

BURST-SUPPRESSION EVENTS AND FAST, LARGE-AMPLITUDE, SHARP WAVES
IN THE CORTICAL EEG DURING DEEP ISOFLURANE COMA

by

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Abstract

Carefully monitoring the depth of anesthesia throughout the surgical procedures is crucial during *in vivo* neurophysiological studies. Although the variability in delivering inhalational anesthetics among the anesthetic vaporizers is very likely, this issue has been commonly ignored. Such variability could produce incongruities in results obtained from *in vivo* studies that can be erroneously interpreted largely due to inherent biological error. The purpose of this study, therefore, was to investigate the depth of anesthesia using the cortical EEG recording technique with carefully increased concentrations of isoflurane (ISO), a common inhalational anesthetic agent. The present study demonstrates the existence of concentration-dependent distinct changes in EEG waveform activity during the shift from light to deep ISO anesthesia in rats. The study was performed across three cohorts of male Sprague-Dawley rats (cohort I - control, cohort II - sleep-deprived continuously for 9 h and cohort III - recovery for 48 h). Initially, a relatively low isoflurane concentration (ISO%) produced continuous large-amplitude slow-wave baseline EEG activity within the delta and theta bandwidths (0-8 Hz). As the ISO% was increased the slow-wave activities fragmented into a burst-suppression pattern. As the concentration of ISO increased further the number of burst-suppression events decreased further and disappeared leaving an isoelectric EEG pattern. Further increases in ISO% (3% relative ISO) resulted in the emergence of FLAS-waves during very deep ISO%. Increasing the ISO% exerted a dose-response curve for burst events and FLAS-waves. This suggests that the cortical EEG can serve as a reliable biomarker of stable and consistent distinct planes of anesthesia by monitoring the desired number of burst events per minute. Since sleep and anesthesia share similar characteristics, it was appropriate to test whether sleep deprivation exerted any effect on the EEG

activities during ISO anesthesia. The present study demonstrated that the dose-response curve for ISO on burst events was shifted to the right following sleep deprivation. Additionally, the finding of FLAS-waves in rats during very deep ISO anesthesia following burst-suppression events and the subsequent isoelectric period suggest a new arena of the brain physiology that may have multiple implications for the study of pain, coma, aging, cognition, etc.

Preface

A portion of the data presented in this M.Sc. dissertation has been published as a Society for Neuroscience abstracts and presented in poster format at the Society for Neuroscience Annual Meeting. In order to conduct the present research the certificate number obtained from the Research Ethics Committee, UBC is A14-0243. I performed all the experiments and data analyses described in the thesis. Dr. Peter Soja is the supervisor and principal investigator on the CIHR grant that funded this research project.

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List of Symbols and Abbreviations

ANOVA	analysis of variance
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AM	ante-meridiem
AP	anterior-posterior distance from bregma
bpm	beats per minute
CBR	cerebral blood flow
cm	centimeter
CMRO	cerebral metabolic rate of oxygen
CMR	cerebral metabolic rate
CNS	central nervous system
Co ₂	carbon dioxide
EEG	electroencephalogram
fMRI	functional magnetic resonance imaging
FLAS	fast, large-amplitude, sharp waves
GABA	gamma aminobutyric acid
h	hour(s)
HFS	high frequency stimulation
Hz	hertz
ID	inner diameter
IPSP	inhibitory postsynaptic potentials
IR	infrared
ISO	isoflurane
ISO%	concentration of isoflurane
LORR	loss of righting reflex

LTD	long-term depression
LTP	long-term potentiation
ML	lateral distance from the midline
mm	millimeter(s)
min	minute(s)
ms	millisecond(s)
NMDA	N-methyl-D-aspartic acid
NREM	non-rapid-eye-movement
OD	outer diameter
O ₂	oxygen
PET	positron emission tomography
PM	post-meridien
PWL	paw withdrawal threshold
Relative ISO%	concentration of isoflurane relative to slow-wave
REM	rapid eye movement
RTn	reticular thalamic neurons
s	second
SD	sleep-deprived
SEM	standard error of mean
TC	thalamocortical
VPL	ventral posterior lateral nucleus of thalamus

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Chapter 1: Introduction

Electrophysiological studies of the CNS (central nervous system) often require extensive and invasive surgical procedures that may expose the spinal cord, cortex and/or deeper brain structures. General anesthetics are therefore necessary and widely used agents to induce a loss of consciousness in *in vivo* neurophysiological studies of animals. The reliability of experimental results obtained from experiment to experiment requires a stable anesthetic plane in animals across all experiments. Monitoring the depth of the general anesthetic state is critical in being able to provide a constant surgical plane of anesthesia in routine *in vivo* studies of the CNS. However, although pre-clinical surgeries and *in vivo* experimental procedures in animal models are common, there is a dearth of knowledge and even in most published literature a complete lack of attention paid to monitor the depth and stability of anesthesia.

The general concern applies especially for the studies on acute and chronic pain mechanisms. One particularly interesting pathophysiological mechanism of chronic pain is long-term potentiation (LTP) of spinal neurons. LTP is a long-lasting potentiation of synaptic transmission between neurons (Bliss and Collingridge, 1993). LTP has been claimed as a crucial mechanism for development of the transition from acute to chronic pain, and generally occurs in the synapses between primary afferent neurons and secondary neurons present in the lamina I of the spinal cord (Nichols et al., 1999; Ikeda et al., 2003). Randic et al. were the first to report that high-frequency stimulation (HFS) of primary afferent fibers resulted in LTP of the C-fiber evoked field potential in the dorsal horn of the rat spinal cord (Randic et al., 1993). Besides, high-frequency synchronous stimulation of primary afferent neurons, natural low-frequency

asynchronous noxious stimulation during peripheral inflammation or nerve injury also induces spinal LTP (Sandkuhler and Liu, 1998; Ikeda et al., 2006).

The VPL (ventral posterolateral nucleus) thalamic neurons are thought to relay non-nociceptive and nociceptive information from the spinal cord to the cerebral cortex (Sherman, 2005). Animal models of chronic injury and acute inflammatory pain display increased pain sensitivity and enhanced excitability of neurons in the VPL of the thalamus (Fischer et al., 2009; Hains et al., 2005; Wang and Thompson, 2008). For example, when diabetic neuropathic pain is induced under general anesthesia in rats, mechanical allodynia occurs following anesthetic recovery. In this model, thalamic VPL neurons become hyperactive to peripheral stimulation that are reflected by increased spontaneous spike discharge activities (Fischer et al., 2009). Similarly, spinal cord transected rats develop mechanical allodynia and thermal hyperalgesia in conjunction with increased spontaneous activities of the neurons in the VPL (Hains et al., 2005). Other studies have also reported that hyperalgesia occurs in rodents following inflammatory pain induced by the intraplantar injection of formalin or carageenin, which are also associated with an increased excitability of VPL neurons (Guilbaud et al., 1986; Miyata et al., 2003).

Despite the wealth of *in vivo* studies elaborated directed at pain research and neurosciences, very few, if any, studies have elaborated on efforts to monitor the depth of anesthesia during surgical preparation and/or during the course of electrophysiological testing, recording and data collection. The only reported indices of adequate anesthetic depth include a lack of reflex withdrawal to pinch and “stable” blood pressure (Zhang et al., 2004; Xin et al., 2006; Drdla and Sandkuhler, 2008). Rather

surprisingly, no studies have monitored and analyzed the depth of anesthesia using conventional EEG recordings of the animals.

Recent studies reported by Sanoja et al., (2013) observed a shift of EEG activity from burst-suppression events towards continuous large-amplitude slow-waves, following high-frequency stimulation (HFS) of the sciatic nerve (Sanoja, 2013) that produces spinal LTP in rats (Sandkuhler, 2007). Sanoja et al., (2010, 2011) reported that during inflammatory pain induced by intraplantar injection of formalin, the EEG also shifts from burst-suppression activity during isoflurane anesthesia to the slow-wave delta (0-4Hz) oscillation (Sanoja and Soja, 2010; Sanoja et al. 2011). Thalamic VPL neurons which make reciprocal synaptic connections with cortical neurons were also enhanced in terms of their response magnitude to non-noxious and noxious peripheral stimuli in conjunction with long-lasting EEG “slowing” following HFS (Sanoja, 2013). The depth of isoflurane anesthesia using cortical EEG was constantly monitored and maintained throughout all the experiments.

A paper by Friedberg et al., (1999) reported that marked changes in the receptive fields of thalamic neurons occurs when the concentration of halothane anesthesia is slightly adjusted (Friedberg et al., 1999). This finding has never been followed up in the field of pain research. Friedberg et al., work suggests that experiments focusing on pathophysiological alterations of the CNS such as chronic pain may indeed reveal true plastic changes in the pain matrix or that could also be due to experimental error caused by variable anesthetic planes during the course of experiments.

Calibration of the anesthetic vaporizers used in nearly all scientific laboratories can vary greatly and very little attention has been paid to this as few studies addressing this

issue exist in the literature. Even at the same “numerical setting”, different vaporizers in the same laboratory or across laboratories could regulate different concentrations of anesthetic agents. Although a given concentration of anesthetic agent, as indicated by vaporizers is typically reported in literature during surgical preparation before *in vivo* electrophysiological recording paradigms (e.g., field potentials, spontaneous activities, evoked potentials) the actual anesthetic level delivered to the animal under study maybe much different than the vaporizer reading. Across the various studies in the literature little or no analyses of input gas concentration have been reported. Thus, variability among anesthetic vaporizers could produce discrepancies in the results that could lead to misinterpretation of collected data. This would, therefore, seen to be a commonly ignored major source of systematic error of data collected in neuroscience.

It is, therefore, necessary to determine if the EEG can provide a reliable means of portraying anesthetic depth following changes in the anesthetic concentration. Although many *in vivo* electrophysiological and pain studies are present in the literature (Sanoja, 2013) where anesthetics are used, no information is currently available where cortical EEG is constantly monitored to determine the depth of anesthesia around baseline and conditioning stimuli. At best, only the concentration marked on the vaporizer dial seems to have been reported. Subtle adjustments in anesthetic depths are often incorporated in the methods of electrophysiological studies, which are rarely noticed. These changes and their consequences can be considered as experimental confounds that could impact interpretation of data collected (Friedberg et al., 1999).

The purpose of this study, therefore, was to investigate the depth of anesthesia using the cortical EEG recording technique and the concentration-dependent actions of isoflurane, a common inhalational anesthetic agent. The present study investigated the

concentration-dependent EEG activities of isoflurane in an effort to document the surgical plane of anesthesia from across three cohorts of animals.

In the present study, low isoflurane concentrations produced continuous large-amplitude slow-wave EEG activity within delta and theta bandwidth range (0-8Hz). As the isoflurane concentration was increased in a stepwise fashion the slow wave activities fragmented and shifted to a burst-suppression pattern. With further increased ISO concentration, the number of burst-suppression events decreased gradually, which in turn was followed by the presence of a distinct isoelectric period that varied in length from one experiment to another. Additional increases in isoflurane concentration (3% relative isoflurane) resulted in the *de-novo* emergence of fast, large-amplitude, sharp (FLAS) waves during very deep stage of anesthesia. These findings suggest that the cortical EEG can provide a reliable means of discriminating between light, deep and extremely deep planes of anesthesia in a concentration-dependent manner. Furthermore, a stable and desired depth of anesthesia can be achieved by determining the desired number of burst-suppression events per minute from the dose-response curve. Using an approximate number of burst-suppression events at the ED₅₀ or ED₉₀ of an anesthetic agent would be a useful target in *in vivo* neuropharmacology experiments. Moreover, in the present study, the presence of fast large-amplitude sharp waves in rats opens up a new area of the brain physiology that has multiple venues for exploration from the viewpoint of pain, trauma, coma, aging, cognition, etc.

Background Literature Review

1.1 Mechanisms of General Anesthetic Actions

General anesthesia is a chemically induced, reversible state that consists of distinct behavioral features: unconsciousness, amnesia, analgesia, lack of movement, and diminished autonomic responses (Evers et al., 2006; Schwartz et al., 2010). Over last 130 years several hypotheses on mechanisms of general anesthetics have been proposed. In 1847 Bernard proposed the first theory of general anesthetics. He worked on "isolated nerve-muscle preparation" with several anesthetics and proposed a theory of general anesthesia based on the fact that chemical constituents mainly proteins of nerve and muscle undergo "reversible coagulation" (Leake, 1971).

In the late 19th century, Meyer and Overton proposed the "lipid hypothesis" of general anesthesia. They observed that the potency of an individual volatile anesthetic is correlated with its lipid solubility. The basis of the "Meyer-Overton lipid theory" was that anesthetics act by their structural modification in the lipid bilayer membrane. The lipid hypothesis was accepted for many years until being challenged by the 'protein theory' of Franks and Lieb in the late 20th century. They proposed that anesthetic agents act by binding to different kind of proteins including serum, ion channel and membrane proteins (Franks and Lieb, 1998). Studies on anesthetic action have mostly concentrated on a variety of ion channels situated on the neuronal membrane (Rudolph and Antkowiak, 2004). Several voltage-gated ion channels and ligand-gated ion channels have been focused on as the targets of general anesthesia. Voltage-gated calcium channels play a significant role in the presynaptic action of general anesthesia (Urban, 2002). Clinically used higher concentrations of volatile anesthetics act on the voltage-gated sodium and

potassium channels located on peripheral nerves (Urban, 1993; Rehberg et al., 1996). Among all ligand-gated ion channels, GABA_A receptors serve a predominant role as the common targets in the CNS for most anesthetic agent (Mashour et al., 2005). A wide range of general anesthetics enhance GABA_A mediated inhibitory postsynaptic potentials (IPSP) beyond the normal level of GABA_A inhibitory responses on neuronal excitation (Jones et al., 1992; Krasowski and Harrison, 2000). In the brain, modulation of GABA_A receptors by the anesthetics are concentration-dependent (Gyulai et al., 2001). Volatile anesthetics such as enflurane enhance the inhibitory action of glycine and inhibits the postsynaptic excitatory responses mediated by AMPA and NMDA receptors (Cheng and Kendig, 2000). Some inhaled anesthetics including non-halogenated anesthetic alkanes, per-halogenated non-immobilizing alkanes, and isoflurane exert inhibitory actions on nicotinic cholinergic receptors in hippocampal neurons, which maybe responsible for the amnesic property of general anesthesia (Flood and Role, 1998; Raines et al., 2002).

1.2 Sleep and Anesthesia

Lydic and Baghdoyan defined sleep as a “biologic rhythm that is actively generated by the brain” (Lydic and Baghdoyan, 2005). In 1953, Aserinsky and Kleitman were the first to report that humans experience distinct categories of sleep, and they described these categories as non-rapid eye movement sleep (NREMS) and rapid eye movement sleep (REMS) (Aserinsky and Kleitman, 1953). Within NREM sleep, there are distinct stages consisting of stages I, II, III and IV. Stages I and II are lighter sleep stages whereas stage III and IV are deeper sleep stages (Diekelmann and Born, 2010), and these stages along with REM sleep episode recur in roughly 90 minute cycles throughout the night (Aserinsky and Kleitman, 1953). Normal sleep patterns at night are composed of 80%

NREM sleep and 20% REM sleep (Borbély, 1982). NREM sleep (stages I to IV) is usually present in the early portion of the sleep cycle whereas REM sleep appears approximately 1.5 hours after the onset of sleep (Aserinsky and Kleitman, 1953). The cortical EEG during NREM sleep comprises a variety of rhythms of different bandwidths: delta (0-4 Hz), theta (4-8 Hz), alpha (8-12 Hz) and sleep spindles (11-16 Hz). K-complexes, large-amplitude spike waves also occur between stage I and II NREM sleep. The EEG of the REM sleep stage is comprised of low-amplitude high-frequency waves. Stage III NREM sleep EEG is characterized by the slowest and largest amplitude waves in the delta bandwidth (Steriade, 2006). REM sleep is often considered as “paradoxical” since the EEG waveform at this stage is similar to waking states (Jouvet, 1965). However, unlike during the awake period, muscle tone is absent during REM sleep (Siegel, 2005; Okura, 2006) due to the presence of glycine-mediated postsynaptic inhibition operating on motor neurons (Soja et al., 1991).

