terminal for support. Active zones are areas in the presynaptic terminal where large amounts of acetylcholine (ACh) containing vesicles are clustered, ready to be released.

The nerve and muscle membrane are aligned tightly by the basal lamina of the neuromuscular junction.

The sarcolemma at the neuromuscular junction is corrugated, forming invaginations (folds) at both a primary and secondary level. The primary peaks of the primary folds are in line with the active zone of the prejunctional terminal (Figure 1).

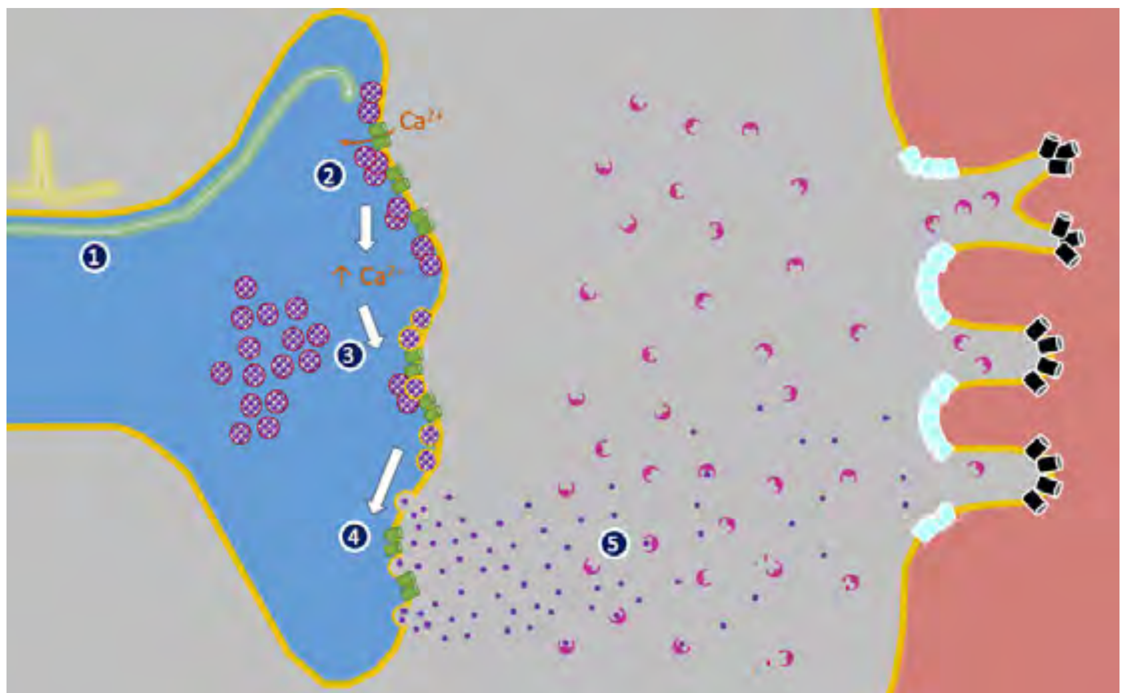



**Figure 2:** Diagram depicting prejunctional events related to neuromuscular transmission. 1) Action potential arrival at the motor terminal depolarises the prejunctional membrane adjacent to the active sites. 2) Voltage-gated $Ca^{2+}$ channels respond to the depolarisation by opening, which results in calcium influx. 3) The resulting increase in calcium concentration acts on the primed ACh-containing vesicles to produce fusion of the vesicle and cell membrane. 4) This produces expulsion of the contents of the vesicle (exocytosis), and 5) diffusion of ACh across the neuromuscular cleft.

### 1.1.2. Nerve action potential

As an action potential arrives at the axon terminal, depolarising membrane potentials open voltage-gated calcium channels leading to ACh release (Figure 2). The amount of ACh released is greatly dependant on the magnitude of the calcium influx, which in turn is dependant on the frequency of action potentials received from the cell body.

The voltage-gated calcium channels in the presynaptic terminal are heavily concentrated at the active zone to ensure release of neurotransmitter upon calcium influx. (Figure 1)

The calcium channels responsible for quantal (released from vesicle through exocytosis) transmitter release in mammals are primarily of the P-type [2-4], and are located directly adjacent to the active zones [5]. (Figure 1)

In addition to calcium channels, potassium channels are also present that rapidly hyperpolarise the membrane to limit the duration the calcium channel open time [6].

Due to the strong reliance of vesicle fusion on calcium influx, the blockade of the P/Q-type calcium channels by other bivalent cations such as magnesium (as well as the aminoglycoside antibiotics) can lead to a reduction in the quanta of ACh released [7, 8].

### 1.1.3. Vesicle release and recycling

ACh-containing vesicles in the prejunctional element exist in two pools - the reserve pool and the readily releasable pool (Figure 1). The readily releasable pool of vesicles are bound to the active zones, docked and primed, awaiting the arrival of an action potential. The vesicles of the reserve pool are bound to the cytoskeleton of the nerve terminal, unable to release their content until they are translocated to the active zones.

Upon action potential-induced calcium influx, calcium is sensed by the vesicle membrane associated protein synaptotagmin, which leads to fusion of the vesicle and cell membranes [7, 9]. This forms a pore between the interior of the vesicle and the extracellular space, allowing ACh to be expelled into the synaptic cleft through a process known as exocytosis. (Figure 2)

The full description of the synaptic vesicle cycle is beyond the scope of this dissertation, however a comprehensive review of the underlying mechanisms is described by Siegelbaum et al. [9].

In certain conditions, such as high frequency stimulation, the vesicles of the reserve pool can be called upon to move into the readily releasable pool for to replace depleting vesicles, in a process known as translocation. The initial mechanism for activating translocation is thought to be mediated by high frequency stimulation increasing intracellular calcium concentration due to the influx outcompeting efflux. This leads to activation of $Ca^{2+}$-dependant enzymes that would break the vesicle-cytoskeletal bonds allowing vesicles to migrate and dock into the readily releasable pool. Prejunctional (nicotinic and muscarinic) receptors [10] also induce translocation of reserve pool vesicles to the active zones, as a means to sustain vesicle output during high frequency stimulation. The receptors often activate the same second messenger pathways discussed above.

Some of these enzymes include: $Ca^{2+}$-Calmodulin and its associated protein kinase (CaMKII) [11-13], protein kinase A [14-16], protein kinase C [11, 16-18] and phospholipase C [19].

Replenishing ACh vesicles is also essential for continued transmitter release. Reuptake of choline by presynaptic reuptake transporters and synthesis of acetate needs to occur before ACh is replenished by acetyltransferase and filled vesicles are transported to the active zone once more [20].

**Figure 3:** Diagram depicting postjunctional events related to neuromuscular transmission. At the postjunctional membrane, when two ACh molecules (●) bind the nAChR ( ), the receptor ion channel opens, creating a MEPP through sodium influx, which when summated to reach threshold at the secondary folds activates voltage-gated sodium channels ( ) which produces an action potential that is propagated down the muscle membrane and into the myocyte.

### 1.1.4. Receptors of the neuromuscular junction

Propagation of neuromuscular signalling is primarily by the action of nicotinic cholinergic receptors.

The nicotinic cholinergic receptors (nAChR) are primarily heterogenic (made up of multiple different subunits) at the neuromuscular junction, with their subunits forming an ion channel (i.e. Inotropic receptor) that is permeable to sodium and potassium ions and (to a minor degree) calcium ions [1].

The muscarinic receptors (mAChR) are metabotropic, G protein-coupled receptors, that when activated initiate second messenger systems to produce more prolonged changes in the cell.

Purinergic receptors respond to purine nucleotides (ATP) or nucleosides (adenosine).

#### 1.1.4.1. Postjunctional receptors

The cholinergic receptors of the postjunctional division of the NMJ are of the nicotinic type, and are concentrated at the peaks of the primary folds of the muscle membrane. The postsynaptic nicotinic receptors are those responsible mainly for the reception of the neural signal onto the muscle membrane, and activation of which is generally considered to mediate the twitch height of a muscle contraction. The junctional isoform (normal adult type) of these receptors are composed of two alpha subunits, one beta subunit, one delta subunit and an epsilon subunit ($\alpha_2\beta\delta\varepsilon$) [21]. The alpha subunit contains the binding site for ACh and thus the postjunctional nicotinic receptors contain two binding sites for ACh. It is well established that binding of two ACh molecules simultaneously is necessary for activation of this channel [22].

When the receptor is activated, there is a conformation change that opens the ion channel, allowing free flow of cations. This results in sodium and calcium influx and potassium efflux, which creates short lasting miniature end plate potentials (MEPPs). These will summate with neighbouring MEPPs produced by adjacent receptors to form an end plate potential (EPP) which will propagate into the secondary folds of the sarcolemma. If the summated EPPs are sufficient to reach threshold, voltage-gated sodium channels in the troughs of the secondary folds will open to produce an action potential that propagates down the sarcolemma and ultimately initiates the contraction of the muscle cell [1]. (Figure 3)

It should noted that studies on rats [23] and mice [24] have both shown selective blockade of the postjunctional nAChRs by $\alpha$-bungarotoxin and $\alpha$-conotoxin GI to be capable of producing fade in twitch response to repetitive nerve stimulation, a phenomenon previously thought to be purely prejunctionally mediated.

### 1.1.4.2. Prejunctional receptors

The prejunctional receptors function to regulate the release of ACh during different frequencies of stimulation, either through inhibition or facilitation of vesicle release.

#### 1.1.4.2.1. Nicotinic receptors

The prejunctional nAChR is made up of three alpha subunits and two beta subunits ($\alpha_3\beta_2$) [1]. The prejunctional nAChRs function as a positive feedback system, facilitating increased mobilisation of the reserve pool of ACh vesicles into the active zone by breaking their cytoskeletal anchors. This occurs especially during high (50 Hz) frequency stimulation [12]. It has been suggested that activation of this receptor leads to increased calcium influx through activation of CaMKII.

#### 1.1.4.2.2. Muscarinic receptors

There also exist two mAChR subtypes at the prejunctional division of the NMJ. The mAChR subtype 1 (M1) is believed to facilitatory in nature, by activating PKC and PLC second messenger cascades which lead to an increase in calcium currents through the P/Q-type VGCCs [16, 18, 25], as well as increasing the translocation and priming of vesicles from the reserve pool to the readily releasable pool by modulating the vesicle-priming protein Munc13-1 [19]. The mAChR subtype 2 (M2) is believed to be inhibitory in nature by blocking the effect of M1 signalling on its targets [14, 15].

#### 1.1.4.2.3. Purinergic receptors

Receptors that respond to purines exist on the prejunctional membrane.

The natural ligand of the P2Y receptor is adenosine triphosphate (ATP). The function of the P2Y receptor appears to be modulation of non-quantal (spontaneous) transmitter release through phospholipase C activation [11, 26].

The $A_1$ and $A_{2A}$ receptors respond to adenosine as their ligand. It is known that they function together with prejunctional muscarinic receptors to modulate neurotransmitter release,

although the exact dynamics of interaction have yet to be conclusively determined [14, 15, 27-29].

### 1.1.5. Acetylcholinesterase

The catabolic enzyme of ACh, acetylcholinesterase (AChE), is a type B carboxylesterase enzyme that converts ACh into choline and acetic acid. It is produced in the muscle, beneath the endplate region, after which it is released into the neuromuscular junction, however still anchored to the basement membrane of the muscle cell by collagen fibres [21].

One AChE enzyme has six active capable of breaking down ACh. Each site has two active binding sites – namely the anionic and the esteratic sites. The quaternary nitrogen group on ACh binds the anionic site, while the esteratic site binds and forms a covalent bond with the carbamate group of ACh. Within 100 µs, hydrolysis of ACh has occurred and acetate and choline are release from the active site [30].

AChE is one of the most efficient catabolic enzymes known to mankind, with most literature citing that all released ACh molecules are invariably broken down 1 millisecond after it is released from the nerve terminal [1], and just under 50% of released ACh is broken down before it even reaches the postjunctional membrane [31].

### 1.1.6. Safety margin of the NMJ

The safety margin (or factor) of the neuromuscular synapse can be regarded as "an expression of how much greater an effect the nerve has on the muscle fibre than is required to generate an action potential" [32]. This is because the prejunctional component of the NMJ releases far greater amounts of ACh than that ultimately required to produce muscle contraction. The need for such a high safety margin stems from the NMJ's difference from central synapses. Muscles cells being innervated by only one motor neuron have only one input, in contrast to the central nervous system (CNS) where neurotransmission which is dependant on multiple inputs to form a signal.

## 1.2. Neuromuscular block

Neuromuscular block (NMB) is a pharmacological technique whereby skeletal muscle relaxation is obtained through inhibition of neuromuscular transmission.

### 1.2.1. Indications

Neuromuscular block is used when skeletal muscle relaxation is needed for a variety of clinical applications. The most common use is prior to endotracheal intubation, where indicated, usually at induction of general anaesthesia. Relaxation is usually maintained in order to allow smooth mechanical ventilation of the lungs during surgery or in the intensive care unit. Neuromuscular block additionally provides favourable operating conditions for surgery, such as lack of abdominal muscle tone for laparoscopic procedures [33] or orthopaedic surgery [34]. It also can be used to suppress any muscle movements for delicate procedures for example as seen in ophthalmology, although it should be emphasised that neuromuscular block is never a replacement for balanced anaesthesia.

### 1.2.2. Classification

A neuromuscular blocking agent (NMBA) can be of either the depolarising or non-depolarising type, depending on the mechanism of inducing neuromuscular block. Since this study focuses on non-depolarising NMB, for the purpose of ease, when referring to NMB in this text, non-depolarising neuromuscular block is implied.

Either the chemical class they belong to or their duration of action generally classifies NMBAs.

Beyond the introduction chapter of this thesis, when a NMBA used in this study is referred to, rocuronium is implied.

#### 1.2.2.1. Chemical class

Most NMBAs can be classified (with examples of clinically available agents) into either the benzylisoquinoline class (atracurium, cisatracurium and mivacurium), or the aminosteroid class (pancuronium, vecuronium and rocuronium).

#### 1.2.2.2. Duration of onset and duration of effect

NMBAs are also divided into long acting (defined as acting longer than 50 minutes), intermediate acting (between 20-50 minutes) and short acting (10-20 minutes).

Long-acting NMBAs include pancuronium and d-tubocurarine. Intermediate-acting NMBAs include vecuronium, rocuronium, atracurium and cisatracurium. The only short-acting non-depolarising NMBA is mivacurium [34].

### 1.2.3. Pharmacodynamics

The non-depolarising neuromuscular blockers are competitive antagonists of the nicotinic cholinergic receptors, primarily of those found in the neuromuscular junction. By acting as an antagonist to even one of the binding sites, the receptor is prevented from being activated even if ACh occupies the other binding site. This makes the neuromuscular blockers antagonists of the nicotinic cholinergic receptors. Binding of NMBA to the postjunctional receptors is probably the most important mechanism to produce neuromuscular block [35]. On the other hand, it should be noted, that a large proportion of the postsynaptic receptors (70-75%) could be blocked, with no measureable impairment of neuromuscular transmission [36]. This however reflects the high safety factor of neuromuscular transmission rather than the potency of the NMBA.

Binding of the NMBAs has been shown *in vitro* on Xenopus laevis oocytes expressing the prejunctional nAChRs [37]. This is often cited as the mechanism by which fade of twitch height was observed during repetitive stimulation. However, despite evidence of binding to the receptor, selective blockade of the prejunctional nAChRs was not able to produce tetanic or TOF fade in rats [23], without also decreasing the safety margin with elevated magnesium concentrations.

### 1.3.4. Pharmacokinetics

Since it is the only NMBA used in this study, the pharmacokinetics of only rocuronium will be discussed. Rocuronium is excreted unchanged in the urine (10-25%), and the liver (>70%) by a carrier-mediated active transport mechanism [34]. Its onset time is approximately 90

seconds with a standard dose (0.9mg/kg) with an average duration of action of 40 minutes [38].

## 1.3. Neuromuscular monitoring

In order for an anaesthetist to responsibly administer a balanced anaesthetic (including muscle relaxation), the depth of NMB should be able to be deepened or maintained prior to functional recovery of muscle tone or spontaneous breathing, which might otherwise provide unfavourable operating conditions for the surgeon [33]. Additionally, most NMB reversal agents, in order to be most effective, should be administered only after a certain degree of spontaneous recovery from NMB [39, 40]. Following reversal, it would also be prudent to ensure the neuromuscular transmission had recovered to a satisfactory level prior to extubation, and before discharge to the post-anaesthesia care unit (PACU). All of these scenarios require us to be able to measure depth of neuromuscular block.

### 1.3.1. Methods of neuromuscular monitoring

There are three generally accepted categories of neuromuscular monitoring measures: clinical measures of muscle weakness (clinical neuromuscular monitoring), qualitative measurement of evoked muscle twitches (qualitative neuromuscular monitoring) and quantitative measurement of evoked muscle twitches (quantitative neuromuscular monitoring).

#### 1.3.1.1. Clinical neuromuscular monitoring

Clinical measures of muscle weakness include evaluating the ability of the patient to: smile, speak, or perform a sustained head (or leg) lift for five seconds. A study by Cammu et al. [41] clearly illustrates the inadequacies of these measures in their sensitivity, specificity, positive and negative predictive values. Despite these findings, the use of clinical measures is still common for determination of time of reversal agent administration as well as extubation [42-47].

#### 1.3.1.2. Measurement of evoked muscle twitches

These methods work on the principle of a controlled stimulation of a peripheral nerve producing a contraction of the innervated muscle. Fade of this muscle response with repetitive nerve stimulation is a measure of neuromuscular transmission, and thus depth of neuromuscular block.

##### 1.3.1.2.1. Qualitative neuromuscular monitoring

Qualitative neuromuscular monitoring makes use of peripheral nerve stimulators to evoke muscle twitches, with the clinician measuring fade in muscle response with their own senses. This can be accomplished by the clinician either visually – by observing changes in the movement produced by the muscle contraction, or tactilely – by, for example, placing their hand on the thumb to detect twitch strength [48]. Three stimulation patterns can be used in qualitative monitoring. Fade in the muscle twitch response to Train-of-Four (TOF) stimulation (discussed below), tetanic stimulation and double burst stimulation are all used to evaluate moderate to mild neuromuscular block. Inconsistency in the ability to detect fade (by these methods) at clinically relevant depths of neuromuscular block (TOF-R<0.9) has been demonstrated in many studies [49-55], illustrating the unreliability of these subjective measures.

##### 1.3.1.2.2. Quantitative neuromuscular monitoring

The use of transducers to measure the magnitude of the muscle response to nerve stimulation has grown to become the standard for neuromuscular monitoring. This is most likely due to the lack of variability compared to qualitative measures, as well as the objective values that can be used as guides to timing of reversal and extubation.

###### 1.3.1.2.2.1. Method of twitch detection

There are multiple methods of detecting the evoked muscle twitch produced from nerve stimulation, all of which vary in their reliability, practicality and ease of use. Within the scope of this study and other studies discussed herein, mechanomyography (MMG),

electromyography (EMG) and acceleromyography (AMG) will be discussed, however alternative methods do exist, such as: kinemyography and phonomyography. For more detail on these methods, see the review by Fuchs-Buder et al. [56].

###### 1.3.1.2.2.1.1. Mechanomyography (MMG)

This technique involves the measurement (by a force transducer) of change in tension on a preloaded muscle (isometric) produced by the muscle twitch. Its main advantage is its high sensitivity, coupled with low background noise (with proper technique). However, it requires considerable effort to set up since the hand (adductor pollicis is the muscle most often used) needs to be immobilised with a preload applied to the muscle. This makes this method bulky to set up in an operating theatre, with any movement of the setup potentially disturbing the recordings. However, in experimental models of neuromuscular transmission and NMB, MMG has remained the gold standard for decades [57].

###### 1.3.1.2.2.1.2. Electromyography (EMG)

This method relies on the measurement of compound action potentials across the affected muscles that are produced by nerve stimulation. Its advantages include the ability to measure from a much larger variety of muscles, not requiring a preload to the muscle and relatively compact and quick setup. Disadvantageous include the need for thorough preparation of the skin underlying the recording electrodes as well as maintaining a near constant skin temperature, so as to ensure good a quality signal. Poor quality or drift of the signal can also occur, as well as interference from nearby electronic devices [58].

###### 1.3.1.2.2.1.3. Acceleromyography (AMG)

This method uses a piezo-electric crystal attached to an extremity moved by a single muscle (usually the thumb and abductor pollicis) and will detect the isotonic acceleration associated with evoked muscle twitches through nerve stimulation. Its advantages include easy setup and the lack of need for preload or immobilisation. Disadvantages include being limited mostly for use on the adductor pollicises, with the precision of its ability to measure

neuromuscular block decreasing with smaller muscle movements [58]. Most modern day neuromuscular monitoring devices utilise this method (e.g. TOF Watch®, Organon, Roseland, NJ, USA).

###### 1.3.1.2.2.2. Stimulation patterns and measures

The use of different stimulation parameters and measures allows detection of different depths of neuromuscular block. In this section a review of the measures involved in this study will be described, namely single twitch height (T1) and the Train-of-Four Ratio (TOF-R). For a compete review of possible neuromuscular measures, see the review by Fuchs-Buder et al. [56]

###### 1.3.1.2.2.2.1. Single twitch (T1)

This is a single pulse delivered to the nerve applied either manually or at a maximum frequency of 1 Hz. Impairment of neuromuscular transmission will result in suppression of the twitch height. In order to effectively monitor neuromuscular block depth, calibration of the device is needed prior to any NMBAs in order to measure twitch height as a percentage of the baseline value (%T1). While suppression of T1 is a good indicator of moderate neuromuscular block, it is insufficient to detect shallow or residual neuromuscular block. Generally, suppression of single twitch height is considered the result from blockade of postjunctional nAChRs, reflecting reduced recruitment of motor units.

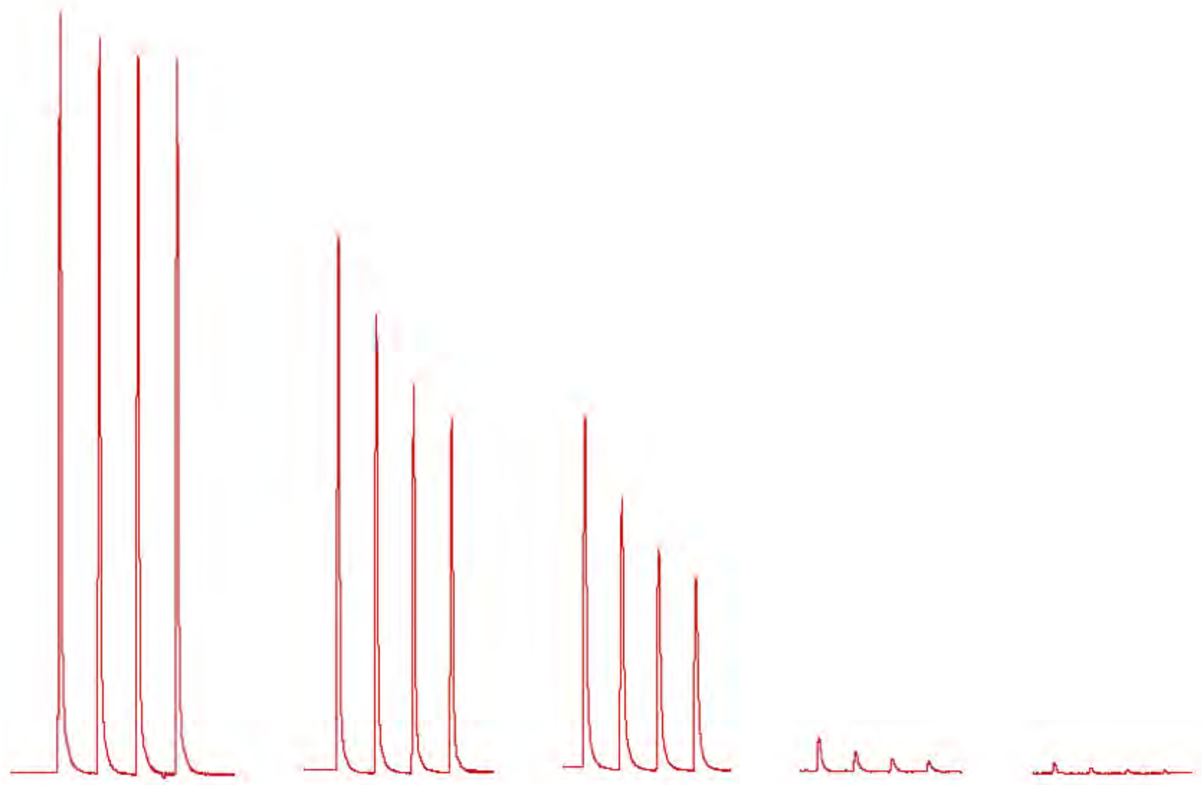


**Figure 4**: Traces illustrating Train-of-Four fade, with the $4^{th}$ and $5^{th}$ train's twitches generally being considered undetectable. Diagram adapted from recordings from this study.

1.3.1.2.2.2.2. Train-of-Four ratio (TOF-R)

Train-of-Four (TOF) stimulation is characterised by four stimulations over 2 seconds (2 Hz for 2 seconds). This train can be repeated at intervals of every 15 seconds or longer, or manually triggered. The measure that TOF stimulation produces is Train-of-Four fade ($TOF_{fade}$), which is observed as a smaller twitch height with each subsequent twitch of the train. (Figure 4) This can be quantified with the TOF-R by dividing the T4 (twitch 4 height) by the T1 (twitch 1 height), giving one a value between 0 and 1, which can then be used as an index of neuromuscular transmission. It is generally considered that $TOF_{fade}$ is a prejunctional phenomenon due to reduced ACh release upon each stimulation [1, 21, 35, 59, 60]. Blockade of the prejunctional cholinergic receptors would inhibit the facilitation of translocation of ACh vesicles from the reserve pool to the active zone, thus making fewer cholinergic vesicles available for release with each subsequent twitch of the train.

However, recent *in vivo* studies in rodents have shown that selective antagonists of postjunctional nicotinic cholinergic receptors are also able to produce $TOF_{fade}$, while

selective antagonists of the prejunctional nicotinic receptors were not able to produce fade, except when combined with magnesium [23, 24].

The TOF-R has become the standard of detecting residual neuromuscular block (rNMB), as well as defining satisfactory recovery from NMB. Volunteer studies have shown an impaired hypoxic ventilatory drive and profound muscle weakness symptoms at a TOF-R of 0.7, decreased pharyngeal muscle function, decreased upper airway volumes and forced inspiratory flow rates at a TOF-R of 0.8 and impaired oesophageal sphincter tone at a TOF-R of 0.9 [61]. From these and other studies described [61], governing bodies within the field of anaesthesia generally agree that a TOF-R of greater than 0.9 indicates satisfactory recovery from neuromuscular block that would allow the patient to breath spontaneous and protect their airway.

It is important to reiterate that it has been shown with a TOF-R equal to 0.9, up to 75% of post-synaptic receptors on the motor end plate may remain occupied by relaxant molecules, reflecting the safety margin of the NMJ [36], and thus recovery to 0.9 (or 90% as often stated) does not imply 90% removal of the NMBA from the NMJ.

### 1.4. Neuromuscular block reversal

Given neuromuscular block's exclusive need to facilitate optimal surgical operating and ventilation conditions, its presence postoperatively is generally unnecessary, unpleasant for the patients and potentially harmful. Therefore, after NMB is no longer needed, methods have been developed to ensure restoration of neuromuscular transmission to allow recovery of spontaneous ventilation, protection of the airway and voluntary muscle control [40].

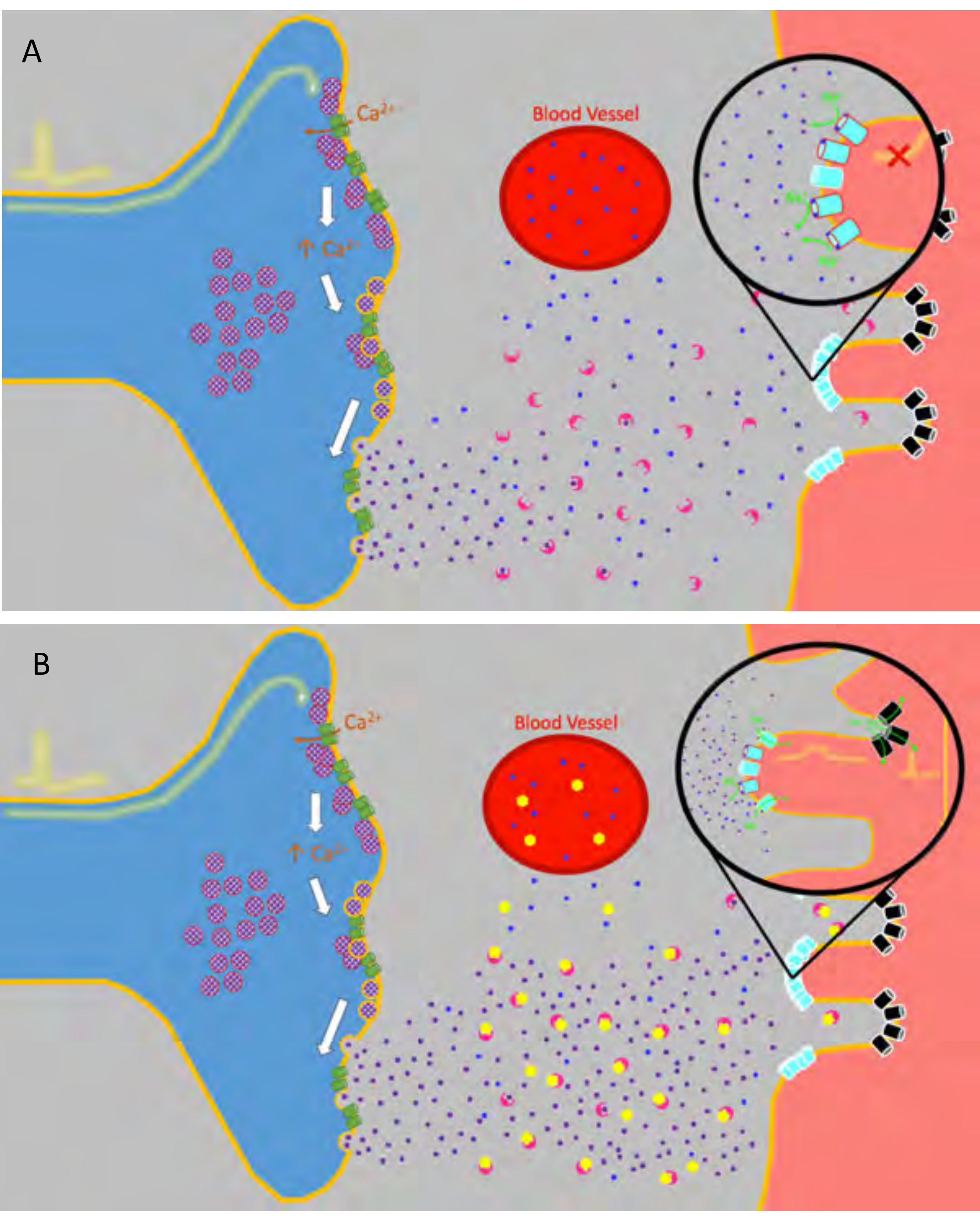



**Figure 5**: Mechanism of AChE inhibitor-induced reversal of NMB. **(A)** Prior to reversal the NMBA (●) dominates the competitive occupation of the postjunctional nAChR. (**B)** Inhibition of the catabolic enzyme of ACh by neostigmine (⬢) leads to an accumulation (and thus increase in concentration) of junctional ACh, which can competitively displace NMBA molecules from the nAChR and reinstate neuromuscular transmission.

### 1.4.1. Acetylcholinesterase inhibitors

The discovery that inhibition of the enzyme AChE could antagonise the neuromuscular impairment of curare-like drugs was first described in an in vivo frog model [62].

Suppression of AChE's activity leads to a reduction in the breakdown of ACh, thus elevating the concentration of ACh in the neuromuscular cleft. This elevated concentration makes ACh competitive for binding to the receptor, effectively displacing the NMBA from the binding site, and thus reinstating neuromuscular transmission. Drugs such as neostigmine and pyridostigmine (being oxydiaphoretic AChE inhibitors) mimic ACh in their binding, however the covalent bond formed is stronger than that formed with ACh. This leads to a vastly longer occupation of the active site (neostigmine's half-life of binding to AChE can be in the order of 7 minutes). During this time ACh cannot be bound or broken down, thus elevating its junctional concentration [40]. (Figure 5)

It is important to note that this mechanism does not increase the removal of the NMBA from the neuromuscular junction [30]. Any alteration of ACh concentration after AChE inhibitor reversal could potentially allow the NMBA to rebind the receptor and reinstate NMB.

Due to the AChE inhibitor's inability to inhibit ACh breakdown at the NMJ selectively, cholinergic transmission is elevated systemically, with the exception of the central nervous system, as the agents used for neuromuscular block reversal are not able to penetrate the blood brain barrier. This systemic increase in ACh leads to widespread parasympathetic activation – accounting for the side effect profile of AChE inhibitors including bradycardia, increased respiratory secretions and gastrointestinal mobility. To counteract these effects, whenever NMB is reversed with AChE inhibitors, an antagonist to the muscarinic receptor, such as atropine or glycopyrrolate, is administered [30].

### 1.4.2. Selective relaxant binding agents

The only clinically available drug is sugammadex (Bridion®, MSD). Sugammadex is a $\gamma$-cyclodextrin molecule, a cyclic ring of sugar molecules with a central pore.

The discovery that neuromuscular block could be reversed through encapsulation and binding of a NMBA was first described in 2002 [63, 64].

The exact pharmaco-chemical interactions that underlie the encapsulation and binding of the steroidal NMBA are covered in detail by Epemolu et al. [64], as they are beyond the scope of this study.

Selective encapsulation and irreversible binding of rocuronium (and other aminosteroid NMBA) by sugammadex in the plasma creates a concentration gradient of rocuronium between the plasma and the extracellular fluid of the NMJ. This gradient facilitates diffusion of unbound rocuronium from the NMJ and into the plasma, where if there are still unbound sugammadex molecules it will also be bound. This essentially makes sugammadex a soluble plasma bound receptor that is able to draw the neuromuscular blocking agent away from the nicotinic receptors (i.e. substantially lowering the concentration of rocuronium in the NMJ). This allows restoration of neuromuscular transmission by reinstating ACh's competitive binding to the nicotinic receptors, without altering its concentration in the NMJ. (Figure 6)

It should however be emphasised that in order to reverse neuromuscular block satisfactorily, not every molecule of rocuronium needs to bound by sugammadex or even extracted from the NMJ. Only enough rocuronium needs to be bound to reduce the junctional concentration sufficiently to allow ACh to bind (or effectively competitively displace rocuronium) and activate 25 – 30% of the postjunctional receptors, as this is all that is needed to produce a TOF-R > 0.9, the standard of adequate NMB reversal.

The use of acyclic cucurbit[n]uril-type molecules has recently been described to be able to bind neuromuscular blocking agents (both steroidal and benzylisoquinoline) and reverse neuromuscular block *in vivo* [65, 66], potentially being the second drug to join this class of neuromuscular blocking agents. Data on human use, however, is not currently available.

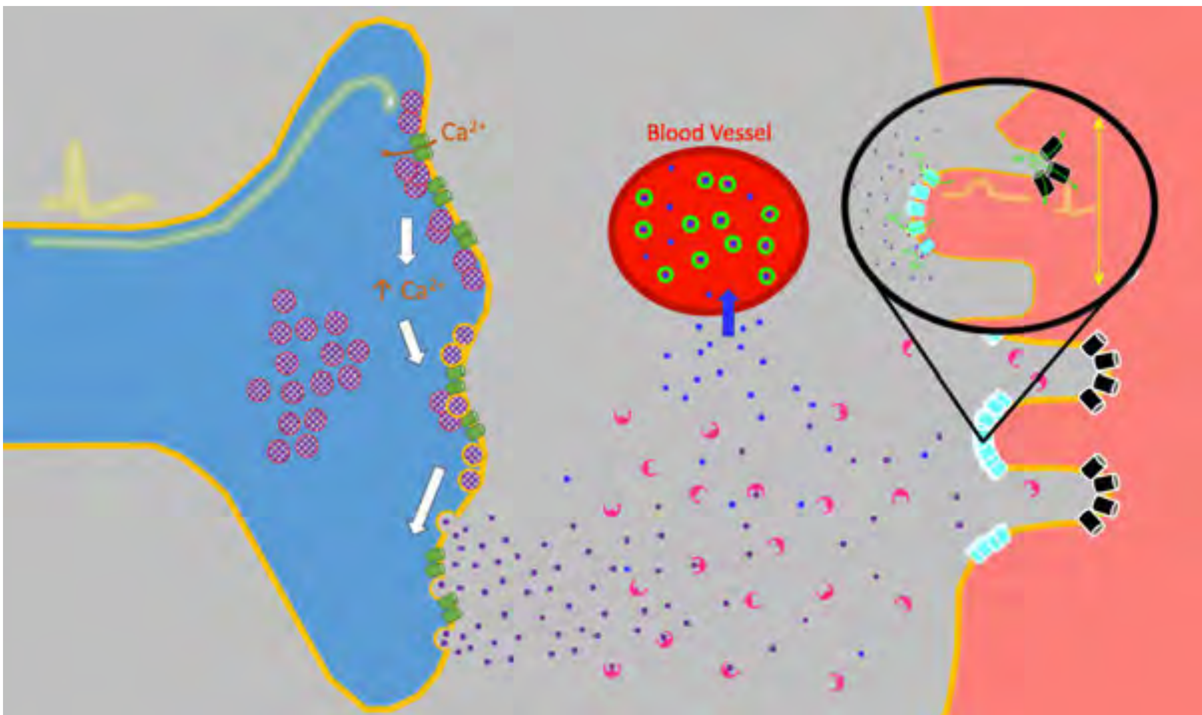



**Figure 6:** The mechanism of sugammadex reversal of rocuronium neuromuscular block. After sugammadex (O) is administered, rocuronium in the intravascular compartment is encapsulated and bound. This creates a rocuronium concentration gradient between the blood and the NMJ, promoting rocuronium movement out of the NMJ. This frees up the postjunctional receptors to allow ACh to rebind the receptor and reinstate neuromuscular transmission.

## 1.5. Residual neuromuscular block (rNMB)

An integral component of the safe and responsible practice of administering anaesthesia is ensuring the restoration of neuromuscular transmission after muscle relaxation is no longer required. rNMB can thus be defined as an impairment of neuromuscular transmission caused by inadequate recovery from neuromuscular block. This is most common postoperatively in the post-anaesthesia care unit (PACU) or even later on the ward [61].