Neuronal networks similar to those responsible for sleep regulation also modulate the behavioral characteristics and the states of sleep and general anesthesia (Lydic and Baghdoyan, 2005). Similarities between a brain in NREM sleep and general anesthesia have been found through imaging studies (Braun et al., 1997; Kajimura et al., 1999; Maquet, 2000., Franks, 2008). The cerebral blood flow (CBF) was found to be decreased in the thalamus, brainstem, basal forebrain, basal ganglia, anterior cingulate, precuneus and posterior cingulate cortex when compared between NREM sleep and waking states (Franks, 2008). Studies with different anesthetics such as propofol, sevoflurane and xenon also showed decreased CBF in the thalamus, midbrain, cerebellum, precuneus, posterior cingulate cortex and some areas of frontal cortex during anesthesia (Fiset et al., 1999; Bonhomme et al., 2001; Kaisti et al., 2003; Franks, 2008). However, ketamine exerts the opposite effect on CBF (Långsjö et al., 2005).

Neurons in the ventral lateral preoptic area marked by c-Fos expression are also found to be active during both natural sleep (NREM) and isoflurane anesthesia when compared to wakefulness (Scammell et al., 2001; Moore et al., 2012).

The spindle (11-16 Hz) and delta (0-4 Hz) waves present in EEG slow-wave pattern produced by lower concentrations of most anesthetics including propofol and halothane is reminiscent of the EEG during NREM sleep (Keifer et al., 1996; Franks, 2008; Murphy et al., 2011). The generation of this common EEG pattern during both sleep and anesthesia suggests a direct involvement of thalamus (Franks, 2008) which infers that sleep and unconsciousness produced by anesthetics share similar network mechanisms (Franks, 2008). Generally, sensory information proceeds to the cortex through the thalamus during wakefulness (Franks, 2008). Anesthetics activate two-pore-domain K^+ (2PK) channels and potentiate $GABA_A$ receptors, which in turn, hyperpolarize thalamocortical (TC) neurons (Sugiyama et al., 1992; Ries and Puil, 1999; Jurd et al., 2003). The spindle and delta waves are generated when thalamic neurons are hyperpolarized by the anesthetics (Bal et al., 1995). During the shift from wakefulness to sleep, the EEG spindle waves are generated by the thalamus that involves a reciprocal interaction between thalamocortical and GABAergic inhibitory reticular thalamic neurons (RTn) (Steriade et al., 1993a; Bal et al., 1995; McCormick and Bal, 1997). As sleep progress to the NREM stage the 'low-frequency' bursts are developed by the thalamic neurons (Franks, 2008). The 'bursting pattern', which is intrinsic to TC neurons, spreads over the large area of thalamus and cortex (Bal et al., 1995; Franks, 2008) which, in turn, results in large-amplitude EEG delta wave (0-4 Hz) activities that are hallmark of deep NREM sleep (Franks, 2008). These delta wave activities are thought to be responsible for the blockade of ascending sensory information during NREM sleep (Franks, 2008).

While sleep and general anesthesia share similar behavioral features, they can be distinguished by some differences in physiological and behavioral traits (Shafer, 1995). Sleep occurs naturally whereas general anesthesia is a drug-induced state and is not interrupted by noxious stimuli (Tung and Mendelson, 2004). The EEG in different sleep stages is also different from distinct EEG patterns during anesthesia, which do not recur in cycles like in sleep (Tung and Mendelson, 2004). For example, burst-suppression events typically occur with an increase in anesthetic concentration and follow fragmentation of slow-wave activity during lighter phase of anesthesia (Kroeger et al., 2013). Burst-suppression events are not a normal feature of the EEG during naturally occurring NREM or REM sleep (Schwartz et al., 2010)

1.3 Sleep Deprivation and its Interaction with Nociception and Anesthesia

Mounting evidence suggests that deep anesthesia induced by propofol decreases sleep need, and recovery (REM and NREM sleep) from sleep restriction may occur during propofol anesthesia (Tung et al., 2004). In contrast to propofol-induced anesthesia, recovery from REM sleep restriction does not occur during isoflurane-induced anesthesia (Nelson et al., 2010). Thus, different anesthetic agents differentially affect recovery from sleep deprivation. Interestingly, the loss of righting reflex (LORR), an indicator of anesthetic sensitivity, occurs significantly faster in rats that were sleep-deprived for 24 h in comparison to those that slept normally (Tung et al., 2002). This phenomenon of quicker LORR occurs when either isoflurane or propofol is used to induce anesthesia. Sleep-deprived rats also display a delayed recovery to awakening from propofol and isoflurane anesthesia when compared with control rats. This suggests that neuronal mechanisms involved in sleep may be operating during the state of anesthesia and that the sleep-deprived brain is more susceptible to propofol and

isoflurane anesthesia than the normal rested brain (Tung et al., 2002).

Several studies have clearly demonstrate that sleep deprivation influences pain perception in both animals, e.g., rats, as well as humans. For example, disruption of slow-wave sleep for 3 nights results in decreased pain threshold at the diagnostic points for fibromyalgia (Lentz et al., 1999). Similarly, 40 h of total sleep deprivation in healthy male subjects decreased threshold for pressure pain (Onen et al., 2001), and one night of sleep deprivation resulted in decreased heat pain thresholds, which had rebound effects following sleep recovery (Kundermann et al., 2004). Hicks et al., (1978) were first to investigate the effect of sleep disruption on pain perception in animals, in which they reported that REM sleep deprivation in rats increased the sensitivity to electrical stimuli in tail flick test (Hicks et al., 1978). Subsequent studies found that four days of REM sleep deprivation decreases pain threshold (Hicks et al., 1979), and three days of REM sleep deprivation led to decreased vocalization threshold produced by mechanical stimuli-induced pain in rats (Onen et al., 2000). Moreover, REM sleep deprivation for three days in rats produced a significant increase in pain sensitivity as measured by a mechanical stimulus, thermal stimulus and electrical tail shock test (Hakki Onen et al., 2001; Sanoja, 2013).

1.4 EEG Characteristics of Isoflurane Anesthesia

The changes that occur in cortical EEG reflect clear-cut changes in the depth of anesthetic plane as a function of anesthetic concentration. This was elegantly characterized by studies conducted on cats by Amzica and colleagues (Kroeger et al., 2013). A relatively low dose (1%) of isoflurane produces continuous slow-wave EEG activity which is similar to the slow-wave EEG activities during NREM sleep where delta

waves (0-4 Hz) predominate (Kroeger et al., 2013; Sanoja, 2013) (Steriade et al., 1993b; Friedberg et al., 1999). As the dose of isoflurane is further increased, the continuous slow-wave EEG activity fragments into a burst-suppression pattern (Kroeger et al., 2013; Sanoja, 2013). EEG burst-suppression comprises a period of high-amplitude activities referred to as the burst, followed by period of flat EEG called suppression (Swank and Watson, 1949; Friedberg et al., 1999; Urrego et al., 2014). The duration of burst portion varies from 0.4-1.4 s (Akrawi et al., 1996). EEG burst-suppression is also found during certain states that affect brain function, such as coma, hypoxia, drug-overdose, childhood encephalopathies, hypothermia, after cardio-respiratory arrest (Leake, 1971; Silverman, 1975; Brenner, 1985; Michenfelder and Milde, 1991; Weissenborn et al., 1991; Nakashima et al., 1995; Niedermeyer, 1999). Burst-suppression is commonly observed when injectable (propofol) or inhaled (e.g., isoflurane, sevoflurane, desflurane) anesthetics are used (Akrawi et al., 1996; Franks and Lieb, 1998; Murrell et al., 2008).

Neuronal mechanisms responsible for burst-suppression during the state of anesthesia are not completely understood (Rudolph and Antkowiak, 2004; Kroeger and Amzica, 2007). Steriade and Amzica (1994) were the first to attempt to investigate the cellular mechanisms of burst-suppression during anesthesia in cats (Steriade et al., 1994). The authors reported that 60-70% thalamic cells were silent prior to the onset of EEG burst-suppression, and these cells became entirely silent during the flat EEG period. The remaining 30-40% of monitored thalamic cells exhibited 'rhythmic spike bursts' activities during the flat EEG period, and when the flat period of EEG activities became longer than 30 s, all the thalamic cells stopped firing (Steriade et al., 1994). Kroeger and Amzica later reported that bursting activities can be evoked by micromechanical external stimuli, which suggests a 'state of cortical somatosensory

hyperexcitability' (Kroeger and Amzica, 2007). Blocking NMDA receptors significantly reduced the duration and amplitude of these evoked bursts induced by ascending inputs.

Thalamo-cortical networks were thought to be responsible for the burst activities in anesthetized patient (Steriade et al., 1993a; Steriade, 2006). However, Ching and Brown (2012) suggested that in fact thalamo-cortical networks are not necessarily required to generate burst-suppression but rather intrinsic cortical activity alone might be able to produce burst-suppression events (Ching et al., 2012). Nonetheless, the synchronization of burst-suppression throughout the whole brain is not present when the network is disconnected (Henry and Scoville, 1952; Lazar et al., 1999). It is possible that the intrinsic activity within the cortical network is necessary to produce the burst-suppression events; however, the network activities might be modulated by the external input (Urrego et al., 2014).

Ching and Brown (2012) developed a neurophysiological metabolic model for burst-suppression events using only cortical interneurons and pyramidal cells. The authors suggested an association between reduced cerebral metabolism and burst-suppression events, which is modulated by the ATP-gated potassium channels (K_{ATP}). In their model, K_{ATP} regulates cortical neuronal discharge as a function of ATP generation. The rate at which ATP is generated is directly regulated by the cerebral metabolic rate of oxygen consumption. When a burst occurs, the ATP concentration is reduced by 25% and this is thought to result in an increase in K_{ATP} conductance (gK_{ATP}). As K_{ATP} channels open, hyperpolarization occurs which leads to the EEG suppression. As the concentration of ATP slowly starts to recover, the gK_{ATP} reduces at the same time down to the level where neuronal spikes could appear again (Ching et al., 2012; Urrego et al., 2014). This model suggests that the gradual decrease in burst-suppression events during a state of

profound anesthetic-induced brain inactivation might be due to a progressive reduction of cerebral metabolism (Ching et al., 2012). This would occur in a concentration-dependent manner (Ramani et al., 1992).

During isoflurane anesthesia, increasing the anesthetic dose leads to a decrease in burst events and an increase in the suppression duration until an isoelectric line (flat EEG) is achieved (Aserinsky and Kleitman, 1953; Tomoda and Mori, 1993). In clinical settings, a prolonged isoelectric line is one of the hallmarks associated with criteria for defining brain death and diagnosing a fatal condition in comatose patients (JAMA, 1981; Borbély, 1982; Kroeger et al., 2013). In rat neocortical brain slice preparations, the glutamate receptor antagonist (CNQX) abolished micro-EEG burst-suppression like events leading isoelectric activity (Aserinsky and Kleitman, 1953; Lukatch and MacIver, 1996), suggesting a glutamatergic basis for this phenomenon.

Recently Kroeger and Amzica (2013) have reported on novel brain activity in a deeply anesthetized human epileptic patient that was refractory to antiseizure medications. They recapitulated this novel brain activity in cats during a very deep stage of isoflurane anesthesia. In this study, they recorded the cat's EEG at different concentrations of isoflurane. Large amplitude slow-wave EEG activities occurred at a very low concentration of isoflurane (%). As the concentration was increased, slow-wave EEG activity fragmented into burst-suppression events, which in turn, decreased in number as the isoflurane concentration was further increased. Eventually isoelectricity followed the reduction in burst events. Additional increases in the isoflurane concentration resulted in the appearance of high-amplitude "fast" waves in the EEG record. This latter unique brain activity, which was reflected in the EEG after a period of isoelectricity, was referred to as 'Nu-complexes' by the authors. The authors suggested that this finding

presented a new property of brain physiology. These investigators performed *in vivo* intracellular recordings in the hippocampus and cortex simultaneously and observed that Nu-complexes first appeared in the hippocampus compared to other subcortical or cortical regions. The hippocampal Nu-complexes displayed higher amplitude and occurred at least 10ms prior to cortically recorded Nu-complexes. The cortical Nu-complexes were therefore thought to originate in the hippocampus which projects to the cortex (Steriade, 2006; Kroeger et al., 2013). Furthermore, the authors suggested that this depth of isoflurane anesthesia where Nu-complexes develop represents the 'deepest form of coma' to date and indicates that the brain can be functioning past the isoelectric line. Also, the finding of this novel brain state could be an important aspect to consider toward future suggestions to modify the rigid criteria of determining clinical brain death (Jouvet, 1965; Kroeger et al., 2013).

This recent finding of Kroger and Amzica (2013) raised the question whether the novel brain activity produced at very deep stage of anesthesia is species-specific or is a common property in all species. Moreover, it is not known if the dose-related effect in EEG events, such as, continuous slow-wave activity, followed by burst-suppression events etc., induced by increasing concentrations of isoflurane, is independent of pre-existing changes in brain state before anesthesia. A classic example here would be sleep deprivation.

Based on the above findings, the hypothesis of the present thesis is:

Isoflurane will produce a dose-related decrease in the number of EEG burst events, which will be followed by a period of isoelectricity. The isoelectric period will subsequently be replaced by the de novo appearance of fast, large-amplitude spike-like (FLAS) waves at very deep stage of anesthesia in rats.

A corollary to this hypothesis is:

The dose-related decrease in the EEG burst-suppression event activities in rats induced by isoflurane will not be affected by marked changes in the animal's behavioral state before the induction of anesthesia.

Chapter 2: Materials and Methods

All experimental procedures involving the use of animals reported in the present thesis were approved by the Animal Care Committee of University of British Columbia and carried out according to the guidelines of Canadian Council of Animal Care and University of British Columbia Animal Care Committee. Adult male Sprague- Dawley rats (400-480g) were used for this thesis project. The animals were obtained from the Modified Barrier Facility in the University of British Columbia. Animals were housed in 12 h light: 12 h dark cycles. Standard rodent food and water were supplied *ad libitum*.

In the present study, animals were divided into 3 cohorts: cohort I - control rats were allowed to sleep normally. Cohort II - rats were sleep-deprived by gentle handling continuously for 9 h (typically between 7 AM – 4 PM). Cohort III - rats that were subjected previously to 9 h of sleep restriction were allowed to recover subsequently for 48 h sleep.

2.1 Surgical Procedures

Standard aseptic techniques were maintained during all surgical procedures.

2.1.1 Anesthetic Induction

To induce anesthesia, each rat was placed in an induction chamber with a continuous flow of 4% ISO (Ohio 100 vaporizer) and oxygen (2L/min). Loss of consciousness of the rat was confirmed when the animal's loss of righting reflex (LORR) occurred. The time required to LORR was noted for each animal. Once the animal lost consciousness, it was removed from the chamber and placed on a heated blanket in a supine position. ISO was lowered to 3% and delivered through a custom-made nose cone. A rectal

temperature probe was lubricated and inserted about 2 cm into the rectum, and body temperature was maintained $37 \pm 5^\circ$ using a feedback-controlled heating blanket throughout the experiment. During surgical procedures, the heart rate and end tidal CO₂ were constantly monitored and kept within physiological limits (heart rate: 300-350 bpm and end tidal CO₂ saturation: 1.5 to 2 and O₂ at 90-99%) was maintained using a pulse oximeter/capnograph (Model V9004, Surgivet, Harvard apparatus, Massachusetts, USA).

2.1.2 Tracheotomy

Immediately prior to conducting any surgical procedures, the surgical plane of anesthesia was confirmed by the lack of a toe pinch reflex. The ventral part of the neck was shaved and skin was appropriately prepared by “triple surgical scrub” (70% Isopropyl alcohol). A 1-cm anterior to posterior vertical incision was made on the shaved skin. Using the blunt dissection technique the subcutaneous tissue and sternohyoideus muscles were separated and the underlying trachea was exposed. The connective tissue around the trachea was carefully dissected. A suture thread was tied between two tracheal rings. A partial transverse incision was made between two tracheal rings rostral to the tied thread. An angle-ended custom-made polyethylene cannula (OD: 2.2 mm, ID: 1.28 mm) was used for tracheal intubation. After the transverse incision was made, the angled end of the cannula was promptly and carefully inserted into the trachea through the incision and secured in place with sutures. The other end of the cannula was attached to a mechanical respirator (Inspira; Harvard apparatus, Holliston, MA) by a Y connector. A tissue adhesive (3M Vetbond) was used to seal the tracheal incision.

2.1.3 Electrode Implantation

A stereotaxic frame (Model 1430, David Kopf Instruments, Tujunga, Canada) was used to hold the rat securely in place. The rat head was fixed firmly using the ear bars and palate bar. The head was shaved and the exposed skin was aseptically prepared by a “triple surgical scrub”. To expose the calvarium, a 2 cm anterior to posterior midline incision was made from the frontal bone to the alanto-occipital joint. The subcutaneous tissues were cleaned using a cotton tip applicator and gauze to clearly expose the bregma. Two bilateral trephinations in the parietal bone (AP = -1, ML = +/- 3) and a unilateral trephination in the frontal bone were made to fix stainless steel miniature screw EEG electrodes. Dental cement (Lang Dental Manufacturing Co., Inc, USA) was used to seal around the electrodes and to keep the scalp dry. Finally, custom-made EEG leads were connected to the screw electrodes.

2.2 Electroencephalogram (EEG) Recording Procedure

The cortical EEG was recorded and monitored at various increasing concentrations of ISO throughout the experiment. Actual ISO concentrations, referred to subsequently as ISO% were measured and calibrated accordingly using a Capnomac gas analyzer beforehand. A custom-made amplifier (Model 440, Brownlee Precision Instrumentation Amplifier, gain 10,000X) was used with an AC-coupled filter setting between 0.1 Hz and 30 Hz. Spike acquisition and analysis software (Spike2 V.8.0, Cambridge Electronics Design; Cambridge, UK) was used to record EEG signals at a sampling rate of 256 Hz. Low ISO% (1%) is known to produce high-amplitude slow-wave EEG activity. When the ISO% is increased further, the EEG shifts to a burst-suppression pattern (Siegel, 2005; Okura, 2006; Kroeger et al., 2013). In the present thesis, 1% ISO was used as starting concentration. ISO was then increased in 0.5% steps on the vaporizer up to a maximum

of 5%. At each increasing step of ISO%, the EEG was recorded for 30 min. During the first few experiments the occurrence of slow-wave EEG activity at low ISO% was found to vary. The slow-wave activity occurred at more than one isoflurane concentration (marked on the dial of the anesthetic vaporizer) and these concentrations were different from one animal to other. Due to this variability of slow-wave activity, the ISO% that exerted a minimum of 20 min of continuous slow-wave activity just before the appearance of reliable burst-suppression activity was assigned as the baseline ISO% for each animal. This procedure was employed, to normalize the ISO% values across the animals. According to their corresponding baseline ISO concentration, ISO% is expressed as 'relative ISO% to slow-wave' EEG activity. Off-line analyses of the EEG activities were conducted using a customized script written for Spike 2. This script allowed the user to tag burst-suppression events, determine their individual and cumulative durations, duration of suppression period between each burst event and to export the data to an excel base statistics program.

2.3 Sleep Deprivation and Recovery

Rats were sleep-deprived for 9 h by gentle handling in a cage. From 7am to 4pm rats were kept awake by mild tapping of the cage and gentle prodding with a brush. For the recovery experiments, sleep deprived rats were returned to their holding cage and allowed to recover for 48 h.

2.4 Assessment of Sensory Threshold

von Frey filaments and Hargreaves plantar test were used to assess mechanical and thermal sensory thresholds respectively of each rat. Mechanical and thermal sensory thresholds were assessed before and after 9 h of sleep deprivation and again after 48h of recovery of sleep loss.

2.4.1 von Frey Hair Test

A set of calibrated von Frey hairs (monofilament) of variable stiffness was used to measure the mechanical sensory threshold of the fore and hind paws of the rats. The filaments are arranged in a logarithmic scale of the actual force used to bend a given filament. Rats were placed in a transparent plexiglass chamber (15 X 15 X 15 cm³) where the floor consisted of a metal mesh (wire diameter = 1mm, 8X8 mm square grid). Before each sensory testing session, each rat was placed in the chamber for 5 min and allowed to habituate to the testing environment. The forces of calibrated von Frey filaments used were 1, 1.4, 2, 4, 6, 8, 10, 15, and 26 g. Each filament was applied from below the metal mesh to the plantar surface of the rat paw until the filament bent. The filament was held in its bent position for 4-5 sec or until the animal withdrew its paw in response to the stimuli. Testing always began with the 4 g filament and testing progressed by using the “up down” method as described by Chaplan et al (1994). In this method, the positive response (withdrawal response) specified the previous lower force of filament to be used and a negative response (no withdrawal response) indicated the next higher filament to be used until the positive response was observed. After the first positive response was approached four additional filaments were used which then determined the response pattern of the rat. The testing was stopped when the lowest possible force of filament (1.4 g) or the highest possible force of filament (26 g) was used. Paw withdrawal threshold (PWT) was calculated by using the adjustment factors established on the pattern of response of the rat (Chaplan et al., 1994; Lydic and Baghdoyan, 2005). The response pattern and used filament forces in grams were recorded in an Excel spreadsheet where the PWT was calculated using adjustment factors modified by Chaplan et al. from Dixon’s look-up tables (Dixon, 1965; Chaplan et al., 1994; Braun et al., 1997; Kajimura et al., 1999; Maquet, 2000).

2.4.2 Thermal Planter Test

Thermal sensory thresholds were determined using a thermal plantar test, known as Hargreaves method (Hargreaves et al., 1988; Franks, 2008). A plantar test device (Ugo Basile, Model 7370, Italy) was used to measure the paw withdrawal threshold of fore- and hind-paws of the rat. A movable radiant infrared (IR) heat source was placed underneath an elevated glass table. A clear plexiglass chamber (15 X 15 X 15 cm³) for single rat was placed on the glass tabletop. The chambers were made at the workshop in the Department of Zoology, UBC. During each testing session, the rat was placed in the chamber and was allowed to habituate for 5 min to the testing environment. Testing began following the habituation period when the animal was immobile. A radiant IR beam generated by the heat source of the device was focused through the glass onto the planter surface of the rat paw. A built in timer in the device measured the paw withdrawal latency in seconds. The IR beam was switched off automatically as soon as the paw was fully removed from the glass top in response to the heat stimuli. To prevent tissue injury, an automatic cutoff point of 30 s was set. The IR beam intensity was set at the middle point of emission range (50 units). The plantar test was performed twice for each paw with a 5 min period of resting time in between. The mean value of two trials was considered as final PWL.

2.5 Statistical Analysis

In all cases, statistical analyses were performed using GraphPad Prism software. A one-way analysis of variance (ANOVA) was performed to analyze differences in mean values in cohort 1. A two-way repeated measures ANOVA was performed to analyze differences in mean values when compared between different cohorts. Tukey's post-hoc test was performed in cases where the ANOVA result was significant. In all cases

p<0.05 was considered as statistically significant. All values in the results were expressed as mean ± SEM.

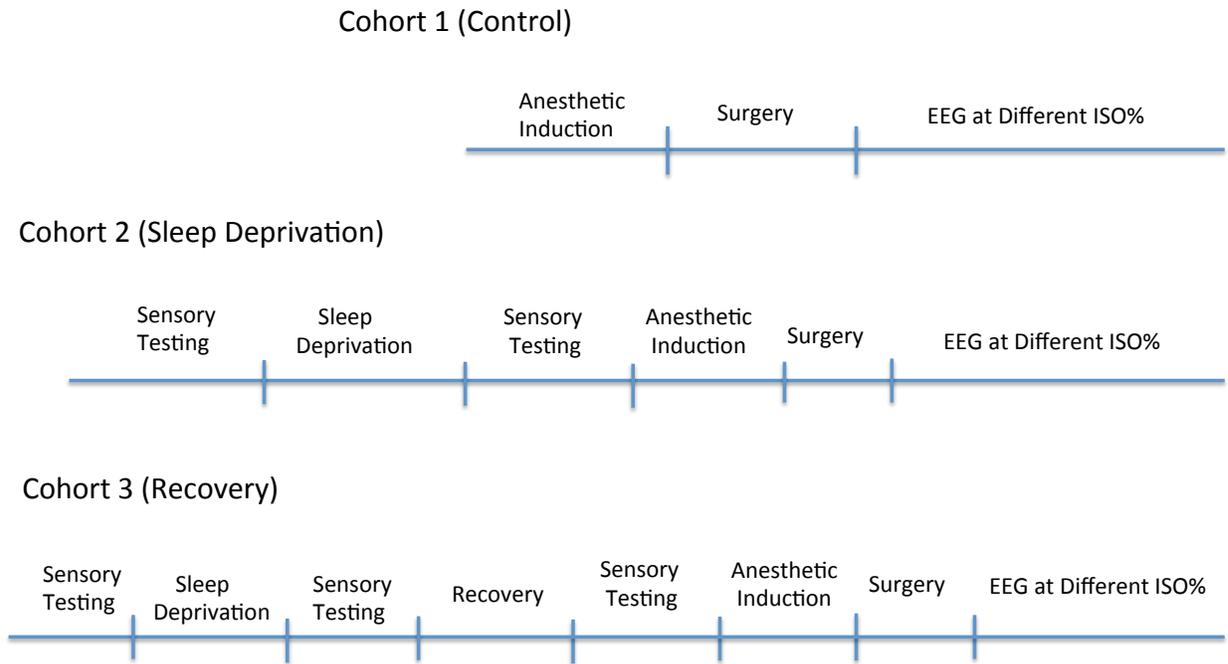


Figure 1. General experimental procedure

Chapter 3: Results

The purpose of the following experiments was to characterize dose-response changes in EEG activities under ISO anesthesia, how this EEG response varies in sleep-deprived animals, and whether the EEG characteristics reverts following sleep recovery. The experimental results for the present study were obtained from a total of 18 Sprague-Dawley rats, which were divided into three cohorts as follows: cohort I (n=6) - rats slept normally, cohort II (n=6) - rats were sleep-deprived continuously for 9 h, cohort 3 - rats that were subjected previously to 9 h of sleep restriction were allowed to recover for 48 h sleep following sleep-deprivation. To characterize EEG in response to increasing ISO%, cortical EEG was recorded for ~ 5 h. At low ISO%, 20 min of continuous large-amplitude slow-wave EEG activity prior to the appearance of a reliable burst-suppression activity were ascribed as baseline ISO% for each rat. ISO was then increased in 0.5% steps and EEG activities were recorded for 30 min at each step. As the ISO% was increased the baseline slow-wave EEG activity fragmented into burst-suppressions, which was then followed by isoelectricity. Further increases in ISO% resulted in the appearance of fast, large-amplitude spikes (FLAS). In the present study, ISO% is reported as “relative ISO% to slow-wave” as described in the Methods chapter. The results were quantified by calculating the number of bursts and FLAS, duration of bursts and suppression, and the distribution of bursts and FLAS-waves. The main findings of the present study are discussed in the following sub sections:

3.1 Number of Burst Events at Relative ISO% (Control- Cohort I)

The number of burst events was counted in the last 10 min of 30 min EEG recording. As mentioned on page 20 chapter 2, ISO% is expressed as 'relative ISO% to slow-wave activity' in the present study. The mean number of bursts/min quantified at each relative ISO% gradually decreased with an increase in ISO%. The mean number of bursts/min at 0.5, 1, 1.5 and 2 ISO% measured 20.5 ± 1.5 , 9.8 ± 2.3 , 3.5 ± 0.7 , and 0, respectively (figure 18). A Tukey's post hoc test following one-way ANOVA revealed a significant difference between the means at 0.5, 1, and 1.5 ISO% ($p < 0.05$).

3.2 Number of Fast, Large-Amplitude, Sharp (FLAS) Waves at Relative ISO% (Control- Cohort I)

As ISO was increased further by 0.5%, the burst events started to disappear and the FLAS-waves coined as Nu-complexes by Kroger et al., 2013 began to emerge. The FLAS-waves started to appear at 2% relative ISO in the control animals. The mean number \pm SEM of FLAS-waves per minute at relative ISO% 1.5, 2, 2.5, and 3 measured 0, 2.1 ± 0.4 , 2.8 ± 0.9 , and 7.5 ± 2.5 , respectively (figure 19). A post hoc Tukey's test following one-way ANOVA revealed a significant difference between 1.5 and 3 ISO%, 2, and 3 ISO% in cohort I.

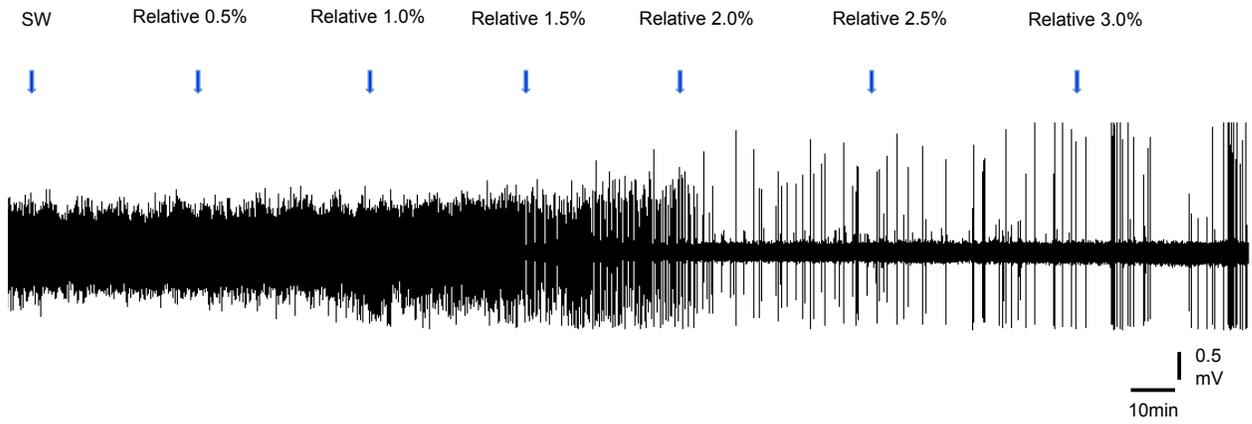


Figure 2. EEG activity trace. Arrows represent the point where relative ISO% was changed.