It is thus imperative for clinicians to be able to monitor NMB depth, and to have measures within said method that indicates satisfactory recovery of neuromuscular transmission. This would allow extubation and/or discharge to the PACU of the patient with a degree of confidence in the recovery from neuromuscular block.

A variety of measures have been described in the above section: 'Neuromuscular Monitoring'.

### 1.5.1. Incidence

There have been many studies on the incidence of rNMB in hospitals across the world reporting varied results. A review by Murphy & Brull [61] compared the incidence of rNMB (definition TOF-R<0.9) reported by 15 studies published between 2000 and 2008, and found the range to be between 3.5 and 85%. The large range reported is believed to be primarily due to the differences in intraoperative (choice of NMBA, routine reversal etc.) management between the studies.

Even since the publication of the above review, and various editorials [67-71] emphasising the importance of preventing rNMB, the incidence persists in recent literature.

A multicentre study in Portugal conducted in 2013 reported an incidence of 26% in the PACU [72]. A 2015 Canadian multicentre study reported rNMB to occur in 68.5% and 56.5% of patients at time of extubation and time of arrival at the PACU respectively [73]. A prospective audit of 64 children who received NMBAs in an Australian tertiary hospital found rNMB incidence to be 28.1% [74]. An observational study of 415 patients who received intermediate-acting NMBAs in 2010 observed a rNMB incidence of 43% [47]. A multicentre observational Chinese study of 1571 adult patients showed rNMB to have an incidence of 57.8% [75].

### 1.5.2. Risk factors/causes

The absolute risk factor for rNMB is obviously the use of NMBA. A landmark study of 599,548 surgical patients between 1948 and 1952 found that mortality rates were increased six-fold with the use of NMBA [76], which since then has almost certainly been attributed to rNMB [61]. Additionally the use of long acting NMBA were more likely to produce rNMB than intermediate or short acting agents [39].

Risk factors thereafter seem to be related primarily to the perioperative management of the patient.

Inadequate reversal of neuromuscular block was found to result in inadequate recovery of neuromuscular transmission. A study on 526 patients in who the intubating dose of NMBA was not reversed found an rNMB incidence in the PACU to be 45%. A study found that

patients who were routinely reversed had a lower incidence of rNMB compared to patients in whom reversal was omitted [77].

Reversal of NMB should be based on objective endpoints, such as a specific TOF count or TOF-R. The recommended guidelines for dosing of neostigmine are: 0.07 mg/kg at a TOF count of 2 or 3 and 0.04 – 0.05 mg/kg at TOF-R > 0.4. It is recommended that at a TOF-R > 0.7, pharmacological reversal is omitted, due to the risk of AChE-inhibition impairment of neuromuscular transmission [40]. According to the dose recommendations provided by the manufacturer, sugammadex dosing is as follows: 2 mg/kg at a TOF count > 2, 4 mg/kg at 1 – 2 post-tetanic counts and TOF count = 0 and 16 mg/kg for reversal of rocuronium immediately after IV administration [78].

The class of reversal agent used appears to play a role in rNMB. Sugammadex reversal has been shown to reduce the incidence of rNMB compared to neostigmine [79] and the omission of NMB reversal [79].

### 1.5.3. Complications

The risks of postoperative morbidity and mortality have in the past largely been attributed to anaesthetic complications [76, 80, 81]. It was revealed that respiratory complications, such as respiratory depression or arrest and upper airway obstruction, are among the largest contributors to these postoperative events. As discussed above, the incidence of rNMB has been found to be high (although this varies from study to study) in the postoperative recovery room and theorised to be the major contributing factor [61]. Inadequate respiratory ventilation and impaired pharyngeal function are dangerous conditions in the postoperative period (especially if the patient has already been extubated, and airway protection is diminished), and can lead to hypoxia and aspiration pneumonia respectively. In addition to respiratory weakness, generalised skeletal muscle weakness would also most likely be evident (depending on the degree of rNMB), which could significantly impair their ability to mobilise on the ward. Injury to the patient aside, impaired neuromuscular recovery delays discharge from the PACU (and ultimately from the facility), a factor that contributes significantly to the costs of hospitalisations [82].

### 1.5.4. Prevention/management

Prevention of rNMB is predominantly aimed at reducing use of long-acting NMBAs, routine monitoring of NMB using quantitative methods (including intraoperatively – to dictate the need for top up of NMBA) and the appropriate reversal of NMB - i.e. appropriate dosing according to depth of NMB to be reversed [39, 61]. Additionally, the perioperative use of other drugs that act on the neuromuscular junction has been shown to produce rNMB, such as volatile anaesthetics [83-85] or magnesium sulphate [86-95].

## 1.6. Magnesium

Magnesium is an alkali earth metal, which takes the form of a divalent cation ($Mg^{2+}$) when in solution. It is a widely used ion in normal physiological (in all living things) processes, as well as medicine and just about any division of industry.

### 1.6.1. Physiology

The $Mg^{2+}$ ion is the fourth most common cation in the body, and second most common intracellularly. Less than 1% of all magnesium in the body (total body magnesium – TBM) however is found circulating in the blood - dissolved in serum (0.3% of TBM) or within erythrocytes. Serum magnesium can either be ionised (62% of serum $Mg^{2+}$), plasma protein bound (33% of serum $Mg^{2+}$) or bound to serum anions (5% of serum $Mg^{2+}$). The remaining magnesium is within the bone (53% of TBM) and the intracellular fluid (46% of TBM). Excretion of magnesium is performed and regulated by the kidney, where it is filtered through the glomerulus and reabsorbed by the ascending division of the loop of Henle [96]. Serum magnesium is also affected by Vitamin D which regulates the absorption of $Ca^{2+}$ (and with it, $Mg^{2+}$) from the gut [97]. Serum magnesium is maintained at approximately 0.7 – 1 mmol/L.

For a more comprehensive review of the systemic physiology of magnesium, see the review by Fawcett, Haxby and Male [96].

#### 1.6.1.1. Neuromuscular effects

It has been known since the early 1950s that magnesium was capable of negatively affecting neuromuscular transmission. Studies performed by del Castillo [98, 99], Engbaek [98], Katz [99] and Jenkinson [100] on the frog sartorius nerve-muscle preparation showed that magnesium produced a decrease in ACh release by competitively inhibiting calcium influx into the prejunctional terminal.

Further studies have also illustrated impairment in neuromuscular transmission (or neuromuscular block) with intravenous magnesium sulphate, as seen in a guinea pig hypogastric nerve-vas deferens preparation [101], pig MMG and EMG model [102] and in human parturients at clinically relevant doses [103].

The P- (and to a lesser extent Q-) type VGCCs are the primary VGCC at the neuromuscular junction of mammals [2, 3, 104-107]. It has been determined that the neuromuscular block produced certain members of the aminoglycoside class of antibiotics is mediated by competitive blockade of these calcium channels [108]. Magnesium reportedly produces similar impairment of calcium currents to those produced by the aminoglycoside antibiotics [8].

An *in vitro* electrophysiological study [109] related depression of prejunctional ACh release with repetitive nerve stimulation (fade) to reduced $Ca^{2+}$ influx, which in turn lowers the probability of vesicle release.

Taken together, it would be reasonable to assume that the depression of ACh release (and potentially the observed fade) produced by magnesium is by competitive inhibition of calcium influx through the P/Q-type VGCC, as is generally reported in the literature [110].

Another observation made of magnesium neuromuscular block in the 1950s was inhibition of postjunctional potentials and depression of muscle cell excitability, although these effects were described as minor compared to its prejunctional effects [99, 100]. A more recent study by Wang et al. possibly explains the inhibitory effect on postjunctional potentials. They were able to demonstrate that magnesium decrease the conductance of the postjunctional nAChR to the depolarising action of ACh [111] using an *in vitro* patch-clamp technique in cells expressing adult mouse muscle type nAChR. It should, however once again, be emphasised that magnesium concentrations considerably higher than those seen

clinically were needed to produce relatively low inhibition of conductance (6mmol/L to produce 20% inhibition).

### 1.6.2. Interaction with NMBAs

Magnesium's ability to potentiate the neuromuscular blocking effects of the curare-like drugs is also well documented; including shortening the duration of onset [112, 113], prolonging recovery [91, 92, 94] and reinstating NMB [89, 90, 93, 95].

The patch-clamp study mentioned above, by Wang et al., found magnesium to potentiate vecuronium-induced depression of conductance of postjunctional nAChR [111]. This potentiation effect was induced at clinically relevant doses of magnesium (1 and 3 mmol/L).

### 1.6.2. Clinical use

The use of the magnesium ion as a drug is close to ideal for a number of reasons.

It is already a component of nearly every living thing (including humans), meaning hypersensitivity is 'impossible'.

The human body has already developed physiological mechanisms to increase elimination of the ion through renal filtration, thus when administered in excess, excretion can be increased accordingly.

Clinically relevant side effects, as a result of hypermagnesaemia, only begin to appear at plasma concentrations of 5 mmol/L, well above the therapeutic range of 2 - 3.5 mmol/L [114]. These include muscle (including respiratory) weakness and CNS depression at plasma concentrations between 6 – 8 mmol/L and cardiac conduction abnormalities at 7 mmol/L [115]. It should however be noted that these toxicity levels are when magnesium is administered alone. Muscle weakness would most likely be observed at lower plasma levels in the presence of NMBAs or aminoglycoside antibiotics.

Finally, magnesium sulphate makes for an incredibly economical therapeutic. It's low cost being very simply explained by a quote by Professor M. James: "Magnesium is dirt cheap, because its made from dirt" [116].

#### 1.6.2.1. Indications

Magnesium sulphate ($MgSO_4$) has been used therapeutically for decades. Despite the wide variety of indications for which oral administration is applicable, this section will briefly focus on some of the conditions for which intravenous magnesium therapy is used that are applicable to the perioperative period.

The most common use of intravenous magnesium is in the management of pre-eclampsia and eclampsia, for which it is the first line treatment [117]. Target therapeutic plasma levels for management of eclampsia is 2 - 3.5 mmol/L and is usually given as an intravenous infusion of 4 – 6 g over 15-20 minutes, followed by 1 – 4 g/h [114].

The use of magnesium sulphate in the anaesthetic management of phaeochromocytoma resection was first described in 1985 by James [118]. Successful use of magnesium in phaeochromocytoma resections has continued to be reported by James [119-122] and others [123-127].

Magnesium has also been shown to be effective in the treatment of certain cardiac arrhythmias (especially in the perioperative period) such as torsade de pointes [128-134] and atrial fibrillation [135-137].

Additional uses for magnesium infusion include: hemodynamic control during intubation [138, 139] or after electroconvulsive therapy [140], as an adjuvant to opioids for postoperative pain management [141-143] and for severe manifestations of asthma where it is used as a second-line treatment [144-146].

### 1.6.3. Recurarisation

Recurarisation is a reinstatement of neuromuscular block, after a degree of recovery (or reversal) of neuromuscular transmission. Although there are many possible mechanisms to produce recurarisation, incidents described in the literature are mostly drug induced, with the most mentioned drugs being able to produce a decrease in ACh release, such as magnesium (described below) and the aminoglycoside antibiotics [147].

Another possible mechanism of recurarisation is if a long-acting NMBA is reversed with AChE inhibitor with a shorter duration of action [148]. This would cause junctional ACh concentrations to drop when the reversal agent wears off, allowing the NMBA to rebind the receptor.

The magnesium recurarisation effect was first reported in 1996 in isoflurane anaesthetised patients, after spontaneous recovery from vecuronium NMB [89]. In this study, magnesium sulphate 60 mg/kg was given either at the recovery of TOF-R to 0.7 or one hour thereafter. Significant depression of T1 and TOF-R was seen in the former group.

A case report published in the British Journal of Anaesthesia (2003), also reported recurarisation after magnesium administration in a neostigmine/glycopyrrolate-reversed patient [90].

Magnesium recurarisation following sugammadex reversal was described following what can be considered inadequate dosing of reversal and overdose of magnesium. In an *in vivo* guinea-pig model, rocuronium was reversed with 330 nmol/kg (approximately 0.7 mg/kg) sugammadex to a %T1 of 99% and was followed by 86.6 mg/kg of magnesium sulphate. This produced a decrease in %T1 of 97%. Ninety percent recovery was obtained after 34 minutes post-magnesium injection. This effect could be reduced to a reduction of 21% of %T1, and recovery after 13 minutes, by increasing the dose of sugammadex used for reversal two-fold [110]. This dose of magnesium used was far greater and the doses of sugammadex substantially less than those used in our study.

A 20-person study published in the European Journal of Anaesthesiology showed magnesium recurarisation after a dose of 50 mg/kg administered at a TOF-R of 0.9 in patients who had spontaneous recovered from rocuronium NMB [93]. This was an important repetition of the similar study conducted by Fuchs-Buder et al., as the definition of sufficient recovery from NMB had since then changed from a TOF-R > 0.7 [149-152] to > 0.9 [153, 154].

Unterbachner et al. published a case report wherein they reported magnesium recurarisation with a dose of 60 mg/kg of magnesium, after a patient was reversed with sugammadex [95]. Additionally, recovery of the TOF-R to baseline values was incomplete, as it plateaued out.

#### 1.6.3.1. Previous recurarisation data

In a previous study performed by the author of this thesis in 2012 using a similar rat model as described in the current study, recurarisation was observed upon administering 30 mg/kg magnesium sulphate after reversal of rocuronium neuromuscular block with either sugammadex (2 mg/kg) or neostigmine/glycopyrrolate, and showed no significant differences between the reversal groups [155]. The results of this study were perplexing at the time, as a lack of recurarisation was expected in sugammadex-reversed rats, on the basis of the sugammadex mechanism of removing residual rocuronium.

## 1.7. Problem identification

Presently, there are no controlled animal studies comparing magnesium recurarisation between all the current reversal regimes available. Our preliminary data [156] taken together with the case report published by Unterbachner et al.[95] brings into question the degree to which sugammadex is able to remove rocuronium from the NMJ, and the stability of neurotransmission after reversal to alteration in ACh release.

However, due to the unexpected result observed in the previous study, certain questions were raised that brought into question the validity of data:

Was the dose of magnesium administered (30 mg/kg) perhaps sufficiently high to produce neuromuscular impairment itself, instead of the result of reinstatement of rocuronium NMB? The highest intravenous dose of magnesium sulphate that did not produce detectable neuromuscular impairment in the absence of NMBA would need to be determined.

Was the dose of sugammadex used, albeit the standard dose, not high enough to sequester all the rocuronium from the NMJ? If the dose used for reversal was increased, would this result in a reduction in the magnitude of the recurarisation effect.

In order to fully characterise magnesium recurarisation, it was believed to be necessary to measure the magnesium recurarisation effect after neuromuscular block was omitted, in addition to when pharmacological reversal.

## 1.8. Aims

Based on the aforementioned preliminary data and the questions subsequently raised, the aims of the current are presented below.

### 1.8.1. Dose-effect experiment

To investigate the neuromuscular effects of a range of intravenous magnesium sulphate doses, in the absence of NMBAs, in this rat model of neuromuscular transmission, and determine the highest dose that does not produce detectable neuromuscular depression.

### 1.8.2. Magnesium recurarisation between reversal regimes

To investigate the magnesium recurarisation effect after neostigmine/glycopyrrolate, sugammadex (4 mg/kg) or sugammadex (16 mg/kg) reversal of rocuronium neuromuscular block, or if reversal is omitted and magnesium is administered after spontaneous recovery. The recurarisation effect would be further analysed in terms of its magnitude, its onset as well as the recovery from the recurarisation effect.

## 1.9. Hypothesis

### 1.9.1. Magnesium dose-effect experiment

$H_0$: Increasing dose of intravenous magnesium sulphate produces no observable depression of neuromuscular transmission

$H_1$: Increasing dose of intravenous magnesium sulphate produce a dose-dependant depression of neuromuscular transmission

### 1.9.2. Magnesium recurarisation experiment

$H_0$: No significant differences exist in the recurarisation produced by magnesium, between the different reversal regimes.

$H_1$: Significant differences exist between the recurarisation produced by magnesium, between the different reversal regimes.

# 2. MATERIALS & METHODS

## 2.1. Drugs

### 2.1.1. Supply

The following were supplied from Lakato (Pty) Ltd.:

| | |
|---|---|
| • Isoflurane | Isofor®, Safeline Pharmaceuticals |
| • Sodium Pentobarbitone 200 mg/mL | Euthanaze ®, Bayer Healthcare |
| • Neostigmine Methylsulphate 0.5 mg/mL | Bodene |
| • Glycopyrrolate 0.2 mg/mL | Robinul®, Bodene |
| • Magnesium Sulphate 50% Solution | Kyron Laboratories |

The University of Cape Town, Department of Anaesthetics, donated the following:

| | |
|---|---|
| • Rocuronium Bromide 10 mg/mL | Esmeron®, MSD |
| • Sugammadex 100 mg/mL | Bridion®, MSD |

### 2.1.2. Drug preparation and storage

#### 2.1.2.1. Magnesium Dose-effect Experiment

0.5 mL aliquots of Magnesium sulphate were prepared in 0.9% saline to concentrations of 0, 10, 30, 100 mg/mL in 1.5 mL labelled eppendorfs and stored at room temperature until use. On the day of use, the solution was drawn into a 1 mL syringe at least 3 hours before intravenous injection.

#### 2.1.2.2. Magnesium recurarisation experiment

50 mL of magnesium sulphate (30 mg/mL) was prepared in 0.9% saline and stored in a 50 mL centrifuge tube at room temperature. On the day of use, the solution was drawn into a 1 mL syringe at least 3 hours prior to intravenous injection.

10 mL of neostigmine/glycopyrrolate formulation (0.04 mg/mL/0.001 mg/mL) was prepared in 0.9% saline and stored in a 10 mL centrifuge tube at room temperature. On the day of use, the solution was drawn into a 1 mL syringe at least 3 hours prior to intravenous injection.

1 mL of rocuronium (0.5 mg/mL) was prepared in 0.9% saline in a 1.5 mL labelled eppendorf and drawn up into a 1 mL syringe at least 3 hours prior to intravenous injection, after which it was stored at 5°C. The syringe was removed from the fridge approximately 10 minutes prior to its anticipated injection, to allow it to equilibrate with room temperature. Rocuronium was prepared on the day of use and any unused prepared rocuronium was discarded 10 hours after dilution. Stock vials of rocuronium were stored at 5°C according to manufacturers guidelines.

1 mL of sugammadex (4 mg/mL or 16 mg/mL) was prepared in 0.9% saline in a 1.5 mL labelled eppendorf and drawn up into a 1 mL syringe at least 3 hours prior to intravenous injection and stored at room temperature. Sugammadex was prepared on the day of use and any unused sugammadex was discarded 10 hours after dilution.

## 2.2. Animals

Male Sprague-Dawley (SD) rats were used in this study weighing between 280 and 400g. Rats were housed under standard laboratory rat husbandry conditions with a 12-h light–dark cycle (100 – 300 lux) and allowed free access to commercial pellet food and tap water.

To limit possible confounding effect of hormonal fluctuations during the females' four-day oestrus cycle, and to reduce uncontrolled variables, only male SD rats were used in this study.

6 rats were used to refine surgical technique and characterise use of new and/or modified equipment. 25 rats were used for the magnesium dose-effect experiment. 25 rats were used for magnesium recurarisation experiment.

The University of Cape Town Animal Ethics Committee authorised this study. (AEC Reference: 013/006)

## 2.3. Surgical and pre-experimental preparation of the model

All rats underwent surgical and pre-experimental preparation of the model as outlined below, followed by the drug administration protocol of assigned experiment.

### 2.3.1. Anaesthesia

Figure 7 shows a diagram of the layout of the gas flow/anaesthetic setup.

Induction of anaesthesia was performed in an induction chamber (Figure 7: F) prefilled for 2 minutes with 4% isoflurane from a calibrated Isoflurane vaporiser (Isotec 4, GE Healthcare, Little Chalfont, UK) (Figure 7: D) in 100% oxygen at 2000 mL/min flow rate measured using a gas flow meter (Figure 7: C). Actual isoflurane concentration from vaporiser output was measured using an infrared anaesthetic gas analyser (Vamos, Dräger, Lübeck, Germany) (Figure 7:F). Induction of anaesthesia was rapid and judged by the loss of the righting reflex, upon which the rat was quickly removed from the induction chamber and transferred to the surgical platform, placed on it's back, with it's head placed within a nose cone – through which anaesthesia was maintained (Figure 7: J). The isoflurane dose was titrated to the surgical plane of anaesthesia, judged by loss of the pedal withdrawal reflex. The rat's body temperature was maintained by a thermostatically controlled heating plate (set at 37 $^{o}$C). Temperature feedback was through a probe placed between the animal and the metal plate.

Adequacy of oxygenation was ensured by monitored by ensuring sufficient oxygen gas flow and ventilation was satisfactory. Observation of the colour of the rat's mucous membranes was also monitored as an indirect measure of blood oxygen saturation.

Anaesthetic depth, by means of the pedal withdrawal reflex, was monitored throughout the surgical preparation procedure and recorded on a monitoring sheet (Appendix A-1), in accordance of guidelines agreed to with the UCT Faculty of Health Sciences Animal Ethics Committee.

### 2.3.2. Tracheostomy and Ventilation

Hair over the neck area was removed using a veterinary grade hair trimmer (Model D-4D, Andis Professional, Sturtevant, WI, USA). A midline incision was made along the ventral surface of the neck. Blunt dissection of fascia and sharp dissection of the sternohyoid muscle was used to expose and visualise the trachea. Using silk thread, (CliniSilk 3/0, CliniSut, Port Elizabeth, South Africa) a loose fitting ligature was placed around the trachea for quick securing of the tracheostomy tube, and a selection of polypropylene tracheostomy tubes (made in-house, Department of Human Biology, Cape Town, South Africa) were prepared beforehand and matched to the size of the trachea. An adapted 18G cannula was inserted into the tracheostomy tube to act as an introducer for insertion. An incision was made in the trachea using fine scissors, and the tracheostomy tube (Figure 7: M) was inserted and secured within the trachea by tying off the ligature rostral to the flared tip of the tube. (Figure 8: A) The tracheostomy tube was connected to the tracheostomy adaptor apparatus (Figure 7: L, Figure 8: C) (Described in Appendix A-2) and secured in placed with the tightening screws. Anaesthetic gas flow was directed from the nose cone to a rodent ventilator (Model 683, Harvard Apparatus, Holliston, MA, USA) (Figure 7: K), by adjusting tap 3 (Figure 7:I) and gas flow rate was decreased to approximately 200 mL/min. The ventilator was set to ventilate at a rate of 65 strokes per minute and a stroke volume of 1.5 – 1.75 mL. The stroke volume settings for average rat size used in this model were determined by direct observation of lung expansion during mechanical ventilation *in situ* in a rat of a previous study.

The isoflurane concentration was then adjusted gradually to the reappearance of the pedal reflex; after which it was increased to the last concentration to supress the pedal reflex.

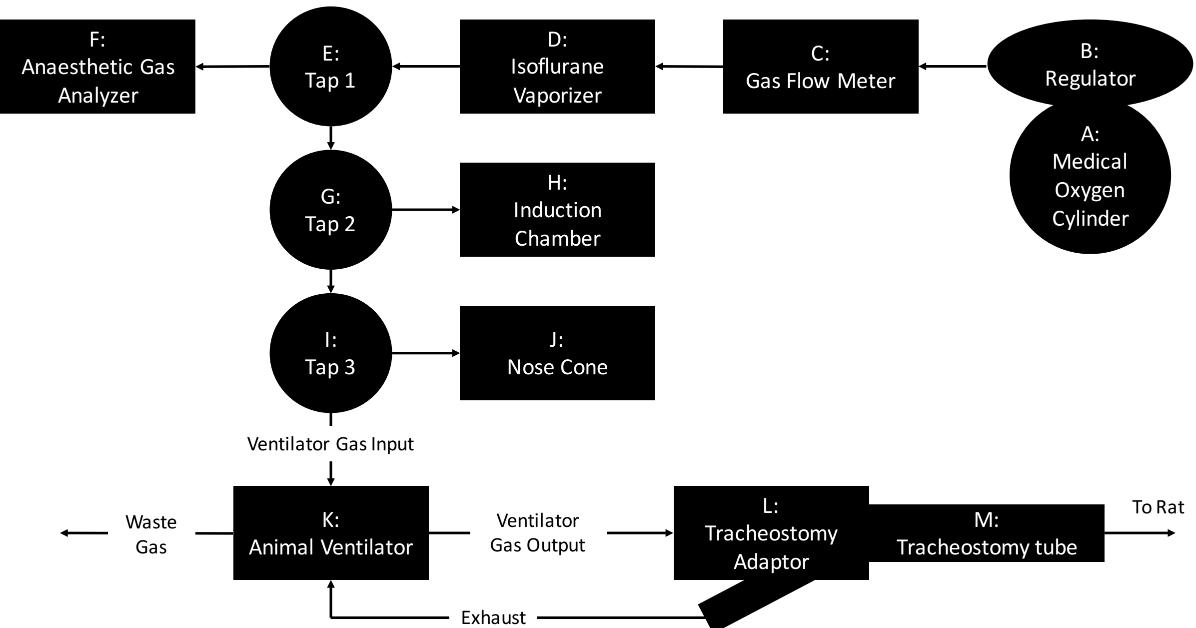



**Figure 7:** Flow Diagram to show Oxygen-Isoflurane gas flow setup used for induction and maintenance of anaesthesia. Oxygen cylinder **(A)** supplying oxygen to anaesthetic vaporiser **(D)** through regulator **(B)** and gas flow meter **(C)**. Tap 1 **(E)** directs a portion of the anaesthetic-oxygen mixture to either the anaesthetic gas analyser **(F)** while the remainder goes on to Tap 2 **(G)**. Tap 2 directs either to the induction chamber **(H)** or to Tap 3 **(I)**, which directs the mixture to either the nose cone **(J)** or the animal ventilator **(K)**. The animal ventilator supplies anaesthetic-oxygen mixture to the rat through the tracheostomy tube **(M)** connected to the adaptor **(L)**. End-tidal gas is removed through the ventilator by a one-way valve through the exhaust branch of the tracheostomy adaptor.

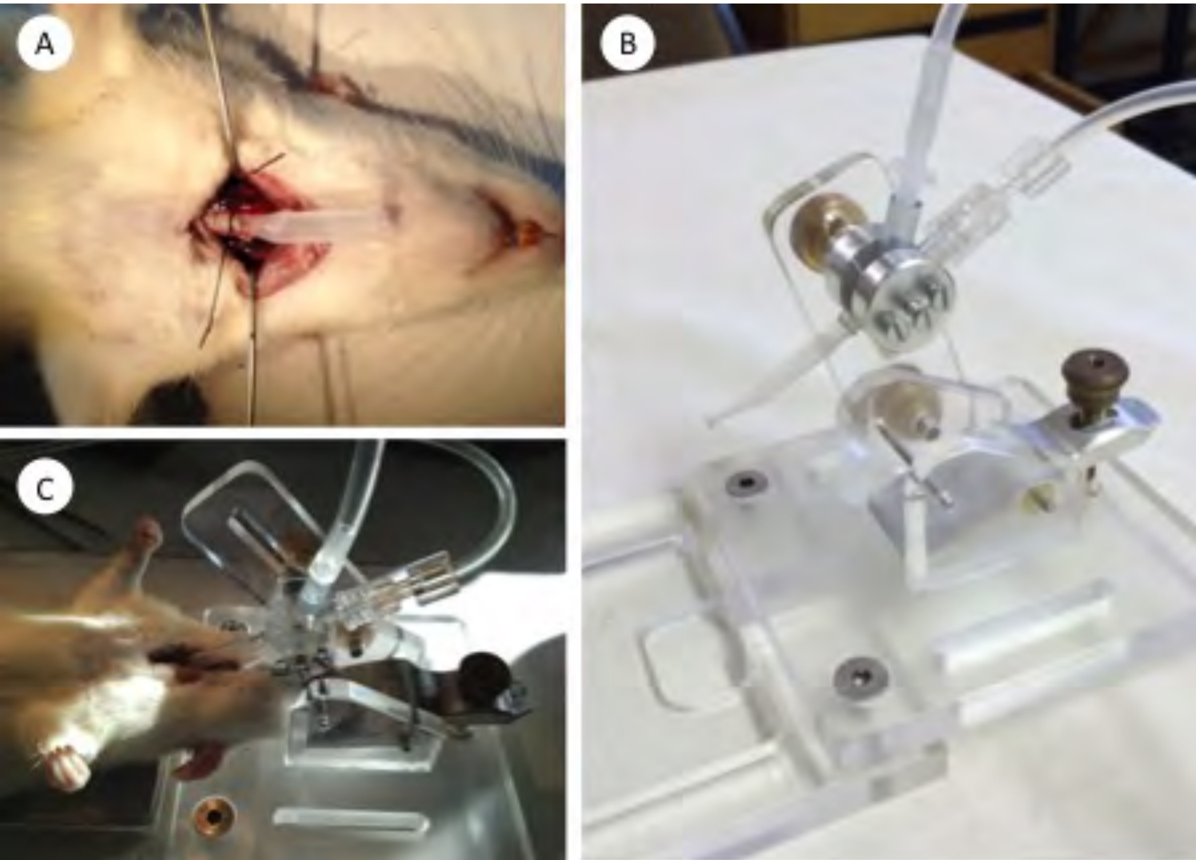


**Figure 8**: Tracheostomy and Mechanical Ventilation **(A)** A tracheostomy was performed and secured using a silk ligature. **(B)** The tracheostomy tube was connected to the tracheostomy apparatus. **(C)** This secured the tracheostomy in place, but allowed free movement of the rat during procedures and into the experimental box without disturbing mechanical ventilation.

### 2.3.3. Femoral vein cannulation

Hair over the ventromedial surface of the right hind limb was removed using a veterinary grade hair trimmer. An incision was made along the ventral surface of the right hind limb, and blunt dissection was used to isolate the femoral vein. Using 3/0 silk thread, proximal and distal ligatures were placed on the femoral vein. Using iridectomy scissors, a small snip was made in the vein, and a 24G winged intravenous catheter (Introcan-W, B Braun Medical (Pty) Limited, Northriding, South Africa) was introduced into the vein through the aperture. (Characterisation of this method is found in Appendix A-3) The catheter was secured in the vein by tying off the proximal ligature and was further secured to the vein using the distal ligature. The incision was closed with interrupted sutures using 3/0 silk suture in order to limit dehydration and to protect cannula from mechanical interference (Figure 9). The catheter was periodically flushed with 1 mL of saline to prevent clot formation and ensure

adequate hydration of the rat. All intravenous test drugs were administered through the femoral vein catheter followed by a saline flush of approximately 1.5 mL. This volume more than compensated for the cannula dead space, ensuring accurate delivery of the bolus dose.

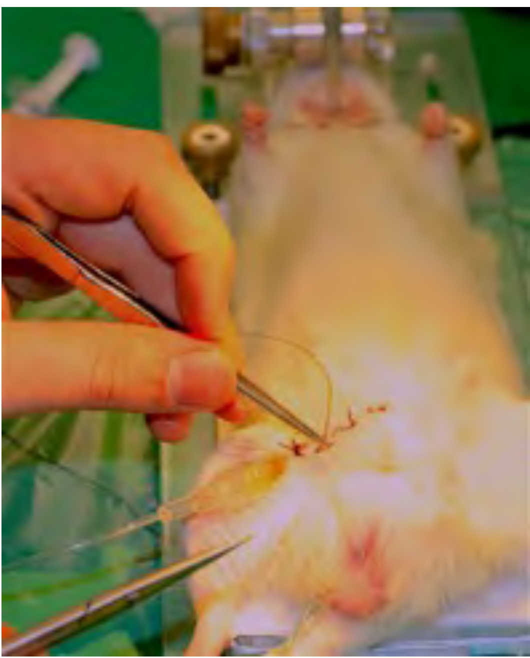


**Figure 9**: Securing of femoral vein cannula with interrupted sutures.

### 2.3.4. Sciatic nerve stimulation

Two wire hook electrodes were constructed from 1mm thick silver wire threaded into a 22G intravenous catheter and bent to form a hook (Appendix A-4). Hair of the left hind limb was removed using a veterinary grade hair trimmer. An incision was made along the skin of the groin and extended laterally towards the lower leg. Muscle separation was performed to expose the sciatic nerve in the gluteal space. The electrodes were hooked over the nerve and secured to underlying muscle using 3/0 silk suture. This ensured separation of electrodes, minimising short circuitry. (Figure 10)

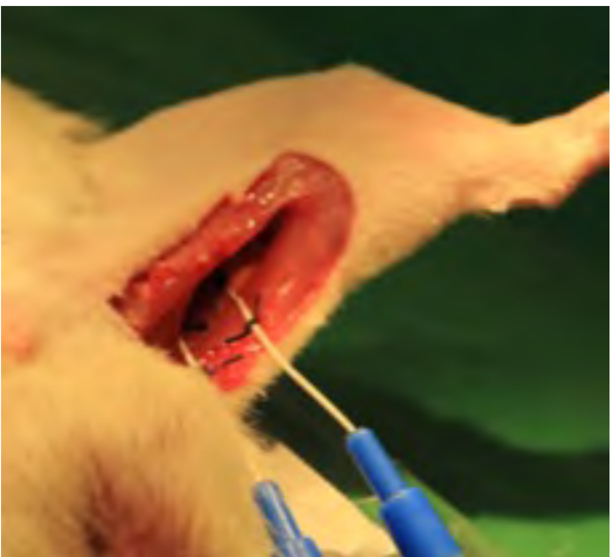


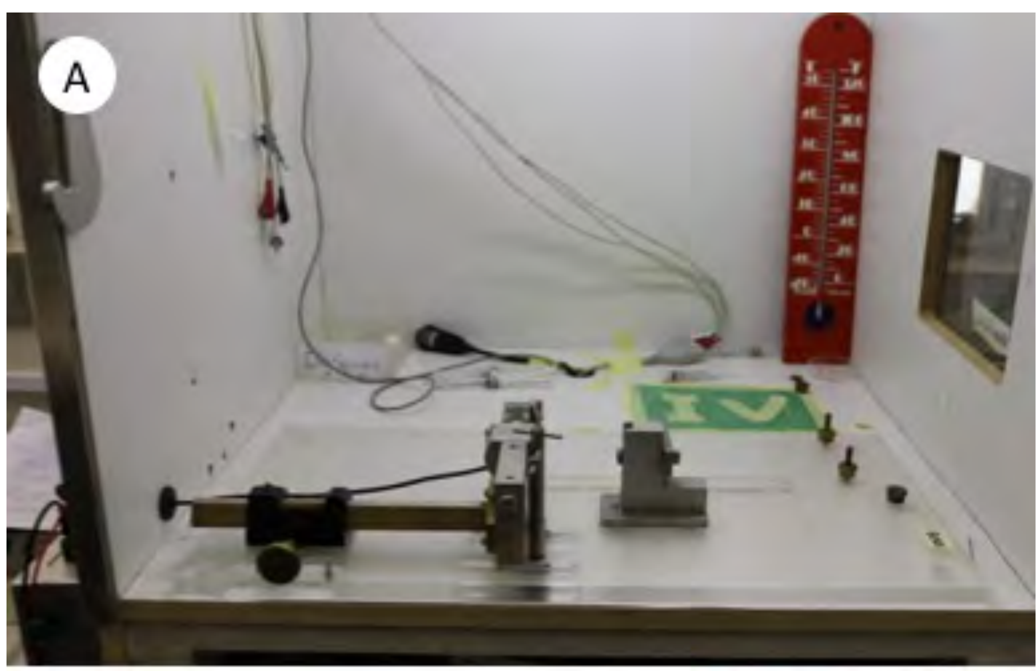
**Figure 10**: Sciatic nerve electrodes placed and secured to underlying muscle with silk sutures.

### 2.3.5. Tendon isolation

The left common calcaneal tendon was exposed and securely ligated with 3-0 silk thread. Ensuring secure tying of the ligature was crucial to prevent slipping of the ligature once connected to the force transducer and applied with a preload. The tendon was severed distally to the ligature and freed from immediate surrounding tissue.

### 2.3.6. Transfer and Setup

Once all surgical procedures had been completed the rat was transferred to a specifically constructed box housing the strain gauge force transducer (Human Biology Electronics Workshop). (Figure 11: A) The tendon was attached to the strain gauge needle by the ligature. A 20G needle was inserted through the tendon capsule of the knee joint, which was used to anchor the knee to the pivotal point. The pull against the strain gauge was adjusted to ensure moderate tension on the tendon. Nerve electrodes were connected to a GRASS Stimulator (Model S48, GRASS Technologies) through a stimulus isolation unit (Model PSIU6, GRASS Technologies). (Figure 11: B)



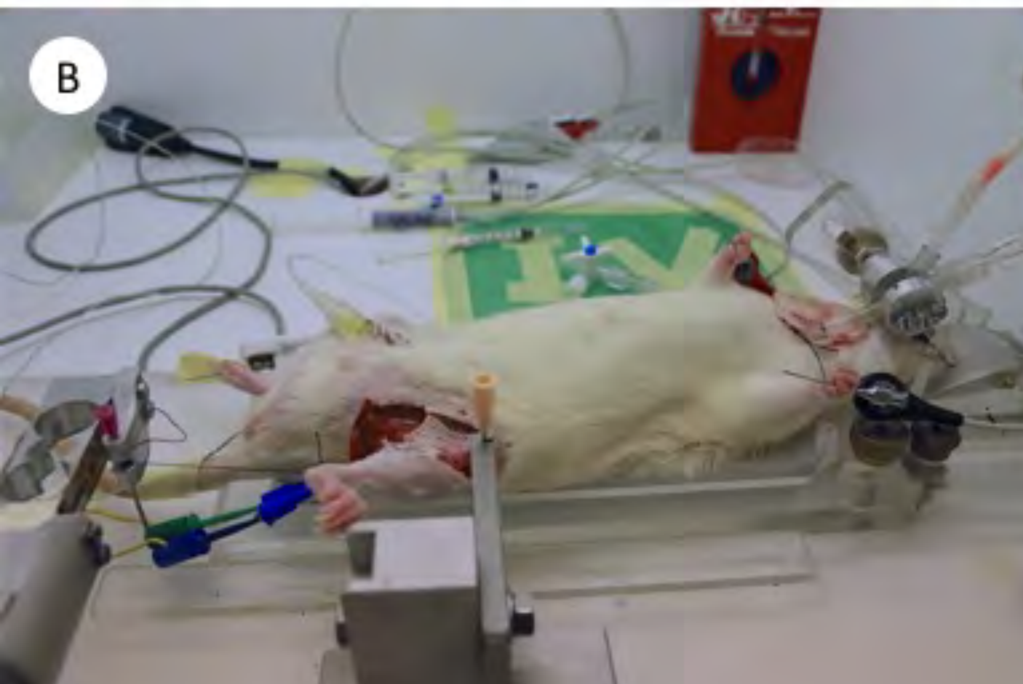
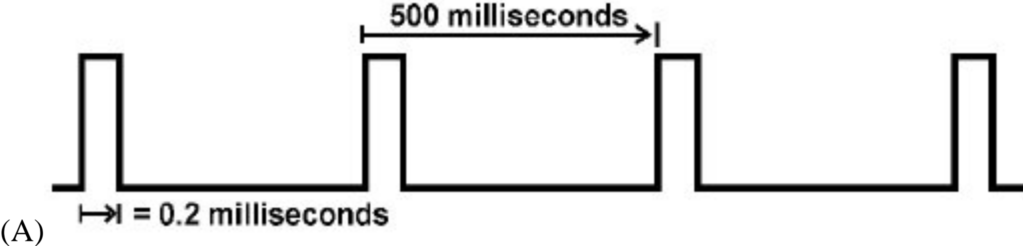




**Figure 11**: Experimental Box **(A)** Prior to transfer of rat – showing the strain gauge, pivotal point, ECG needles and connecting leads to the stimulus isolation unit. **(B)** After transfer of rat to experimental box – showing securing of the tracheostomy apparatus to the box, attachment of the ligature to the strain gauge, stimulus isolation unit connecting leads attached to electrodes and ECG electrodes set up in a Lead I orientation.

### 2.3.7. Train-of-Four stimulation

The GRASS stimulator was set to deliver Train-of-Four (TOF) stimulations (Figure 12: A) every 20 seconds. The force transducer was connected to a specifically constructed

amplifier (Department of Human Biology Electronics Workshop), which was connected to the Powerlab Unit (Model 26T, ADInstruments) for recording by LabChart 7 Pro software (ADInstruments). Detection settings and Channel calculation were set up to generate optimum muscle twitch recordings and real-time Train-of-Four Ratio (Figure 12: B) calculations. Muscle tension was adjusted using a ratchet mechanism attached to the strain gauge mount, and stimulation voltage was adjusted on the GRASS stimulator to generate maximal muscle twitch force with minimal baseline $TOF_{fade}$ of elicited muscle twitch heights. Voltage was then set to stimulate at twice the voltage needed to produce maximal twitch height in order to achieve supramaximal stimulation conditions.

(A) 


(B) $$Train - of - Four\ Ratio\ (TOF - R) = \frac{Twitch\ 4\ Height\ (T4)}{Twitch\ 1\ Height\ (T1)}$$

**Figure 12:** Train-of-Four stimulation and calculations **(A)** Train-of-Four nerve stimulation parameters. Train stimulation was repeated every 20 seconds. [picture taken from: http://symbiosbilling.com][56] **(B)** Equation for calculation of the Train-of-Four Ratio.

### 2.3.8. Electrocardiogram (ECG) / Heart Rate Calculations

A three lead ECG was set up using needle electrodes inserted into the foot pads and connected to the BioAmp port of the Powerlab. (Figure 11: B) ECG was analysed using LabChart Pro ECG Analysis Module (ADInstruments) to generate a 5 beat-averaged heart rate, from ECG Lead II, in real time. A baseline heart rate, during confirmed surgical plane of anaesthesia, was calculated based on a 5 minute ECG trace. Greater than 10% increase from the baseline value was used as an indicator for inadequate depth of anaesthesia, in the period where neuromuscular block was present thus suppressing any visible pain reflexes (for e.g. pedal twitches). Additionally a heart rate alarm was set up to alert when heart rate increased above 10% of the baseline rate, and would signal for an increase in isoflurane concentration. It should be noted however, that adjustment of the delivered anaesthetic

concentration was never needed in any of the experiments after transfer to the experimental box, indicating more than adequate anaesthetic depth during the period of neuromuscular block.

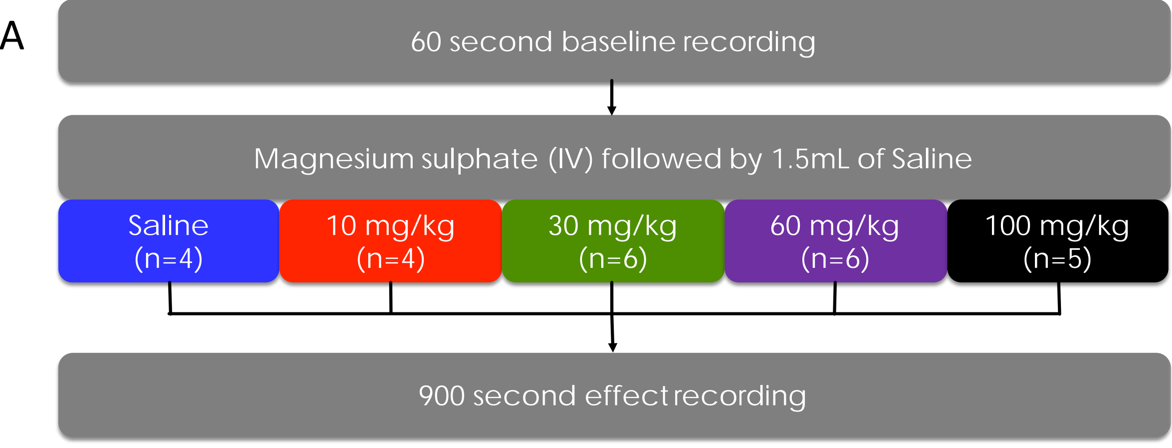
### 2.3.9. Animal Endpoint

After the experimental recording had ended and the nerve stimulation terminated, 200 mg/kg of sodium pentobarbitone was injected into the intravenous cannula extension tube followed by 1mL of saline flush. The ECG trace was observed for asystole. After 5 minutes of observed asystole the rat was cervically dislocated to confirm death.

All aspects of the surgical preparation and experiment protocol were executed with great care and specific attention.

## 2.4. Experimental drug protocols and recordings

A




B

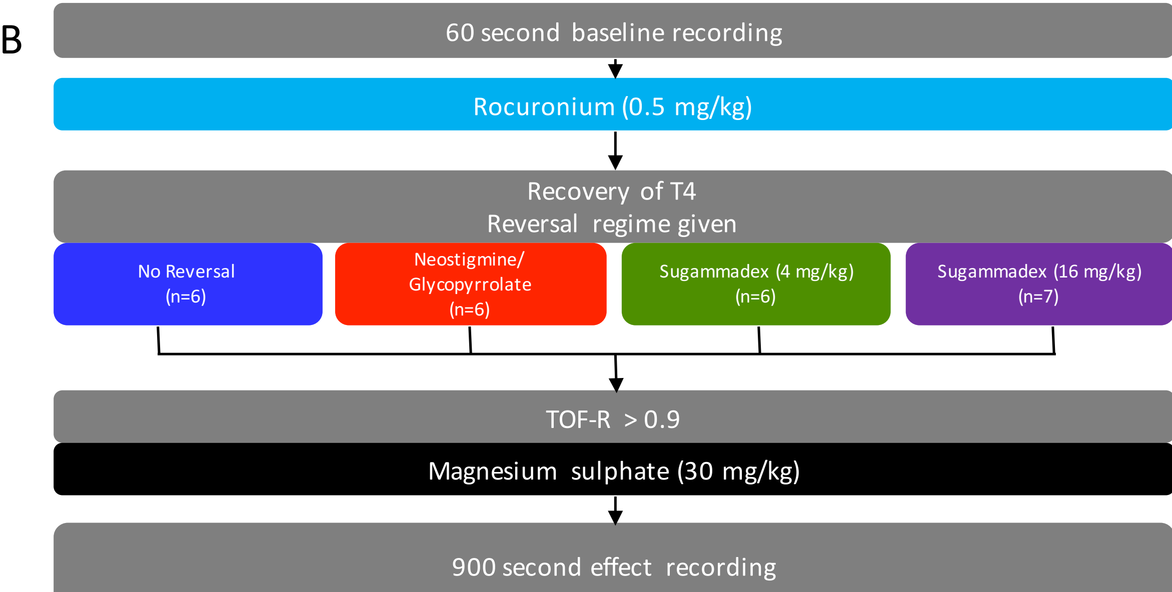



**Figure 13**: Group assignment to experimental drug protocols **(A)** Magnesium dose-effect experiment **(B)** Magnesium recurarisation experiment

### 2.4.1. Magnesium dose-effect experiment

Rats were assigned to 0, 10, 30 or 100 mg/kg dose group (Figure 13: A). After pre-experimental preparation and 60 seconds of baseline recording had been completed, 0.1 mL per 100 g body weight of the test solution was injected into the intravenous cannula extension tube. Using the LabChart software, a marker was placed on the recording and the intravenous line was flushed with 1.5 mL of saline over 15 seconds upon the start of the next TOF stimulation. The end of the experimental recording was signalled after 20 minutes after the injection of the saline flush, after which the Train-of-Four stimulation was stopped and the animal endpoint was reached.

### 2.4.2. Magnesium recurarisation experiment

Rats were assigned to one of four neuromuscular block reversal groups (NEO, SUG4, SUG16 and SPON) (Figure 13: B): neostigmine/glycopyrrolate combination (NEO), 4 mg/kg sugammadex (SUG4), 16 mg/kg (SUG16) or a group where reversal was omitted and whose NMB was allowed to recover spontaneously (SPON).

After pre-experimental preparation and 200 seconds of baseline TOF recordings had been completed, a comment marker was placed on the recording after which 0.1 mL per 100 g body weight (0.5 mg/kg) of rocuronium was injected into the intravenous cannula extension tube upon the next TOF stimulation. The intravenous line was flushed with 1.5 mL of saline over 20 seconds upon the start of the next TOF stimulation. The recording was observed for the disappearance of the fourth twitch of the TOF twitches.

When recovery of fourth twitch of the Train-of-Four twitches had occurred, a comment marker was placed on the recording after which 0.1 mL per 100 g body weight of the assigned neuromuscular block reversal agent was injected into the intravenous cannula extension tube. The intravenous line was flushed with 1.5 mL of saline over 15 seconds upon the start of the next TOF stimulation. In the case of the SPON group, where neuromuscular block reversal was omitted, no intravenous injection was made at this point.

When the Train-of-Four ratio had recovered to above 0.9 or had remained at a value at least > 0.85 for longer than 2 minutes, a comment marker was placed on the recording, after which 0.1 mL per 100 g body weight (30 mg/kg) of magnesium sulphate was injected into the intravenous cannula extension tube. The intravenous line was flushed with 1.5 mL of saline over 15 seconds upon the start of the next TOF stimulation.

Train-of-Four stimulation was stopped 15 minutes after the saline flush had been.

## 2.5. Data extraction

All collected data for each rat was exported to its own worksheet on a Microsoft Excel file entitled 'Raw Data', that contained the extracted data of all rats used for this study.

### 2.5.1. Rat, anaesthetic and surgical data

For both experiments, the following data was collected from the anaesthetic monitoring and surgical Record sheet (Appendix A-1): rat number, treatment group, rat weight, date of experiment, concentration of administered isoflurane stabilised after mechanical ventilation commenced, time of induction of anaesthesia and time of transfer to experimental box.

### 2.5.2. Recorded data

For both experiments files were saved and then duplicated to produce an analysis copy. In the analysis copy, recordings from before the baseline recordings and after animal endpoint were deleted.

LabChart's "multiple add to datapad" was used to automatically extract the following values from each TOF stimulation; time, T1 twitch height and train-of-four ratio. (Appendix B-3)

Time points of drug administration and spontaneous recovery of T1, T2, T3, and T4 were also determined if applicable.

## 2.6. Data analysis

Analysis workbooks were created in Microsoft Excel for the sole purpose of analysis of a specific extracted recorded parameter (i.e. filename: MgDoseResponse - %T1).

### 2.6.1. Magnesium dose-effect experiment

#### 2.6.1.1. Pre-experimental variables

These are variables that, if significantly different between the experimental groups, could potentially contribute towards differences observed in the outcome (hypothesis answering) variables.

The weight of the rat ($W_{rat}$), maintenance dose of isoflurane ($[Iso]_{ave}$) administered after starting mechanical ventilation, and the baseline heart rate ($HR_{base}$) prior to administering the magnesium were compared between experimental groups.

Time from induction of anaesthesia ($T_{anaesth}$) to administration of magnesium was calculated and compared between experimental groups to account for variations in duration exposure to the anaesthetic influencing the outcome variables.

The necessity for baseline correction of T1 eliminates the raw value of T1 (as measured in mV), and thus also the measure of tension (strength of contraction) produced by the muscle twitch. Comparison of the raw baseline T1 value ($T1_{raw}$) between experimental groups was performed to assess these potential variations.

### 2.6.1.2. Experimental parameters

#### 2.6.1.2.1. T1 baseline correction

A baseline average T1 was calculated from the 60 second recording prior to magnesium administration. All T1 values were then converted to a percentage of the averaged baseline value (%T1).

#### 2.6.1.2.2. Magnitude of neuromuscular impairment

The neuromuscular impairing effect of magnesium was measured by the lowest TOF-R ($TOF_{min}$) and %T1 ($\%T1_{min}$) reached within 200 seconds of administering the magnesium.

Additionally, the maximum decrease in TOF-R ($\Delta TOF$) and %T1 ($\Delta\%T1$) were calculated by subtracting the $TOF_{min}$ and $\%T1_{min}$ respectively from the corresponding value at the time of magnesium administration.

#### 2.6.1.2.3. Onset of neuromuscular impairment

The peak onset time of magnesium induced neuromuscular impairment was calculated from the difference in time from magnesium administration to the time of $TOF_{min}$ ($TOF_{onset}$) and $\%T1_{min}$ ($\%T1_{onset}$). Only dose groups shown to have produced neuromuscular impairment after magnesium administration were included in this analysis.

#### 2.6.1.2.4. End of experiment TOF-R

To assess the recovery profile from the impairment produced by the different doses of magnesium, the TOF-R ($TOF_{900}$) at the end of experimental period (i.e. 900 seconds after magnesium was administered) was calculated.

### 2.6.2. Magnesium recurarisation experiment

#### 2.6.1.1. Pre-experimental variables

The weight of the rat ($W_{rat}$), maintenance dose of isoflurane ($[Iso]_{ave}$) administered after starting mechanical ventilation, and the baseline heart rate ($HR_{base}$) prior to administering the rocuronium were compared between experimental groups.

Time from induction of anaesthesia ($T_{anaesthesia}$) to administration of rocuronium was calculated and compared between experimental groups to account for variations in duration exposure to the anaesthetic influencing the outcome variables.

The necessity for baseline correction of T1 eliminates the raw value of T1 (as measured in mV), and thus also the measure of tension (strength of contraction) produced by the muscle twitch. Comparison of the raw T1 value at the time of magnesium administration ($T1_{base}$) between experimental groups was performed to assess these potential variations.

To assess whether rats in different experimental groups were exposed to a similar degree of neuromuscular block, the spontaneous recovery from NMB was assessed and compared between the experimental groups.

The time taken from the administration of rocuronium to the recovery of the four TOF twitches (R-T1, R-T2, R-T3 and R-T4) to detectable levels was calculated.

The TOF-R value measured at the recovery of T4 (TOF@T4) was compared to assess the degree of neuromuscular transmission at the time of reversal agent administration (at the time of T4 recovery).

#### 2.6.1.2. Neuromuscular block reversal

To compare the differences in the rate of neuromuscular block reversal between the different reversal regimes, the time taken from recovery of T4 to magnesium administration (T4-Mg) was calculated and compared between the experimental groups, as this reflects the time from reversal to the recovery of TOF-R to greater than 0.9.

Given that the spontaneous experimental group did not receive a reversal agent, and in 3 rats did not even recover to a TOF-R > 0.9, the spontaneous group was excluded from this analysis.

#### 2.6.1.3. Magnesium recurarisation

The phenomenon of magnesium recurarisation was assessed in its magnitude and onset to maximum impairment (as measured by TOF-R and %T1) and its recovery profile (as measured by TOF-R).

##### 2.6.1.3.1. T1 baseline correction

The raw T1 value at the time of magnesium administration was recorded. All T1 values were then baseline corrected to a percentage of this value (%T1).

##### 2.6.1.3.2. Magnitude of recurarisation

The degree of impairment of neuromuscular transmission produced by magnesium administration was as the lowest TOF-R ($TOF_{min}$) and %T1 ($\%T1_{min}$) reached within 200 seconds of administering the magnesium.

In order to account for variation in the actual TOF-R at the time of magnesium administration (especially in the SPON group), maximum decrease in TOF-R ($\Delta TOF$) and %T1 ($\Delta \%T1$) were calculated by subtracting the $TOF_{min}$ and $\%T1_{min}$ respectively from the corresponding value at the time of magnesium administration.

#### 2.6.1.3.3. Onset of recurarisation

The peak onset time of recurarisation was calculated from the difference in time from magnesium administration to the time of $TOF_{min}$ ($TOF_{onset}$) and $\%T1_{min}$ ($\%T1_{onset}$).

#### 2.6.1.3.4. Recovery from recurarisation

In order to assess the degree to which neuromuscular transmission had recovered 15 minutes after magnesium was administered, the TOF-R at the end of the experiment ($TOF_{900}$) was calculated and compared between experimental groups.

To assess the time course of recovery from magnesium recurarisation (R-%) the time taken to recover 25% (R25%) and 50% (R50%) of the recurarisation magnitude was calculated and compared between experimental groups.

## 2.7. Statistical analysis

All descriptive statistics, normality testing and hypothesis testing was performed on Stata SE (Version 13, StataCorp, College Station, TX, USA).

### 2.7.1. Power analysis

A power calculation was performed on the basis of a preliminary study using this model in which 30 mg/kg magnesium sulphate produced a reduction in TOF-R from 0.9 to 0.5 (SD 0.19) after reversal of NMBA with neostigmine.  On the assumption that increased doses of sugammadex would prevent any recurarisation, this gave a sample size of 6 rats per group to test the primary hypothesis.

For the magnesium dose-effect experiment no power calculation was performed as it was an observational study, but agreement with the ethics committee that 6 rats per group should provide adequate data for the higher magnesium groups.  For the saline and 10 mg/kg groups, the data scatter proved to be so small that only 4 rats per group were used in order to minimise animal usage.

### 2.7.2. Normality

All experimental variables were tested for normality, using the Shapiro Wilk Test. Data were considered normally distributed if the p-value was greater than 0.05.

In the event Shapiro Wilk test returned a p-value less than 0.05, the data was log-transformed by converting each value to it's logarithm (log with base 10) and then reanalysed for normality. If the log-transformed data returned a p-value greater than 0.05, the data was considered normally distributed, and the log-transformed values were used for all parametric statistical hypothesis testing. If the log-transformed data returned a p-value less than 0.05, the data was considered not normally distributed, and the non-transformed values were used in non-parametric statistical hypothesis testing.

### 2.7.3. Descriptive statistics and graphs

For each variable, the mean, standard deviation (SD), standard error of the mean (SEM) and sample size (n) was calculated.

Within the results section, in-text description of results was reported as mean ± SD, while all graphs illustrated data as mean with the error bars representing the SEM.

### 2.7.4. Comparison between groups

#### 2.7.4.1. Parametric tests

In the event of a variable being normally distributed, parametric tests were used to determine if there were statistically significant differences between experimental groups.

If comparing greater than 2 experimental groups for a particular variable, a one-way ANOVA was used to assess if there were differences between the compared groups ($p<0.05$). If the ANOVA returned a p-value of less than 0.05, a Bonferroni posthoc test was applied to determine between which groups the differences were significant ($p<0.05$).

If comparing only two experimental groups for a particular variable, an unpaired t-test was used to assess if differences in the means of the compared groups were significant ($p<0.05$).

This test was only used to test significant differences in the $TOF_{onset}$ and $T1_{onset}$ variables of the magnesium dose-effect Experiment.

#### 2.7.4.2. Non-parametric tests

In the event of a variable not being normally distributed, non-parametric tests were used to determine if there were statistically significant differences between experimental groups.

If comparing greater than 2 experimental groups for a particular variable, a Kruskall-Wallis ANOVA was used to assess if there were differences between the compared groups ($p<0.05$). If the Kruskall-Wallis ANOVA returned a p-value of less than 0.05, a Bonferroni posthoc test was applied to determine between which groups the differences were significant ($p<0.05$).

# 3. RESULTS

This chapter presents analysed and statistically tested outcome measures for the two experiments: Magnesium dose-effect and magnesium recurarisation. The results of the non-outcome measures for both experiments are outlined in Appendix D.

## 3.1. Magnesium dose-effect experiment

The magnesium dose-effect experiment assessed the capacity of different magnesium sulphate doses to produce impairment in neuromuscular transmission as measured by TOF-R and T1. However, it's aim for this project was to assess the impairment effect of the 30 mg/kg dose of magnesium sulphate in the absence of other neuromuscular blocking agents. This was in response to perplexing results obtained in a preliminary study conducted in this laboratory, showing magnesium-induced (30 mg/kg) recurarisation after sugammadex reversal of NMB, as outlined in '1.8. Aims'.

### 3.1.1. Magnitude of neuromuscular impairment

#### 3.1.1.1. TOF measures

The mean (±SD) $TOF_{min}$ obtained after the test dose of magnesium being administered was 0.925 (0.0036) for 0 mg/kg group, 0.943 (0.0263) for 10 mg/kg group, 0.935 (0.0189) for 30 mg/kg group, 0.696 (0.1413) for 60 mg/kg group and 0.452 (0.1507) for 100 mg/kg group. Significant differences were observed between the 100 mg/kg group and the groups 0 mg/kg ($p<0.001$), 10 mg/kg ($p<0.001$), 30 mg/kg ($p<0.001$) and 60 mg/kg ($p=0.006$), as well as between the 60 mg/kg group and the groups 0 mg/kg ($p=0.018$), 10 mg/kg ($p=0.009$) and 30 mg/kg ($p=0.004$). (Figure 14: A)

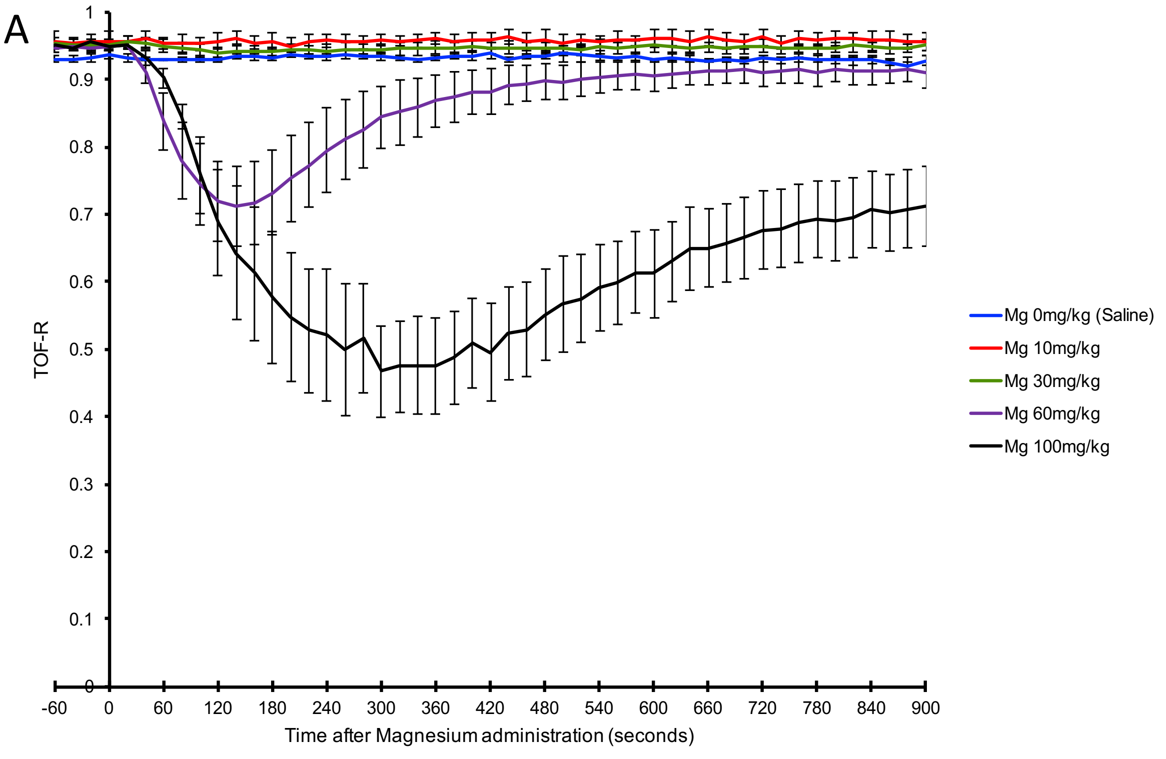
The $TOF_{min}$ achieved corresponded to a mean (±SD) $\Delta TOF$ (Figure 14: B) of 0.012 (0.0077) for 0 mg/kg group, 0.0142 (0.0032) for 10 mg/kg group, 0.017 (0.0155) for 30 mg/kg group, 0.2528 (0.1376) for 60 mg/kg group and 0.4979 (0.1551) for 100 mg/kg group. Significant differences were observed between the 100 mg/kg group and the groups 0 mg/kg

(p<0.001), 10 mg/kg (p<0.001), 30 mg/kg (p<0.001) and 60 mg/kg (p=0.005), as well as between the 60 mg/kg group and the groups 0 mg/kg (p=0.011), 10 mg/kg (p=0.012) and 30 mg/kg (p=0.005).

#### 3.1.1.2. T1 measures

The mean $\%T1_{min}$ obtained after the test dose of magnesium being administered was 90.73% (±1.544%) for 0 mg/kg group, 94.36% (±1.352%) for 10 mg/kg group, 93.69% (±2.082%) for 30 mg/kg group, 75.52% (±18.078%) for 60 mg/kg group and 41.45% (±18.128%) for 100 mg/kg group. Significant differences were only observed between the 100 mg/kg group and the groups 0 mg/kg (p<0.001), 10 mg/kg (p<0.001), 30 mg/kg (p<0.001) and 60 mg/kg (p=0.002). (Figure 15: A)

The $\%T1_{min}$ achieved corresponded to a mean $\Delta\%T1$ (Figure 15: B) of 8.48% (±1.726%) for 0 mg/kg group, 5.34% (±1.382%) for 10 mg/kg group, 5.70% (±1.551%) for 30 mg/kg group, 24.02% (±17.862%) for 60 mg/kg group and 58.02% (±18.145%) for 100 mg/kg group. Significant differences were observed only between the 100 mg/kg group and the groups 0 mg/kg (p<0.001), 10 mg/kg (p<0.001), 30 mg/kg (p<0.001) and 60 mg/kg (p=0.002).



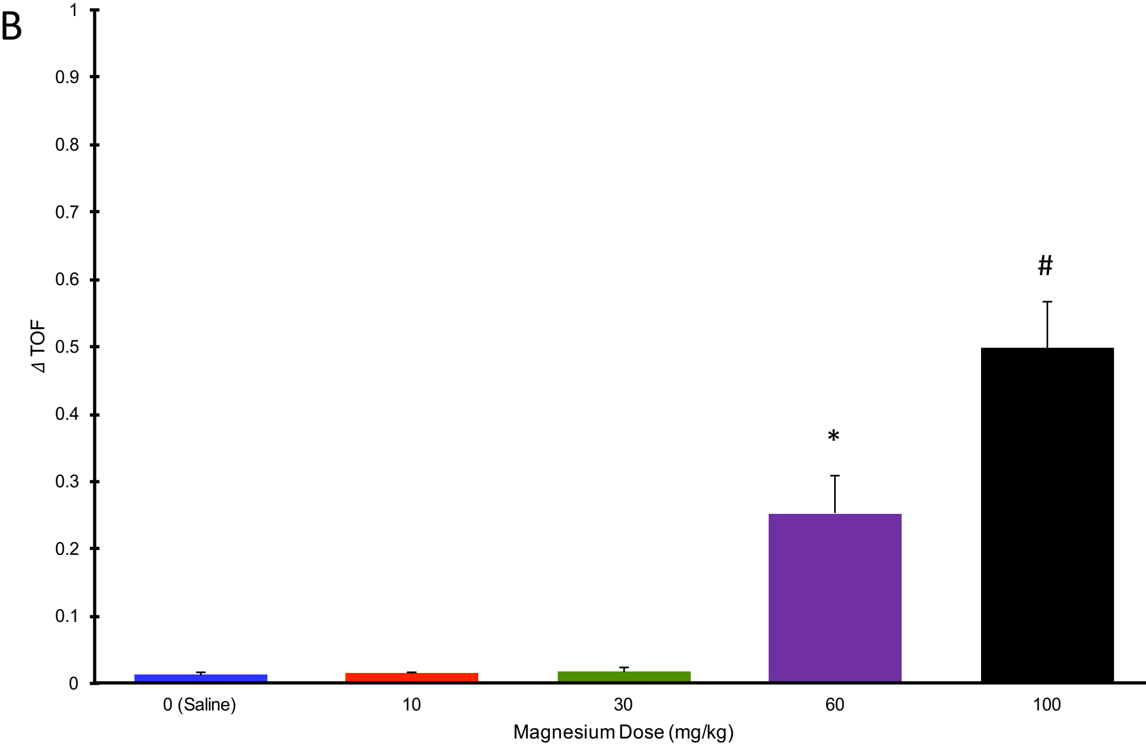




**Figure 14:** Magnesium-induced neuromuscular impairment as measured by TOF-R. **(A)** Time course of TOF-R depression. Mean ± SEM **(B)** Showing greatest reduction in TOF-R ($\Delta\mathrm{TOF}$). Mean ± SEM. * significant difference from 0, 10 ($p<0.05$), 30 and 60 ($p<0.01$) mg/kg dose groups. # significant difference from 0, 10, 30 ($p<0.001$) and 60 ($p<0.01$) mg/kg dose groups.

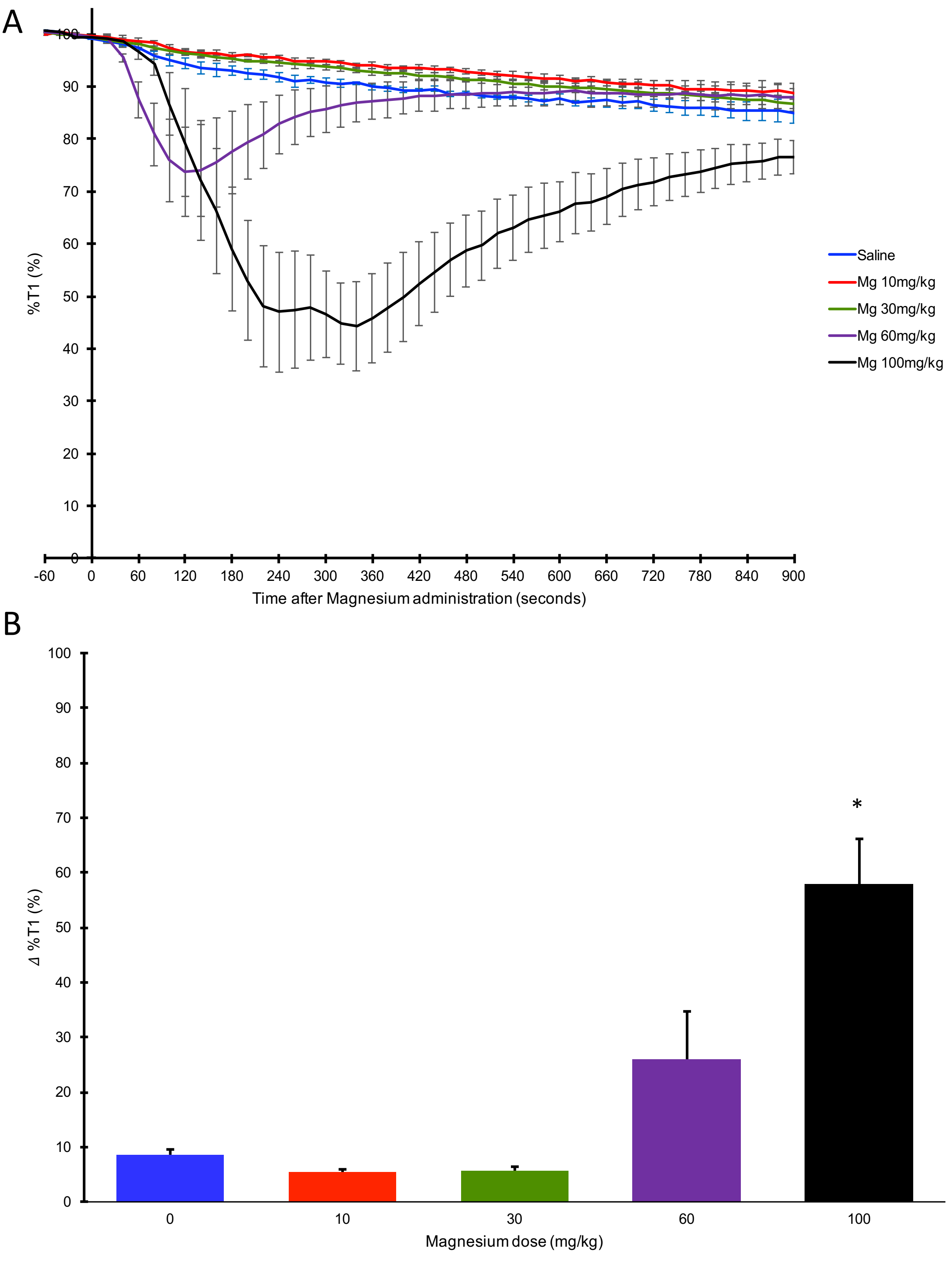



**Figure 15:** Magnesium-induced neuromuscular impairment as measured by %T1. **(A)** Time course of %T1 depression. Mean ± SEM **(B)** Showing greatest reduction in %T1 ($\Delta$%T1). Mean ± SEM. * significant difference from 0, 10, 30 (p<0.001) and 60 (p<0.005) mg/kg dose groups.

### 3.1.2. Onset of neuromuscular impairment

Time to onset of maximum impairment was only tested in the 60mg and 100 mg/kg groups as these were the only groups to show impairment in neuromuscular transmission compared to the 0 mg/kg (saline) group.

The mean time taken for TOF-R to reach it's minimum ($TOF_{min}$) after the test dose of magnesium was administered was 140.0 seconds (±31.0 seconds) in 60 mg/kg group and 248 seconds (±33.5 seconds) in 100 mg/kg group, with a significant difference between these two groups ($p=0.0004$). (Figure 16)

The mean time taken for %T1 to reach it's minimum ($\%T1_{min}$) after the test dose of magnesium was administered was 133.3 seconds (±31.0 seconds) in 60 mg/kg group and 264.0 seconds (±21.9 seconds) in 100 mg/kg group, with a significant difference between these two groups ($p<0.0001$). (Figure 16)

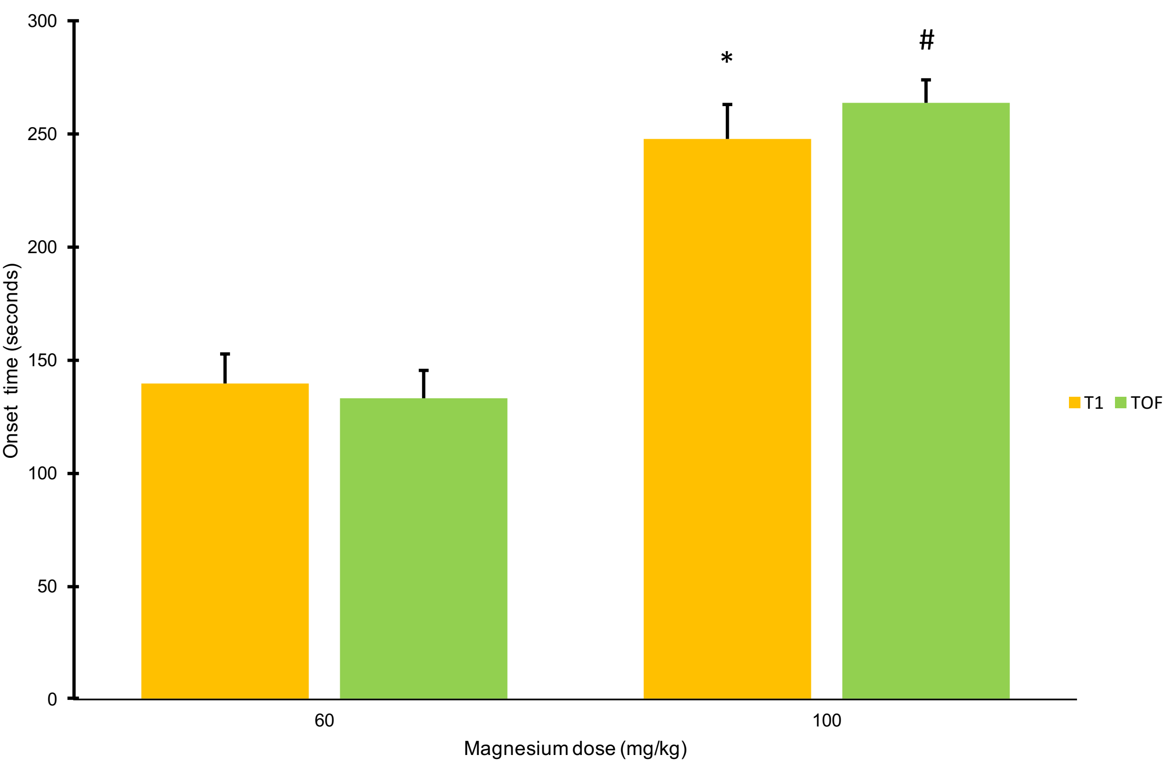



**Figure 16:** Time to onset of maximum impairment of neuromuscular transmission as measured by %T1 (orange) and TOF-R (green). Mean ± SEM * significant difference from corresponding measure of the 60 mg/kg dose group ($p<0.0005$). # significant difference from corresponding measure of the 60 mg/kg dose group ($p<0.0001$).

### 3.1.3. End of experiment TOF-R

The mean $TOF_{900}$ (TOF-R 15 minutes after magnesium administration) (Figure 17:B) was found to be 0.924 (±0.0143) in the 0 mg/kg group, 0.957 (±0.0268) in the 10 mg/kg group, 0.952 (±0.0205) for 30 mg/kg group, 0.913 (±0.0492) for 60 mg/kg group and 0.712 (±0.1303) for 100 mg/kg group. Significant differences were observed only between the 100 mg/kg group and the groups 0 mg/kg ($p=0.001$), 10 mg/kg ($p<0.001$), 30 mg/kg ($p<0.001$) and 60 mg/kg ($p=0.001$).