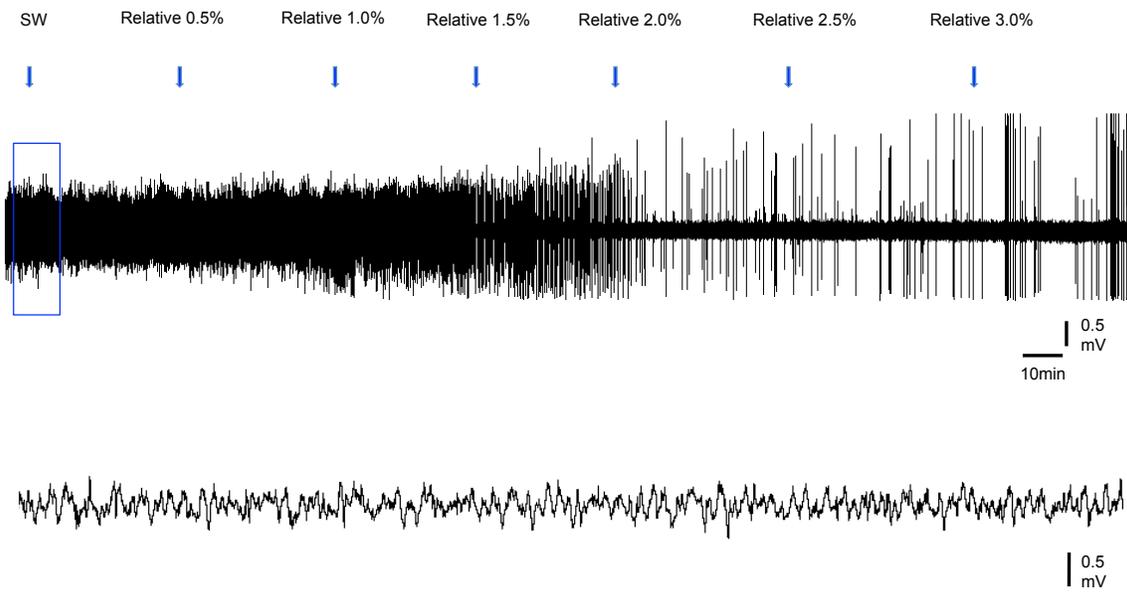


Figure 3. Slow-wave activity trace (30s).

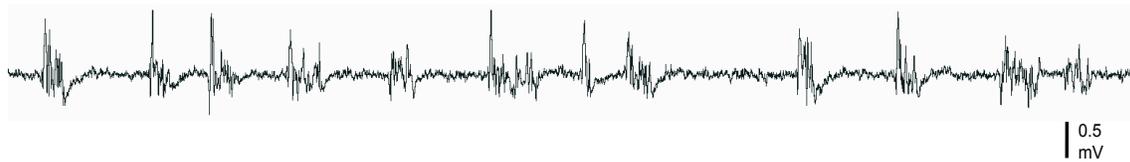
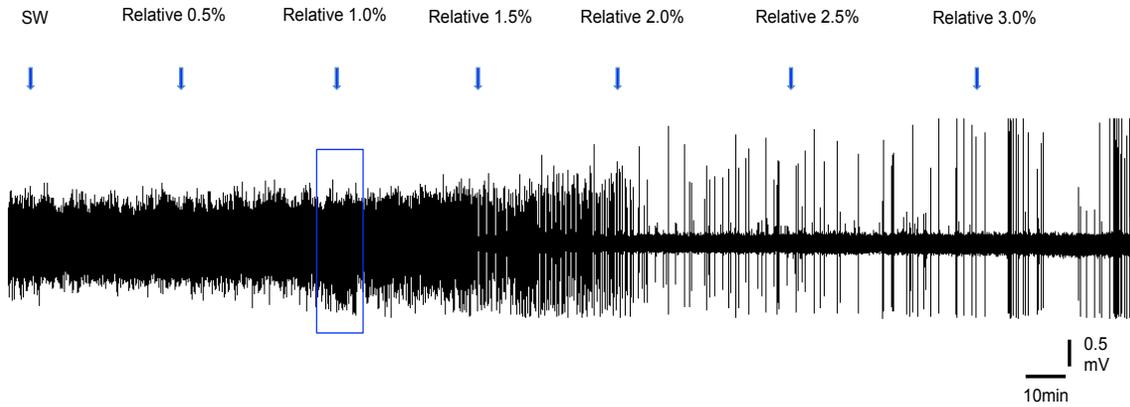


Figure 4. Burst-suppression traces (30s).

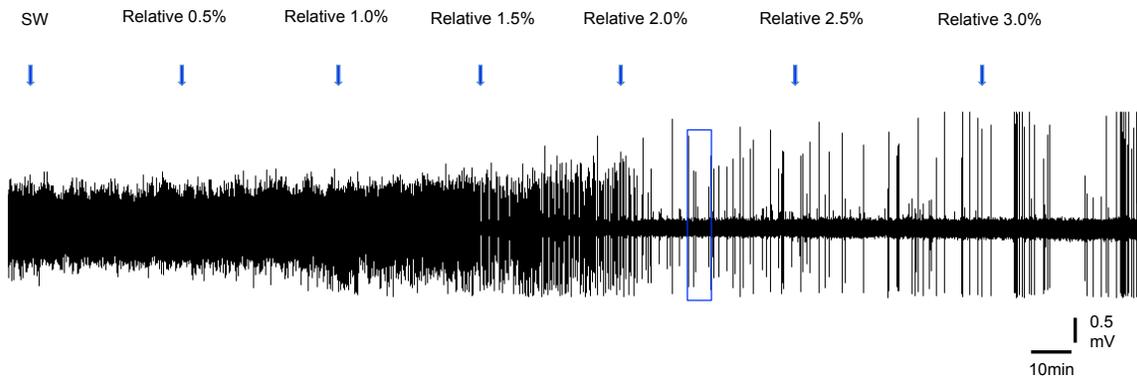


Figure 5. Isoelectric line trace (30).

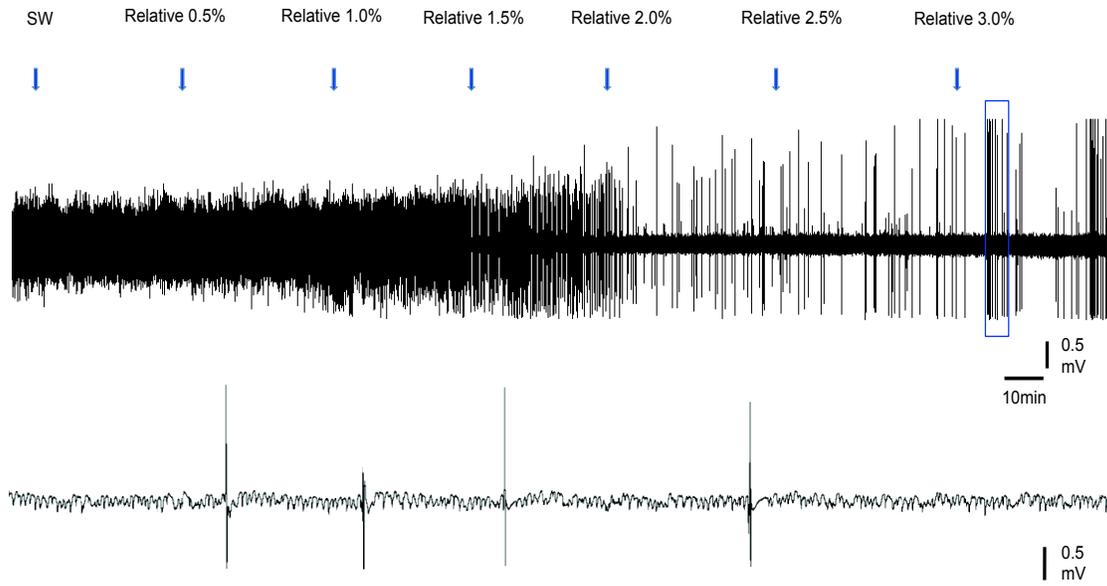


Figure 6. Fast, large-amplitude, sharp (FLAS) waves trace (30s)

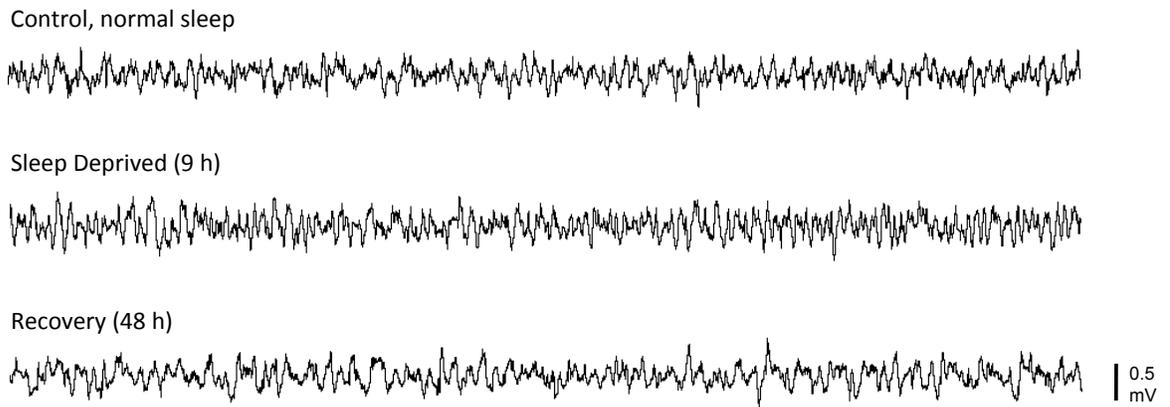


Figure 7. Slow-wave activity traces (30s) in control, test (SD) and recovery group.

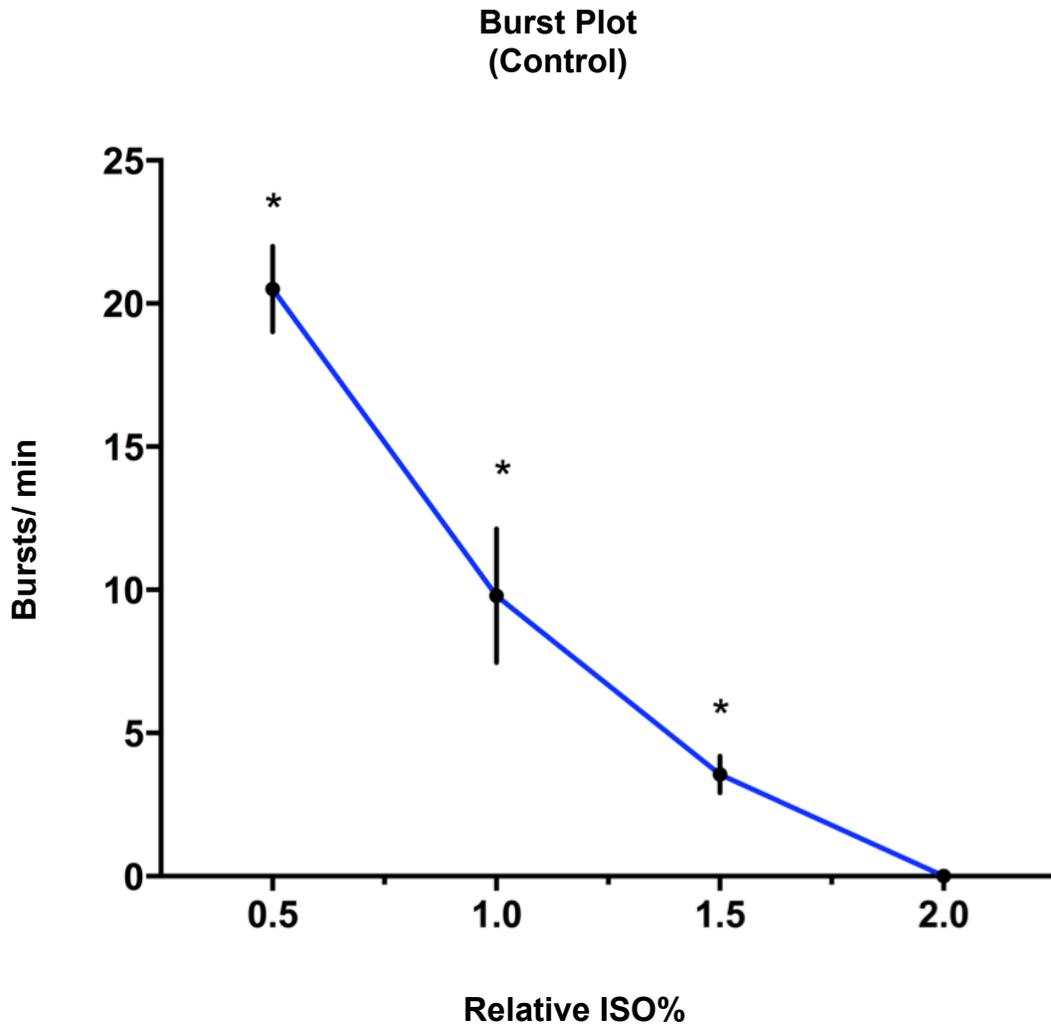


Figure 8. Effects of relative ISO% on the number of bursts/min. The mean number of bursts/min at 0.5, 1, 1.5 and 2 ISO% measured 20.5 ± 1.5 , 9.8 ± 2.3 , 3.5 ± 0.7 , and 0 respectively. A Tukey's test following one-way ANOVA revealed significant difference between 0.5,1 and 1.5 ISO%. Each data point represents mean ($n=6$) \pm SEM; * $p < 0.05$.

Fast, Large-Amplitude, Sharp Waves (Control)

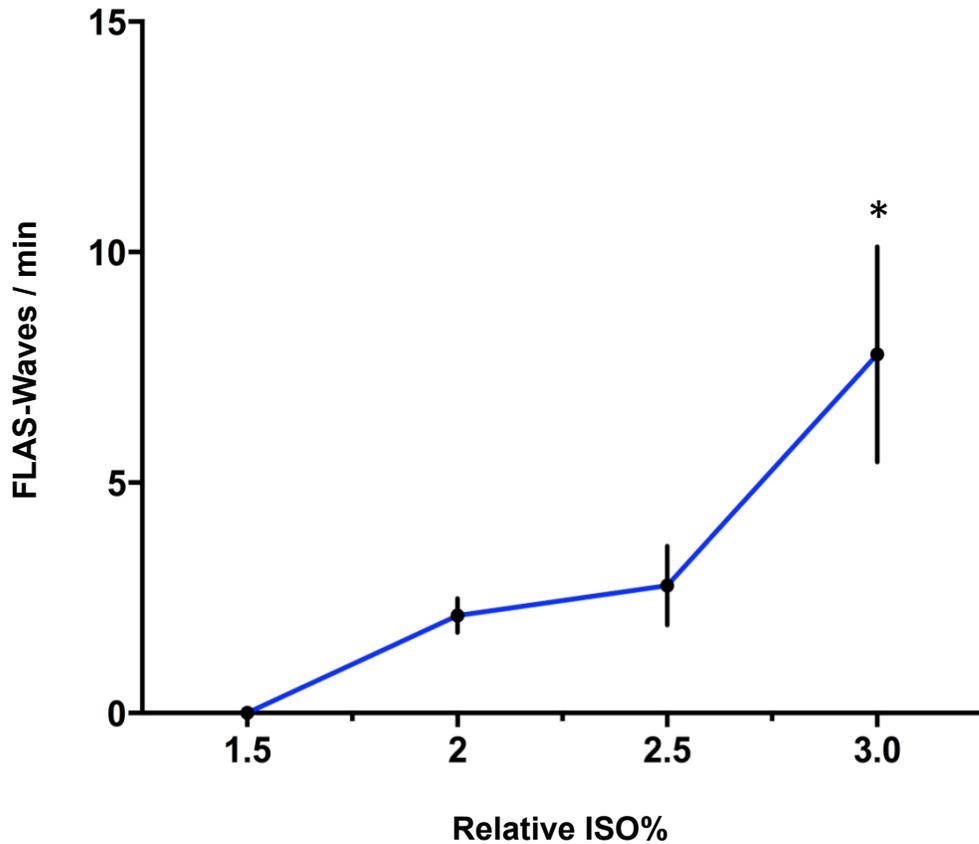


Figure 9. Effects of relative ISO% on the number of FLAS-waves/min. The mean number \pm SEM of FLAS-waves/min at relative ISO% 1.5, 2, 2.5 and 3 measured 0, 2.1 ± 0.4 , 2.8 ± 0.9 , and 7.5 ± 2.5 respectively. A Tukey's test following one way ANOVA revealed a significant difference between 1.5 and 3 ISO%, 2 and 3 ISO%. Each data point represents, mean ($n=6$) \pm SEM; * $p < 0.05$.