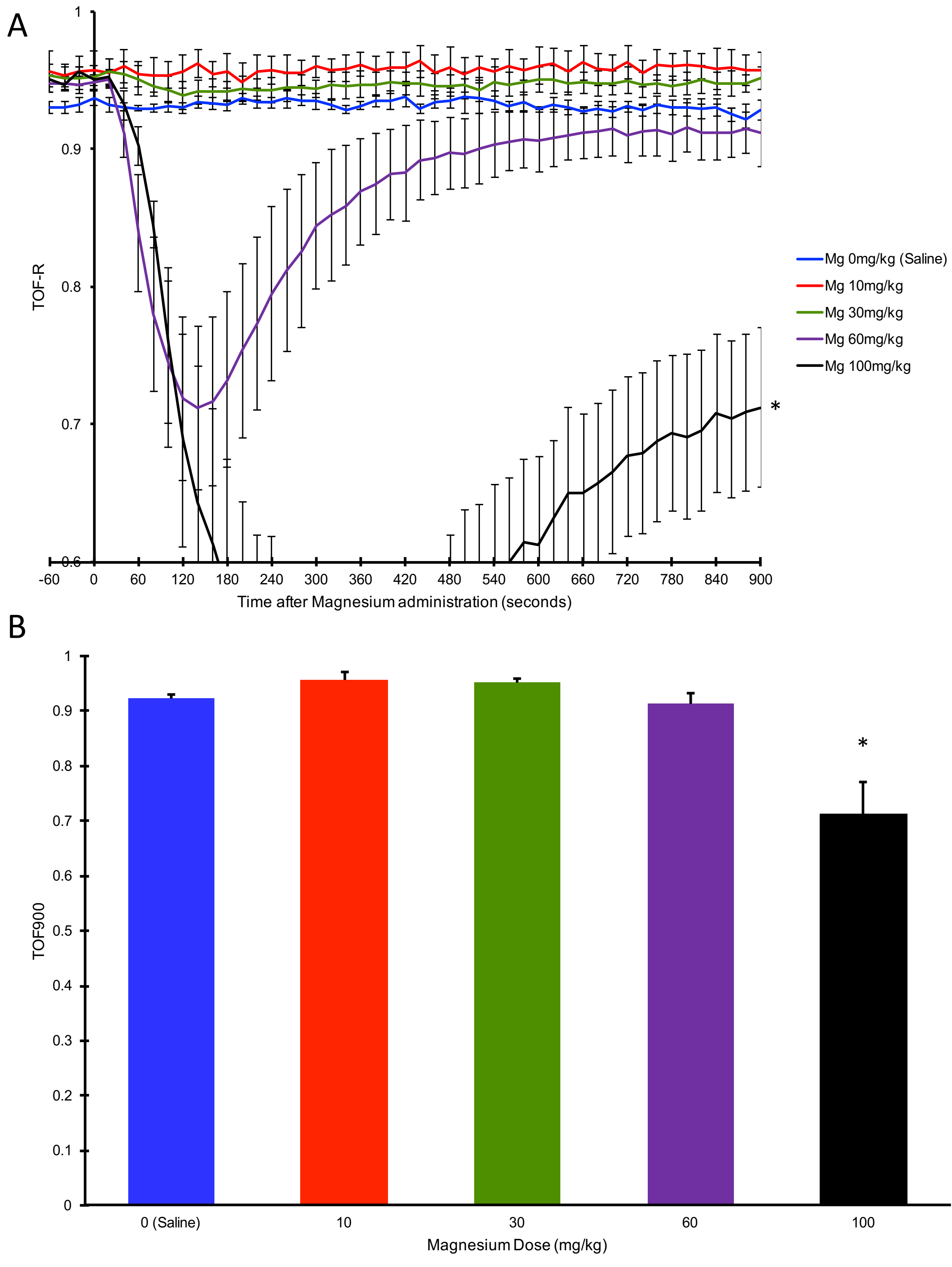



**Figure 17:** Recovery to baseline after administration of magnesium. **(A)** Time course of TOF-R after magnesium illustrating the recovery of TOF-R after neuromuscular impairment. Mean ± SEM **(B)** TOF-R value 900 seconds (TOF900) after magnesium administration. Mean ± SEM * significant difference from 0, 10, 30 ($p<0.001$) and 60 ($p<0.005$) mg/kg dose group.

## 3.2. Magnesium recurarisation experiment

The aim of the magnesium recurarisation experiment was to explore the interaction of residual NMBA at the NMJ and magnesium-induced depression of ACh release on neuromuscular transmission after reversal of NMB. This study sought to investigate any differences with different choices of NNB reversal regime on the magnitude, onset time and recovery profile of the recurarisation effect.

### 3.2.1. Neuromuscular block reversal

The mean T4-Mg (Time from recovery of T4 to time of magnesium administration) (Figure 18: B) was found to be 493.3 seconds (±77.63 seconds) in the NEO group, 223.3 seconds (±42.74 seconds) in the SUG4 group and 154.3 seconds (±19.02 seconds) in the SUG16 group. Significant differences were observed only between the NEO group and the groups SUG4 ($p<0.001$) and SUG16 ($p<0.001$).

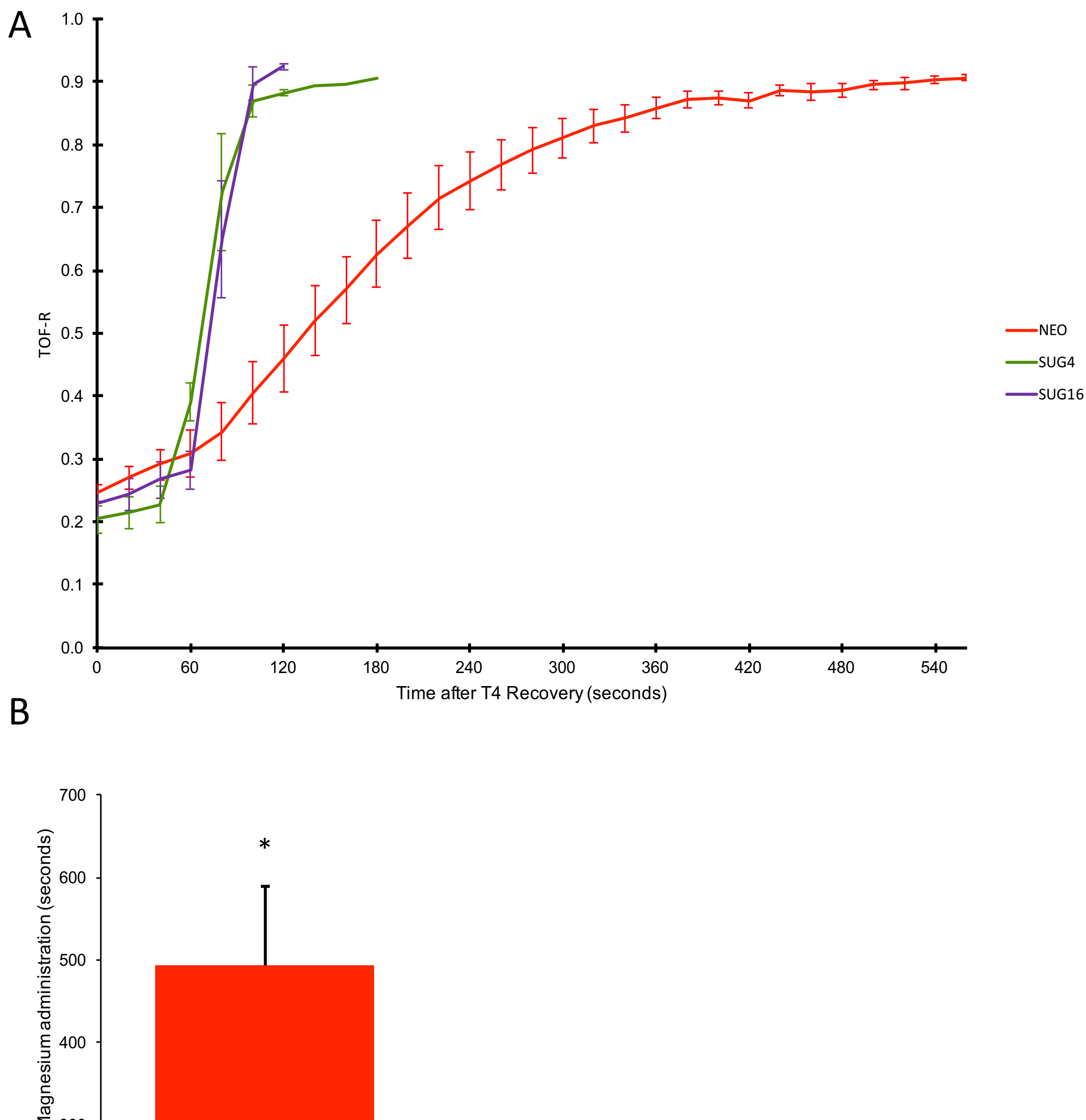



**Figure 18:** Rate of Neuromuscular block reversal. **(A)** Time course of TOF-R recovery after reversal agent administration (i.e. T4 Recovery). Mean ± SEM **(B)** Time from reversal agent administration (time of T4 Recovery) to TOF-R > 0.9 (i.e. time of magnesium administration). Mean ± SEM * significant difference from SUG4 ($p<0.001$) and SUG16 ($p<0.005$) reversal groups.

### 3.2.2. Magnitude of recurarisation

#### 3.2.2.1. TOF measures

The mean $TOF_{min}$ obtained after magnesium was administered was 0.418 (±0.1641) for the SPON group, 0.731 (±0.0712) for the NEO group, 0.616 (±0.1399) for the SUG4 group and 0.821 (±0.0938) for the SUG16 group. Significant differences were observed between the SUG16 group and the groups SUG4 (p=0.038) and SPON (p<0.001), as well as between the SPON and NEO groups (p=0.001). (Figure 19: A)

The $TOF_{min}$ achieved corresponded to a mean $\Delta\mathrm{TOF}$ (Figure 19: B) of 0.451 (±0.1412) for the SPON group, 0.174 (±0.0717) for the NEO group, 0.280 (±0.1164) for the SUG4 group and 0.096 (±0.0848) for the SUG16 group. Significant differences were observed between the SUG16 group and the groups SUG4 (p=0.031) and SPON (p<0.001), as well as between the SPON and NEO groups (p=0.001).

#### 3.2.2.1. T1 measures

The mean $\%T1_{min}$ obtained after magnesium was administered was 63.8% (±21.80%) for the SPON group, 94.6% (±3.25%) for the NEO group, 87.6% (±9.60%) for the SUG4 group and 94.7% (±5.50%) for the SUG16 group. Significant differences were observed between the SPON group and the groups NEO (p=0.003), SUG4 (p=0.030) and SUG16 (p=0.001). (Figure 20: A)

The $\%T1_{min}$ achieved corresponded to a mean $\Delta\%\mathrm{T1}$ (Figure 20: B) of 36.2% (±21.80%) for the SPON group, 5.4% (±3.25%) for the NEO group, 12.4% (±9.60%) for the SUG4 group and 5.3% (±5.50%) for the SUG16 group. Significant differences were observed between the SPON group and the groups NEO (p=0.014) and SUG16 (p=0.005).

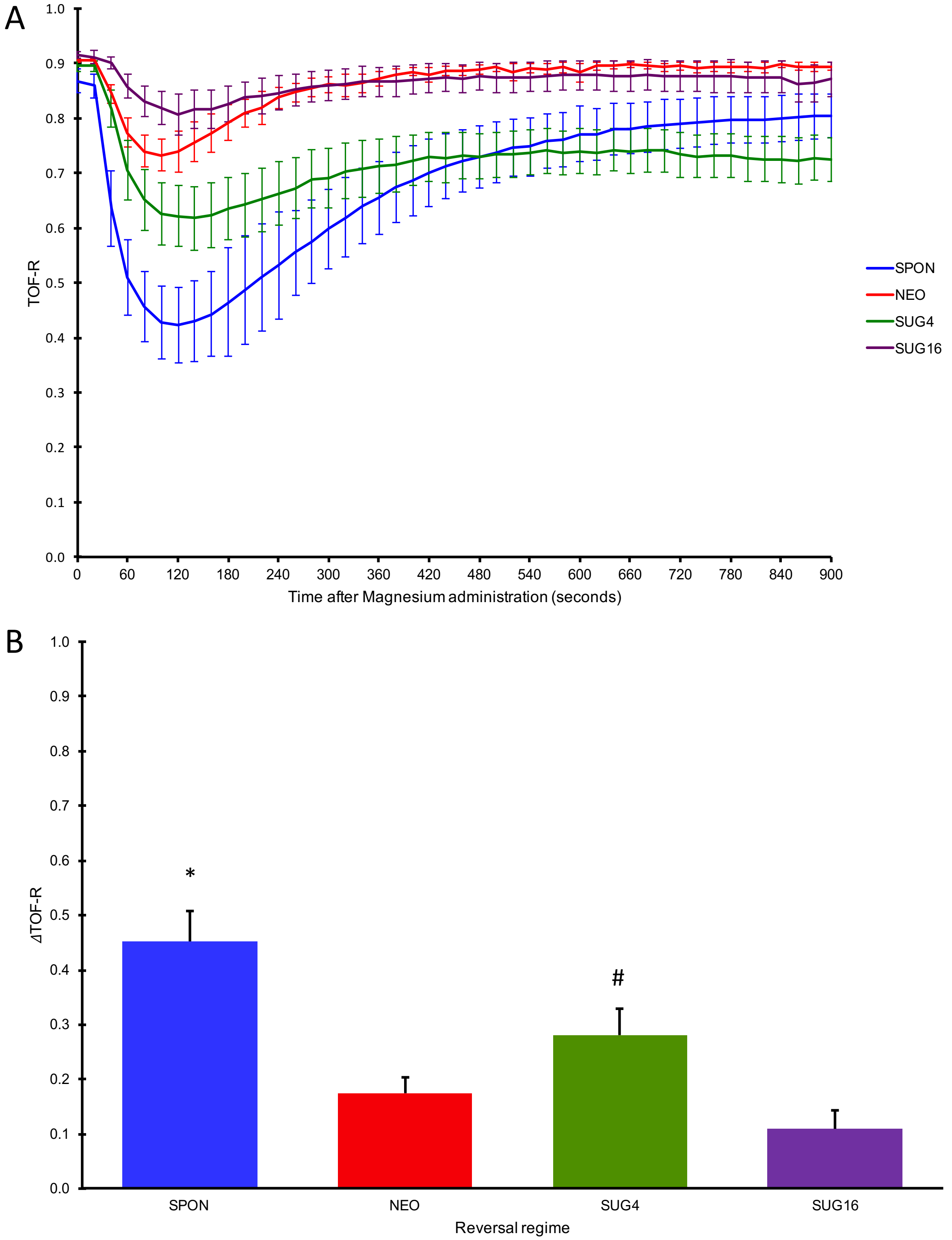



**Figure 19:** Magnesium recurarisation compared between neuromuscular block reversal regimes, as measured by TOF-R. **(A)** Trace showing magnitude and time course of magnesium recurarisation. Mean ± SEM. **(B)** Maximal depression of TOF-R (*Δ*TOF) induced by magnesium compared by neuromuscular block reversal regime. Mean ± SEM. *significant differences from NEO ($p<0.005$) and SUG16 ($p<0.001$) reversal groups. #significant difference from SUG16 ($p<0.05$) reversal group.

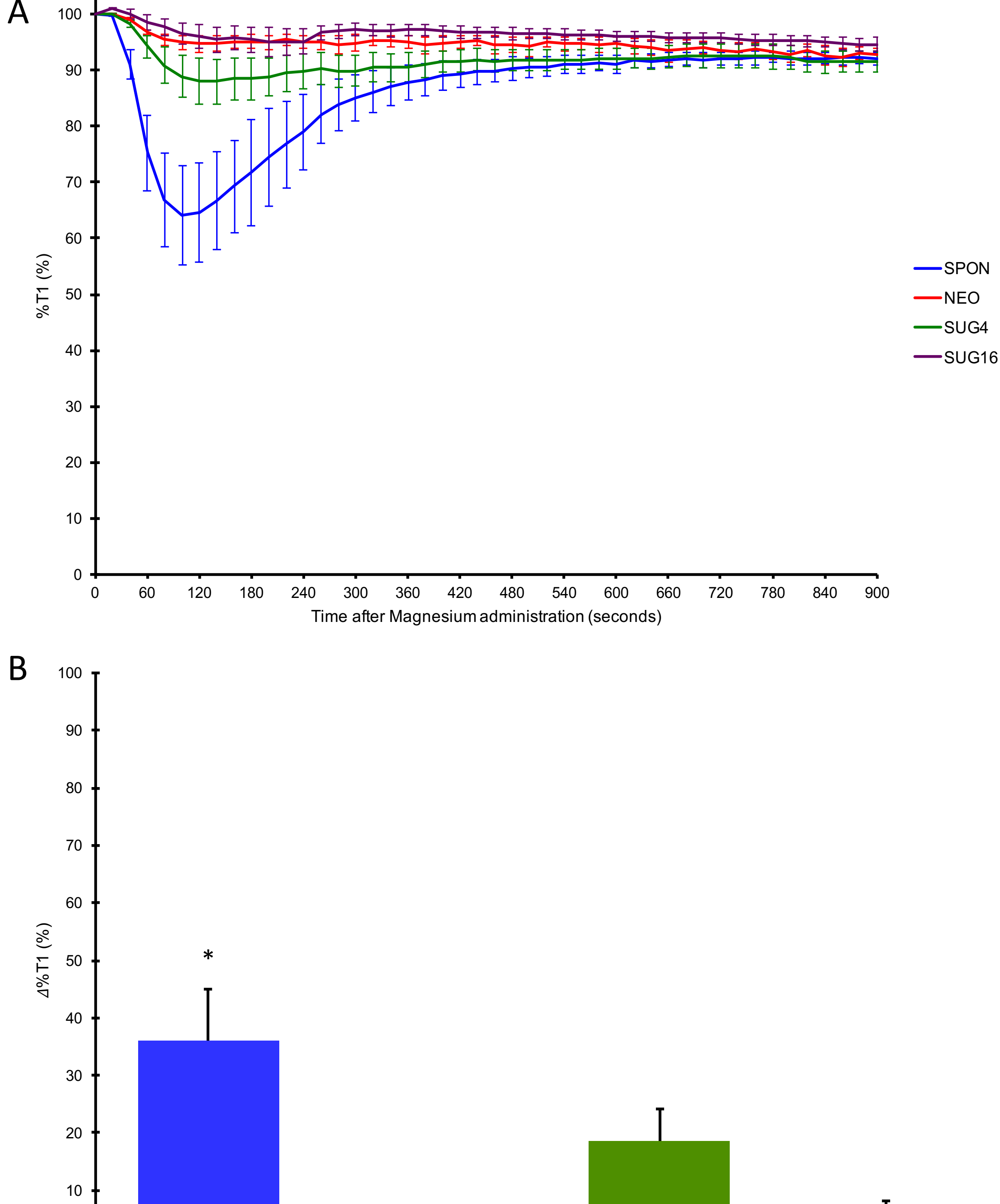



**Figure 20:** Magnesium recurarisation compared between neuromuscular block reversal regimes, as measured by %T1. **(A)** Trace showing magnitude and time course of magnesium recurarisation. Mean ± SEM. **(B)** Maximal depression of %T1 ($\Delta\%T1$) induced by magnesium compared by neuromuscular block reversal regime. Mean ± SEM. *significant differences from NEO ($p<0.05$) and SUG16 ($p<0.01$) reversal groups.

### 3.2.3. Onset of recurarisation

The mean time taken for TOF-R to reach it's minimum ($TOF_{min}$) after magnesium administration was 120.0 seconds (±17.89 seconds) in SPON group, 103.3 seconds (±15.06 seconds) in NEO group, 130.0 seconds (±10.95 seconds) in SUG4 group and 111.4 seconds (±10.69 seconds) in SUG16 group, with significant differences only observed between the SUG4 group and NEO group (p=0.019). (Figure 21)

The mean time taken for %T1 to reach it's minimum ($\%T1_{min}$) after magnesium administration was 103.3 seconds (±15.06 seconds) in SPON group, 116.0 seconds (±26.1 seconds) in NEO group, 143.3 seconds (±29.44 seconds) in SUG4 group and 160.0 seconds (±25.30 seconds) in SUG16 group, with significant differences observed between the SUG16 group and the groups SPON (P=0.005) and NEO (p=0.047). (Figure 21)

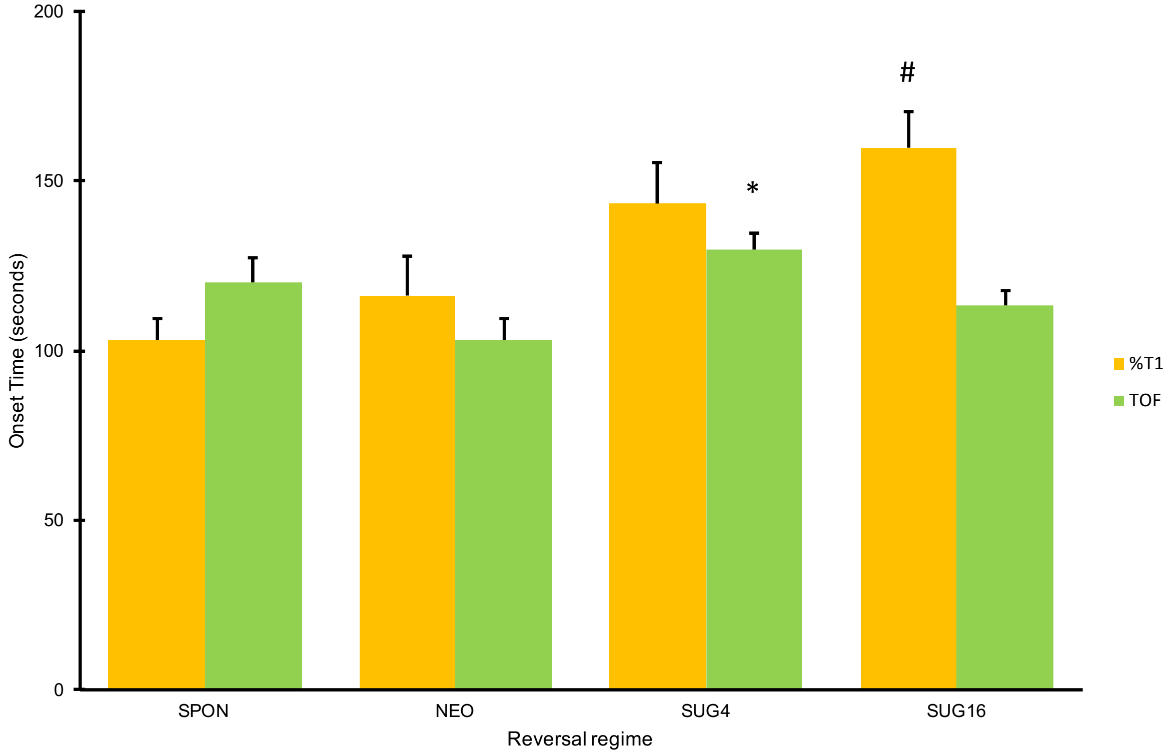



**Figure 21:** Time to onset of maximum recurarisation between reversal groups, as measured by %T1 (orange) and TOF-R (green). Mean ± SEM * significant difference from corresponding measure of the NEO (p<0.05) reversal group. #significant difference from corresponding measure of the SPON (p<0.01) and NEO (p<0.05) reversal group.

### 3.2.4. Recovery from recurarisation

#### 3.2.4.1. End of experiment TOF-R

The mean $TOF_{900}$ (TOF-R 15 minutes after magnesium administration) (Figure 22: B) was found to be 0.804 (±0.0991) in the SPON group, 0.895 (±0.0171) in the NEO group, 0.725 (±0.0988) for the SUG4 group and 0.882 (±0.0739) for the SUG16 group. Significant differences were observed between the SUG4 group and groups NEO (p=0.008) and SUG16 (p=0.011).

#### 3.2.4.2. Recovery time course (R%) from recurarisation

The mean R25% (Time taken to recover 25% of the maximum recurarisation effect after time of TOF-$R_{min}$) (Figure 23) was found to be 133.3 seconds (±53.16 seconds) in the SPON group, 90.0 seconds (±30.30 seconds) in the NEO group, 170.0 seconds (±80.75 seconds) for the SUG4 group and 65.7 seconds (±27.60 seconds) for the SUG16 group. Significant differences were observed between the SUG4 and SUG16 groups (p=0.011).

The mean R50% (Time taken to recover 50% of the maximum recurarisation effect after time of TOF-$R_{min}$) (Figure 23) was found to be 230.0 seconds (±95.29 seconds) in the SPON group, 153.3 seconds (±56.10 seconds) in the NEO group, 333.3 seconds (±94.52 seconds) for the SUG4 group and 120.0 seconds (±63.25 seconds) for the SUG16 group. Significant differences were observed between the SUG4 and groups NEO (p=0.023) and SUG16 (p=0.006).

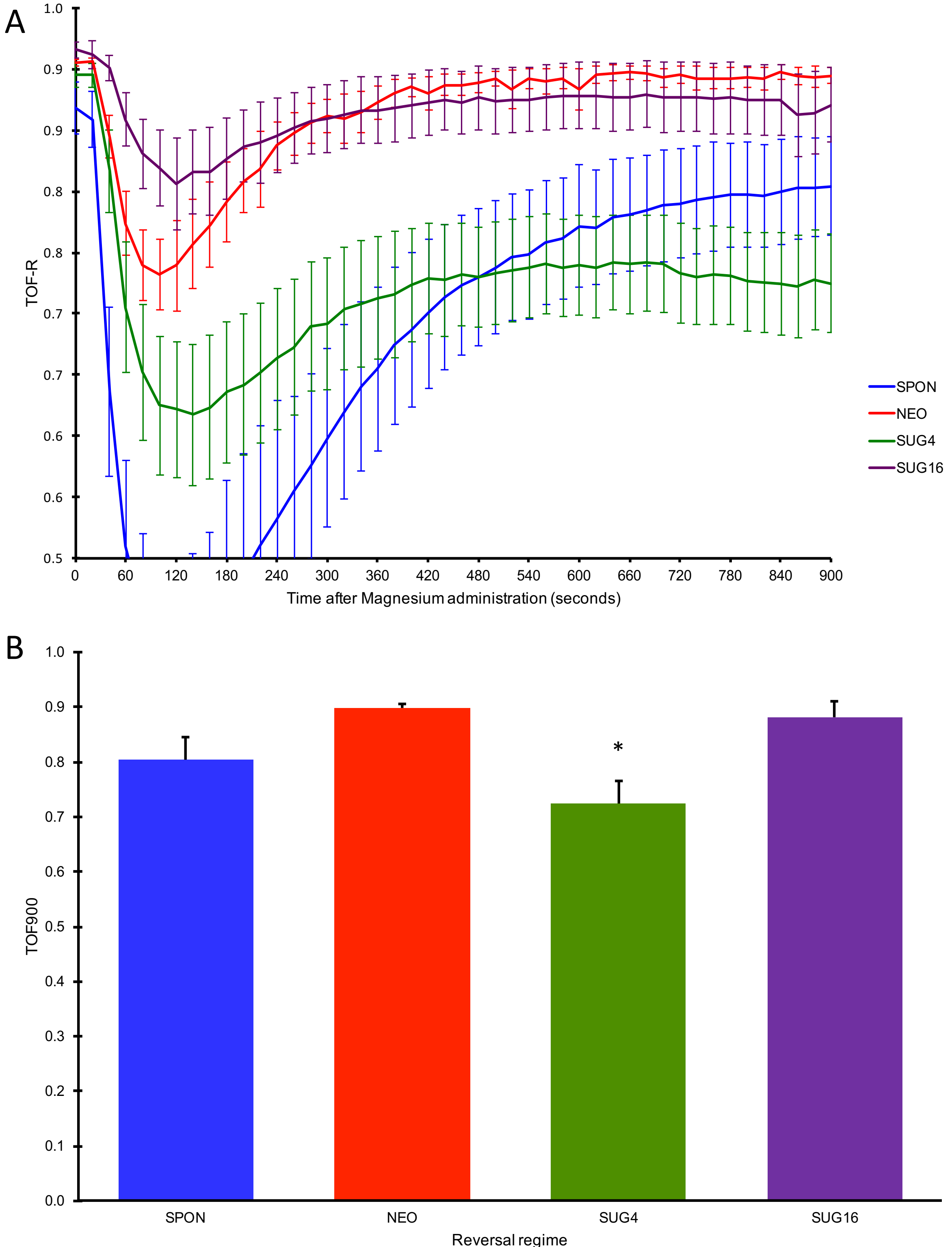



**Figure 22:** Recovery to baseline after magnesium recurarisation. **(A)** Time course of TOF-R after recurarisation illustrating the recovery of TOF-R. Mean ± SEM **(B)** TOF-R value 900 seconds (TOF900) after magnesium administration. Mean ± SEM * significant difference from NEO ($p<0.01$) and SUG16 ($p<0.05$) reversal groups.

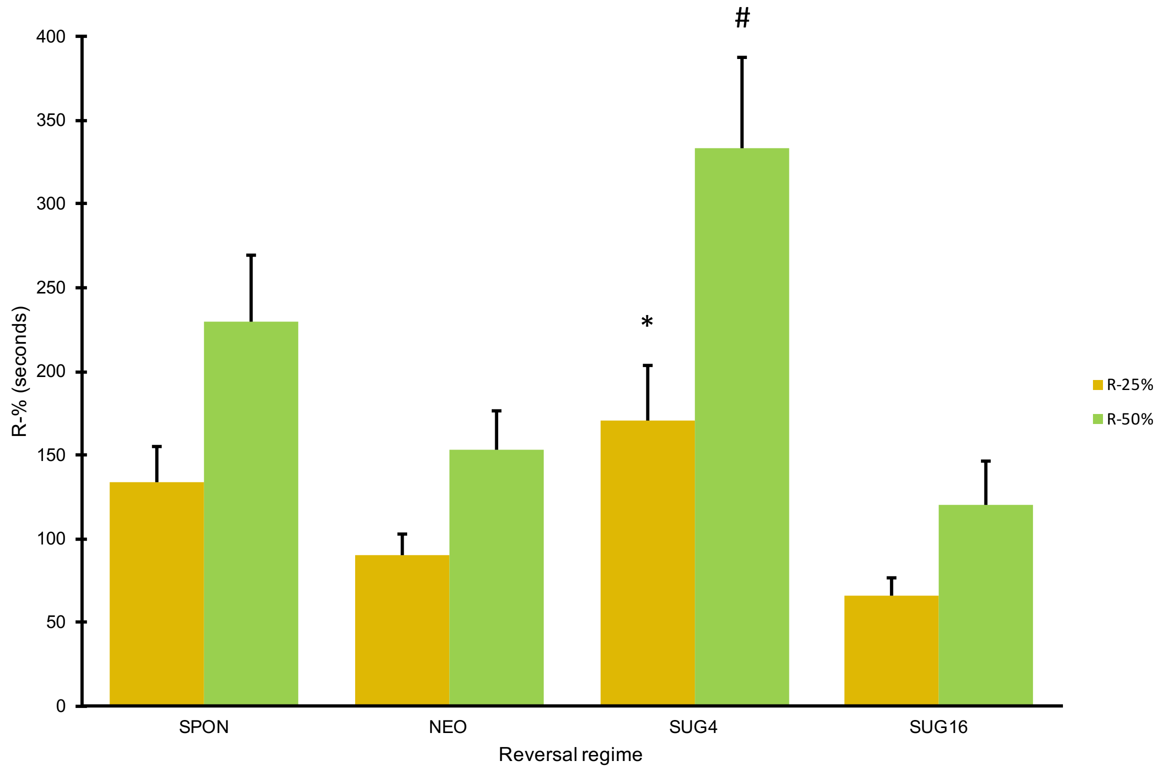



**Figure 23:** Time course of TOF-R recovery from recurarisation using recovery to % of maximum depression of TOF-R. Mean ± SEM * significant difference from corresponding measure of SUG16 group ($p<0.05$). # significant differences from corresponding measure of NEO ($p<0.05$) and SUG16 ($p<0.01$) groups.

# 4. DISCUSSION

## 4.1. Refinement of model

As stated in the methods (section 2.2), rats were allocated within this study to the characterisation of modified equipment (tracheostomy, heating plate, IV cannula and nerve electrodes) and the techniques used with these modified procedures. These modifications were made as a way to reduce the surgical preparation time (and thus time of exposure to anaesthesia) as well as to improve the stability of the procedure/technique to provide optimal experimental conditions – as identified from challenges encountered in previous use of this model. A significant contribution of this work is refinement of the model. The exact modifications that were made are described in Appendix A: Supplementary Methods.

## 4.2. Magnesium dose-effect experiment

This experiment sought to investigate the neuromuscular impairing effect of a range of intravenous magnesium sulphate doses in this *in vivo* rat model of neuromuscular transmission. The results of the magnesium dose-effect experiment and it's implications for the rest of the study, as well as its potential applications towards clinical practice will be discussed in this section.

In this experiment it was shown that intravenous magnesium sulphate was able to produce significant (compared to saline) neuromuscular impairment, as measured by TOF-R, at the doses of 60 mg/kg and 100 mg/kg. This effect was also elicited with a dose of 100 mg/kg when measured by T1. Doses up to and including 30 mg/kg were not able to produce any measurable neuromuscular impairment, shown by either TOF-R or T1.

The onset of maximum depression of neuromuscular function in these two dose groups was found to be inversely proportional to the dose. The time taken to reach $TOF_{min}$ and $\%T1_{min}$ was significantly faster in the rats given 60 mg/kg compared to those given 100 mg/kg of magnesium. As the slope of the decrease in neuromuscular transmission appeared similar

between these two groups, this may just reflect a greater time to reach a larger level of depression.

Recovery from neuromuscular impairment 15 minutes after magnesium was found to be inadequate (TOF-R<0.9) only in rats treated with 100 mg/kg magnesium, while the impairment produced by 60 mg/kg recovered satisfactorily at approximately 9 minutes post-magnesium.

The chief mechanism through which magnesium inhibits neuromuscular transmission is by competitive inhibition of calcium influx through P/Q-type VGCCs and thus reducing the quantal release of ACh [98-100]. This may be explained by reduction of the safety factor of neuromuscular transmission by decreasing the amount of ACh that reaches the postjunctional membrane and its receptors.

A secondary mechanism of magnesium's neuromuscular impairing effect has been shown to occur at the postjunctional division of the NMJ. Decreased postjunctional potentials were observed [99, 100], although they were thought to be of little significance compared to the prejunctional effects magnesium produces. A recent study appears to have elaborated on this by showing that magnesium decreases cation conductance at the postjunctional nAChRs, that which produces the MEPP, by acting as a non-competitive antagonist to the receptor [111]. The $IC_{50}$ of magnesium for this effect (concentration of the drug necessary to produce 50% inhibition of the target function) is 29.2mmol/L however, a concentration beyond what can reasonably be assumed to have been reached in our animal model, or as the authors concede, with therapeutic use of magnesium. In fact, in order to produce a 20% suppression of ACh's stimulation effect, a magnesium concentration of 6mmol/L was necessary.

Although magnesium's ability to impair neuromuscular transmission is well established; the exact effect a 30 mg/kg dose of magnesium as used in this *in vivo* rat model has not been published before. For this reason, an experiment exploring its neuromuscular effect in the absence of NMBAs was performed, before investigating how it interacts with them. In addition, while there are many clinical studies looking at magnesium and neuromuscular function, they predominantly examine how it affects the onset or recovery from the NMBA

[87, 89, 91-94, 157-159]. A study investigating the neuromuscular response (using TOF-R and T1) to a range of magnesium doses has as of yet, not been published.

It is important to point out that while no neuromuscular impairment was observed in the magnesium dose groups of 10 mg/kg and 30 mg/kg, this does not imply a lack of decrease in ACh released. The neuromuscular junction's safety margin protects the efficiency of its transmission by releasing far greater amounts of ACh than is necessary to produce an action potential, even taking into consideration the substantial portion hydrolysed by AChE. In order to observe a prejunctionally mediated decrease in TOF-R or T1 (measures of mild-moderate neuromuscular block), a substantial decrease in the release of ACh would thus have to occur. If a stimulation method of detecting shallow neuromuscular block was used, such as tetanic fade [160] one might be able to detect neuromuscular impairment at these lower doses.

The major finding of this dose characterising study has clearly shown that 30 mg/kg of magnesium is not able to produce detectable neuromuscular impairment when administered alone, as measured by T1 or TOF-R. It is suspected that any decrease in neuromuscular transmission in the recurarisation model after the administration of 30 mg/kg of magnesium, should therefore reflect an interaction of the magnesium's mechanism of action with any residual NMBA. In other words, 30 mg/kg of magnesium can be used as a "probe" of residual NMBA occupancy of the nAChR in the NMJ.

Additionally, the results in this rat model showing that doses up to and including 30 mg/kg of magnesium (given as a bolus) show no neuromuscular effect, indicate that (from a neuromuscular point of view) it might be possible to safely administer these doses to the clinical patient, with relatively little concern for neuromuscular compromise. While the 60 mg/kg dose of magnesium produced neuromuscular impairment, this effect was mild and transient, especially given the fact that it was given as a bolus. Since magnesium is usually administered clinically as an infusion over several minutes, the effect seen with the 60 mg/kg dose may be even smaller if given in this manner.

Although these data indicate a safe range of magnesium doses for clinical use, it is very important to be cognisant of the limitations of animal models. Direct extrapolation from animal models to the clinical scenario is not possible due to differences between human and

rodent physiology. These results should however, be used as the basis of clinical studies, in order to verify the results obtained in this study and whether they can be reproduced in humans.

## 4.3. Magnesium recurarisation experiment

This study sought to validate and further explore the results obtained in the previous honours studies conducted in this laboratory in 2012 [156] (See Section 1.6.3.1. Previous recurarisation data), by exploring the phenomenon of magnesium-induced reinstatement of NMB (magnesium recurarisation) after NMB reversal. The aim was to compare this effect between the choice of reversal regime, namely reversal with: neostigmine/glycopyrrolate, two different doses of sugammadex (4 mg/kg and 16 mg/kg) and when reversal was omitted. This section will discuss the results of the magnesium recurarisation experiment i.e. the differences between reversal regimes, the recurarisation effect itself, as well as recovery from recurarisation. The implications of these results on our understanding of neuromuscular block pharmacology and neuromuscular physiology will also be discussed, as well as how this may apply to the clinical management of a patient.

From the results of the magnesium dose-effect experiment in this study (Results: Section 3.1, Discussion: 4.1) that showed 30 mg/kg of magnesium was not able to produce neuromuscular block in the absence of NMBAs, it was concluded that the neuromuscular impairment observed in the previous honours study [156] after administration of 30 mg/kg of magnesium post-sugammadex reversal had to reflect the rebinding of the rocuronium to the postjunctional nAChR. It was hypothesised that with sugammadex reversal, only sufficient rocuronium is removed to liberate enough receptors to produce a TOF-R > 0.9, leaving behind significant residual rocuronium with the potential to rebind the receptor if conditions were changed. To test this, higher doses of sugammadex (2- and 8- fold the standard dose used in the previous study) were used for reversal, the rationale being to extract even greater amounts of rocuronium, to determine if the magnesium recurarisation effect could be reduced or eliminated.

### 4.3.1. Magnitude of recurarisation effect

This study has shown that 30 mg/kg of magnesium sulphate (a clinically relevant dose) can produce recurarisation after reversal of NMB, regardless of the method/ agent of reversal.

The magnitude of magnesium recurarisation was greatest in the rats that received no reversal agent and were allowed to recover spontaneously from NMB. The next largest recurarisation effect was observed in rats reversed with a sugammadex (4 mg/kg), followed by reversal with neostigmine/ glycopyrrolate, while the group of rats reversed with sugammadex (16 mg/kg) showed the smallest effect.

The most likely explanation of these findings probably relates to the safety margin of the neuromuscular junction and the pharmacology of neuromuscular blocking agents such as rocuronium. It is generally accepted that at a TOF-R of 0.9, 70 - 75% of the nicotinic cholinergic receptors in the NMJ may still be occupied by NMBA molecules [36]. This means that only 25 - 30% of the motor endplate's receptors need to be free in order to produce an action potential that generates muscle cell contraction. Any alteration at this point in ACh concentration would be able to shift the balance of competitive binding to the nicotinic receptor in favour of the residual NMBA in NMJ.

Magnesium's ability to impair neuromuscular transmission is well established and occurs by two mechanisms previously discussed (Section 1.6.1.1.). The primary mechanism is however leads to a decrease in ACh release, and thus a corresponding decrease in the concentration at the receptor site. If, as was the case in this study, there is residual NMBA present at the point when extracellular concentrations of magnesium rise, ACh competitive binding affinity for the receptor would decrease, and the likelihood of a NMBA molecule binding the receptor instead of ACh would increase, thus reinstating neuromuscular block.

The second mechanism of magnesium's neuromuscular impairing effect is through diminished conductance through the ion channel of the nAChR in response to ACh activation [111]. This effect was unlikely to be relevant to the neuromuscular impairment observed in the magnesium dose-effect experiment of this study, where NMBAs were not present. However in the same study, in the presence of vecuronium, a non-depolarising NMBA like rocuronium, magnesium at doses as low as 1- or 3- mmol/L (that did not significant depress conductance through the channel) were able to enhance the vecuronium

blockade of ACh stimulation by as much as 20%. Given the continued presence of rocuronium in the neuromuscular cleft at the time of magnesium administration in this study, and magnesium's ability the enhance the antagonistic effect of NMBA at the postjunctional nAChR, magnesium's postjunctional effects (and to what degree it contributes towards the recurarisation effect observed) is worth considering.

#### 4.3.1.1. Recurarisation after reversal was omitted

A profound magnesium recurarisation effect occurred after neuromuscular block reversal was omitted, and rocuronium neuromuscular block recovered spontaneous to TOF-R > 0.9. Fuchs-Buder et al. first reported magnesium recurarisation in a clinical study on patients who received magnesium (50 mg/kg) after spontaneous recovery (i.e. received no reversal) from NMB [89]. Hans et al. also published a clinical study comparing recurarisation between magnesium (50 mg/kg) and to saline control, administered after spontaneous recovery from rocuronium to TOF-R > 0.9 [93]. They found a decrease to a TOF-R value of 0.49 in the magnesium group, a value similar to the TOF-R value of 0.42 found in this study in rats given similar doses (30 mg/kg).

#### 4.3.1.2. Recurarisation after reversal with neostigmine

In this study, reversal of neuromuscular block with a combination of neostigmine/ glycopyrrolate was also not able to prevent magnesium recurarisation, with significant impairment in neuromuscular transmission, that returned to near baseline values within 7 - 8 minutes. These results were expected, since similar findings were described in a case report by Fawcett and Stone [90]. The case report describes a patient who was administered 20 mg/kg of magnesium for a rapid atrial arrhythmia after reversal of cisatracurium NMB with 2 standard doses of neostigmine/ glycopyrrolate. The patient, who at the time had no neuromuscular monitoring, progressed to respiratory arrest and required tracheal intubation and assisted ventilation for 20 minutes until no neuromuscular impairment was detected using T1 or tetanic fade. This means that the present study is the first to show magnesium recurarisation after neostigmine reversal of NMB in a controlled *in vivo* animal experiment, confirming the observations reported by Fawcett and Stone [90].

Neostigmine's mechanism is the inhibition of hydrolysis of released ACh, with the resulting increased concentration of ACh overpowering the competitive binding affinity of rocuronium for the nicotinic cholinergic receptor. It is thus not surprising that magnesium's ability to decrease ACh release is able to cause rebinding of residual rocuronium to the receptor, as the action of neostigmine is to maintain a increased concentrations of the transmitter, but does not affect the quantity of transmitter released.

#### 4.3.1.3. Recurarisation after reversal with sugammadex

The magnesium recurarisation result observed in the sugammadex 4 mg/kg group was significant both in its magnitude and recovery profile from the recurarisation effect. It is noteworthy however that when NMB was reversed was a higher dose of sugammadex (16 mg/kg) the magnitude of recurarisation was significantly reduced.

Sugammadex reverses rocuronium NMB by binding it in the plasma and promoting its diffusion out of the NMJ [63, 161]. It has been shown that sugammadex is more rapid in its reversal, and is generally accepted as more predictable and reliable, compared to neostigmine reversal [82, 162-169].

A study by Czarnetzki et al. showed that sugammadex is able to reverse rocuronium NMB in patients pre-treated with magnesium [158]. On the basis of this data, it was suspected that reversal with sugammadex would be robust to magnesium's neuromuscular effects. Due to the near irreversible complex formed between rocuronium and sugammadex [64], and the speed at which neuromuscular block was reversed [63, 163, 170]; it is believed that very little rocuronium would remain in the neuromuscular junction after reversal that could then rebind the receptor.

The results of this study, however, reveal a noteworthy limitation in the suggested mechanism of sugammadex reversal. Up to a dose of 4 mg/kg, sugammadex does bind enough rocuronium to cause sufficient movement thereof to the intravascular compartment to free up at least 25 - 30% of the nicotinic receptors. This results in restoration of satisfactory neuromuscular transmission (TOF-R > 0.9), allowing the patient to be extubated, as they would then be able to protect their airway and breath spontaneously.

In perusing the clinical and pharmacological literature, this concept in the mechanism of NMB and its reversal appears be understated.

A significant residual amount of rocuronium still appears to remain in the NMJ (at least in this rodent model), which has the potential to rebind the nicotinic receptor. Additionally, due to sugammadex's lack of effect on AChE, ACh concentrations remain unenhanced, and thus neuromuscular transmission is constantly dependent on the physiological concentration of ACh outcompeting the residual concentration of rocuronium for occupancy of the nicotinic receptor. Thus, any decrease in the release of ACh (such as caused by magnesium) will diminish the safety margin of neuromuscular transmission in such a way that favours the binding of the rocuronium over ACh to the receptor, thus reinstating NMB.

### 4.3.2. Recovery profile from recurarisation

Further inspection of the data revealed that the recovery profile from magnesium recurarisation after reversal with sugammadex (4 mg/kg) showed a gradual recovery over the first seven minutes after magnesium and eventual levelling out at a sub-baseline value, while a similar magnitude of recurarisation after neostigmine reversal showed a consistent return to baseline (TOF-R>0.9) at approximately 6 minutes post-magnesium, which was shown to be significantly different to sugammadex. The recovery profile of half the recurarisation magnitude (time taken to recover 50% of the recurarisation effect after the peak effect) was also found to be significantly slower after sugammadex (4 mg/kg) reversal compared to neostigmine reversal. This could be interpreted to reflect sugammadex's lack of effect on the availability of ACh at the motor endplate, in contrast to neostigmine, which through inhibition is able to elevate these levels, and at least partially antagonise the neuromuscular effects of magnesium [171].

It is noteworthy that when the sugammadex dose used for reversal was substantially increased (from 4 mg/kg to 16 mg/kg), in addition to the magnitude of the magnesium recurarisation effect being reduced and the recovery profile (in terms of recovery of 25 and 50% of the recurarisation effect) also significantly improved. This reflects a greater extraction of rocuronium out of the NMJ and liberation from the postjunctional nicotinic

receptors, increasing the safety margin of neuromuscular transmission by shifting the competitive binding even greater in favour of the ACh.

### 4.3.3. Reversal of neuromuscular block

These data showed that, as expected, the speed of recovery of neuromuscular transmission (in increasing order) between NMB reversal agents (i.e. reversal rate) was: neostigmine/ glycopyrrolate < sugammadex (4 mg/kg) < sugammadex (16 mg/kg). Significant differences were observed between neostigmine/ glycopyrrolate and both sugammadex doses, however no significant differences were observed between the two sugammadex doses.

It is worth noting that the occurrence of recurarisation in the sugammadex group may be in part due to one of its properties that make it so popular as a reversal agent, namely its faster time of reversal. The time taken to reverse NMB from T4 recovery to a TOF-R > 0.9 was much shorter with sugammadex reversal compared to neostigmine reversal, during which time less spontaneous elimination of rocuronium from the plasma and NMJ had occurred compared to neostigmine reversed rats. It could however be argued, that the sugammadex-induced diffusion of rocuronium from the NMJ to the intravascular compartment would compensate for this, as its mechanism essentially resembles an accelerated spontaneous recovery.

### 4.3.4. Onset of recurarisation

Although differences between reversal regime groups in onset to maximum recurarisation were found to be significantly different, the maximum difference in between groups was approximately one minute, bringing into question the clinical (and in this model, experimental) significance of the differences in onset of recurarisation, compared to the magnitude of the effect.

## 4.4. Limitations of the study

A number of limitations related to the model, experimental design and analysis and how these may impact on the validity of the results described above.

### 4.4.1. Extrapolation from animal studies

As with all animal studies, the direct extrapolation of this study's results to humans and the clinical scenario is not possible. While *in vivo* animal studies can reveal much about the effects and interactions of various drugs on physiology or pathology, the results obtained should be used to infer mechanisms and create further hypotheses. Animal studies should certainly not be used to dictate (or change) clinical practice, however they form an important step towards answering a clinical hypothesis, hence the alternative name "pre-clinical studies".

### 4.4.2. Anaesthetic agent

One of the more obvious limitations in this study is the continual use of a volatile (i.e. isoflurane) anaesthetic. The choice of anaesthetic was for ethical reasons, as it provided the best stability of maintaining anaesthetic depth, due to continuous administration through a small animal ventilator, with relatively easy adjustment in the event anaesthetic depth became too light or too deep.

The use of total intravenous anaesthesia (for example with propofol/fentanyl, pentobarbital or ketamine/xylazine) was considered to negate the confounding neuromuscular effects of the volatile anaesthetic. However, due to ethical concerns related to animal welfare and regulation of anaesthetic depth maintenance – the use of volatile anaesthesia was chosen.

Isoflurane, and other volatile anaesthetics, have been shown to exert depressive effects on the NMJ. It is not known, if, or to what degree, these effects may influence the validity of this study, however the following information may assist in rationalising the interpretation of the results.

Karis et al. concluded from their work on *in vitro* frog nerve-muscle that the volatile anaesthetics action on the NMJ is postjunctionally mediated [172]. Kobayashi et al. came to a similar conclusion after *in vitro* investigations using rat phrenic nerve-hemidiaphragm preparations [173]. There have been studies that have shown volatile anaesthetics to inhibit calcium currents through a variety of VGCCs [174, 175] in the brain, including the P-type channel that is also present in the NMJ. No studies, however, have been published specifically showing decreased ACh at the NMJ due to these anaesthetics.

A study by de Castro Fonseca et al. reported depressive effects on the prejunctional release of ACh at the mouse NMJ, through inhibition of prejunctional voltage-gated sodium channels [176].

The ability of volatile anaesthetics to potentiate the neuromuscular impairment effect of the NMBAs is well documented in clinical studies, usually showing a prolongation of the duration of NMB [83, 177-181]. These anaesthetic agents have also been shown clinically to impair the NMB reversal ability of AChE inhibitors [182, 183], while no such effect has been able to be demonstrated when NMB is reversed with sugammadex [166, 184, 185].

The mechanism of the interaction of volatile anaesthetics with NMBA has also been investigated; with many studies concluding that agents such as isoflurane potentiate the inhibitory effects of non-depolarising NMBA [186, 187].

This was especially a limitation in this study, as isoflurane was still being administered to the rat at the time of magnesium administration. This is in contrast to the clinical scenario this study was attempting to mimic, namely magnesium administration in the PACU (i.e. after anaesthesia had been terminated). However, one can infer the results of this study as mimicking the worst-case clinical scenario – for example, recurarisation occurring after magnesium was being administered to the patient just a few minutes after anaesthetic delivery was discontinued.

Additionally, significant differences in the average isoflurane concentration ($[Iso]_{ave}$) were observed in the magnesium dose-effect experiment between the saline group and groups 10, 30, 60 and 100 mg/kg of magnesium. This could potentially be a confounding variable, as the question of whether the lack of neuromuscular impairment observed in the saline control group is due to the lack of magnesium or the reduced isoflurane concentration. However, given that the rats in the 10 mg/kg magnesium dose group also showed no signs of neuromuscular impairment, and the concentration of isoflurane did not differ significantly with those in the groups 30 and 100 mg/kg of magnesium, it is unlikely that this difference impacted significantly on the obtained results. Significant differences in $[Iso]_{ave}$ were also observed between 10 and 60 mg/kg dose groups, however upon visual inspection of the differences in $[Iso]_{ave}$, the actual difference in concentration administered is relatively small, and could potentially be of little experimental significance.

#### 4.4.3. Lack of blood sampling

The study design did not incorporate blood sampling, for the following reasons. This is a technical limitation, as concern of compromising the rat was based on: repeated blood sampling protocols in a model with a relatively small blood volume, as well an additional invasive procedure (arterial cannulation). One of the other major obstacles was the lack of availability of the equipment or services to analyse said rodent blood samples. This will be considered for all future studies performed in this laboratory on magnesium and the NMJ.

##### 4.4.3.1. Lack of serum magnesium level measurements

The lack of data on serum magnesium levels prior to and a few minutes after magnesium administration in this study makes it difficult to correlate degree of neuromuscular impairment produced with the concentration of magnesium at the NMJ induced by the magnesium bolus.

It should however be noted that the objective of these experiments was to determine the neuromuscular impairing effect of a specified dose of magnesium, not to the plasma magnesium concentration reached. In clinical perioperative anaesthetic practice, magnesium is rarely titrated to serum levels, but rather infused or injected according to dose-response relationships already established.

##### 4.4.3.2. Lack of blood gas analysis

A further limitation was not taking into account possible pH effects in the face of the rat being ventilated. The concern here would be alkalosis, however great care was taken to ventilate the rat as close to true physiological conditions as possible (see section 2.3.2.). The additional of pH (and blood gas analysis) would require additional blood sampling that as discussed above, could potentially compromise the condition of the rat. The author is aware of the concerns of pH state at the time of recording, and in future studies every effort will be made to address these variables.

### 4.4.4. Lack of intravenous infusions

The vast majority of clinical and animal studies cited in this text regarding magnesium and the neuromuscular junction, as well as most *in vivo* animal studies cited investigating NMBA pharmacology, utilise intravenous infusions of test drugs compared to the intravenous boluses used in this study. This was taken into account but not included in this study protocol. While effects of magnesium and other drugs tend to produce larger effects when given as a fast bolus, compared to infusion, it is believed that the use of boluses may provide a situation, as discussed above, of a worst-case clinical scenario.

## 4.5. Clinical applications

The results obtained in this study seem to suggest that prevention of magnesium recurarisation cannot be guaranteed based on the pharmacological agent used to reverse the patient's NMB. The recurarisation effect observed when NMB reversal is omitted seems to suggest that routine reversal is generally in the patient's best interest, and this is certainly consistent with recommendation in the literature [39, 61, 188].

Magnesium recurarisation after neostigmine, might perhaps not be surprising given the case report by Fawcett and Stone [90] however confirmation of this effect in a controlled experiment certainly adds confidence to the observed clinical phenomenon.

The observation of recurarisation after reversal with sugammadex might initially seem counterintuitive, especially given all the literature (discussed above) that shows that magnesium does not affect NMB reversal when using sugammadex. This can potentially give clinicians a false sense of security of the safety margin of neuromuscular transmission after reversing with sugammadex. The results shown in this study show that even when reversing with double the recommended dose of sugammadex (4 mg/kg), the neuromuscular junction is still susceptible to reinstatement of neuromuscular block, if the conditions change so as to favour the binding of the NMBA to the nAChR as opposed to its natural ligand, ACh. Factors that can potentially influence the safety factor of the NMJ are not limited to magnesium, but also to aminoglycoside antibiotics and many other drugs [189].

While sugammadex did appear to produce a dose-dependant protective effect from recurarisation, the dose required (16 mg/kg) for routine reversal would be 8-fold the normal

dose, and thus 8-fold the price. This makes prophylactic reversal of NMB with high-dose sugammadex unfeasible. If magnesium recurarisation does occur in a patient, however, the results of this study suggest that the administration of sugammadex in high doses (16 mg/kg) could potentially extract sufficient rocuronium to accelerate recovery from the recurarisation effect, and rapidly restore neuromuscular transmission (rescue treatment). Additional studies are needed to confirm these suggestions however, before these types of recommendations can be put into common clinical practice.

The results of this study, from a clinical perspective, point out a possibly understated weakness in the reversal of NMB with sugammadex. The rate of neuromuscular block obtained with sugammadex may give the clinician the false impression of complete removal of NMBA in the NMJ. Residual NMB in the PACU, as discussed earlier (Section 1.5.1.), continues to persist (with great variability in reported incidences) in hospitals around the world. It is up to the anaesthetist and other perioperative healthcare professionals to be cognisant of the concept of the safety factor of neuromuscular transmission, and its delicate nature after recovery from neuromuscular block.

## 4.6. Future studies

With the results obtained in this animal study, new questions have arisen regarding magnesium's interaction with the NMBAs and the recurarisation it produces.

Firstly, given that magnesium was able to produce recurarisation in rats reversed with either sugammadex or neostigmine/glycopyrrolate, and that the mechanisms by which these agents reverse NMB are completely different, it is possible that the co-administration of these two agents for reversal of NMB might be able to protect against magnesium recurarisation. This is an especially intriguing question given that neostigmine's recovery profile from recurarisation shows complete recovery to baseline, compared to the recovery profile observed in the sugammadex group showing a plateau at suboptimal levels. Could the combined use of both neostigmine and sugammadex allow each reversal agent to make up for the weakness in reversal of the other agent? Neostigmine could compensate for sugammadex's lack of effect on the concentration of ACh, while sugammadex could make up for neostigmine's lack of effect on the rocuronium concentration. This could potentially

result in a state of the NMJ (after reversal) where the concentration of rocuronium has been lowered by the sugammadex, and what residual rocuronium is left near the receptor site is substantially overpowered by the increase in ACh produced by neostigmine.

Secondly, it was observed that in this animal model, sugammadex was able to dose-dependently reduce the magnesium recurarisation effect. However, routine reversal of NMB with high-doses of sugammadex on the basis of preventing recurarisation when magnesium is administered is most likely an expensive decision to make in clinical practice, especially when magnesium administration is not anticipated. It is then possibly more appropriate to investigate in this model (and perhaps thereafter, clinically) the use of reversal agents to accelerate recovery from recurarisation once it has already occurred, i.e. rescue treatment.

If the reversal agent used prior to magnesium administration (and thus recurarisation) was sugammadex, what would the effect of subsequent neostigmine administration, or an additional dose of sugammadex, be on the recurarisation effect?

Finally, since magnesium's mechanism of producing recurarisation is hypothesised to occur due to competitive blockade of the prejunctional VGCC, what effect on already instated recurarisation would occur by increasing the extracellular calcium concentration, thus re-establishing prejunctional calcium currents? In other words, could the administration of intravenous calcium chloride after magnesium recurarisation has occurred, accelerate recovery from the effect so as to minimise the clinical impact of the reinstated NMB.

The questions posed above can all be investigated in the rat model described in this study, giving valuable information to guide the design of clinical studies. However, as mentioned above, certain limitations of the model need to be addressed in order to strengthen the results obtained, and thus their ability to be extrapolated to the clinical scenario.

The use of an alternative to isoflurane as the anaesthetic used in this study would be ideal, particularly to one that has been shown to have minimal effects at the NMJ. Urethane (ethyl carbamate), at concentrations needed to produce a surgical plane of anaesthesia (10 mmol/kg body weight) has been shown *in vitro* to produce no decrease in ACh release at the NMJ [190]. Additionally, urethane is an ideal maintenance anaesthetic in terminal rodent experiments, as it produces a long duration of surgical anaesthesia (between 8 and 10

hours). This is of particular use in NMB research where commonly used methods of monitoring anaesthetic depth utilise motor reflexes (such as the toe pinch-pedal reflex), are of little use given the blockade of neuromuscular transmission. Its main disadvantage however, is that of danger to the persons administering the agent including carcinogenicity, cytotoxicity and immunosuppression [191]. These can be minimised however with training on the careful and proper technique in it's handling and use. Since the toxic effects are often harmful to the rat, the use of urethane is only appropriate in terminal (non-survival) animal experiments. This makes urethane an ideal maintenance anaesthetic for the model described in this study.

For all future studies, conducted in this laboratory, investigating magnesium's effects at the NMJ, techniques for arterial blood sampling will be investigated for implementation into this animal model, if deemed appropriate. Methods for the quantification of serum magnesium concentration will be examined, so as to better relate the magnesium effect to the concentration in the extracellular fluid. Means to analyse blood samples for blood gases and pH will also be sought to verify that the acid-base state of the rat during the experiment was within physiological limits.

If deemed appropriate and/or necessary for the realisation of the aim of the experiment, the use of intravenous infusions of experimental drugs will be considered, characterised and subsequently employed for use in this animal model.

# 5. CONCLUSION

## 5.1. Magnesium dose-effect experiment

In conclusion, in this rat model, a bolus dose of 30 mg/kg displayed no detectable impairment on neuromuscular transmission, as detected by TOF-R or T1.

On the basis that this result is relatively consistent with clinical data published on this topic, the data suggest that an intravenous bolus dose of magnesium sulphate up to and including 30 mg/kg is not producing impairment in this rat model is also unlikely to produce clinically relevant muscle weakness in patients with an otherwise normal safety margin of neuromuscular transmission i.e. absence of NMBA or neuromuscular pathology. Further clinical studies however are needed in order to verify these findings in humans before magnesium at these doses can be administered without concern of neuromuscular impairment.

This study has also provided the basis for the use of a dose of 30 mg/kg magnesium sulphate that will not, of itself, impair neuromuscular transmission and thus validates the use of this dose as a “probe” to investigate residual receptor occupancy at the NMJ after the use of NMBAs.

## 5.2. Magnesium recurarisation Experiment

In conclusion, magnesium recurarisation, in this animal model, was observed after all tested reversal regimes: standard neostigmine/glycopyrrolate reversal, reversal with sugammadex at both moderate and high doses, as well as when reversal was omitted.

The recurarisation effect observed in the neostigmine group, while significant, displayed a rapid recovery to baseline conditions. However after reversal with a moderate dose of sugammadex, poor recovery from recurarisation was evident.

A dose-dependant reduction in the magnitude and improvement of the recovery profile was evident with the use of sugammadex for reversal in this model, however the high doses

clinically required for routine reversal to achieve this prophylactic effect makes it economically unfeasible.

The results of this study are the first of its kind, in that it is the first controlled study (clinical or animal), to our knowledge, that has compared magnesium recurarisation between different regimes of NMB reversal. While caution is required in directly extrapolating the results of this animal study to the clinical scenario, these data do provide information that cautions clinicians against presuming that reversal of NMB fully restores the safety margin of neuromuscular transmission, irrespective of which agent is used for reversal.

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# APPENDIX A: SUPPLEMENTARY METHODS

Appendix A-1: Anaesthesia

**SURGICAL AND ANAESTHETIC RECORD**
**PROJECT NUMBER: 013/006**
*"REVERSAL COMPARISON" EXPERIMENT*

Date of Experiment: __ / __ / ____ Rat Weight: _____ . __ g

Reversal Regime: Neo / Sug (X in applicable box)

Rat Label

Signature: ____________________
MAURITS VAN DEN BERG

**PROCEDURE RECORDS**

| Anaes. Procedure | Time | % Iso | $O_2$ Flow Rate |
|---|---|---|---|
| Habituation | : | | |
| Induction | : | | |
| Move to Nose Cone | : | | |
| General Anaesthesia | : | | |
| Mechanical Vent | : | | |
| Transfer to Box | : | | |
| Rocuronium IV | : | | |
| Reversal agent IV | : | | |
| Magnesium IV | : | | |
| Animal Endpoint | : | | |
| Time of Death | : | | |

**Baseline Heart Rate:**

__ __ __ BPM

**10% Increase HR Limit:**

__ __ __ BPM

**ANAESTHETIC MONITORING**

| Time | % Iso | $O_2$ Flow Rate | HR | Event/Note: | Action/Change: |
|---|---|---|---|---|---|
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**ANAESTHETIC DEPTH MONITORING GUIDELINES:**

**Pedal Reflex:** Tested by squeezing the foot of the rat with increasing pressure. If this elicits reflex movement of the limb it would confirm inadequate depth of anaesthesia signaling for an increase in anaesthetic dose.
**Corneal Reflex:** Tested by lightly touching the cornea with a piece of tissue paper. If a reflex blink is elicited, this would suggest inadequate depth of anaesthesia signaling for an increase in anaesthetic dose.
**Heart Rate:** If the heart rate increases by a value of 10% of the baseline value then anaesthesia dose will be increased and heart rate observed for return to baseline rate.

**Appendix Figure 1:** Example of surgical record and anaesthetic monitoring sheet

Appendix A-2: Tracheostomy & mechanical ventilation

The aim of this modification was to allow the rat to be moved on the surgical table as needed and safe transfer of the rat to the experimental box, without concern of dislodging the tracheostomy tube from the trachea, or disconnecting the tracheostomy tube from the Y-connector (that connected the tube to the ventilator).

An apparatus (tracheostomy-heating plate apparatus) was constructed (Appendix Figure 2) from Perspex that held the heating plate, on which the rat was placed for the duration of the experiment, imbedded in it.

An inverted incisor bar was used to secure the rat's head and prevent the rat from slipping off the heating plate, during movement or transfer to the experimental box.

A Y-connector for IV use was firmly secured with screws to the tracheostomy holder, which in turn was connected by means of a mobile Perspex bar, connected to the base plate by a set of hinges that allowed a wide range of movement to adjust the tracheostomy holder to the position of the tracheostomy tube. Attachment of the tracheostomy holder to the base plate ensured that the tracheostomy always moved with the rat, vastly preventing the accidental dislodging of the tracheostomy (after this modification was made, no complications related to dislodging of the tracheostomy were noted).

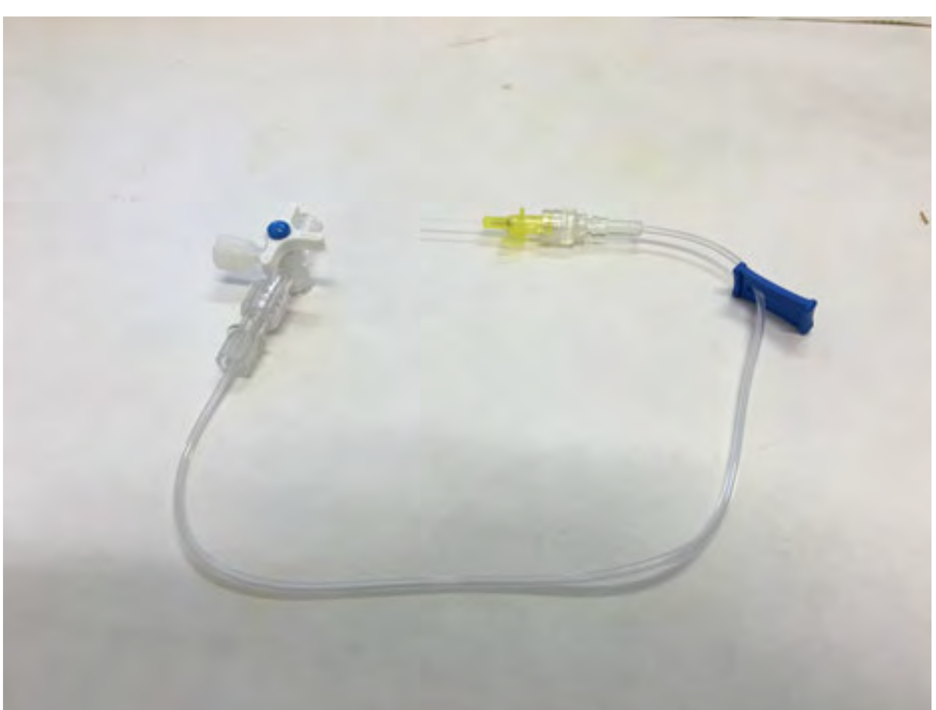
**Appendix Figure 2:** Photograph of the tracheostomy-heating plate apparatus

Appendix A-3: Femoral vein cannulation

Modifications to the technique and equipment used for femoral vein cannulation was deemed necessary in order to reduce the time spent on this procedure, thus reducing the time the rat was exposed to anaesthesia. Additionally, a need was determined, from previous experience with this model, for a method to secure the cannula firmly to the cannulation site to prevent dislodging it with the weight of the extension set and 3-way tap.

A range of commercially available intravenous cannulas were tested in rat carcases (killed for unrelated reasons to this project and with permission of the ethics committee) for ease of insertion and stability after securing to underlying muscle.

The winged 24G intravenous cannula Introcan-W (B Braun Medical (Pty) Limited, Northriding, South Africa) was selected and connected by a 30 cm mini-bore extension set (B Braun Medical (Pty) Limited, Northriding, South Africa) to a 3-way tap (Appendix Figure 3). The material of the cannula proved to be durable, yet flexible, making insertion through the (surgically created) venous aperture easy and reliable. The extension set used provided a low dead-space (0.235 mL), reducing the volume of saline flush needed after each experimental drug injection.

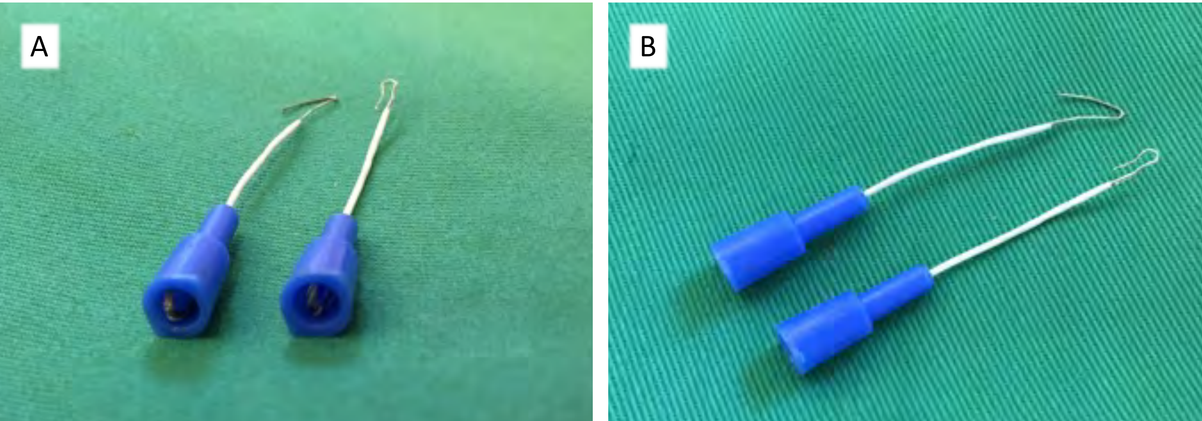


**Appendix Figure 3**: Photograph showing the assembled IV cannula, extension set and 3-way tap.

Appendix A-4: Sciatic nerve electrode construction & stimulator parameters

The need for insulation of the nerve electrodes was found to be necessary from experience in this model with previous experiments. Contact between the two electrodes, especially once the experimental recording had begun, was often the cause of sudden changes in the quality of evoked-twitches that could not be attributed to experimental drug administration.

In order to facilitate accurate and uninterrupted sciatic nerve stimulation, two partially insulated silver wire electrodes were constructed as follows. The first 4 cm of an 8 cm length of silver wire was coiled around a small wooden stick to create a loop to which the hook connector clips of the output leads of the stimulus isolation unit could easily be attached (Appendix Figure 4: A). The remaining length of the silver wire was threaded through a 22G intravenous cannula to provide insulation and protection. This was especially important when placed *in situ* next to the other electrode, and if contact was made could result in short circuiting of the stimulation. The length of wire the protruded from the cannula tip was bent into a hook shape to allow secure attachment to the sciatic nerve (Appendix Figure 4: B).




**Appendix Figure 4:** Construction of nerve stimulation electrodes **(A)** Showing the constructed loops for attachment of hook connector clips. **(B)** Showing the fully constructed electrodes, and the hook portion for placement around the sciatic nerve.

The GRASS stimulator settings were configured and adjusted (Appendix Figure 5) until TOF-R stimulation parameters were obtained as determined by an output to the Powerlab.

**Appendix Figure 5**: GRASS Stimulator configured settings to obtain TOF-R stimulation conditions. Train rate: 0.05 trains per second (TPS), Train duration: 2000 ms, Stimulation rate: 2 Hz, Delay: 0 ms, Duration: 0.2 ms, Volts: variable – here shown at 140 V.

# APPENDIX B: POWERLAB/LABCHART

Appendix B-1: Input

Channel 1: Muscle Tension (Powerlab Input 1) - Signal from strain gauge-amplifier (Range 500mV, Low Pass Filter: 20Hz, sample rate: 1000 $s^{-1}$): (Appendix Figure 6: A)

Channel 7: ECG Lead 1 (BioAmp Port 3) – ECG Signal (Range: 20mV, Low Pass Filter: 50Hz, High Pass Filter: 10Hz, sample rate: 1000 $s^{-1}$): (Appendix Figure 8: A)

Channel 10: Stimulus (Powerlab Input 2) – Indicator output from GRASS stimulator indicating start of train stimulus (Range: 20V, sample rate: 1000 $s^{-1}$): (Not shown)

Appendix B-2: Train-of-Four ratio calculation

Channel 2: Twitch Height – Twitch heights calculated using Peak Analysis Module (Detection threshold (Two-sided height): 1mV, Peak Window: 100ms, Baseline: average between peaks) (Appendix Figure 6: B)

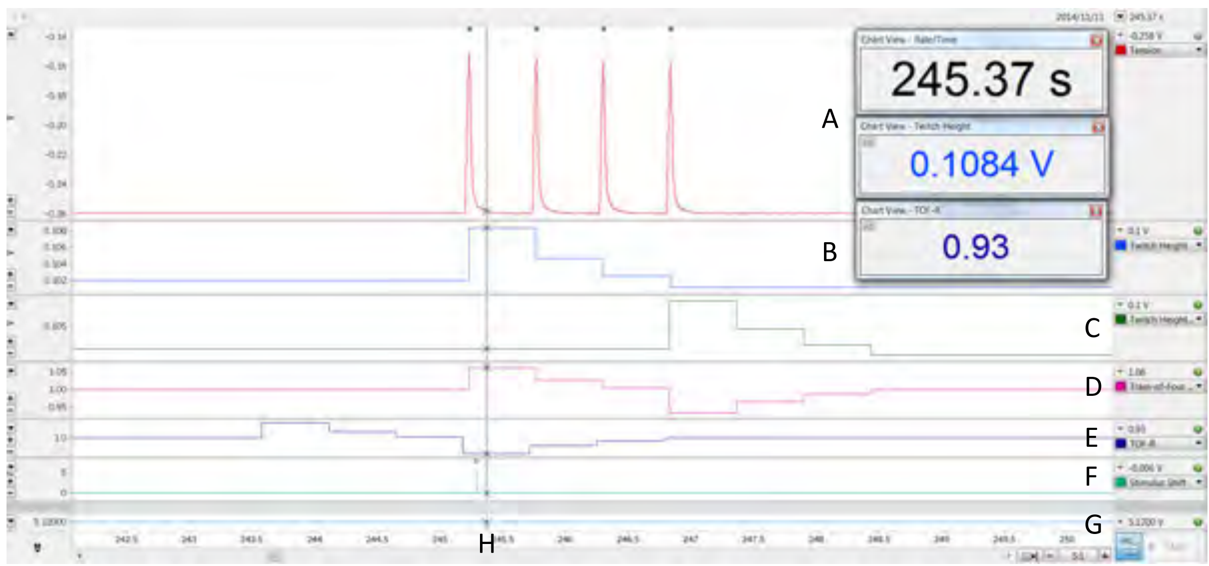
Channel 3: Twitch Height Shift – Channel 2 Recording shifted 1.64s to the right (Appendix Figure 6: C)

Channel 4: TOF Ratio Calculation – Arithmetic "= Channel 2/Channel 3" (Appendix Figure 6: D)

Channel 5: TOF Ratio Shift – Channel 4 Recording shifted 1.64s to the left (Appendix Figure 6: E)

Placement of the Recording Bar at position H (Appendix Figure 6: H) generates the Train-of-Four Ratio and T1 height values in their respective DVMs for the selected TOF train.

Note: Due to nature of Peak Analysis software during recording, T4 twitch was only detected and its height was only plotted in Channel 2 when the next train had been delivered. For this reason, TOF Ratio could only be recorded upon delivery of the next train.




**Appendix Figure 6:** LabChart muscle recording channel setup and display. Channel recordings are **(A)** Muscle tension, **(B)** Twitch height, **(C)** Twitch height shift, **(D)** TOF Ratio calculation, **(E)** TOF Ratio shift, **(F)** Stimulus shift and **(G)** Stimulus detection. **(H)** Placement of measurement line for measurement of TOF Ratio and T1.