3.3 Distribution of Number of Bursts

Number of burst was calculated in each minute of the last 10 min of EEG recordings comprising burst suppression events at relative ISO% 0.5, 1.0 and 1.5 in the control (cohort I) group. This shows that the number of bursts is reliably distributed in the last 10 min of EEG recordings at each relative ISO% across all the animals in the control group. The mean (n=6) number of bursts \pm SEM in the 1st to 10th min at relative ISO% 0.5 measured 18.7 ± 2.2 , 21.8 ± 1.9 , 21.3 ± 1.5 , 21.8 ± 1.2 , 21.3 ± 1.7 , 21.8 ± 1.8 , 20.5 ± 1.5 , 22.0 ± 1.4 , 18.8 ± 2.2 and 16.9 ± 2.4 . The mean (n=6) number of bursts \pm SEM in the 1st to 10th min at relative ISO% 1.0 measured 8.3 ± 2.5 , 9.5 ± 2.4 , 8.8 ± 2.5 , 8.8 ± 2.4 , 10.5 ± 2.7 , 10.7 ± 2.7 , 9.3 ± 2.5 , 10.3 ± 1.9 , 10.0 ± 2.3 and 9.2 ± 2.1 . The mean (n=6) numbers of bursts \pm SEM in the 1st to 10th min at relative ISO% 1.5 measured 3.7 ± 0.8 , 3.8 ± 0.9 , 3.5 ± 0.8 , 3.9 ± 0.9 , 3.3 ± 0.5 , 4.3 ± 1.0 , 3.3 ± 1.0 , 2.8 ± 0.5 , 3.0 ± 0.9 and 3.8 ± 0.7 . Repeated measures one-way ANOVA was performed among 1st to 10th time points at relative ISO% 0.5, 1.0 and 1.5. No significant differences was observed in the number of burst in the 1st to 10th min at each relative ISO% in the control group ($p > 0.05$).

Distribution of Bursts (Control-Cohort I)

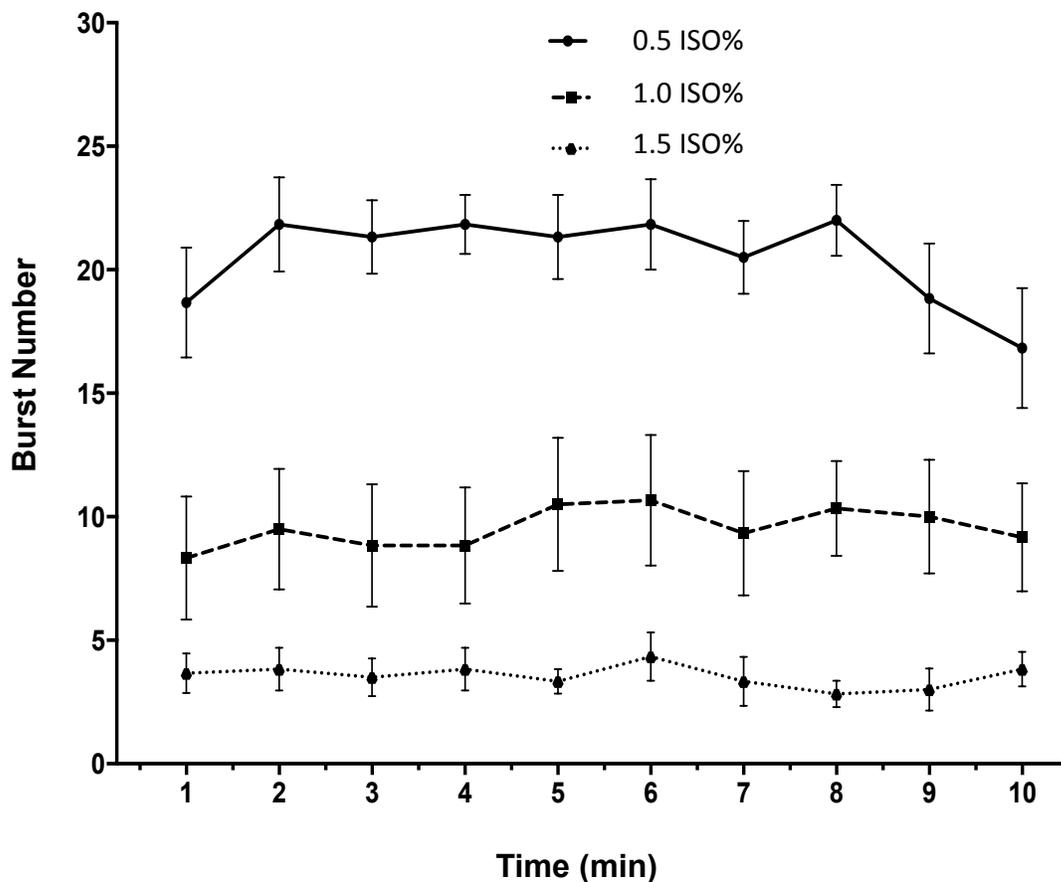


Figure 10. Distribution of bursts in the last 10 min of EEG recording at relative ISO% 0.5, 1.0 and 1.5. Repeated measures one-way ANOVA was performed among 1st to 10th time points at relative ISO% 0.5, 1.0 and 1.5 in the control group. No significant difference was observed in the number of burst in the 1st to 10th min at each relative ISO%. Data point, mean (n=6); error bars, \pm SEM.

3.4 Distribution of Number of Fast, Large-Amplitude, Sharp (FLAS) Waves

Number of FLAS-waves was calculated in each minute in the last 10 min of the EEG recordings comprising FLAS-waves at ISO% 2.0, 2.5 and 3.0 in the control (cohort I) group. The mean (n=6) numbers of FLAS-waves \pm SEM in the 1st to 10th min at relative ISO% 2.0 measured 2.7 ± 0.5 , 1.7 ± 0.4 , 1.8 ± 0.8 , 2.3 ± 0.6 , 2.2 ± 0.6 , 1.7 ± 0.6 , 2.2 ± 0.7 , 2.5 ± 0.6 , 2.50 ± 0.9 and 2.2 ± 0.7 . The mean (n=6) numbers of FLAS-waves \pm SEM in the 1st to 10th min at relative ISO% 2.5 measured 2.7 ± 1.6 , 2.8 ± 1.0 , 2.3 ± 0.8 , 3.5 ± 1.2 , 2.5 ± 1.0 , 2.8 ± 0.9 , 3.0 ± 1.1 , 2.8 ± 1.1 , 2.0 ± 1.1 and 3.2 ± 0.7 . The mean (n=6) numbers of FLAS-waves \pm SEM in the 1st to 10th min at relative ISO% 3 measured 7.7 ± 2.4 , 7.7 ± 2.5 , 7.7 ± 2.9 , 7.7 ± 2.7 , 7.0 ± 2.4 , 8.0 ± 2.6 , 8.0 ± 2.7 , 6.7 ± 2.5 , 6.7 ± 2.7 and 7.8 ± 2.2 . Repeated measures one-way ANOVA was performed among 1st to 10th min at relative ISO% 2.0, 2.5 and 3.0. No significant difference existed in the number of FLAS-waves in the 1st to 10th min at each relative ISO% in the control group.

Distribution of FLAS- Waves (Control-Cohort I)

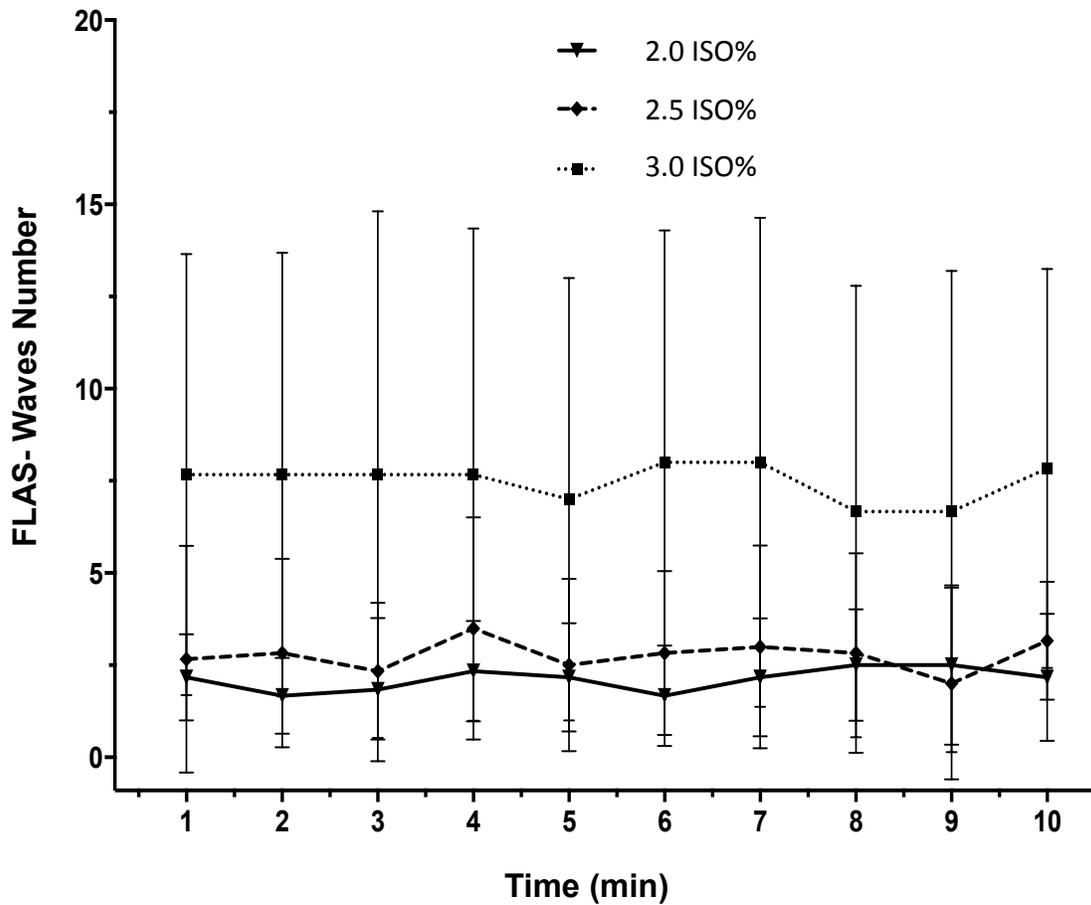


Figure 11. Distribution of FLAS-waves in the last 10 min of EEG recording at relative ISO% 2.0, 2.5 and 3.0. Repeated measures one-way ANOVA was performed among 1st to 10th min at relative ISO% 2.0, 2.5 and 3.0. No significant difference was observed in the number of FLAS-waves in the 1st to 10th min at each relative ISO% in the control group. Data point, mean (n=6); error bars, \pm SEM.

3.5 Sleep Deprivation in the von Frey Hair Test (Cohort II-Test)

The mechanical threshold using von Frey Hair test for fore paws and hind paws were measured before and after 9 h of sleep deprivation. The withdrawal thresholds in all the four paws were decreased significantly after 9 h of sleep deprivation compared to the withdrawal thresholds measured before sleep deprivation. The mean (n=5) paw withdrawal threshold before sleep deprivation for the left forepaws, left hindpaws, right forepaws and right hindpaws were 10.0 ± 0.8 , 16.1 ± 4.0 , 9.8 ± 0.7 , and 13.5 ± 3.1 s respectively. The mean paw withdrawal threshold after sleep deprivation for the left forepaws, left hindpaws, right forepaws and right hindpaws were 2.0 ± 5.6 , 5.6 ± 1.9 , 1.8 ± 0.4 , and 2.7 ± 0.2 s respectively. The withdrawal latency after sleep deprivation decreased in the left forepaws, left hindpaws, right forepaws and right hindpaws by 80%, 65.2%, 81.6%, and 80% respectively. A repeated measures two-way ANOVA indicated significant differences in paw withdrawal thresholds before vs. after 9 h of sleep deprivation ($p < 0.05$). A post hoc Tukey's multiple comparisons revealed a significant difference in the withdrawal thresholds before vs. 9h following sleep deprivation in all four paws in cohort II ($p < 0.05$).

von Frey Hair Test (Cohort II)

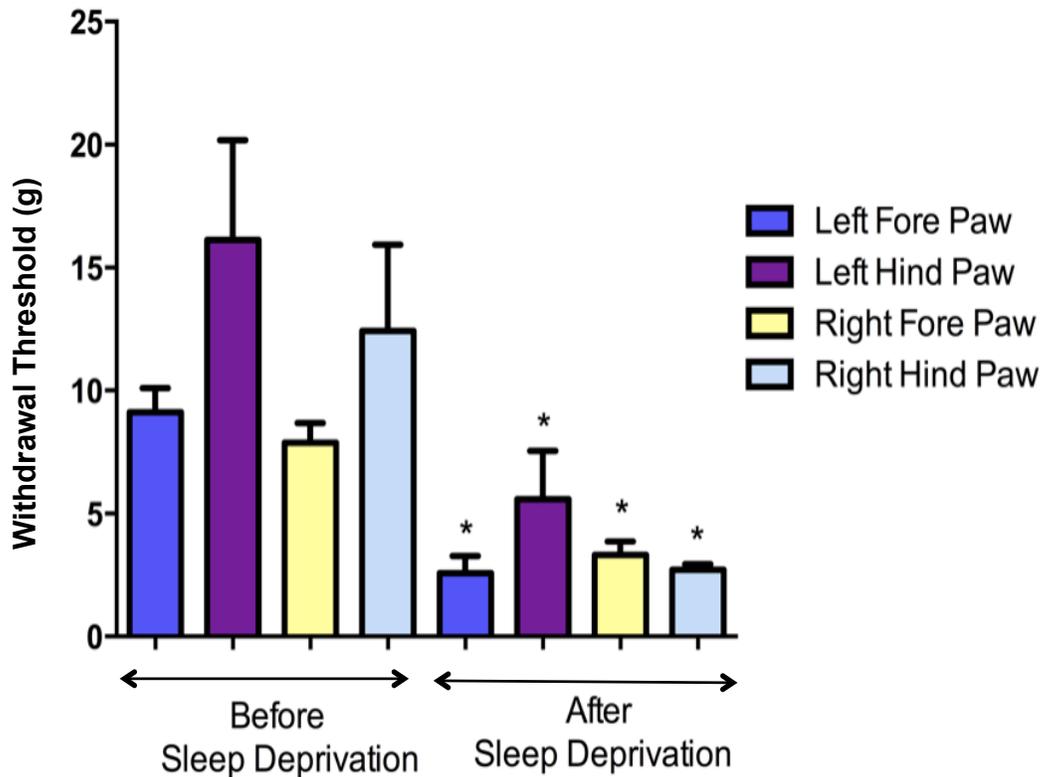


Figure 12. Effect of sleep deprivation on mechanical sensory thresholds in the von Frey hair test. A repeated measures two-way ANOVA indicated significant difference in mean withdrawal threshold between before and after sleep deprivation. A Tukey's multiple comparison test indicated significant differences in all four paws after sleep deprivation compared to before sleep deprivation. Mean withdrawal threshold (g) \pm SEM after sleep deprivation: left fore paw = 2.0 ± 0.7 , left hind paw = 5.6 ± 1.9 , right fore paw = 1.8 ± 0.4 , right hind paw = 2.7 ± 0.2 . The withdrawal threshold after sleep deprivation decreased in the left forepaws, left hindpaws, right forepaws and right hindpaws by 80%, 65.2%, 81.6% and 80%, respectively. Column, mean (n=5); error bars, \pm SEM; * p < 0.05.

3.6 Sleep Deprivation in the Hargreaves Plantar Test (Cohort II- Test)

The paw withdrawal latency using Hargreaves Plantar test for forepaws and hindpaws were measured before and after 9 h of sleep-deprivation. The paw withdrawal latencies in all four paws were decreased significantly after 9 h of sleep-deprivation compared to the paw withdrawal latencies measured before sleep-deprivation. The mean (n=5) paw withdrawal latencies before sleep deprivation for the left forepaws, left hindpaws, right forepaws and right hindpaws were 11.9 ± 0.1 , 12.8 ± 0.5 , 11.7 ± 1.1 , and 14.5 ± 0.6 s, respectively. The mean paw withdrawal latencies after 9h of sleep deprivation for the left forepaws, left hindpaws, right forepaws and right hindpaws were 8.6 ± 0.8 , 9.1 ± 0.6 , 7.9 ± 0.7 , and 9.2 ± 1.3 s, respectively. The withdrawal latency after sleep deprivation decreased in the left forepaws, left hindpaws, right forepaws and right hindpaws by 28%, 29%, 32.5%, and 36.6%, respectively. A repeated measures two-way ANOVA indicated significant differences in paw withdrawal latencies before vs. after 9 h of sleep deprivation ($p < 0.05$). A post hoc Tukey's multiple comparisons test revealed a significant difference in the withdrawal latencies before vs. 9 h following sleep deprivation in all four paws in cohort II ($p < 0.05$).

Hargreaves Plantar Test (Cohort II)

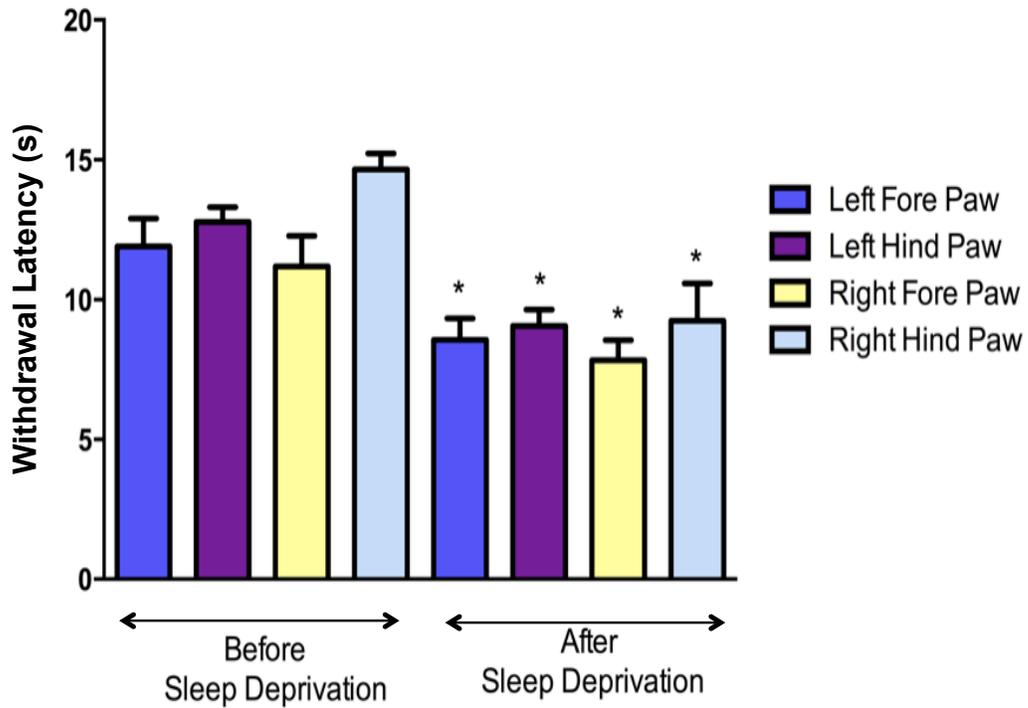


Figure 13. Effect of 9 h of sleep deprivation on paw withdrawal latencies in the Hargreaves Plantar test. Repeated measures two-way ANOVA indicated significant difference in mean withdrawal latencies before vs. after sleep deprivation. A Tukey's multiple comparison tests indicated a significant difference in the withdrawal latencies in all four paws before vs. withdrawal latencies following 9 h of sleep deprivation. Mean withdrawal latency (s) \pm SEM after sleep-deprivation: left fore paw = 8.6 ± 0.8 , left hind paw = 9.1 ± 0.6 , right fore paw = 7.9 ± 0.7 , right hind paw = 9.2 ± 1.3 . The withdrawal latency after sleep deprivation decreased in the left forepaws, left hindpaws, right forepaws and right hindpaws by 28%, 29%, 32.5%, and 36.6% respectively. Column, mean (n=5); error bars, \pm SEM; * p < 0.05.

3.7 Recovery from Sleep Deprivation in the von Frey Hair Test (Cohort III-Recovery)

Rats in the cohort III were sleep deprived for 9 h. The mechanical thresholds for forepaws and hindpaws were measured before and after sleep deprivation. After 9 h of sleep deprivation, rats were allowed to recover for 48 h. After recovery from sleep deprivation the withdrawal thresholds for four paws were measured again. The mean (n=6) paw withdrawal threshold before sleep deprivation for the left forepaws, left hindpaws, right forepaws and right hindpaws measured 12.0 ± 1.2 , 23.4 ± 2.6 , 10.4 ± 0.9 , 20.0 ± 3.0 g, respectively. The mean paw withdrawal thresholds 9 h following sleep-deprivation for the left forepaws, left hindpaws, right forepaws and right hindpaws were 3.2 ± 0.4 , 4.0 ± 1.0 , 2.6 ± 0.4 , 6.5 ± 1.3 g, respectively. The mean paw withdrawal thresholds after 48 h recovery period from sleep-deprivation for the left forepaws, left hindpaws, right forepaws and right hindpaws measured 11.0 ± 0.6 , 20.0 ± 3.0 , 11.4 ± 0.6 , and 16.1 ± 3.1 g, respectively. The withdrawal threshold after sleep-deprivation decreased in the left forepaws, left hindpaws, right forepaws and right hindpaws by 73.3%, 83%, 75%, and 67.5% respectively. The withdrawal threshold after 48 h recovery of the left forepaws, left hindpaws, right forepaws and right hindpaws were 8.3%, 14.5%, -9.6%, and 19.5%, of the control values. Repeated measures two-way ANOVA indicated significant differences in paw withdrawal latencies before vs. 9 h following sleep deprivation vs. 48 h following recovery period ($p < 0.05$). A post hoc Tukey's multiple comparisons indicated significant differences in the withdrawal latencies before vs. 9 h following sleep deprivation and 9 h following sleep deprivation vs. 48h following recovery period in all the four paws in cohort III ($p < 0.05$). No significant differences existed in the paw withdrawal latencies before 9h of sleep deprivation vs. 48h following recovery period. These results suggest that 48 h of recovery from sleep deprivation was associated with a reversible reduction in the mechanical sensory thresholds.

von Frey Hair Test (Cohort III)

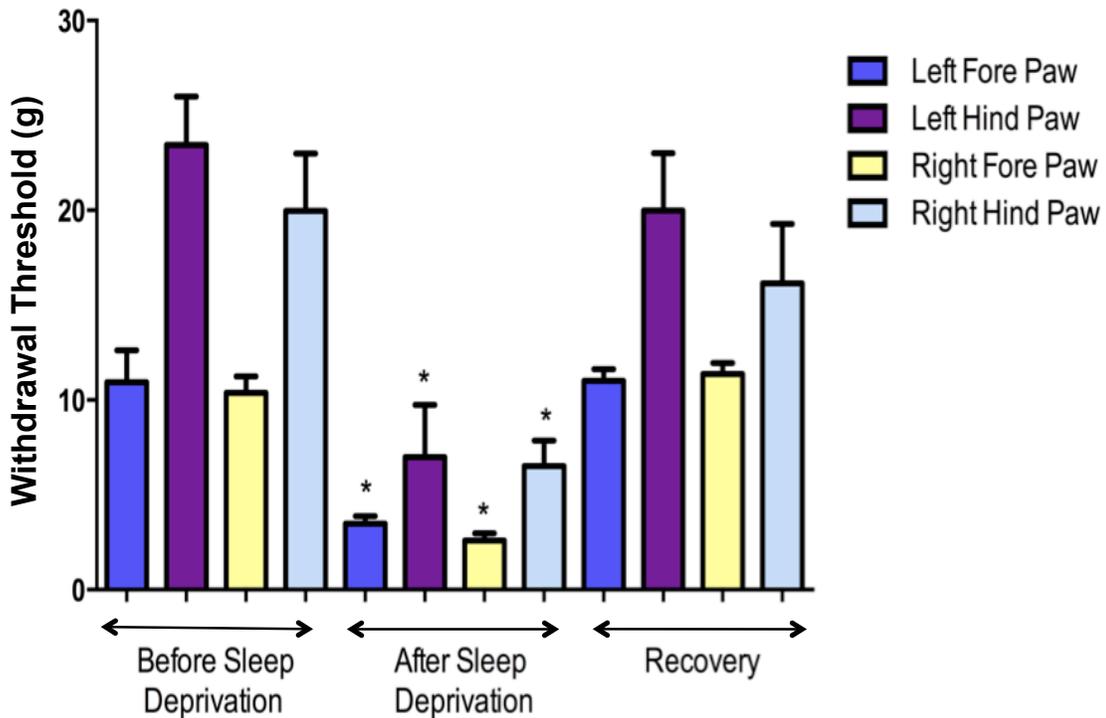


Figure 14. Effect of 48 h recovery following 9 h of sleep deprivation in the von Frey hair test. A repeated measures two-way ANOVA indicated significant differences in the paw withdrawal latencies before vs. 9h following sleep-deprivation vs. 48 h following recovery period ($p < 0.05$). A post hoc Tukey's multiple comparisons revealed significant differences in the withdrawal latencies before vs. 9 h following sleep-deprivation and 9 h following sleep-deprivation vs. 48 h following recovery period in all the four paws ($p < 0.05$). The withdrawal threshold after sleep-deprivation decreased in the left forepaws, left hindpaws, right forepaws and right hindpaws by 73.3%, 83%, 75%, and 67.5% respectively. The withdrawal threshold after 48 h recovery of the left forepaws, left hindpaws, right forepaws and right hindpaws were 8.3%, 14.5%, -9.6%, and 19.5%, of the control values. No significant differences were found in the paw withdrawal latencies before 9 h of sleep-deprivation vs. 48 h following recovery period. Column, mean ($n=6$); error bars, \pm SEM; * $p < 0.05$.

3.8 Recovery from Sleep Deprivation in the Hargreaves Plantar Test (Cohort III-Recovery)

Rats in the cohort III were sleep deprived for 9 h. The paw withdrawal latencies for fore and hindpaws were measured before and after sleep deprivation. After recovery from sleep deprivation the withdrawal thresholds for four paws were measured again. The mean paw withdrawal latencies before sleep deprivation for the left forepaws, left hindpaws, right forepaws and right hindpaws were 11.7 ± 0.9 , 13.1 ± 0.9 , 10.8 ± 0.8 , and 12.5 ± 0.6 s, respectively. The mean paw withdrawal latencies 9 h following sleep deprivation for the left forepaws, left hindpaws, right forepaws and right hindpaws were 6.4 ± 0.6 , 7.3 ± 0.7 , 7.0 ± 0.8 , and 8.2 ± 0.6 s respectively. The mean paw withdrawal latencies 48 h following recovery from sleep deprivation for the left forepaws, left hindpaws, right forepaws and right hindpaws were 11.5 ± 0.7 , 12.0 ± 0.9 , 10.1 ± 0.7 , and 13.0 ± 1.2 s, respectively. In relative terms the thermal threshold after sleep deprivation measured in the left forepaws, left hindpaws, right forepaws and right hindpaws by 45.2%, 44.2%, 34.6% and 34.4%, respectively. In relative terms the withdrawal latency after 48 h recovery of the left forepaws, left hindpaws, right forepaws and right hindpaws were 1.7%, 8.7%, 6.5%, and -4.3%, of the control values. A repeated measures two-way ANOVA indicated significant differences in paw withdrawal latencies before vs. 9 h following sleep-deprivation vs. 48 h following recovery period ($p < 0.05$). A post hoc Turkey's multiple comparisons revealed significant differences in the withdrawal latencies before vs. 9 h following sleep-deprivation and 9 h following sleep-deprivation vs. 48 h following recovery period in all four paws in cohort III ($p < 0.05$). No significant differences were found in the paw withdrawal latencies between the control and recovery groups. These results suggest that 48 h of recovery from sleep deprivation was associated with a reversible reduction in thermal thresholds

Hargreaves Plantar Test (Cohort III)

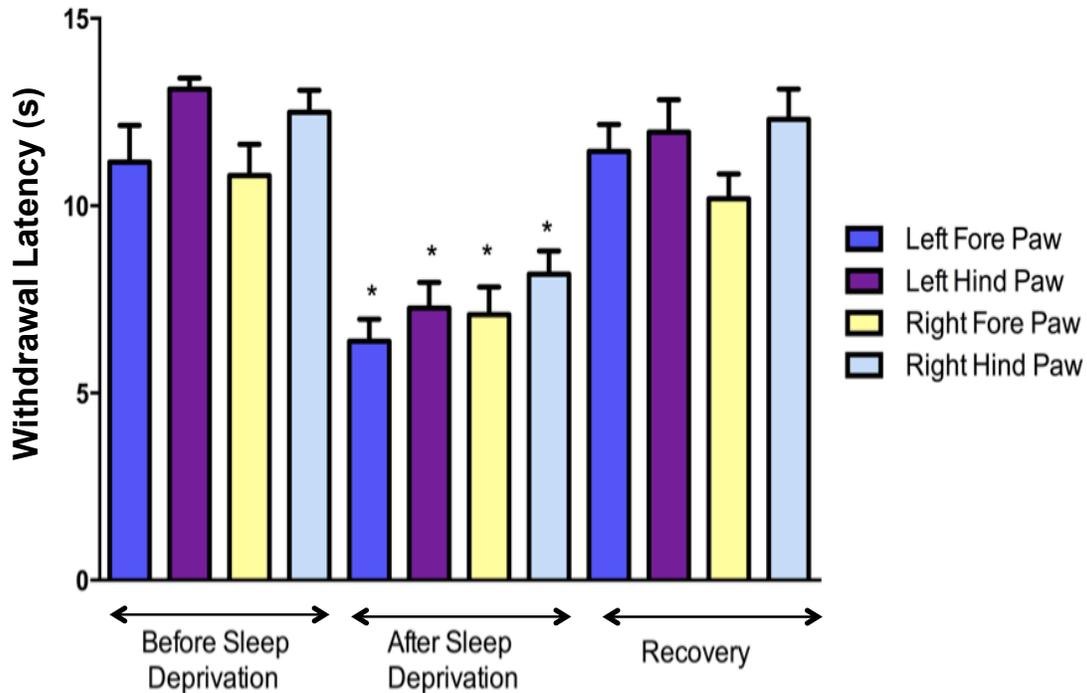


Figure 15. Effect of 48 h recovery following 9 h of sleep deprivation in the Hargreaves Plantar test. A repeated measures two-way ANOVA indicated significant differences in the paw withdrawal latencies before vs. 9 h following sleep deprivation vs. 48 h following recovery period ($p < 0.05$). A post hoc Tukey's multiple comparisons test revealed significant differences in the withdrawal latencies before vs. 9 h following sleep deprivation and 9 h following sleep deprivation vs. 48 h following recovery period in all four paws ($p < 0.05$). The withdrawal latency after sleep deprivation decreased in the left forepaws, left hindpaws, right forepaws and right hindpaws by 45.2%, 44.2%, 34.6% and 34.4% respectively. The withdrawal latency after 48 h recovery of the left forepaws, left hindpaws, right forepaws and right hindpaws were 1.7%, 8.7%, 6.5%, and -4.3%, of the control values. No significant differences were found in the paw withdrawal latencies before 9 h of sleep-deprivation vs. 48 h following recovery period. Column, mean ($n=6$); error bars, \pm SEM; * $p < 0.05$.