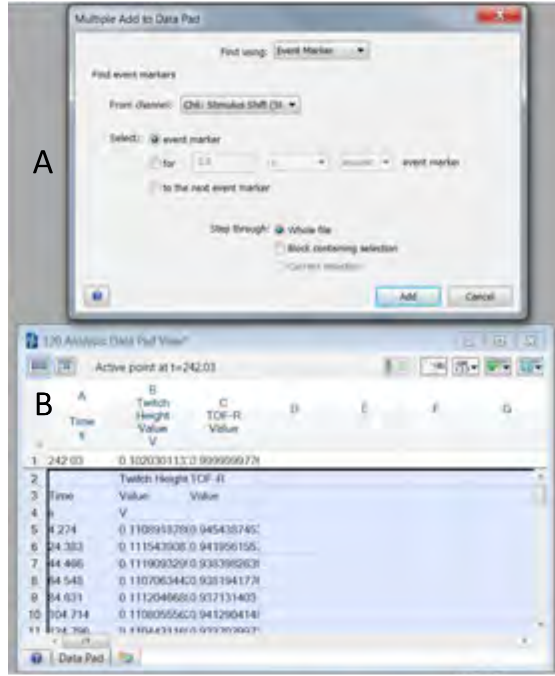
<u>Appendix B-3: Automated transfer of data to data pad</u>

Channel 6: Stimulus Shift – Channel 10 recording shifted 100ms to the right (Appendix Figure 6: F)

Channel 11: Stimulus detection – Cyclic measurements (Height) from Channel 6 – Arbitrary calculation in order to place event markers at time of stimulation. (Appendix Figure 6: G)

Upon completion of recording, data pad was set up to reflect values to be extracted (Appendix Figure 7: B)

“Multiple Add To Data Pad” selected (Appendix Figure 7: A)

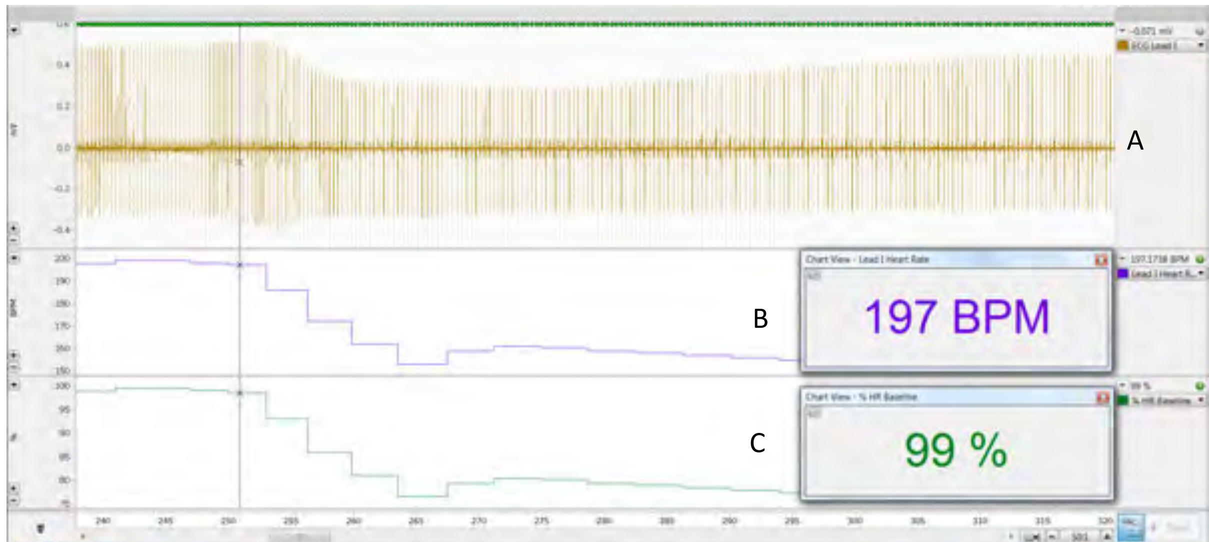



**Appendix Figure 7**: Screenshot of automated data extraction from recording. **(A)** Multiple add to Data Pad, showing settings used. **(B)** Data Pad window showing parameters extracted from each train.

## Appendix B-4: Heart rate calculation

Channel 8: Heart Rate – 5 Beat averaged heart rate using ECG Analysis Module (Standard Powerlab/LabChart Rat ECG Detection Settings (Appendix Figure 8: B)

Channel 9: %HR – HR displayed as a percentage of the average baseline value. (Appendix Figure 8: C)




**Appendix Figure 8**: ECG recording and heart rate calculations. **(A)** Lead I ECG recording **(B)** ECG-calculated heart rate **(C)** Heart rate as % of averaged baseline heart rate.

# APPENDIX C: PROJECT COSTS

Cost of project calculated through expenditure in the following categories:

| **Category/Expenditure:** | **Cost:** |
|---|---|
| **Rats** | **R14 613.36** |
| Order Cost | R10 553.36 |
| Husbandry Costs (cage cleaning, bedding, food etc.) | R4 060.00 |
| **Equipment:** | **R2 000.00** |
| Equipment modifications (modification of existing equipment) | R2 000.00 |
| **Consumables:** | **R8 584.76** |
| Drugs (isoflurane, neostigmine, magnesium, pentobarbitone, etc.) | R5 078.76 |
| Surgery Consumables (sutures, surgical drapes, masking tape, plastic bags etc.) | R2 350.00 |
| IV Consumables (cannulae, extension sets, syringes, needles etc.) | R1 156.00 |
| **Conferences:** | **R8 687.64** |
| Accommodation | R3 126.00 |
| Travel (flights and taxi fare to/from airport) | R4 561.64 |
| Registration | R1 000.00 |
| **TOTAL COST OF PROJECT:** | R33 885.76 |

**Appendix Table 1**: Cost of project according to category

# APPENDIX D: SUPPLEMENTARY RESULTS

D-1. Rat utilisation

| Experiment / Group: | Rats used: | Rats data used for analysis: |
|---|---|---|
| | | |
| **Magnesium dose-effect:** | **30** | **25** |
| 0 (Saline) group | 6 | 4 |
| 10 mg/kg group | 6 | 4 |
| 30 mg/kg group | 6 | 6 |
| 60 mg/kg group | 6 | 6 |
| 100 mg/kg group | 6 | 5 |
| | | |
| **Magnesium recurarisation:** | **36** | **25** |
| No reversal group (SPON) | 9 | 6 |
| Neostigmine group (NEO) | 9 | 6 |
| Sugammadex 4 mg/kg (SUG4) | 9 | 6 |
| Sugammadex 16 mg/kg (SUG16) | 9 | 7 |

**Appendix Table 2:** Rat data used for analysis compared to total rats used for experimentation

D-2. Rat weight

The weight of the rat at the time of induction of anaesthesia, and the weight ultimately used to calculate volume (and thus dose) of drugs administered.

D-2.1. Magnesium dose-effect experiment:

| Magnesium Dose: | Mean Weight (g): | SD (g): | Range (g): | Significance: |
|---|---|---|---|---|
| 0 mg/kg (Saline) | 367.50 | 15.000 | 350 - 380 | |
| 10 mg/kg | 353.75 | 52.182 | 285 - 410 | |
| 30 mg/kg | 323.00 | 66.212 | 270 - 450 | |
| 60 mg/kg | 298.33 | 20.656 | 275 - 325 | |
| 100 mg/kg | 303.00 | 33.091 | 260 - 350 | |
| POOLED | 325.12 | 47.661 | 260 - 450 | ----------------- |

**Appendix Table 3**: Descriptive statistics of rat weight at the time of induction of anaesthesia in the magnesium dose-effect experiment. One-way ANOVA revealed no significant differences between the groups (p=0.0930)

D-2.2. Magnesium recurarisation experiment:

| Reversal Regime: | Mean Weight (g): | SD (g): | Range (g): | Significance: |
|---|---|---|---|---|
| SPON | 332.50 | 21.622 | 300 - 355 | |
| NEO | 339.17 | 6.646 | 330 - 350 | |
| SUG4 | 335.00 | 25.298 | 300 - 375 | |
| SUG16 | 322.14 | 17.762 | 290 - 340 | |
| POOLED | 331.80 | 19.033 | 290 - 375 | ----------------- |

**Appendix Table 4**: Descriptive statistics of rat weight at the time of induction of anaesthesia in the magnesium recurarisation experiment. One-way ANOVA revealed no significant differences between the groups (p=0.4326)

## D-3. Average isoflurane concentration

The dose of isoflurane that was stabilised upon after tracheostomy and switch to mechanical ventilation, and reflects the relative steady state concentration of isoflurane exposed to the neuromuscular junction.

D-3.1. Magnesium dose-effect experiment

| Magnesium Dose: | Mean [Iso] (%): | SD (%): | Range (%): | Significance: |
|---|---|---|---|---|
| 0 mg/kg (Saline) | 1.68 | 0.150 | 1.6 - 1.9 | * |
| 10 mg/kg | 3.33 | 0.330 | 3 - 3.7 | |
| 30 mg/kg | 3.13 | 0.383 | 2.6 - 3.7 | |
| 60 mg/kg | 2.67 | 0.121 | 2.5 - 2.8 | # |
| 100 mg/kg | 3.22 | 0.402 | 2.8 - 3.7 | |
| POOLED | 2.84 | 0.634 | 1.6 - 3.7 | ---------------------- |

**Appendix Table 5**: Descriptive statistics of average isoflurane concentration in the magnesium dose-effect experiment. One-way ANOVA revealed significant differences between the groups (p<0.0001). * Indicates significant difference to 10, 30, 100 (p<0.001) and 60 (p=0.001) mg/kg groups. # Indicates significant difference to 10 mg/kg group (p<0.05).

### D-3.2. Magnesium recurarisation experiment

| Reversal Regime: | Mean [Iso] (%): | SD (%): | Range (%): | Significance: |
|---|---|---|---|---|
| SPON | 2.33 | 0.532 | 1.4 - 2.8 | |
| NEO | 2.65 | 0.105 | 2.5 - 2.8 | |
| SUG4 | 2.80 | 0.283 | 2.4 - 3.2 | |
| SUG16 | 2.70 | 0.183 | 2.4 - 2.9 | |
| POOLED | 2.62 | 0.342 | 1.4 - 3.2 | ----------------- |

**Appendix Table 6**: Descriptive statistics of average isoflurane concentration in the magnesium dose-effect experiment. Kruskall-Wallis ANOVA revealed no significant differences between the groups (p=0.2408).

## D-4. Anaesthesia time (Induction to start of experimental protocol)

This is the time from the induction of anaesthesia to the administration of the first test drug. This measure was compared between groups to account for variability in the duration of time spent under anaesthesia (and thus exposure to isoflurane) prior to starting the experimental protocol.

### D-4.1. Magnesium dose-effect experiment

The anaesthesia time was measured from time of anaesthesia induction to time of magnesium administration.

| Magnesium Dose: | Mean $T_{anaesth}$ (min): | SD (min): | Range (min): | Significance: |
|---|---|---|---|---|
| 0 mg/kg (Saline) | 184.3 | 10.34 | 170 - 194 | |
| 10 mg/kg | 187.0 | 19.17 | 168 - 212 | |
| 30 mg/kg | 175.0 | 30.43 | 130 - 220 | |
| 60 mg/kg | 138.7 | 6.53 | 129 - 149 | |
| 100 mg/kg | 177.4 | 35.32 | 130 - 210 | |
| POOLED | 170.2 | 28.53 | 129 - 220 | ---------------------- |

**Appendix Table 7**: Descriptive statistics of the time from induction of anaesthesia to administration of magnesium, in the magnesium dose-effect experiment. One-way ANOVA revealed significant differences (p=0.0211), however Bonferroni posthoc test showed no significant differences between groups.

D-4.2. Magnesium recurarisation experiment

The anaesthesia time was measured from time of anaesthesia induction to time of rocuronium administration.

| Reversal Regime: | Mean $T_{anaesth}$ (min): | SD (min): | Range (min): | Significance: |
|---|---|---|---|---|
| SPON | 161.8 | 21.64 | 138 - 186 | * |
| NEO | 141.2 | 10.80 | 127 - 157 | |
| SUG4 | 155.5 | 19.23 | 140 - 192 | |
| SUG16 | 134.0 | 11.34 | 121 - 157 | |
| POOLED | 147.6 | 19.02 | 140 - 192 | ----------------- |

**Appendix Table 8:** Descriptive statistics of the time from induction of anaesthesia to administration of rocuronium, in the magnesium recurarisation experiment. One-way ANOVA revealed significant differences (p=0.0174). * Indicates significant difference to SUG16 group (p<0.05).

D-5. Pre-magnesium baseline measures (Dose-effect experiment only)

D-5.1. TOF-R baseline

The Train-of-Four Ratio value at the time of administering magnesium was compared to account for variations in the baseline efficiency of neuromuscular transmission.

Due to the differences in state of neuromuscular physiology at the time of magnesium administration in the magnesium recurarisation experiment, TOF-R was not compared for the recurarisation experiment.

| Magnesium Dose: | Mean $TOF\text{-}R_{base}$: | SD: | Range: | Significance: |
|---|---|---|---|---|
| 0 mg/kg (Saline) | 0.9375 | 0.01056 | 0.9283 - 0.9476 | |
| 10 mg/kg | 0.9574 | 0.02795 | 0.9301 - 0.9956 | |
| 30 mg/kg | 0.9533 | 0.02105 | 0.9188 - 0.9757 | |
| 60 mg/kg | 0.9435 | 0.00692 | 0.9321 - 0.9539 | |
| 100 mg/kg | 0.9503 | 0.00887 | 0.9351 - 0.9580 | |
| POOLED | 0.9485 | 0.01651 | 0.9188 - 0.9956 | ---------------------- |

**Appendix Table 9**: Descriptive statistics of the baseline TOF-R prior to administering magnesium, in the magnesium dose-effect experiment. One-way ANOVA revealed no significant differences (p=0.4301).

### D-5.2. $T1_{raw}$

The raw T1 value at the time of administering magnesium was compared to account for variations in the contraction strength of the muscle.

Due to the gradual fade in T1 height over time, coupled with differences in the time from rocuronium administration to magnesium administration, $T1_{raw}$ was not compared for the recurarisation experiment.

| **Magnesium Dose:** | **Mean $T1_{raw}$ (mV):** | **SD (mV):** | **Range (mV):** | **Significance:** |
|---|---|---|---|---|
| 0 mg/kg (Saline) | 166.62 | 12.738 | 153.9 - 184.3 | |
| 10 mg/kg | 165.73 | 16.860 | 144.9 - 184.0 | |
| 30 mg/kg | 116.72 | 39.446 | 76.9 - 186.1 | |
| 60 mg/kg | 107.14 | 18.398 | 84.4 - 129.5 | |
| 100 mg/kg | 124.32 | 48.058 | 89.4 - 207.1 | |
| POOLED | 131.76 | 38.079 | 76.9 - 207.1 | ---------------------- |

**Appendix Table 10**: Descriptive statistics of the baseline raw T1 value, in the magnesium dose-effect experiment. One-way ANOVA revealed significant differences (p=0.0211), however Bonferroni posthoc test showed no significant differences between groups.

### D-6. Spontaneous recovery of NMB (Pre-reversal parameters)

The duration of NMB (i.e. recovery from NMB) were compared between groups to assess for variations in the degree of neuromuscular block induced and recovery thereof.

D-6.1. Recovery of TOF twitches

The time from the administration of rocuronium to the recovery of T1, T2, T3 and T4 of the TOF stimulation pattern was compared to assess the differences in recovery from NMB.

| Reversal Regime: | Mean $T_{recov}$ (min): | SD (min): | Range (min): | Significance: |
|---|---|---|---|---|
| **T1 Recovery** | | | | |
| SPON | 396.7 | 67.43 | 280 - 480 | |
| NEO | 386.6 | 97.70 | 280 - 560 | |
| SUG4 | 553.3 | 184.90 | 260 - 720 | |
| SUG16 | 442.86 | 96.21 | 260 - 540 | |
| POOLED | 444.80 | 129.39 | 260 - 720 | ----------------- |
| **T2 Recovery** | | | | |
| SPON | 526.7 | 73.39 | 380 - 580 | |
| NEO | 480.0 | 75.89 | 400 - 600 | |
| SUG4 | 693.3 | 199.47 | 420 - 900 | |
| SUG16 | 560.0 | 117.19 | 380 - 740 | |
| POOLED | 564.8 | 142.63 | 380 - 900 | ----------------- |
| **T3 Recovery** | | | | |
| SPON | 593.3 | 80.66 | 440 - 660 | |
| NEO | 553.3 | 97.71 | 460 - 720 | |
| SUG4 | 786.7 | 203.44 | 540 - 1020 | |
| SUG16 | 631.4 | 138.98 | 440 - 860 | |
| POOLED | 640.8 | 156.89 | 440 - 1020 | ----------------- |
| **T4 Recovery** | | | | |
| SPON | 630.0 | 89.22 | 460 - 700 | |
| NEO | 586.7 | 104.05 | 460 - 760 | |
| SUG4 | 840.0 | 218.72 | 600 - 1100 | |
| SUG16 | 660.0 | 140.95 | 480 - 900 | |
| POOLED | 678.4 | 167.82 | 460 - 1100 | ----------------- |

**Appendix Table 11**: Descriptive statistics of the time to recovery of T1, T2, T3 and T4 of the TOF twitches after rocuronium administration. One-way ANOVA of recovery of T1 ($p=0.0898$), T2 ($p=0.0816$) and T3 ($p=0.0609$) revealed no significant differences. One-way ANOVA of recovery of T4 showed significant difference ($p=0.0499$), however Bonferroni posthoc test showed no significant differences between groups.

D-6.2. TOF-R at T4 recovery

The TOF-R value at the time of recovery of T4 to a detectable height was measured in order to compare the efficacy of neuromuscular transmission prior to reversal of NMB.

| **Reversal Regime:** | **Mean $TOF\text{-}R_{@T4}$:** | **SD:** | **Range:** | **Significance:** |
|---|---|---|---|---|
| SPON | 0.224 | 0.0430 | 0.178 - 0.292 | |
| NEO | 0.247 | 0.0318 | 0.198 - 0.278 | |
| SUG4 | 0.205 | 0.0460 | 0.153 - 0.282 | |
| SUG16 | 0.230 | 0.0494 | 0.174 - 0.321 | |
| POOLED | 0.226 | 0.0433 | 0.153 - 0.321 | ----------------- |

**Appendix Table 12**: Descriptive statistics of TOF-R at the time of T4 recovery, in the magnesium recurarisation experiment. One-way ANOVA revealed no significant differences (p=0.4260).

# APPENDIX E: RAW STATISTICAL RESULTS

## E-1. Magnesium dose-effect experiment

### E-1.1. Rat Weight

NORMALITY

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| weight | **25** | **0.93889** | **1.698** | **1.082** | **0.13954** |

NORMALLY DISTRIBUTED

DESCRIPTIVE STATISTICS

-> dose = 0

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| weight | **4** | **367.5** | **15** | **350** | **380** |

-> dose = 10

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| weight | **4** | **353.75** | **52.18157** | **285** | **410** |

-> dose = 30

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| weight | **6** | **323** | **66.21178** | **270** | **450** |

-> dose = 60

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| weight | **6** | **298.3333** | **20.65591** | **275** | **325** |

-> dose = 100

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| weight | **5** | **303** | **33.09078** | **260** | **350** |

COMPARISON OF MEANS

ONE-WAY ANOVA (WITH BONFERRONI POSTHOC TEST)

Analysis of Variance

| Source | SS | df | MS | F | Prob > F |
|---|---|---|---|---|---|
| Between groups | **17241.5567** | **4** | **4310.38917** | **2.31** | **0.0930** |
| Within groups | **37277.0833** | **20** | **1863.85417** | | |
| Total | **54518.64** | **24** | **2271.61** | | |

Bartlett's test for equal variances: chi2(**4**) = **9.4330** Prob>chi2 = **0.051**

NO SIGNIFICANT DIFFERENCES BETWEEN GROUPS

## E-1.2. Average isoflurane concentration

### NORMALITY

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| iso | 25 | 0.94884 | 1.422 | 0.719 | 0.23602 |

NORMALLY DISTRIBUTED

### DESCRIPTIVE STATISTICS

-> dose = 0

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| iso | 4 | 1.675 | .15 | 1.6 | 1.9 |

-> dose = 10

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| iso | 4 | 3.325 | .3304038 | 3 | 3.7 |

-> dose = 30

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| iso | 6 | 3.133333 | .3829709 | 2.6 | 3.7 |

-> dose = 60

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| iso | 6 | 2.666667 | .121106 | 2.5 | 2.8 |

-> dose = 100

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| iso | 5 | 3.22 | .4024923 | 2.8 | 3.7 |

### COMPARISON OF MEANS

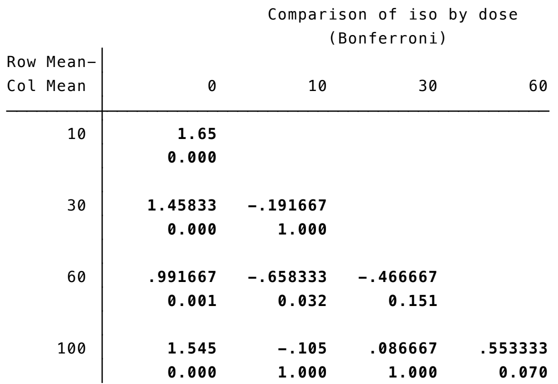
ONE-WAY ANOVA (WITH BONFERRONI POSTHOC TEST)

Analysis of Variance

| Source | SS | df | MS | F | Prob > F |
|---|---|---|---|---|---|
| Between groups | 7.78793334 | 4 | 1.94698333 | 21.05 | 0.0000 |
| Within groups | 1.8496671 | 20 | .092483355 | | |
| Total | 9.63760043 | 24 | .401566685 | | |

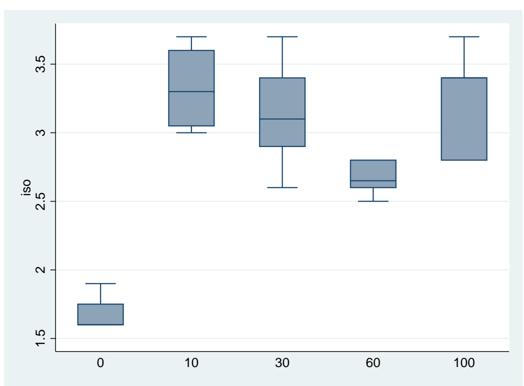
Bartlett's test for equal variances: chi2(4) = 7.6001 Prob>chi2 = 0.107

SIGNIFICANT DIFFERENCES BETWEEN GROUPS (P<0.0001)



Comparison of iso by dose
(Bonferroni)

| Row Mean-Col Mean | 0 | 10 | 30 | 60 |
|---|---|---|---|---|
| 10 | 1.65<br>0.000 | | | |
| 30 | 1.45833<br>0.000 | -.191667<br>1.000 | | |
| 60 | .991667<br>0.001 | -.658333<br>0.032 | -.466667<br>0.151 | |
| 100 | 1.545<br>0.000 | -.105<br>1.000 | .086667<br>1.000 | .553333<br>0.070 |




SIGNIFICANT DIFFERENCES BETWEEN

SAL VS. 10mg (p<0.001)
30mg (p<0.001)
60mg (p=0.001)
100mg (p<0.001)

10MG VS. 60mg (p=0.032)

E-1.3. Anaesthesia time @ magnesium injection

NORMALITY

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| a_time_mg | **25** | **0.93908** | **1.693** | **1.076** | **0.14099** |

NORMALLY DISTRIBUTED

DESCRIPTIVE STATISTICS

-> dose = 0

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| a_time_mg | **4** | **184.25** | **10.34005** | **170** | **194** |

-> dose = 10

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| a_time_mg | **4** | **187** | **19.16594** | **168** | **212** |

-> dose = 30

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| a_time_mg | **6** | **175** | **30.43025** | **130** | **220** |

-> dose = 60

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| a_time_mg | **6** | **138.6667** | **6.531973** | **129** | **149** |

-> dose = 100

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| a_time_mg | **5** | **177.4** | **35.32421** | **130** | **210** |

COMPARISON OF MEANS

ONE-WAY ANOVA (WITH BONFERRONI POSTHOC TEST)

Analysis of Variance

| Source | SS | df | MS | F | Prob > F |
|---|---|---|---|---|---|
| Between groups | **8282.07667** | **4** | **2070.51917** | **3.68** | **0.0211** |
| Within groups | **11257.2833** | **20** | **562.864167** | | |
| Total | **19539.36** | **24** | **814.14** | | |

Bartlett's test for equal variances: chi2(**4**) = **12.2000** Prob>chi2 = **0.016**

SIGNIFICANT DIFFERENCES BETWEEN GROUPS (p=0.0211)

Comparison of a_time_mg by dose
(Bonferroni)

| Row Mean−Col Mean | 0 | 10 | 30 | 60 |
|---|---|---|---|---|
| 10 | **2.75**<br>**1.000** | | | |
| 30 | **-9.25**<br>**1.000** | **-12**<br>**1.000** | | |
| 60 | **-45.5833**<br>**0.075** | **-48.3333**<br>**0.050** | **-36.3333**<br>**0.153** | |
| 100 | **-6.85**<br>**1.000** | **-9.6**<br>**1.000** | **2.4**<br>**1.000** | **38.7333**<br>**0.139** |

NO SIGNIFICANT DIFFERENCES BETWEEN GROUPS

## E-1.4. $T1_{raw}$ baseline value

### NORMALITY

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| t1base | 25 | 0.94161 | 1.622 | 0.989 | 0.16126 |

NORMALLY DISTRIBUTED

### DESCRIPTIVE STATISTICS

-> dose = 0

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t1base | 4 | 166.625 | 12.73876 | 153.9 | 184.3 |

-> dose = 10

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t1base | 4 | 165.725 | 16.86048 | 144.9 | 184 |

-> dose = 30

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t1base | 6 | 116.7165 | 39.4457 | 76.86 | 186.1 |

-> dose = 60

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t1base | 6 | 107.1353 | 18.39814 | 84.4 | 129.5 |

-> dose = 100

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t1base | 5 | 124.32 | 48.05765 | 89.4 | 207.1 |

### COMPARISON OF MEANS

### ONE-WAY ANOVA (WITH BONFERRONI POST HOC TEST)

Analysis of Variance

| Source | SS | df | MS | F | Prob > F |
|---|---|---|---|---|---|
| Between groups | 14749.6117 | 4 | 3687.40293 | 3.68 | 0.0211 |
| Within groups | 20050.0771 | 20 | 1002.50386 | | |
| Total | 34799.6888 | 24 | 1449.98703 | | |

Bartlett's test for equal variances: chi2(4) = 8.2641 Prob>chi2 = 0.082

SIGNIFICANT DIFFERENCES BETWEEN GROUPS (p=0.0211)

Comparison of t1base by dose
(Bonferroni)

| Row Mean-Col Mean | 0 | 10 | 30 | 60 |
|---|---|---|---|---|
| 10 | **-.900002**<br>**1.000** | | | |
| 30 | **-49.9085**<br>**0.240** | **-49.0085**<br>**0.264** | | |
| 60 | **-59.4897**<br>**0.086** | **-58.5897**<br>**0.095** | **-9.58117**<br>**1.000** | |
| 100 | **-42.305**<br>**0.602** | **-41.405**<br>**0.654** | **7.6035**<br>**1.000** | **17.1847**<br>**1.000** |

NO SIGNIFICANT DIFFERENCES BETWEEN GROUPS

## E-1.5. TOF-R baseline value

**NORMALITY**

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| tofbase | 25 | 0.94696 | 1.474 | 0.793 | 0.21395 |

NORMALLY DISTRIBUTED

**DESCRIPTIVE STATISTICS**

-> dose = 0

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tofbase | 4 | .937575 | .0105629 | .9283 | .9476 |

-> dose = 10

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tofbase | 4 | .95735 | .0279538 | .9301 | .9956 |

-> dose = 30

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tofbase | 6 | .95325 | .0210471 | .9188 | .9757 |

-> dose = 60

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tofbase | 6 | .9435167 | .006924 | .9321 | .9539 |

-> dose = 100

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tofbase | 5 | .95028 | .00887 | .9351 | .958 |

**COMPARISON OF MEANS**

ONE-WAY ANOVA (WITH BONFERRONI POSTHOC TEST)

Analysis of Variance

| Source | SS | df | MS | F | Prob > F |
|---|---|---|---|---|---|
| Between groups | .001090905 | 4 | .000272726 | 1.00 | 0.4301 |
| Within groups | .005448288 | 20 | .000272414 | | |
| Total | .006539193 | 24 | .000272466 | | |

Bartlett's test for equal variances: chi2(4) = 9.7147 Prob>chi2 = 0.046

NO SIGNIFICANT DIFFERENCES BETWEEN GROUPS

E-1.6. TOF-$R_{min}$ (lowest TOF-R reached after magnesium injection)

NORMALITY

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| tofmin | 25 | 0.79352 | 5.737 | 3.571 | 0.00018 |

NOT NORMALLY DISTRIBUTED

SHAPIRO WILK (LOG-TRANSFORMATION)

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| tofminlog | 25 | 0.73657 | 7.320 | 4.069 | 0.00002 |

LOG TRANSFORMATION FAILED - DATA NOT NORMALLY DISTRIBUTED

DESCRIPTIVE STATISTICS

-> dose = 0

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tofmin | 4 | .925 | .003587 | .9207 | .9293 |

-> dose = 10

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tofmin | 4 | .94315 | .0263451 | .9204 | .9806 |

-> dose = 30

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tofmin | 6 | .9349833 | .0188816 | .9022 | .9589 |

-> dose = 60

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tofmin | 6 | .6957167 | .1413277 | .4718 | .8736 |

-> dose = 100

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tofmin | 5 | .45232 | .1507308 | .2793 | .5844 |

## COMPARISON OF MEANS

## KRUSKALL WALLIS ANOVA (WITH BONFERRONI POSTHOC TEST)

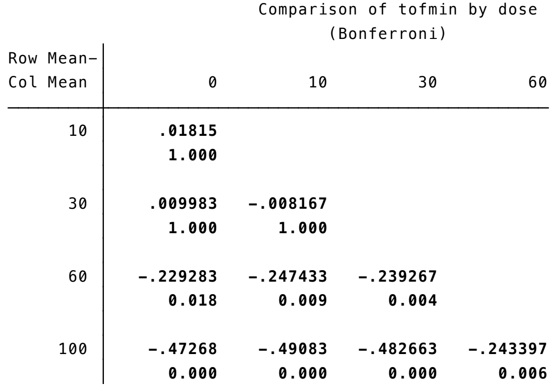
Kruskal-Wallis equality-of-populations rank test

| dose | Obs | Rank Sum |
|---|---|---|
| 0 | 4 | 62.00 |
| 10 | 4 | 79.00 |
| 30 | 6 | 118.00 |
| 60 | 6 | 48.00 |
| 100 | 5 | 18.00 |

chi-squared = 19.675 with 4 d.f.
probability = 0.0006

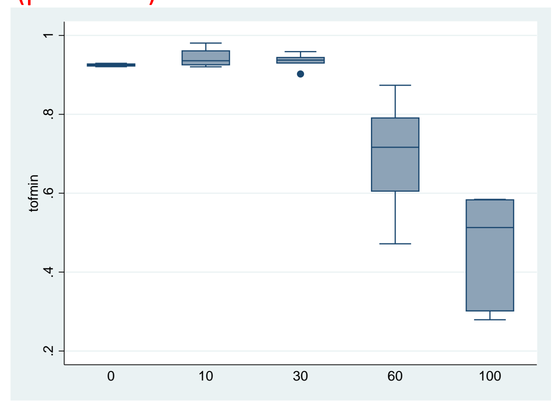
chi-squared with ties = 19.675 with 4 d.f.
probability = 0.0006

SIGNIFICANT DIFFERENCE BETWEEN GROUPS (p=0.0006)



Comparison of tofmin by dose
(Bonferroni)

| Row Mean-Col Mean | 0 | 10 | 30 | 60 |
|---|---|---|---|---|
| 10 | .01815 | | | |
| | 1.000 | | | |
| 30 | .009983 | -.008167 | | |
| | 1.000 | 1.000 | | |
| 60 | -.229283 | -.247433 | -.239267 | |
| | 0.018 | 0.009 | 0.004 | |
| 100 | -.47268 | -.49083 | -.482663 | -.243397 |
| | 0.000 | 0.000 | 0.000 | 0.006 |




SIGNIFICANT DIFFERENCES:

SAL VS. 60mg (p=0.018)
100mg (p<0.001)
10mg vs. 60mg (p=0.009)
100mg (p<0.001)
30mg vs. 60mg (p=0.004)
100mg (p<0.001)
60mg vs. 100mg (p=0.006)

E-1.7. dTOF (Greatest depression of TOF-R after magnesium injection)

NORMALITY

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| dtof | 25 | 0.76862 | 6.429 | 3.804 | 0.00007 |

NOT NORMALLY DISTRIBUTED

SHAPIRO WILK (LOG-TRANSFORMATION)

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| dtoflog | 25 | 0.91007 | 2.499 | 1.872 | 0.03060 |

LOG TRANSFORMATION FAILED - DATA NOT NORMALLY DISTRIBUTED

DESCRIPTIVE STATISTICS

-> dose = 0

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| dtof | 4 | .012575 | .0076678 | .0045 | .0199 |

-> dose = 10

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| dtof | 4 | .0142 | .0031591 | .0097 | .0171 |

-> dose = 30

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| dtof | 6 | .0174667 | .0155065 | .0018 | .0457 |

-> dose = 60

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| dtof | 6 | .2528 | .1375892 | .0992 | .4723 |

-> dose = 100

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| dtof | 5 | .49794 | .1550861 | .352 | .6735 |

## COMPARISON OF MEANS

## KRUSKALL WALLIS ANOVA (WITH BONFERRONI POSTHOC TEST)

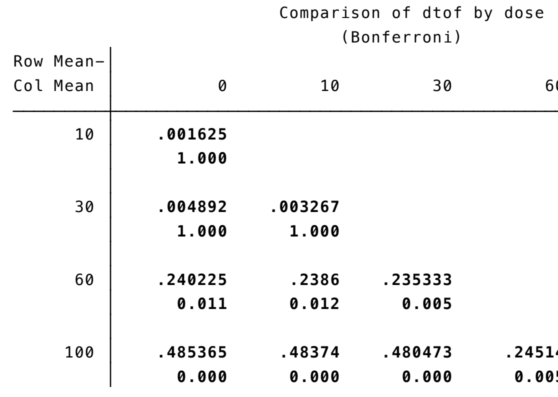
Kruskal-Wallis equality-of-populations rank test

| dose | Obs | Rank Sum |
|---|---|---|
| 0 | 4 | 28.00 |
| 10 | 4 | 31.00 |
| 30 | 6 | 46.00 |
| 60 | 6 | 108.00 |
| 100 | 5 | 112.00 |

chi-squared = **18.770** with **4** d.f.
probability = **0.0009**

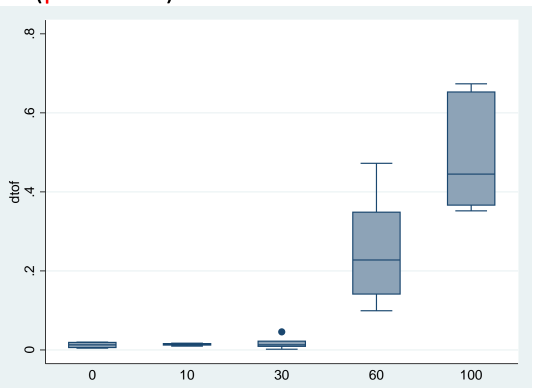
chi-squared with ties = **18.777** with **4** d.f.
probability = **0.0009**

## SIGNIFICANT DIFFERENCES BETWEEN GROUPS (p<0.0009)



Comparison of dtof by dose
(Bonferroni)

| Row Mean-Col Mean | 0 | 10 | 30 | 60 |
|---|---|---|---|---|
| 10 | .001625<br>1.000 | | | |
| 30 | .004892<br>1.000 | .003267<br>1.000 | | |
| 60 | .240225<br>0.011 | .2386<br>0.012 | .235333<br>0.005 | |
| 100 | .485365<br>0.000 | .48374<br>0.000 | .480473<br>0.000 | .24514<br>0.005 |




SIGNIFICANT DIFFERENCES:

| | | |
|---|---|---|
| SAL | vs. | 60mg (p=0.011) |
| | | 100mg (p<0.001) |
| 10mg | vs. | 60mg (p=0.012) |
| | | 100mg (p<0.001) |
| 30mg | vs. | 60mg (p=0.005) |
| | | 100mg (p<0.001) |
| 60mg | vs. | 100mg (p<0.001) |

## E-1.8. %T1$_{min}$ (Lowest %T1 value reached after magnesium injection)

### NORMALITY

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| t1min | 24 | 0.74882 | 6.775 | 3.901 | 0.00005 |

NOT NORMALLY DISTRIBUTED

SHAPIRO WILK (LOG-TRANSFORMATION)

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| t1minlog | 24 | 0.67311 | 8.817 | 4.438 | 0.00000 |

LOG TRANSFORMATION FAILED - DATA NOT NORMALLY DISTRIBUTED

### DESCRIPTIVE STATISTICS

-> dose = 0

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t1min | 3 | 90.72666 | 1.543708 | 88.95 | 91.74 |

-> dose = 10

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t1min | 4 | 94.36 | 1.351614 | 93.16 | 96.27 |

-> dose = 30

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t1min | 6 | 93.69333 | 2.082263 | 90.9 | 96.63 |

-> dose = 60

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t1min | 6 | 75.51667 | 18.07794 | 41.22 | 91.45 |

-> dose = 100

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t1min | 5 | 41.452 | 18.12796 | 20.57 | 61.33 |

## COMPARISON OF MEANS

KRUSKALL WALLIS ANOVA (WITH BONFERRONI POSTHOC TEST)

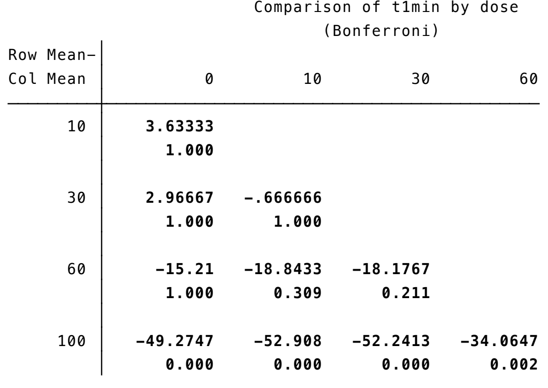
Kruskal-Wallis equality-of-populations rank test

| dose | Obs | Rank Sum |
|---|---|---|
| 0 | 3 | 41.00 |
| 10 | 4 | 78.00 |
| 30 | 6 | 113.00 |
| 60 | 6 | 50.00 |
| 100 | 5 | 18.00 |

chi-squared = 18.819 with 4 d.f.
probability = 0.0009

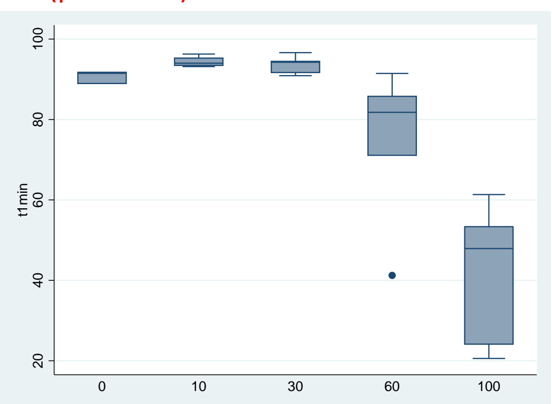
chi-squared with ties = 18.819 with 4 d.f.
probability = 0.0009