3.9 Effects of Relative ISO% on the Number of Bursts (Control vs. Test vs. Recovery)

The number of EEG burst events at each relative ISO% for the rats in control (cohort I), test (cohort II-SD) and recovery (cohort III) groups were calculated using a customized script. In the control, test (SD) and recovery group the number of bursts/min was increased with increasing ISO%. The mean number of bursts in the control group (n=6) at relative ISO% 0.5, 1.0, 1.5 and 2.0 measured 20.5 ± 1.5 , 9.8 ± 2.3 , 3.5 ± 0.7 and 0, respectively. The mean number of bursts in the test (SD) group (n=6) at relative ISO% 0.5, 1.0, 1.5 and 2.0 measured 25.9 ± 1.9 , 20.4 ± 1.8 , 10.2 ± 2.0 , and 1.5 ± 0.5 , respectively. The mean number of bursts in the recovery group (n=6) at relative ISO% 0.5, 1.0, 1.5 and 2.0 measured 18.0 ± 2.5 , 10.3 ± 1.8 , 4.7 ± 0.7 and 0, respectively. Repeated measures two-way ANOVA indicated significant differences in the number of bursts/min. in control vs. test (SD) vs. recovery group ($p < 0.05$). A post hoc Tukey's multiple comparisons test revealed significant differences between control and test (SD) groups at relative ISO% 0.5, 1.0 and 1.5 ($p < 0.05$). Tukey's multiple comparisons test also revealed significant differences between test (SD) and recovery group at relative ISO% 0.5, 1.0 and 1.5 ($p < 0.05$). No significant differences were observed in the mean burst number between control and recovery groups at relative ISO% 0.5, 1.0 and 1.5 ($p > 0.05$). There were also no significant differences in the number of bursts in the control vs. test (SD) vs. recovery at relative ISO% 2 ($p > 0.05$).

**Number of Bursts
(Control vs. Test vs. Recovery)**

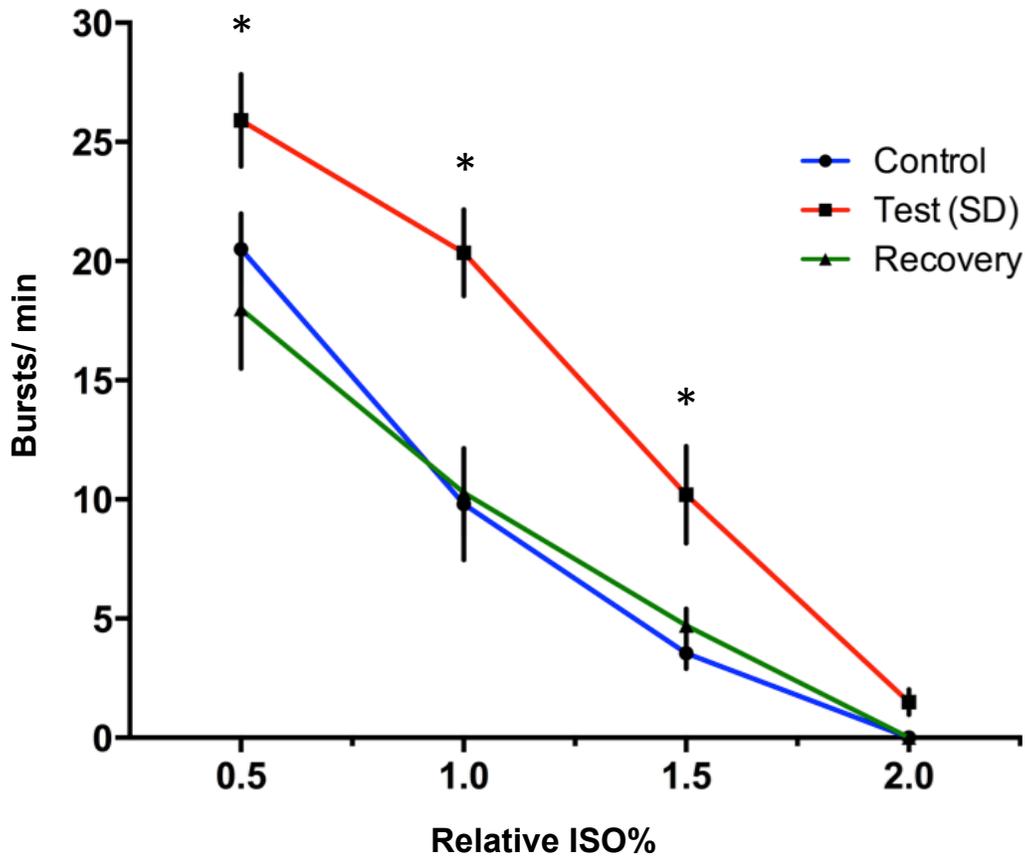


Figure 16. Effect of relative ISO% on the number of bursts/min. Repeated measures two-way ANOVA indicated a significant difference in the number of bursts/min. in control vs. test (SD) vs. recovery group ($p < 0.05$). A post hoc Tukey's multiple comparisons test revealed significant differences in control vs. test (SD) and test (SD) vs. recovery group at relative ISO% 0.5, 1.0 and 1.5 ($p < 0.05$). No significant difference was observed in the burst number in control vs. recovery group at relative ISO% 0.5, 1.0, 1.5 and in the number of bursts in control vs. test (SD) vs. recovery group at relative ISO% 2 ($p > 0.05$). Data point, mean ($n=6$) \pm SEM; * $p < 0.05$.

3.10 Effects of Relative ISO% on the Number of FLAS-Waves (Control vs. Test vs. Recovery)

The number of FLAS-waves/min. was calculated at each relative ISO% for the rats in control (cohort I), test (cohort II- SD) and recovery (cohort III) group. Unlike bursts, the number of FLAS-waves/min was increased with increasing relative ISO% in the control, test (SD) and recovery groups. The mean number of FLAS-waves in the control group (n=6) at relative ISO% 1.5, 2.0, 2.5 and 3.0 measured 0, 2.1 ± 0.4 , 2.8 ± 0.9 , and 7.8 ± 2.3 , respectively. The mean number of FLAS-waves in the test (SD) group (n=6) at relative ISO% 1.5, 2.0, 2.5 and 3.0 measured 0, 3.5 ± 0.9 , 3.4 ± 0.8 , and 8.3 ± 2.0 , respectively. The mean number of FLAS-waves in the recovery group (n=6) at relative ISO% 1.5, 2.0, 2.5 and 3.0 measured 0, 1.6 ± 0.2 , 1.8 ± 0.6 and 5.2 ± 1.7 , respectively. A repeated measures two-way ANOVA indicated no significant differences in the mean number of FLAS-waves/min in control vs. test (SD) vs. recovery group ($p < 0.05$).

**Number of FLAS-Waves
(Control vs. Test vs. Recovery)**

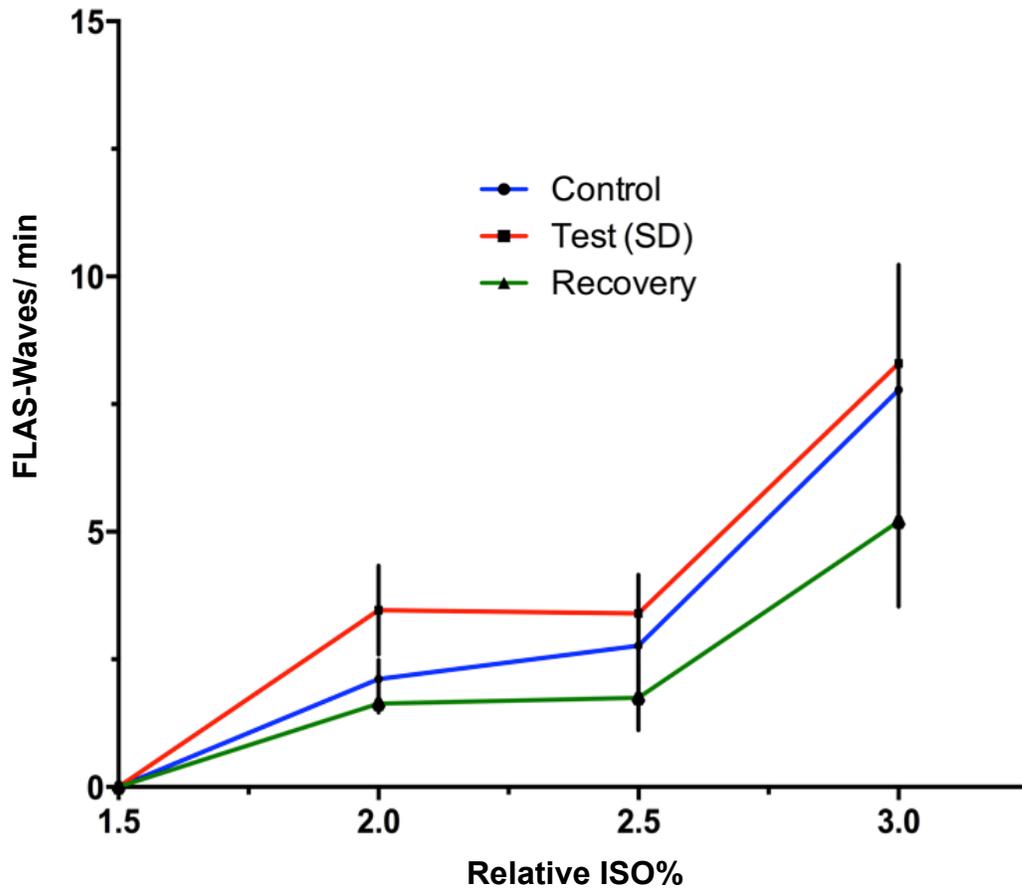


Figure 17. Effects of relative ISO% on the number of FLAS-waves/min. A repeated measures two-way ANOVA indicated no significant differences in the number of FLAS-waves/min. in control vs. test (SD) vs. recovery group ($p > 0.05$). Each data point represents the mean ($n=6$) \pm SEM.

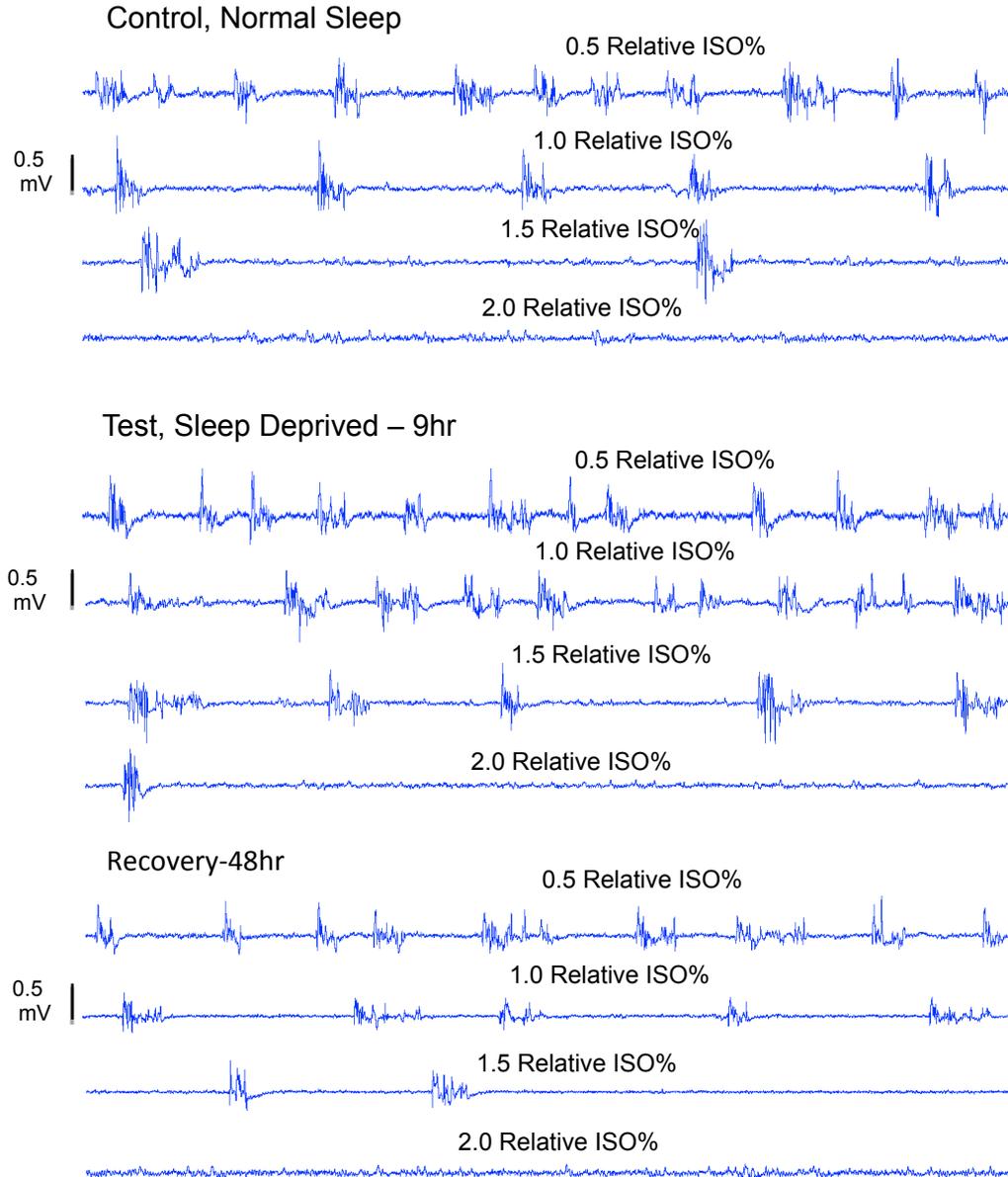


Figure 18. EEG activity traces (30s) representing the changes in the number of bursts at relative ISO% 0.5, 1, 1.5, and 2 in control, test (SD) and recovery group. Note that more burst events were recorded at each relative ISO% in the test group.