SIGNIFICANT DIFFERENCES BETWEEN GROUPS (p=0.0009)



Comparison of t1min by dose
(Bonferroni)

| Row Mean-Col Mean | 0 | 10 | 30 | 60 |
|---|---|---|---|---|
| 10 | 3.63333<br>1.000 | | | |
| 30 | 2.96667<br>1.000 | -.666666<br>1.000 | | |
| 60 | -15.21<br>1.000 | -18.8433<br>0.309 | -18.1767<br>0.211 | |
| 100 | -49.2747<br>0.000 | -52.908<br>0.000 | -52.2413<br>0.000 | -34.0647<br>0.002 |




SIGNIFICANT DIFFERENCES:

100mg vs. Sal (p<0.001)
10mg (p<0.001)
30mg (p<0.001)
60mg (p<0.001)

## E-1.9. dT1 (Greatest depression of %T1 after magnesium injection)

NORMALITY

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| dt1min | 24 | 0.74338 | 6.922 | 3.945 | 0.00004 |

NOT NORMALLY DISTRIBUTED

SHAPIRO WILK (LOG-TRANSFORMATION)

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| dt1minlog | 24 | 0.89580 | 2.811 | 2.107 | 0.01755 |

LOG TRANSFORMATION FAILED - DATA NOT NORMALLY DISTRIBUTED

DESCRIPTIVE STATISTICS

-> dose = 0

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| dt1min | 3 | 8.48 | 1.726007 | 7.39 | 10.47 |

-> dose = 10

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| dt1min | 4 | 5.3375 | 1.381506 | 3.52 | 6.85 |

-> dose = 30

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| dt1min | 6 | 5.703333 | 1.550712 | 3.38 | 7.91 |

-> dose = 60

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| dt1min | 6 | 24.02167 | 17.86151 | 8.54 | 58.07 |

-> dose = 100

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| dt1min | 5 | 58.022 | 18.14471 | 38.38 | 78.59 |

## COMPARISON OF MEANS

## KRUSKALL WALLIS ANOVA (WITH BONFERRONI POSTHOC TEST)

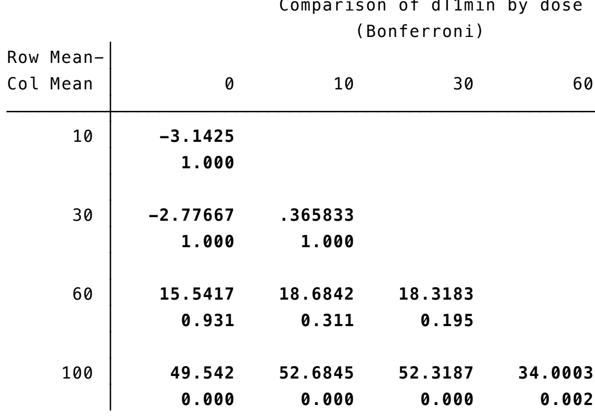
Kruskal-Wallis equality-of-populations rank test

| dose | Obs | Rank Sum |
|---|---|---|
| 0 | 3 | 35.00 |
| 10 | 4 | 21.00 |
| 30 | 6 | 36.00 |
| 60 | 6 | 101.00 |
| 100 | 5 | 107.00 |

chi-squared = 19.491 with 4 d.f.
probability = 0.0006

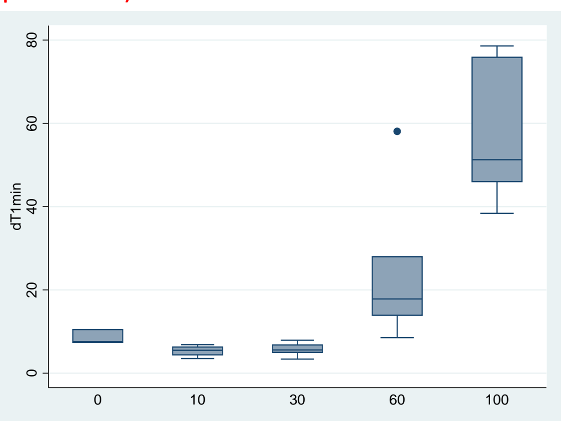
chi-squared with ties = 19.491 with 4 d.f.
probability = 0.0006

SIGNIFICANT DIFFERENCES BETWEEN GROUPS (p=0.0006)



Comparison of dT1min by dose
(Bonferroni)

| Row Mean-Col Mean | 0 | 10 | 30 | 60 |
|---|---|---|---|---|
| 10 | -3.1425<br>1.000 | | | |
| 30 | -2.77667<br>1.000 | .365833<br>1.000 | | |
| 60 | 15.5417<br>0.931 | 18.6842<br>0.311 | 18.3183<br>0.195 | |
| 100 | 49.542<br>0.000 | 52.6845<br>0.000 | 52.3187<br>0.000 | 34.0003<br>0.002 |




SIGNIFICANT DIFFERENCES:

100mg vs. Sal (p<0.001)
10mg (p<0.001)
30mg (p<0.001)
60mg (p=0.002)

E-1.10. $T1_{onset}$ (Time from magnesium injection to $\%T1_{min}$)

Note: Treatment Groups Saline, 10mg and 30mg excluded due to the lack of impairment effect

NORMALITY

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| t1onset | 11 | 0.89951 | 1.627 | 0.907 | 0.18230 |

NORMALLY DISTRIBUTED

DESCRIPTIVE STATISTICS

-> dose = 60

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t1onset | 6 | 140 | 30.98387 | 120 | 200 |

-> dose = 100

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t1onset | 5 | 248 | 33.4664 | 220 | 300 |

COMPARISON OF MEANS

Two-sample t test with equal variances

| Group | Obs | Mean | Std. Err. | Std. Dev. | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 60 | 6 | 140 | 12.64911 | 30.98387 | 107.4844 | 172.5156 |
| 100 | 5 | 248 | 14.96663 | 33.4664 | 206.446 | 289.554 |
| combined | 11 | 189.0909 | 19.32754 | 64.10219 | 146.0265 | 232.1553 |
| diff | | -108 | 19.44413 | | -151.9857 | -64.01433 |

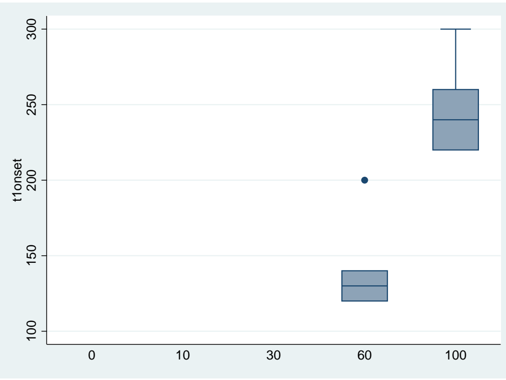
diff = mean(60) - mean(100)    t = -5.5544

Ho: diff = 0    degrees of freedom = 9

Ha: diff < 0    Ha: diff != 0    Ha: diff > 0

Pr(T < t) = 0.0002    Pr(|T| > |t|) = 0.0004    Pr(T > t) = 0.9998

SIGNIFICANT DIFFERENT FROM EACH OTHER (p=0.0004)



E-1.11. $TOF_{onset}$ (time from magnesium injection to $TOF\text{-}R_{min}$)

Note: Treatment Groups Saline, 10mg and 30mg excluded due to the lack of impairment effect

NORMALITY

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| tofonset | **11** | **0.90173** | **1.591** | **0.863** | **0.19405** |

NORMALLY DISTRIBUTED

DESCRIPTIVE STATISTICS

-> dose = 60

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tofonset | **6** | **133.3333** | **30.11091** | **100** | **180** |

-> dose = 100

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tofonset | **5** | **264** | **21.9089** | **240** | **300** |

COMPARISON OF MEANS

Two-sample t test with equal variances

| Group | Obs | Mean | Std. Err. | Std. Dev. | [95% Conf. | Interval] |
|---|---|---|---|---|---|---|
| 60 | **6** | **133.3333** | **12.29273** | **30.11091** | **101.7339** | **164.9328** |
| 100 | **5** | **264** | **9.797959** | **21.9089** | **236.7965** | **291.2035** |
| combined | **11** | **192.7273** | **21.95412** | **72.81359** | **143.8104** | **241.6441** |
| diff | | **-130.6667** | **16.21461** | | **-167.3467** | **-93.98667** |

diff = mean(**60**) - mean(**100**) t = **-8.0586**

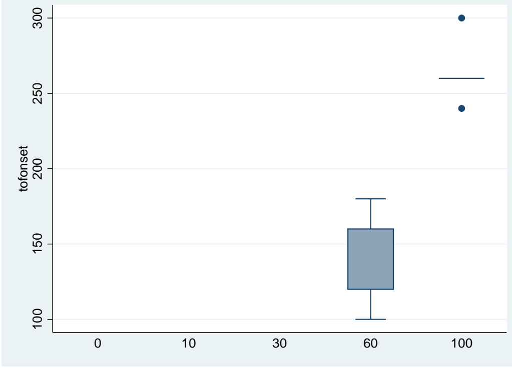
Ho: diff = 0 degrees of freedom = **9**

Ha: diff < 0 Pr(T < t) = **0.0000**

Ha: diff != 0 Pr(|T| > |t|) = **0.0000**

Ha: diff > 0 Pr(T > t) = **1.0000**

SIGNIFICANTLY DIFFERENT FROM EACH OTHER (P<0.0001)



E-1.12. TOF-$R_{900}$ (TOF-R at the end of the experimental period)

NORMALITY

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| tof900 | 25 | 0.71705 | 7.862 | 4.215 | 0.00001 |

NOT NORMALLY DISTRIBUTED

SHAPIRO WILK (LOG-TRANSFORMATION)

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| tof900log | 25 | 0.66018 | 9.443 | 4.590 | 0.00000 |

LOG TRANSFORMATION FAILED - DATA NOT NORMALLY DISTRIBUTED

DESCRIPTIVE STATISTICS

-> dose = 0

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tof900 | 4 | .9236 | .0142927 | .9085 | .9415 |

-> dose = 10

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tof900 | 4 | .957075 | .026805 | .9395 | .997 |

-> dose = 30

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tof900 | 6 | .9515 | .0204581 | .9217 | .9797 |

-> dose = 60

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tof900 | 6 | .9132333 | .0491862 | .8194 | .9663 |

-> dose = 100

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tof900 | 5 | .71218 | .1302933 | .5267 | .848 |

## COMPARISON OF MEANS

## KRUSKALL WALLIS (WITH BONFERRONI POSTHOC TEST)

Kruskal–Wallis equality-of-populations rank test

| dose | Obs | Rank Sum |
|---|---|---|
| 0 | 4 | 46.00 |
| 10 | 4 | 78.00 |
| 30 | 6 | 112.50 |
| 60 | 6 | 72.50 |
| 100 | 5 | 16.00 |

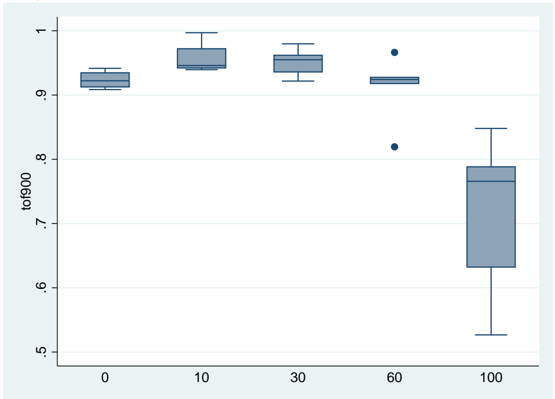
chi-squared = 15.907 with 4 d.f.
probability = 0.0031

chi-squared with ties = 15.913 with 4 d.f.
probability = 0.0031

SIGNIFICANT DIFFERENCES BETWEEN GROUPS (p=0.0031)

Comparison of tof900 by dose
(Bonferroni)

| Row Mean-Col Mean | 0 | 10 | 30 | 60 |
|---|---|---|---|---|
| 10 | .033475<br>1.000 | | | |
| 30 | .0279<br>1.000 | -.005575<br>1.000 | | |
| 60 | -.010367<br>1.000 | -.043842<br>1.000 | -.038267<br>1.000 | |
| 100 | -.21142<br>0.001 | -.244895<br>0.000 | -.23932<br>0.000 | -.201053<br>0.001 |




SIGNIFICANT DIFFERENCES:

100mg vs. Sal (p<0.001)
10mg (p<0.001)
30mg (p<0.001)
60mg (p=0.001)

## E-2. Magnesium recurarisation experiment

### E-2.1. Rat Weight

NORMALITY

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| weight | 25 | 0.97811 | 0.608 | -1.016 | 0.84516 |

NORMALLY DISTRIBUTED

DESCRIPTIVE STATISTICS

-> treatment = N

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| weight | 6 | 339.1667 | 6.645801 | 330 | 350 |

-> treatment = S

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| weight | 6 | 332.5 | 21.62175 | 300 | 355 |

-> treatment = S16

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| weight | 7 | 322.1429 | 17.76165 | 290 | 340 |

-> treatment = S4

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| weight | 6 | 335 | 25.29822 | 300 | 375 |

COMPARISON OF MEANS

ONE-WAY ANOVA (WITH BONFERRONI POSTHOC TEST)

Analysis of Variance

| Source | SS | df | MS | F | Prob > F |
|---|---|---|---|---|---|
| Between groups | 1042.80952 | 3 | 347.603175 | 0.95 | 0.4326 |
| Within groups | 7651.19048 | 21 | 364.342404 | | |
| Total | 8694 | 24 | 362.25 | | |

Bartlett's test for equal variances: chi2(3) = 6.8074 Prob>chi2 = 0.078

NO SIGNIFICANT DIFFERENCES BETWEEN GROUPS (P=0.4326)

## E-2.2. Average isoflurane concentration

### NORMALITY

Shapiro–Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| iso | 25 | 0.85401 | 4.057 | 2.863 | 0.00210 |

NOT NORMALLY DISTRIBUTED

SHAPIRO WILK (LOG-TRANSFORMATION)

Shapiro–Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| isolog | 25 | 0.74528 | 7.078 | 4.001 | 0.00003 |

LOG TRANSFORMATION FAILED - DATA NOT NORMALLY DISTRIBUTED

### DESCRIPTIVE STATISTICS

-> treatment = N

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| iso | 6 | 2.65 | .1048809 | 2.5 | 2.8 |

-> treatment = S

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| iso | 6 | 2.333333 | .531664 | 1.4 | 2.8 |

-> treatment = S16

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| iso | 7 | 2.7 | .1825742 | 2.4 | 2.9 |

-> treatment = S4

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| iso | 6 | 2.8 | .2828427 | 2.4 | 3.2 |

### COMPARISON OF MEANS

KRUSKALL WALLIS ANOVA (WITH BONFERRONI POSTHOC TEST)

Kruskal–Wallis equality–of–populations rank test

| treatm~t | Obs | Rank Sum |
|---|---|---|
| N | 6 | 70.00 |
| S | 6 | 52.50 |
| S16 | 7 | 102.50 |
| S4 | 6 | 100.00 |

chi–squared = 4.036 with 3 d.f.
probability = 0.2576

chi–squared with ties = 4.199 with 3 d.f.
probability = 0.2408

NO SIGNIFICANT DIFFERENCES BETWEEN GROUPS (P=0.2408)

## E-2.3. Anaesthesia time @ Roc injection

### NORMALITY

```
. swilk tanaes
```

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| tanaes | 25 | 0.89857 | 2.818 | 2.118 | 0.01708 |

NOT NORMALLY DISTRIBUTED

SHAPIRO WILK (LOG-TRANSFORMATION)

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| tanaeslog | 25 | 0.92865 | 1.983 | 1.399 | 0.08089 |

LOG TRANSFORMATION SUCCESSFUL – DATA NORMALLY DISTRIBUTED

### DESCRIPTIVE STATISTICS

-> treatment = N

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tanaes | 6 | 141.1667 | 10.7966 | 127 | 157 |

-> treatment = S

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tanaes | 6 | 161.8333 | 21.63716 | 138 | 186 |

-> treatment = S16

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tanaes | 7 | 134 | 11.34313 | 121 | 157 |

-> treatment = S4

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tanaes | 6 | 155.5 | 19.23278 | 140 | 192 |

## COMPARISON OF MEANS

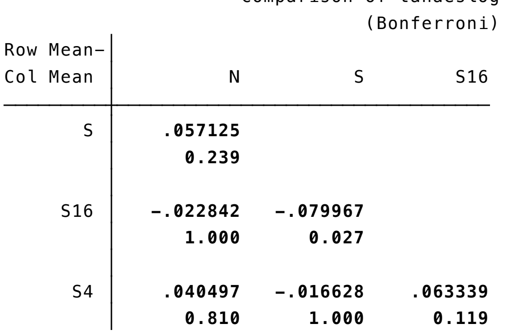
## ONE-WAY ANOVA (WITH BONFERRONI POSTHOC TEST)

Analysis of Variance

| Source | SS | df | MS | F | Prob > F |
|---|---|---|---|---|---|
| Between groups | .025818668 | 3 | .008606223 | 4.23 | 0.0174 |
| Within groups | .04277019 | 21 | .002036676 | | |
| Total | .068588858 | 24 | .002857869 | | |

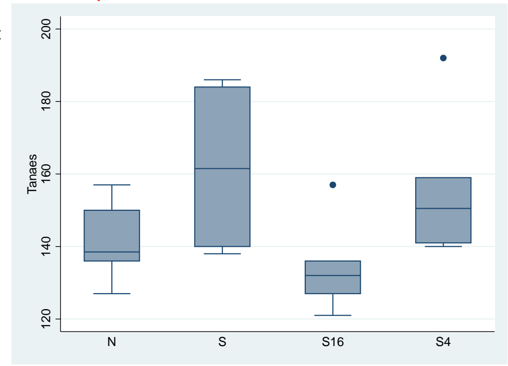
Bartlett's test for equal variances: chi2(3) = 2.1722 Prob>chi2 = 0.537

SIGNIFICANT DIFFERENCES BETWEEN GROUPS (p=0.0174)



Comparison of tanaeslog by treatment
(Bonferroni)

| Row Mean-Col Mean | N | S | S16 |
|---|---|---|---|
| S | .057125<br>0.239 | | |
| S16 | -.022842<br>1.000 | -.079967<br>0.027 | |
| S4 | .040497<br>0.810 | -.016628<br>1.000 | .063339<br>0.119 |




SIGNIFICANT DIFFERENCES:

S vs. S16 (p=0.027)

E-2.4. TOF-R @ T4 recovery

## NORMALITY

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| toft4 | 25 | 0.97879 | 0.589 | -1.080 | 0.86002 |

NORMALLY DISTRIBUTED

## DESCRIPTIVE STATISTICS

-> treatment = N

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| toft4 | 6 | .2467333 | .0318149 | .198 | .2777 |

-> treatment = S

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| toft4 | 6 | .22375 | .0429543 | .1775 | .2916 |

-> treatment = S16

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| toft4 | 7 | .2298429 | .0494143 | .1743 | .3207 |

-> treatment = S4

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| toft4 | 6 | .2045667 | .0459591 | .153 | .2822 |

## COMPARISON OF MEANS

ONE-WAY ANOVA (WITH BONFERRONI POSTHOC TEST)

Analysis of Variance

| Source | SS | df | MS | F | Prob > F |
|---|---|---|---|---|---|
| Between groups | .005465915 | 3 | .001821972 | 0.97 | 0.4260 |
| Within groups | .039498117 | 21 | .001880863 | | |
| Total | .044964032 | 24 | .001873501 | | |

Bartlett's test for equal variances: chi2(3) = 0.9706 Prob>chi2 = 0.808

NO SIGNIFICANT DIFFERENCES BETWEEN GROUPS (p=0.4260)

E-2.5. T1 Recovery

NORMALITY

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| t1 | 25 | 0.93188 | 1.893 | 1.304 | 0.09604 |

DATA NORMALLY DISTRIBUTED

DESCRIPTIVE STATISTICS

-> treatment = N

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t1 | 6 | 386.6667 | 97.70705 | 280 | 560 |

-> treatment = S

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t1 | 6 | 396.6667 | 67.42897 | 280 | 480 |

-> treatment = S16

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t1 | 7 | 442.8571 | 96.21405 | 260 | 540 |

-> treatment = S4

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t1 | 6 | 553.3333 | 184.8964 | 260 | 720 |

COMPARISON OF MEANS

ONE-WAY ANOVA (WITH BONFERRONI POSTHOC TEST)

Analysis of Variance

| Source | SS | df | MS | F | Prob > F |
|---|---|---|---|---|---|
| Between groups | 104881.143 | 3 | 34960.381 | 2.47 | 0.0898 |
| Within groups | 296942.857 | 21 | 14140.1361 | | |
| Total | 401824 | 24 | 16742.6667 | | |

Bartlett's test for equal variances: chi2(3) = 5.3384 Prob>chi2 = 0.149

NO SIGNIFICANT DIFFERENCES BETWEEN GROUPS (p=0.0898)

### E-2.6. T2 Recovery

NORMALITY

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| t2 | **25** | **0.89731** | **2.853** | **2.143** | **0.01604** |

NOT NORMALLY DISTRIBUTED

SHAPIRO WILK (LOG-TRANSFORMATION)

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| t2log | **25** | **0.94303** | **1.583** | **0.939** | **0.17389** |

LOG TRANSFORMATION SUCCESSFUL – DATA NORMALLY DISTRIBUTED

DESCRIPTIVE STATISTICS

-> treatment = N

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t2 | **6** | **480** | **75.89466** | **400** | **600** |

-> treatment = S

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t2 | **6** | **526.6667** | **73.39391** | **380** | **580** |

-> treatment = S16

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t2 | **7** | **560** | **117.1893** | **380** | **740** |

-> treatment = S4

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t2 | **6** | **693.3333** | **199.466** | **420** | **900** |

COMPARISON OF MEANS

ONE-WAY ANOVA (WITH BONFERRONI POSTHOC TEST)

Analysis of Variance

| Source | SS | df | MS | F | Prob > F |
|---|---|---|---|---|---|
| Between groups | **.069843079** | **3** | **.023281026** | **2.57** | **0.0816** |
| Within groups | **.190315812** | **21** | **.009062658** | | |
| Total | **.26015889** | **24** | **.010839954** | | |

Bartlett's test for equal variances: chi2(3) = **3.0685** Prob>chi2 = **0.381**

NO SIGNIFICANT DIFFERENCES BETWEEN GROUPS (p=0.0816)

## E-2.7. T3 Recovery

### NORMALITY

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| t3 | 25 | 0.90454 | 2.653 | 1.994 | 0.02306 |

NOT NORMALLY DISTRIBUTED

### SHAPIRO WILK (LOG-TRANSFORMATION)

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| t3log | 25 | 0.95023 | 1.383 | 0.663 | 0.25376 |

LOG TRANSFORMATION SUCCESSFUL – DATA NORMALLY DISTRIBUTED

### DESCRIPTIVE STATISTICS

-> treatment = N

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t3 | 6 | 553.3333 | 97.70705 | 460 | 720 |

-> treatment = S

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t3 | 6 | 593.3333 | 80.66391 | 440 | 660 |

-> treatment = S16

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t3 | 7 | 631.4286 | 138.9758 | 440 | 860 |

-> treatment = S4

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t3 | 6 | 786.6667 | 203.4371 | 540 | 1020 |

### COMPARISON OF MEANS

### ONE-WAY ANOVA (WITH BONFERRONI POSTHOC TEST)

Analysis of Variance

| Source | SS | df | MS | F | Prob > F |
|---|---|---|---|---|---|
| Between groups | .070389245 | 3 | .023463082 | 2.87 | 0.0609 |
| Within groups | .171875525 | 21 | .008184549 | | |
| Total | .24226477 | 24 | .010094365 | | |

Bartlett's test for equal variances: chi2(3) = 1.8860 Prob>chi2 = 0.596

NO SIGNIFICANT DIFFERENCES BETWEEN GROUPS (p=0.0609)

## E-2.8. T4 Recovery

### NORMALITY

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| t4 | 25 | 0.89766 | 2.844 | 2.136 | 0.01632 |

NOT NORMALLY DISTRIBUTED

SHAPIRO WILK (LOG-TRANSFORMATION)

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| t4log | 25 | 0.95060 | 1.373 | 0.647 | 0.25868 |

LOG TRANSFORMATION SUCCESSFUL – DATA NORMALLY DISTRIBUTED

### DESCRIPTIVE STATISTICS

-> treatment = N

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t4 | 6 | 586.6667 | 104.0513 | 460 | 760 |

-> treatment = S

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t4 | 6 | 630 | 89.21883 | 460 | 700 |

-> treatment = S16

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t4 | 7 | 660 | 140.9492 | 480 | 900 |

-> treatment = S4

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t4 | 6 | 840 | 218.7236 | 600 | 1100 |

### COMPARISON OF MEANS

ONE-WAY ANOVA (WITH BONFERRONI POSTHOC TEST)

Analysis of Variance

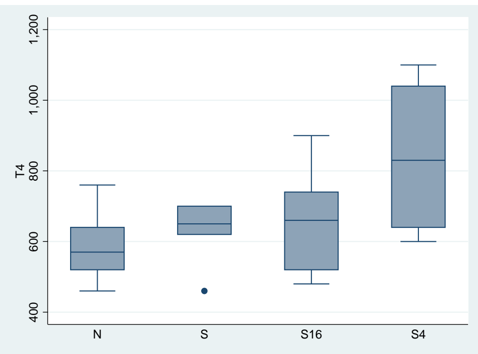
| Source | SS | df | MS | F | Prob > F |
|---|---|---|---|---|---|
| Between groups | .074255379 | 3 | .024751793 | 3.07 | 0.0499 |
| Within groups | .169116893 | 21 | .008053185 | | |
| Total | .243372272 | 24 | .010140511 | | |

Bartlett's test for equal variances: chi2(3) = 1.5428 Prob>chi2 = 0.672

SIGNIFICANT DIFFERENCES BETWEEN GROUPS (p=0.0499)

Comparison of t4log by Treatment
(Bonferroni)

| Row Mean-Col Mean | N | S | S16 |
|---|---|---|---|
| S | .032283<br>1.000 | | |
| S16 | .048212<br>1.000 | .015929<br>1.000 | |
| S4 | .148793<br>0.055 | .11651<br>0.212 | .10058<br>0.342 |




NO SIGNIFICANT DIFFERENCES BETWEEN GROUPS

E-2.9. T4 to Mg (i.e. time to reverse from T4 recovery to TOF-R > 0.9)

Data from Spontaneous Group omitted as data is invalid (some rats did not recover to TOF-R > 0.9

NORMALITY

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| t4mg | 19 | 0.82481 | 3.999 | 2.784 | 0.00268 |

NOT NORMALLY DISTRIBUTED

SHAPIRO WILK (LOG-TRANSFORMATION)

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| t4mglog | 19 | 0.88058 | 2.726 | 2.015 | 0.02198 |

LOG TRANSFORMATION UNSUCCESSFUL - DATA NOT NORMALLY DISTRIBUTED

DESCRIPTIVE STATISTICS

-> reversal = N

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t4mg | 6 | 493.3333 | 77.63161 | 380 | 600 |

-> reversal = S16

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t4mg | 7 | 154.2857 | 19.02379 | 140 | 180 |

-> reversal = S4

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t4mg | 6 | 223.3333 | 42.73952 | 180 | 280 |

COMPARISON OF MEANS

KRUSKALL WALLIS ANOVA (WITH BONFERRONI POSTHOC TEST)

Kruskal-Wallis equality-of-populations rank test

| reversal | Obs | Rank Sum |
|---|---|---|
| N | 6 | 99.00 |
| S16 | 7 | 30.00 |
| S4 | 6 | 61.00 |

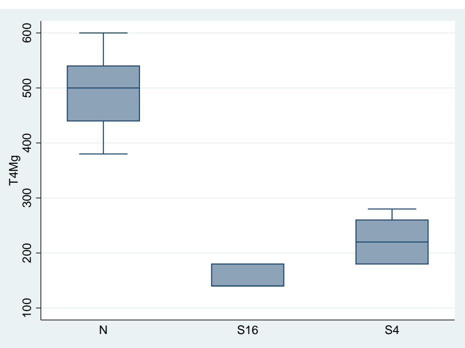
chi-squared = 15.229 with 2 d.f.
probability = 0.0005

chi-squared with ties = 15.501 with 2 d.f.
probability = 0.0004

SIGNIFICANT DIFFERENCES BETWEEN GROUPS (p=0.0005)

Comparison of T4Mg by Reversal
(Bonferroni)

| Row Mean-Col Mean | N | S16 |
|---|---|---|
| S16 | -339.048 | |
| | 0.000 | |
| S4 | -270 | 69.0476 |
| | 0.000 | 0.080 |




SIGNIFICANT DIFFERENCES:

N VS. S4 (P<0.001)
S16 (P<0.001)

E-2.10. $T1_{min}$ (minimum T1 obtained 300 secs after magnesium injection)

NORMALITY

Shapiro–Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| t1min_adjust | 24 | 0.76391 | 6.368 | 3.775 | 0.00008 |

NOT NORMALLY DISTRIBUTED

SHAPIRO WILK (LOG-TRANSFORMATION)

Shapiro–Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| t1min_adju~g | 24 | 0.68338 | 8.541 | 4.373 | 0.00001 |

LOG TRANSFORMATION UNSUCCESSFUL - DATA NOT NORMALLY DISTRIBUTED

DESCRIPTIVE STATISTICS

-> reversal = N

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t1min_adjust | 5 | 94.59788 | 3.24681 | 89.26627 | 97.4 |

-> reversal = S

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t1min_adjust | 6 | 63.82567 | 21.79965 | 33.78397 | 94.1704 |

-> reversal = S16

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t1min_adjust | 7 | 94.71121 | 5.499392 | 84.0421 | 100 |

-> reversal = S4

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t1min_adjust | 6 | 87.55477 | 9.598487 | 75.20564 | 96.06595 |

## COMPARISON OF MEANS

### KRUSKALL WALLIS ANOVA (WITH BONFERRONI POSTHOC TEST)

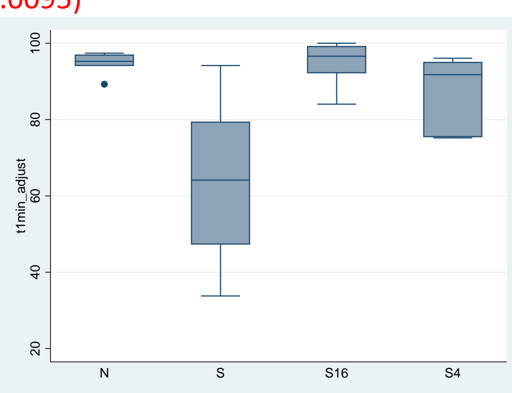
Kruskal-Wallis equality-of-populations rank test

| reversal | Obs | Rank Sum |
|---|---|---|
| N | 5 | 82.00 |
| S | 6 | 31.00 |
| S16 | 7 | 121.00 |
| S4 | 6 | 66.00 |

chi-squared = 11.451 with 3 d.f.
probability = 0.0095

chi-squared with ties = 11.451 with 3 d.f.
probability = 0.0095

SIGNIFICANT DIFFERENCES BETWEEN GROUPS (p=0.0095)




Comparison of t1min_adjust by Reversal
(Bonferroni)

| Row Mean-Col Mean | N | S | S16 |
|---|---|---|---|
| S | -30.7722<br>0.003 | | |
| S16 | .113332<br>1.000 | 30.8855<br>0.001 | |
| S4 | -7.04311<br>1.000 | 23.7291<br>0.020 | -7.15645<br>1.000 |

SIGNIFICANT DIFFERENCES:

Spon. VS. N (P=0.003)
S4 (P=0.020)
S16 (P=0.001)

E-2.11. dT1 (Greatest depression of T1 after magnesium injection)

NORMALITY

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| dt1min | 24 | 0.76391 | 6.368 | 3.775 | 0.00008 |

NOT NORMALLY DISTRIBUTED

SHAPIRO WILK (LOG-TRANSFORMATION)

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| dt1minlog | 24 | 0.97492 | 0.677 | -0.797 | 0.78720 |

LOG TRANSFORMATION SUCCESSFUL – DATA NORMALLY DISTRIBUTED

DESCRIPTIVE STATISTICS

-> reversal = N

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| dt1min | 5 | 5.402118 | 3.246808 | 2.6 | 10.73372 |

-> reversal = S

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| dt1min | 6 | 36.17433 | 21.79965 | 5.829597 | 66.21603 |

-> reversal = S16

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| dt1min | 7 | 5.288786 | 5.499392 | 0 | 15.9579 |

-> reversal = S4

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| dt1min | 6 | 12.44524 | 9.598487 | 3.93405 | 24.79436 |

# COMPARISON OF MEANS

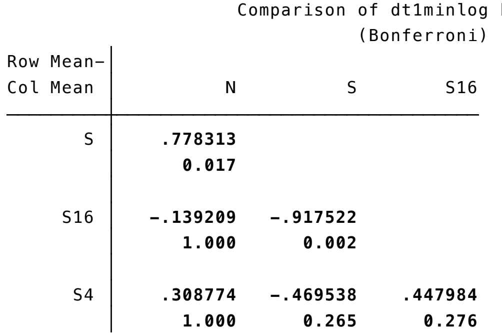
## ONE-WAY ANOVA (WITH BONFERRONI POSTHOC TEST)

Analysis of Variance

| Source | SS | df | MS | F | Prob > F |
|---|---|---|---|---|---|
| Between groups | **3.05978966** | **3** | **1.01992989** | **7.12** | **0.0019** |
| Within groups | **2.86541628** | **20** | **.143270814** | | |
| Total | **5.92520594** | **23** | **.257617649** | | |

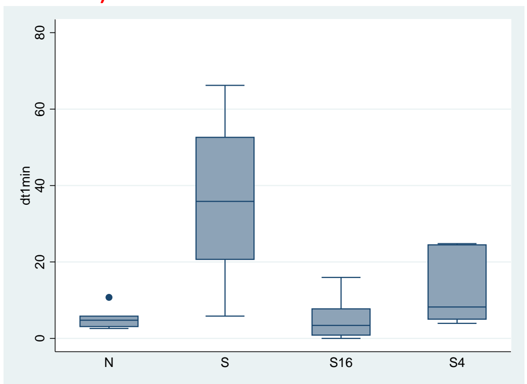
Bartlett's test for equal variances: chi2(**3**) = **1.8224** Prob>chi2 = **0.610**

SIGNIFICANT DIFFERENCES BETWEEN GROUPS (p=0.0019)



Comparison of dt1minlog by reversal
(Bonferroni)

| Row Mean-Col Mean | N | S | S16 |
|---|---|---|---|
| S | **.778313**<br>**0.017** | | |
| S16 | **-.139209**<br>**1.000** | **-.917522**<br>**0.002** | |
| S4 | **.308774**<br>**1.000** | **-.469538**<br>**0.265** | **.447984**<br>**0.276** |




SIGNIFICANT DIFFERENCES:

S VS. N (P=0.017)

S16 (P=0.002)

E-2.12. $T1_{onset}$ (Time from magnesium injection to $\%T1_{min}$)

NORMALITY

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| t1onset | 23 | 0.96255 | 0.980 | -0.042 | 0.51670 |

NORMALLY DISTRIBUTED

DESCRIPTIVE STATISTICS

-> reversal = N

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t1onset | 5 | 116 | 26.07681 | 80 | 140 |

-> reversal = S

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t1onset | 6 | 103.3333 | 15.05545 | 80 | 120 |

-> reversal = S16

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t1onset | 6 | 160 | 25.29822 | 140 | 200 |

-> reversal = S4

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| t1onset | 6 | 143.3333 | 29.4392 | 120 | 180 |

COMPARISON OF MEANS

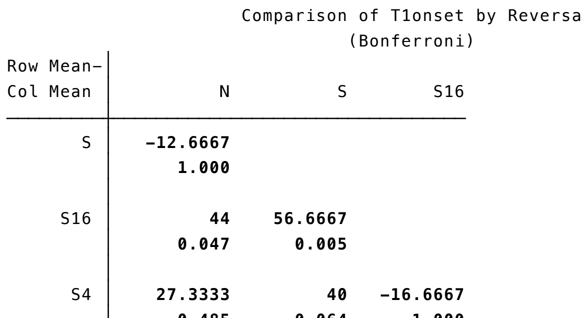
ONE-WAY ANOVA (WITH BONFERRONI POSTHOC TEST)

Analysis of Variance

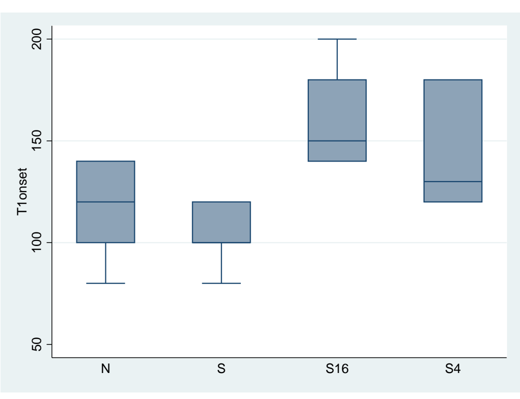
| Source | SS | df | MS | F | Prob > F |
|---|---|---|---|---|---|
| Between groups | 11674.2029 | 3 | 3891.40097 | 6.49 | 0.0033 |
| Within groups | 11386.6667 | 19 | 599.298246 | | |
| Total | 23060.8696 | 22 | 1048.22134 | | |

Bartlett's test for equal variances: chi2(3) = 2.0054 Prob>chi2 = 0.571

SIGNIFICANT DIFFERENCES BETWEEN GROUPS (p=0.4260)


Comparison of T1onset by Reversal
(Bonferroni)

| Row Mean-Col Mean | N | S | S16 |
|---|---|---|---|
| S | -12.6667 | | |
| | 1.000 | | |
| S16 | 44 | 56.6667 | |
| | 0.047 | 0.005 | |
| S4 | 27.3333 | 40 | -16.6667 |
| | 0.485 | 0.064 | 1.000 |




SIGNIFICANT DIFFERENCES:

S16 VS. N (P=0.047)

S (P=0.005)

E-2.13. TOF-$R_{min}$ (Lowest TOF-R reached after magnesium injection)

NORMALITY

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| tofmin | 25 | 0.93494 | 1.808 | 1.211 | 0.11304 |

NORMALLY DISTRIBUTED

DESCRIPTIVE STATISTICS

-> reversal = N

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tofmin | 6 | .7311667 | .0711911 | .649 | .815 |

-> reversal = S

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tofmin | 6 | .4176667 | .1641154 | .229 | .703 |

-> reversal = S16

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tofmin | 7 | .8214286 | .0938205 | .67 | .919 |

-> reversal = S4

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tofmin | 6 | .6158333 | .1399349 | .439 | .791 |

COMPARISON OF MEANS

ONE-WAY ANOVA (WITH BONFERRONI POSTHOC TEST)

Analysis of Variance

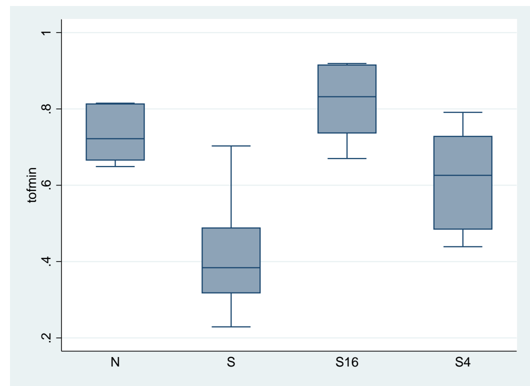
| Source | SS | df | MS | F | Prob > F |
|---|---|---|---|---|---|
| Between groups | .575809564 | 3 | .191936521 | 12.97 | 0.0001 |
| Within groups | .310732727 | 21 | .014796797 | | |
| Total | .886542292 | 24 | .036939262 | | |

Bartlett's test for equal variances: chi2(3) = 3.7761 Prob>chi2 = 0.287

SIGNIFICANT DIFFERENCES BETWEEN GROUPS (p=0.0001)

Comparison of tofmin by reversal
(Bonferroni)

| Row Mean-Col Mean | N | S | S16 |
|---|---|---|---|
| S | -.3135<br>0.001 | | |
| S16 | .090262<br>1.000 | .403762<br>0.000 | |
| S4 | -.115333<br>0.693 | .198167<br>0.061 | -.205595<br>0.038 |




SIGNIFICANT DIFFERENCES:

S VS. N (P=0.001)

S16 (P<0.001)

S4 VS. S16 (P=0.038)

E-2.14. dTOF (Greatest depression of TOF-R after magnesium injection)

NORMALITY

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| dtof | 25 | 0.92446 | 2.099 | 1.516 | 0.06479 |

NORMALLY DISTRIBUTED

DESCRIPTIVE STATISTICS

-> reversal = N

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| dtof | 6 | .1743167 | .071663 | .0847 | .2599 |

-> reversal = S

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| dtof | 6 | .45075 | .1412242 | .1996 | .5591 |

-> reversal = S16

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| dtof | 7 | .0958571 | .0847894 | .0103 | .2366 |

-> reversal = S4

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| dtof | 6 | .2801167 | .1164236 | .1376 | .4321 |

COMPARISON OF MEANS

ONE-WAY ANOVA (WITH BONFERRONI POSTHOC TEST)

Analysis of Variance

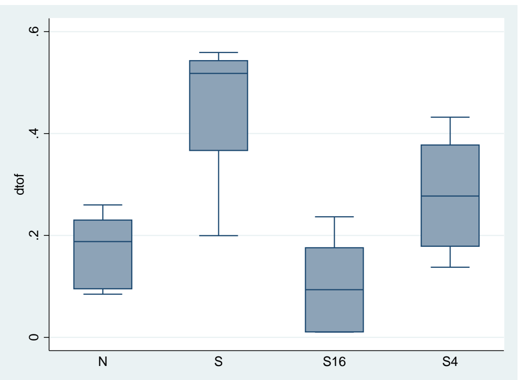
| Source | SS | df | MS | F | Prob > F |
|---|---|---|---|---|---|
| Between groups | .447058415 | 3 | .149019472 | 13.24 | 0.0000 |
| Within groups | .236306981 | 21 | .011252713 | | |
| Total | .683365395 | 24 | .028473558 | | |

Bartlett's test for equal variances: chi2(3) = 2.6095 Prob>chi2 = 0.456

SIGNIFICANT DIFFERENCES BETWEEN GROUPS (p<0.0001)

Comparison of dtof by reversal
(Bonferroni)

| Row Mean-Col Mean | N | S | S16 |
|---|---|---|---|
| S | .276433<br>0.001 | | |
| S16 | -.07846<br>1.000 | -.354893<br>0.000 | |
| S4 | .1058<br>0.593 | -.170633<br>0.066 | .18426<br>0.031 |




SIGNIFICANT DIFFERENCES:

S VS. N (P=0.001)

S16 (P<0.001)

S4 VS. S16 (P=0.031)

E-2.15. TOF900 (TOF-R at the end of the experimental period)

NORMALITY

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| tof900 | **25** | **0.88206** | **3.277** | **2.427** | **0.00762** |

NOT NORMALLY DISTRIBUTED

SHAPIRO WILK (LOG-TRANSFORMATION)

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| tof900log | **25** | **0.85674** | **3.981** | **2.824** | **0.00237** |

LOG TRANSFORMATION UNSUCCESSFUL - DATA NOT NORMALLY DISTRIBUTED

DESCRIPTIVE STATISTICS

-> reversal = N

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tof900 | **6** | **.8948333** | **.0171163** | **.877** | **.92** |

-> reversal = S

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tof900 | **6** | **.8043333** | **.0991073** | **.661** | **.913** |

-> reversal = S16

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tof900 | **7** | **.8815714** | **.0738825** | **.733** | **.946** |

-> reversal = S4

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tof900 | **6** | **.7248333** | **.098834** | **.579** | **.822** |

## COMPARISON OF MEANS

### KRUSKALL WALLIS ANOVA (WITH BONFERRONI POSTHOC TEST)

Kruskal-Wallis equality-of-populations rank test

| reversal | Obs | Rank Sum |
|---|---|---|
| N | 6 | 108.00 |
| S | 6 | 64.00 |
| S16 | 7 | 118.00 |
| S4 | 6 | 35.00 |

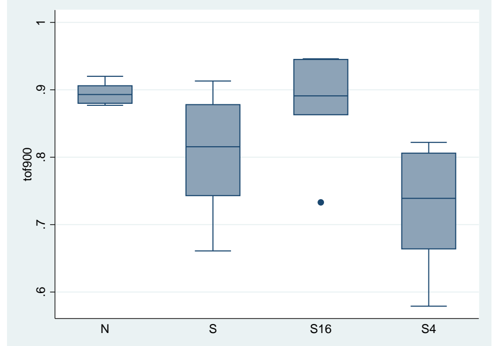
```
chi-squared =     10.984 with 3 d.f.
probability =      0.0118

chi-squared with ties =     10.984 with 3 d.f.
probability =      0.0118
```

SIGNIFICANT DIFFERENCES BETWEEN GROUPS (p=0.0118)

Comparison of tof900 by reversal
(Bonferroni)

| Row Mean-Col Mean | N | S | S16 |
|---|---|---|---|
| S | -.0905<br>0.369 | | |
| S16 | -.013262<br>1.000 | .077238<br>0.568 | |
| S4 | -.17<br>0.008 | -.0795<br>0.584 | -.156738<br>0.011 |




SIGNIFICANT DIFFERENCES:

S4 VS. N (P=0.008)
S16 (P=0.011)

E-2.16. TOF-$R_{onset}$ (Time from magnesium injection to TOF-$R_{min}$)

NORMALITY

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| tofonset | 25 | 0.97614 | 0.663 | -0.840 | 0.79957 |

NORMALLY DISTRIBUTED

DESCRIPTIVE STATISTICS

-> reversal = N

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tofonset | 6 | 103.3333 | 15.05545 | 80 | 120 |

-> reversal = S

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tofonset | 6 | 120 | 17.88854 | 100 | 140 |

-> reversal = S16

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tofonset | 7 | 111.4286 | 10.69045 | 100 | 120 |

-> reversal = S4

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| tofonset | 6 | 130 | 10.95445 | 120 | 140 |

COMPARISON OF MEANS

ONE-WAY ANOVA (WITH BONFERRONI POSTHOC TEST)

Analysis of Variance

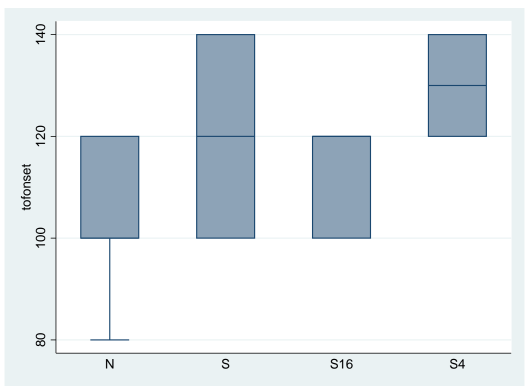
| Source | SS | df | MS | F | Prob > F |
|---|---|---|---|---|---|
| Between groups | 2380.95238 | 3 | 793.650794 | 4.15 | 0.0187 |
| Within groups | 4019.04762 | 21 | 191.38322 | | |
| Total | 6400 | 24 | 266.666667 | | |

Bartlett's test for equal variances: chi2(3) = 1.8624 Prob>chi2 = 0.601

SIGNIFICANT DIFFERENCES BETWEEN GROUPS (p=0.0187)

Comparison of tofonset by reversal
(Bonferroni)

| Row Mean-Col Mean | N | S | S16 |
|---|---|---|---|
| S | 16.6667<br>0.296 | | |
| S16 | 8.09524<br>1.000 | -8.57143<br>1.000 | |
| S4 | 26.6667<br>0.019 | 10<br>1.000 | 18.5714<br>0.150 |




SIGNIFICANT DIFFERENCE:

S4 VS. N (P=0.019)

## E-2.17. Post-recurarisation TOF-R recovery (25% of baseline)

### NORMALITY

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| r25 | 25 | 0.90733 | 2.575 | 1.934 | 0.02658 |

NOT NORMALLY DISTRIBUTED

SHAPIRO WILK (LOG-TRANSFORMATION)

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| r25log | 25 | 0.96700 | 0.917 | -0.178 | 0.57044 |

LOG TRANSFORMATION SUCCESSFUL – DATA NORMALLY DISTRIBUTED

### DESCRIPTIVE STATISTICS

-> reversal = N

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| r25 | 6 | 90 | 30.3315 | 40 | 120 |

-> reversal = S

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| r25 | 6 | 133.3333 | 53.16641 | 40 | 200 |

-> reversal = S16

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| r25 | 7 | 65.71429 | 27.60262 | 20 | 100 |

-> reversal = S4

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| r25 | 6 | 170 | 80.74652 | 100 | 320 |

### COMPARISON OF MEANS

ONE-WAY ANOVA (WITH BONFERRONI POSTHOC TEST)

Analysis of Variance

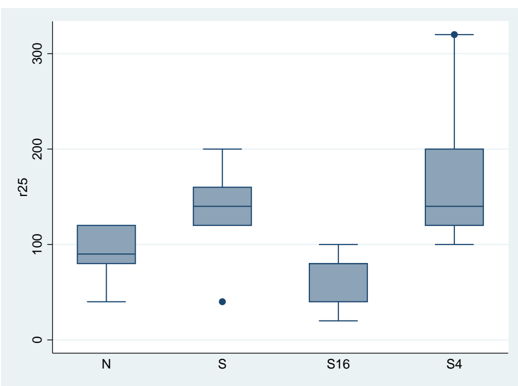
| Source | SS | df | MS | F | Prob > F |
|---|---|---|---|---|---|
| Between groups | .664705496 | 3 | .221568499 | 4.76 | 0.0110 |
| Within groups | .977689572 | 21 | .046556646 | | |
| Total | 1.64239507 | 24 | .068433128 | | |

Bartlett's test for equal variances: chi2(3) = 0.8986 Prob>chi2 = 0.826

SIGNIFICANT DIFFERENCES BETWEEN GROUPS (p=0.0110)

Comparison of r25log by reversal
(Bonferroni)

| Row Mean-Col Mean | N | S | S16 |
|---|---|---|---|
| S | .152007<br>1.000 | | |
| S16 | -.157694<br>1.000 | -.309701<br>0.105 | |
| S4 | .268502<br>0.257 | .116495<br>1.000 | .426196<br>0.011 |




SIGNIFICANT DIFFERENCE:

S4 VS. S16 (P=0.011)

E-2.18. Post-recurarisation TOF-R recovery (50% of baseline)

NORMALITY

Shapiro-Wilk W test for normal data

| Variable | Obs | W | V | z | Prob>z |
|---|---|---|---|---|---|
| r50 | 21 | 0.97098 | 0.711 | -0.689 | 0.75467 |

DATA NORMALLY DISTRIBUTED

DESCRIPTIVE STATISTICS

-> reversal = N

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| r50 | 6 | 153.3333 | 56.09516 | 80 | 220 |

-> reversal = S

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| r50 | 6 | 230 | 95.28903 | 80 | 340 |

-> reversal = S16

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| r50 | 6 | 120 | 63.24555 | 20 | 200 |

-> reversal = S4

| Variable | Obs | Mean | Std. Dev. | Min | Max |
|---|---|---|---|---|---|
| r50 | 3 | 333.3333 | 94.51631 | 260 | 440 |

COMPARISON OF MEANS

ONE-WAY ANOVA (WITH BONFERRONI POSTHOC TEST)

Analysis of Variance

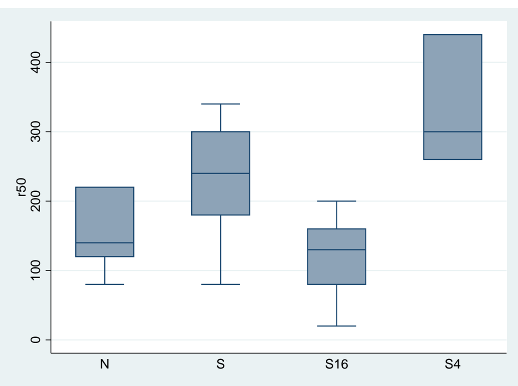
| Source | SS | df | MS | F | Prob > F |
|---|---|---|---|---|---|
| Between groups | 108657.143 | 3 | 36219.0476 | 6.22 | 0.0048 |
| Within groups | 99000 | 17 | 5823.52941 | | |
| Total | 207657.143 | 20 | 10382.8571 | | |

Bartlett's test for equal variances: chi2(3) = 1.6844 Prob>chi2 = 0.640

SIGNIFICANT DIFFERENCES BETWEEN GROUPS (p=0.0048)

Comparison of r50 by reversal
(Bonferroni)

| Row Mean-Col Mean | N | S | S16 |
|---|---|---|---|
| S | **76.6667**<br>**0.599** | | |
| S16 | **-33.3333**<br>**1.000** | **-110**<br>**0.139** | |
| S4 | **180**<br>**0.023** | **103.333**<br>**0.435** | **213.333**<br>**0.006** |




SIGNIFICANT DIFFERENCE:

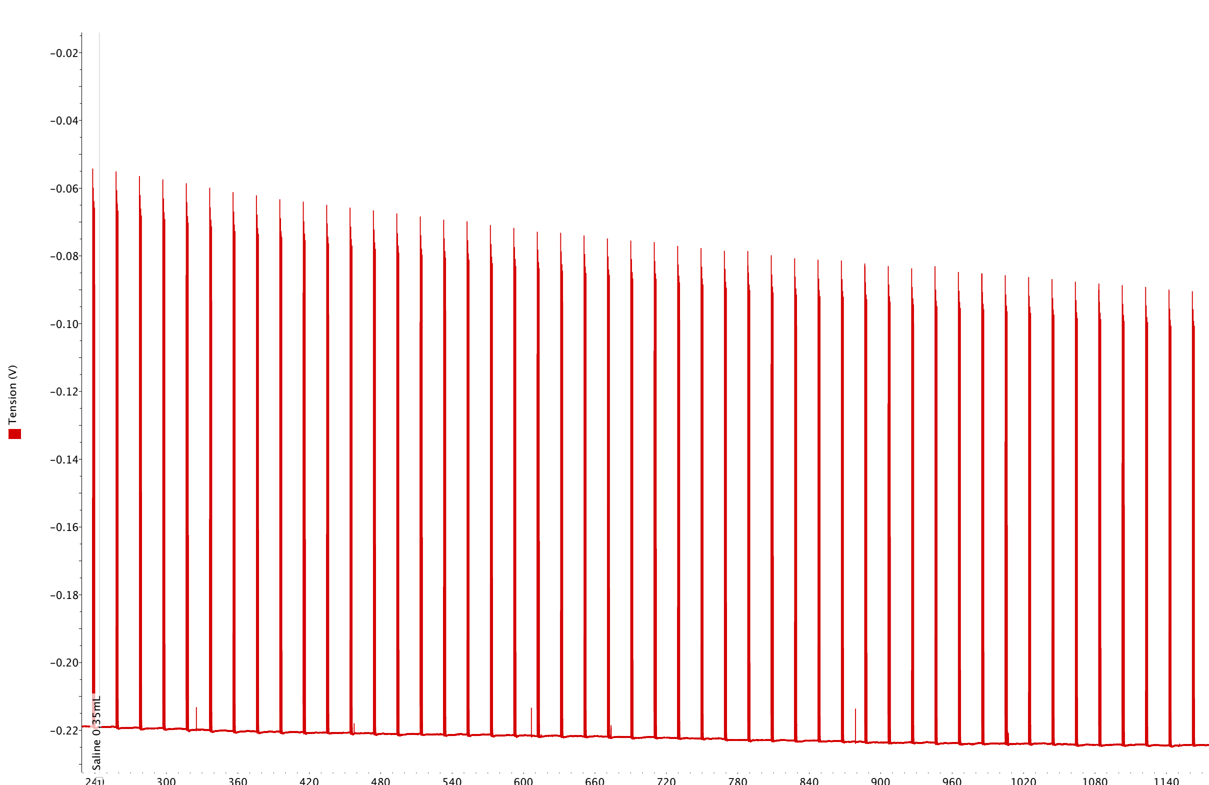
S4 VS. N (P=0.023)

S16 (P=0.006)

# APPENDIX F: REPRESENTATIVE TRACES

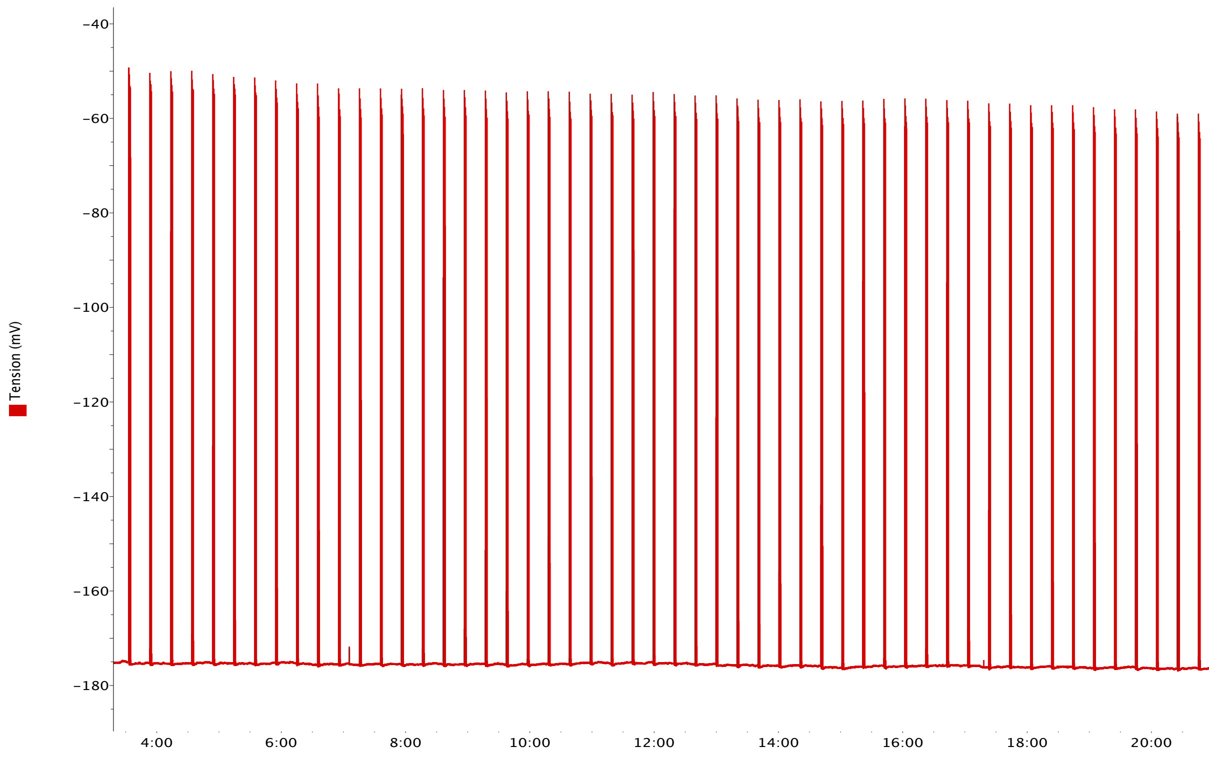
## F-1. Magnesium dose-effect experiment

### F-1.1. Saline treated rat

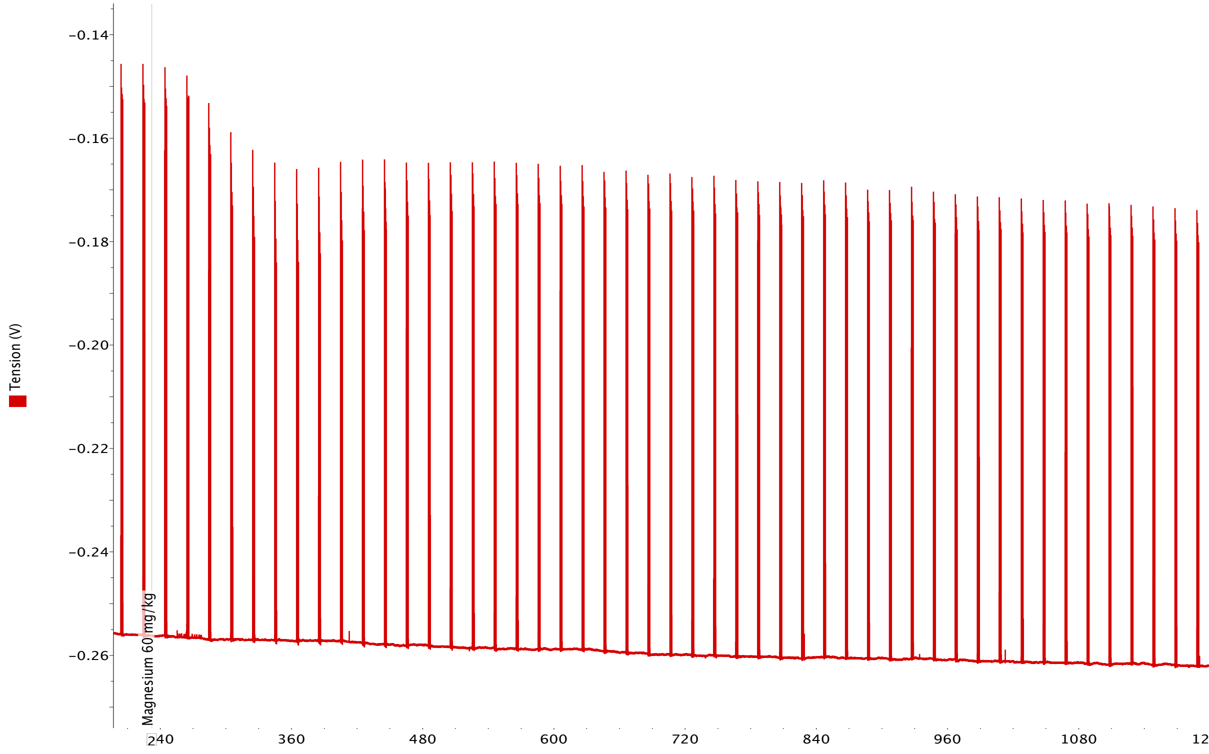


F-1.2. Magnesium 10 mg/kg treated rat

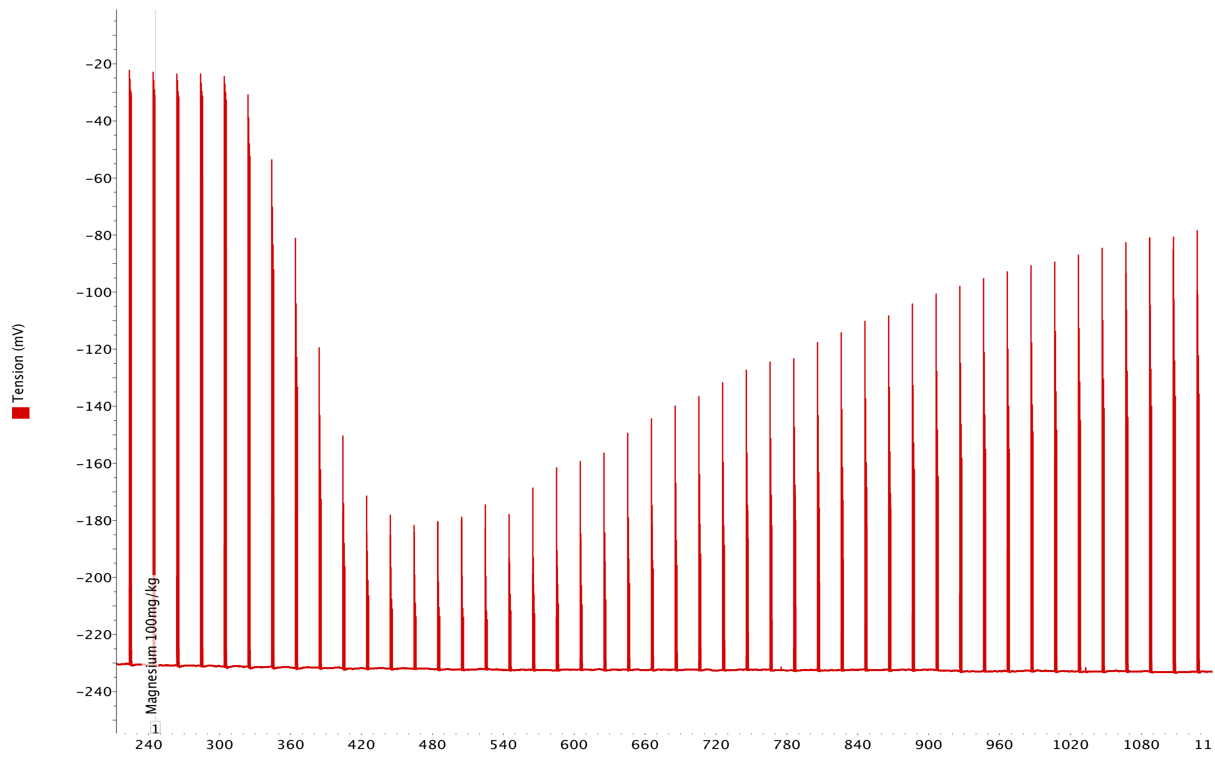
F-1.3. Magnesium 30 mg/kg treated rat



F-1.4. Magnesium 60 mg/kg treated rat

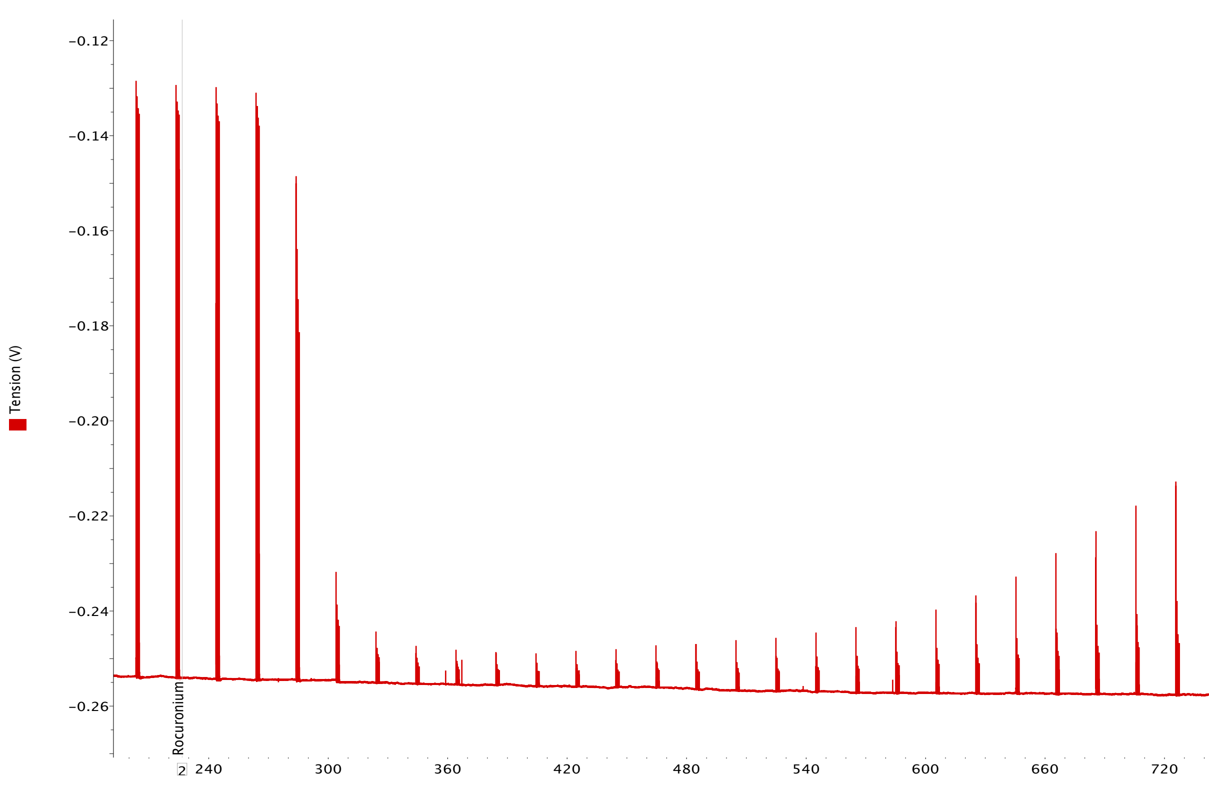



F-1.5. Magnesium 100 mg/kg treated rat

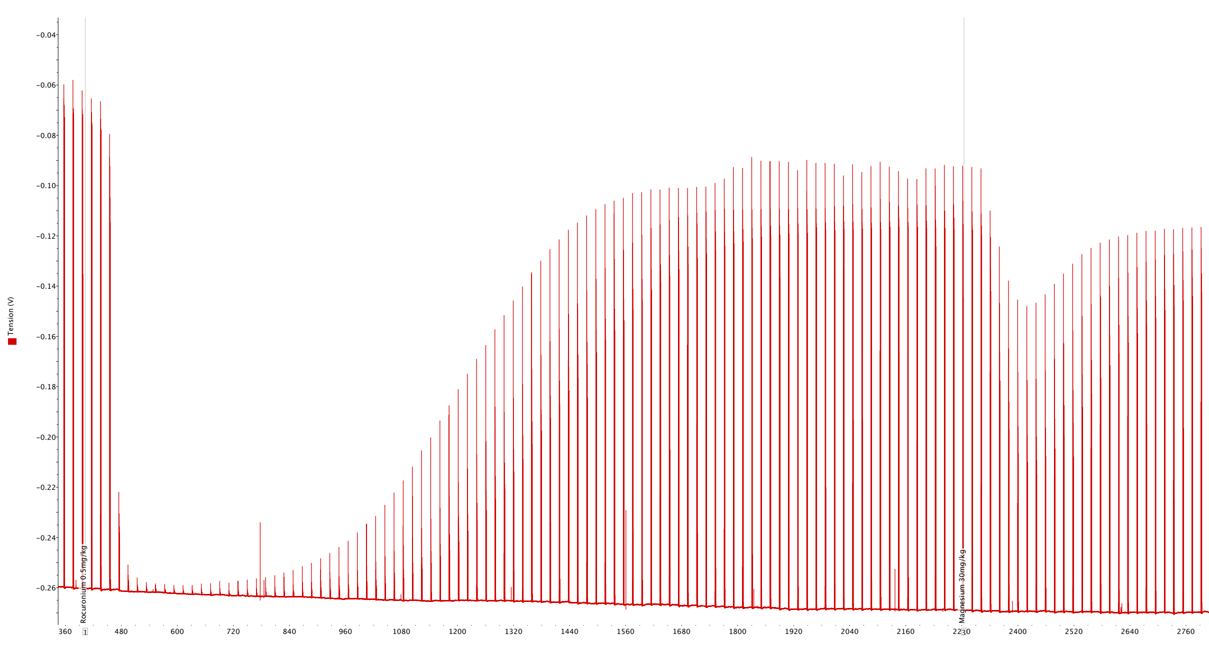


<u>F-2.</u> <u>Magnesium recurarisation experiment</u>

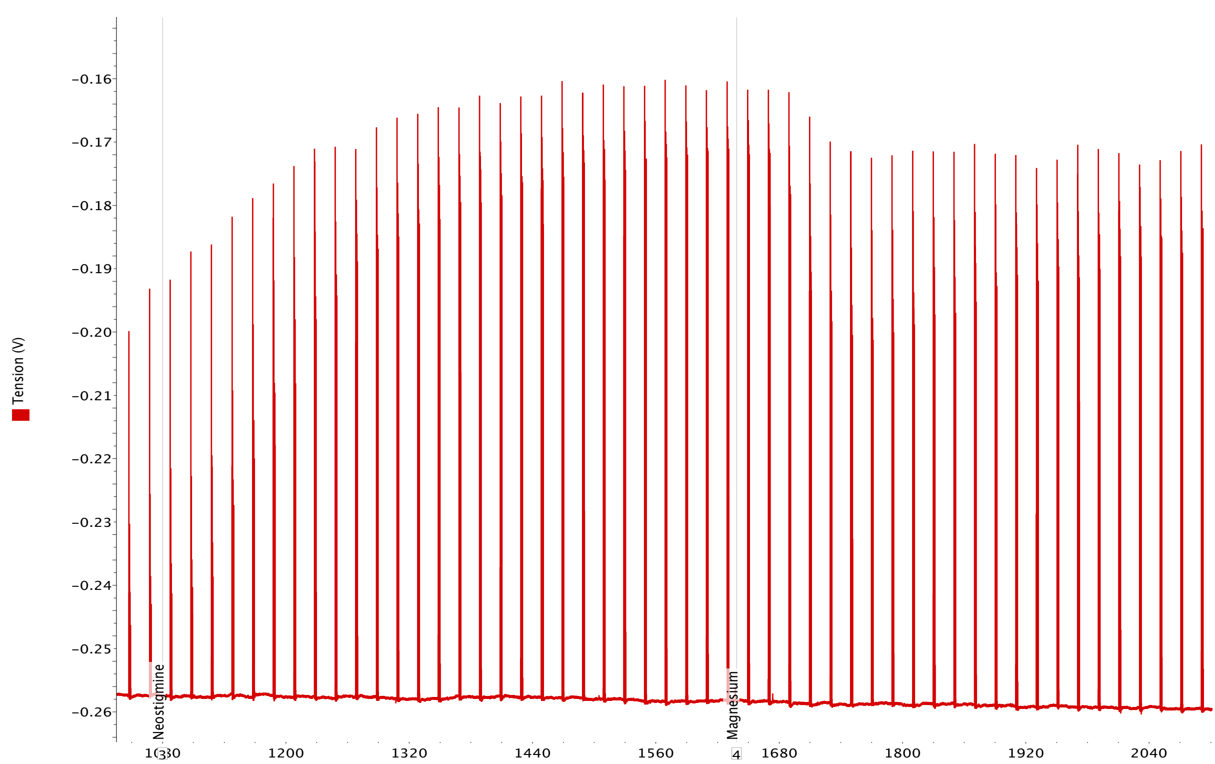
F-2.1. Neuromuscular block induction and spontaneous recovery



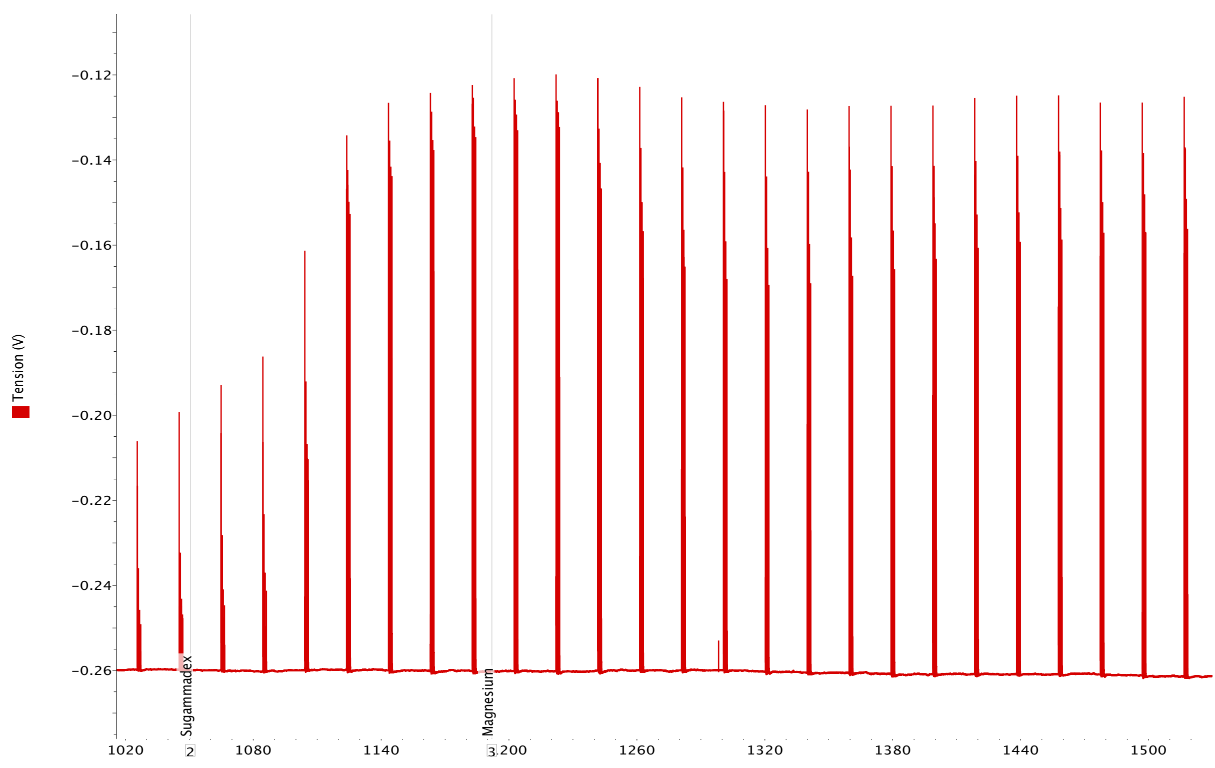

F-2.2. NMB induction, recovery and recurarisation effect: No reversal group (SPON)



F-2.3. Recovery and recurarisation effect: Neostigmine group (NEO)




F-2.4. Recovery and recurarisation effect: Sugammadex 4 mg/kg (SUG4)



F-2.5. Recovery and recurarisation effect: Sugammadex 16 mg/kg (SUG16)