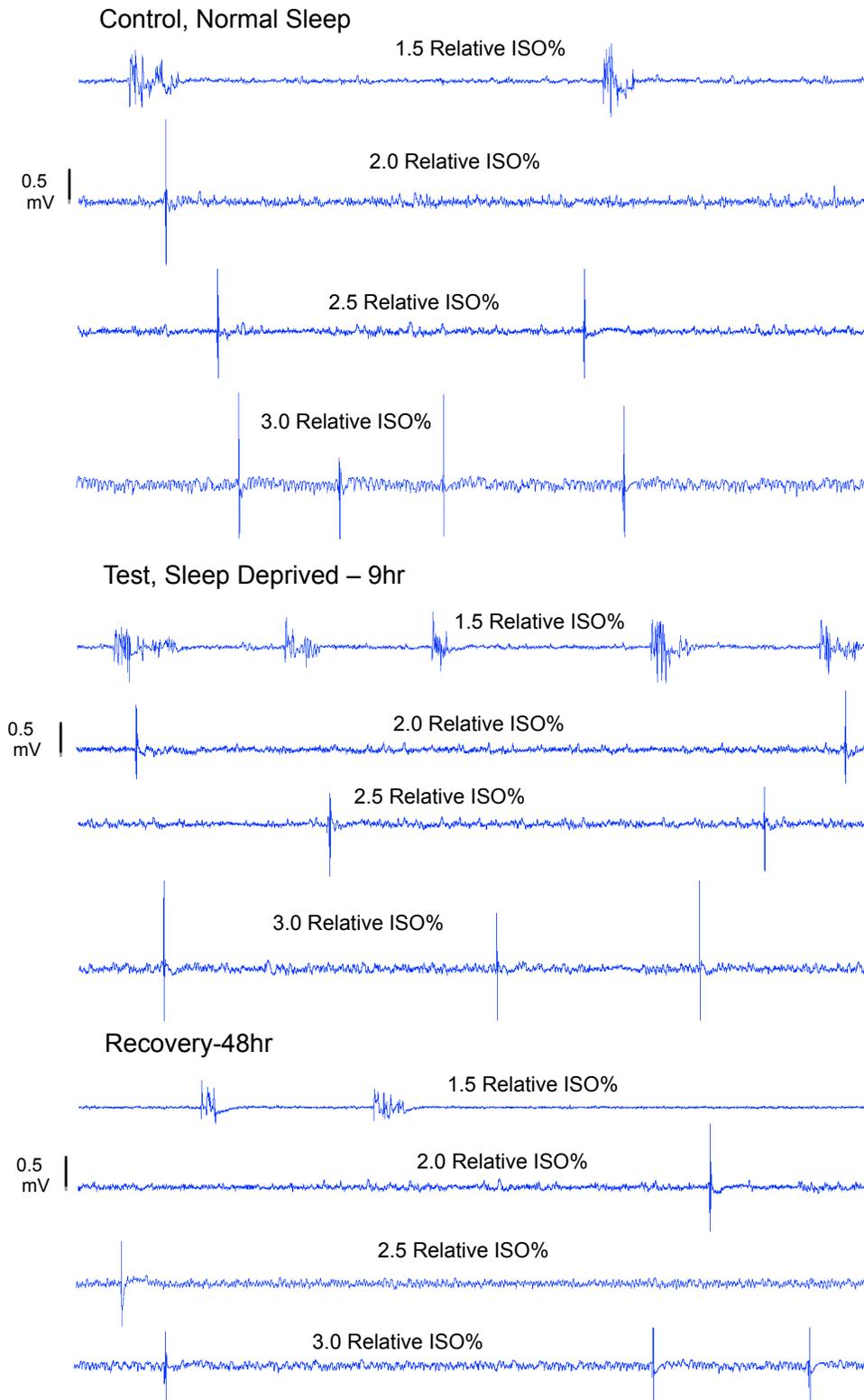


Figure 19. EEG activity traces (30s) representing the changes in the number of bursts and FLAS-waves/min at relative ISO% 1.5, 2, 2.5 and 3 in control, test (SD) and recovery group.

3.11 Effects of Relative ISO% on the Mean Burst Duration

The mean burst duration was calculated at each relative ISO% for the rats in control (cohort I), test (cohort II-SD) and recovery (cohort III) groups. The average duration of EEG bursts was gradually decreased with increasing relative ISO% in the control, test (SD) and recovery groups. The mean burst duration in the control group (n=6) at relative ISO% 0.5, 1.0 and 1.5 are 0.7 ± 0.1 , 0.6 ± 0.2 and 0.3 ± 0.1 respectively. The mean burst duration in the test (SD) group (n=6) at relative ISO% 0.5, 1.0 and 1.5 are 1.1 ± 0.2 , 0.9 ± 0.1 and 0.7 ± 0.1 respectively. The mean number of bursts in the recovery group (n=6) at relative ISO% 0.5, 1.0 and 1.5 are 0.7 ± 0.1 , 0.7 ± 0.1 and 0.5 ± 0.1 respectively. A repeated measures two-way ANOVA indicated no significant differences in the mean burst duration in control vs. test (SD) vs. recovery group ($p < 0.05$).

**Mean Bursts Duration
(Control vs. Test vs. Recovery)**

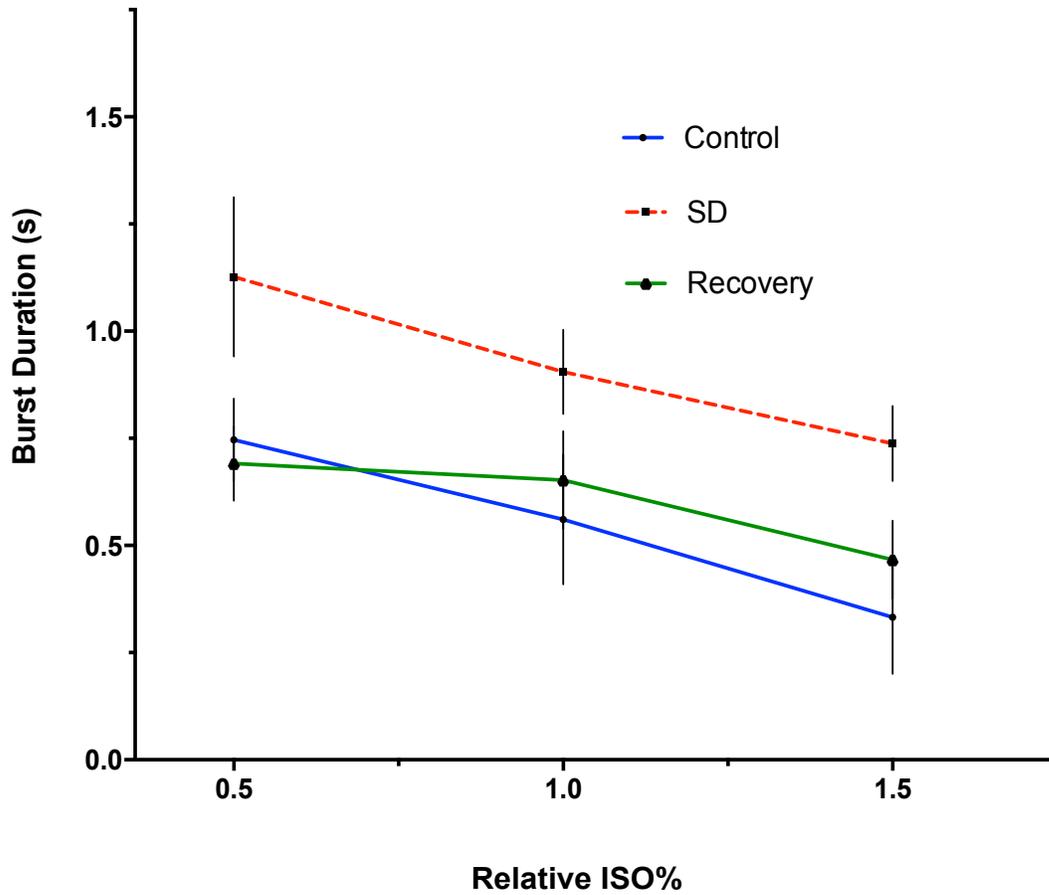


Figure 20. Effects of relative ISO% on the duration of bursts. Repeated measures two-way ANOVA indicated no significant difference in the mean burst durations in control vs. test (SD) vs. recovery group ($p > 0.05$). Each data point represents the mean ($n=6$) \pm SEM.

3.12 Effects of Relative ISO% on the Mean Suppression Duration

The mean suppression duration was calculated at each relative ISO% for the rats in control (cohort I), test (cohort II-SD) and recovery (cohort III) group. The average duration of suppression was increased with increasing relative ISO% in the control, test (SD) and recovery groups. The mean suppression duration in the control group (n=6) at relative ISO% 0.5, 1.0 and 1.5 measured 3.2 ± 0.9 , 9.7 ± 2.3 and 19.1 ± 4.3 s, respectively. The mean suppression duration in the test (SD) group (n=6) at relative ISO% 0.5, 1.0 and 1.5 are 2.4 ± 0.6 , 4.0 ± 1.0 and 6.5 ± 1.4 s, respectively. The mean suppression duration in the recovery group (n=6) at relative ISO% 0.5, 1.0 and 1.5 are 3.3 ± 0.6 , 7.8 ± 1.5 and 16.9 ± 2.9 s respectively. A Tukey's multiple comparisons test following Repeated Measures two-way ANOVA revealed significant differences in control vs. test (SD) and test (SD) vs. recovery group at relative ISO% 1.5 ($p < 0.05$). No significant differences were observed in the mean suppression duration at relative ISO% 0.5 and 1 when compared between control, test (SD) and recovery groups.

Mean Suppression Duration (Control vs. Test vs. Recovery)

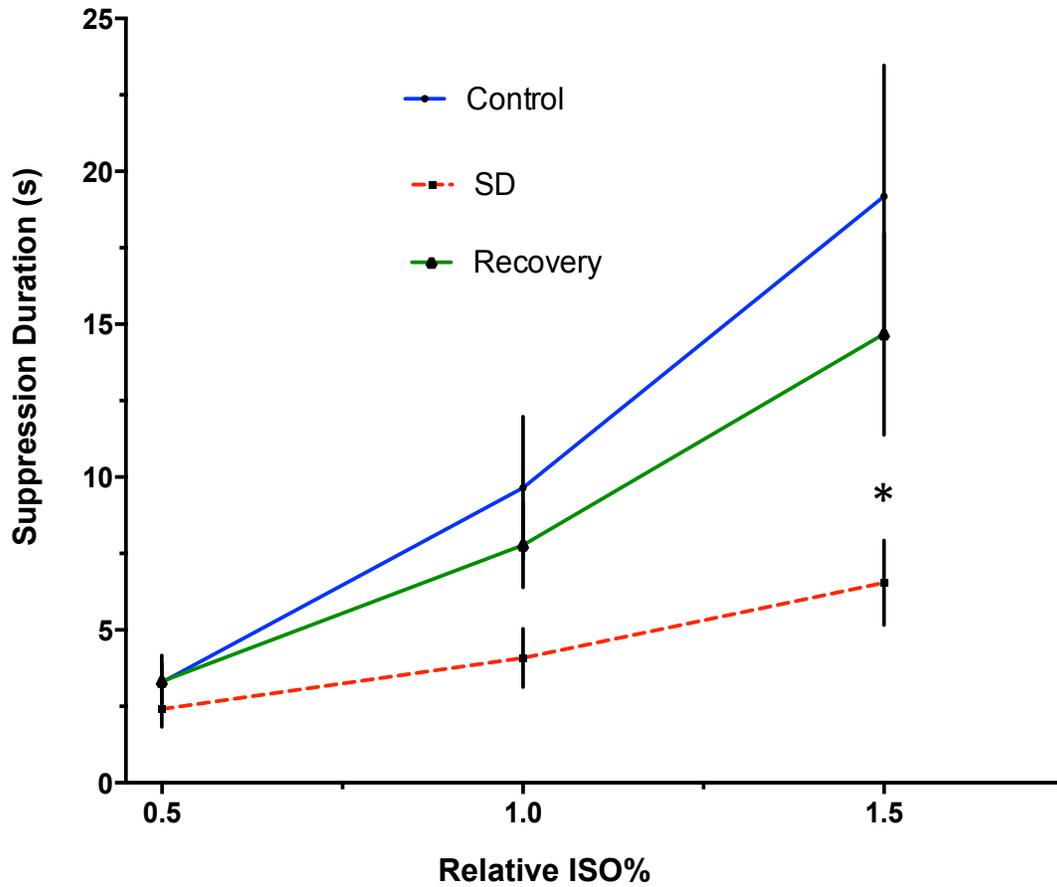


Figure 21. Effects of relative ISO% on the duration of suppression. A post hoc Tukey's multiple comparisons test following repeated measures two-way ANOVA indicated revealed significant differences in control vs. test (SD) and test (SD) vs. recovery group at relative ISO% 1.5. No significant differences were observed in the mean suppression duration at relative ISO% 0.5 and 1 when compared between control, test (SD) and recovery group. Each data point represents the mean ($n=6$) \pm SEM; * $p < 0.05$.

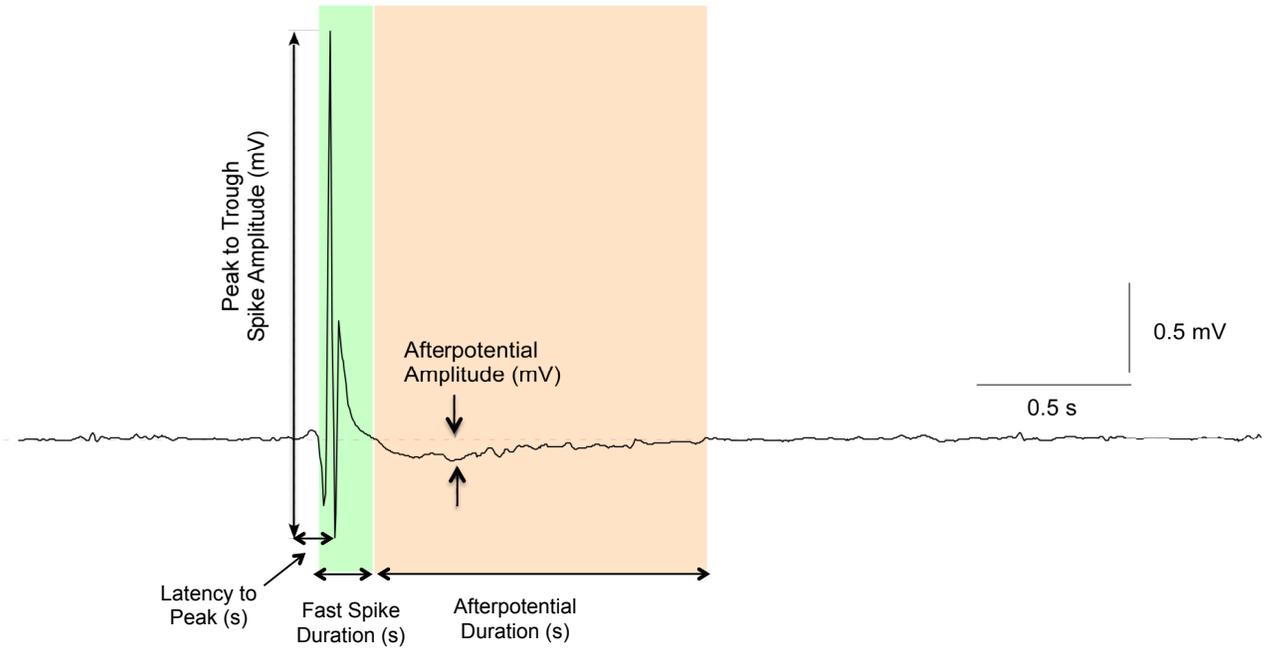


Figure 22. Computer averaged FLAS-waves. The figure shows the characteristics of typical FLAS-waves comprising a sharp fast spike, which are followed by a long after potential.

Cohort	Peak to Trough Amplitude (mV)	Fast Spike Duration (s)	Latency to Peak (s)	Afterpotential Duration (s)	Afterpotential Amplitude (s)	Total Spike Duration (s)
Control (n=6)	2.2 ± 0.3	0.2 ± 0.0	0.1 ± 0.0	1.1 ± 0.1	0.2 ± 0.0	1.3 ± 0.1
Test (n=6)	1.4 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	1.2 ± 0.6	0.1 ± 0.0	1.4 ± 0.1
Recovery (n=6)	2.1 ± 0.3	0.2 ± 0.0	0.1 ± 0.4	1.6 ± 0.1	0.1 ± 0.0	1.7 ± 0.1

Table 1. Characteristics of computer averaged fast, large-amplitude, sharp (FLAS) waves. The table summarizes parameters representing characteristics of FLAS-waves in 3 cohorts. A one-way ANOVA performed for each parameter revealed no significant differences in the control, test (SD) and recovery group ($p > 0.05$). Each parameter represents as the group mean \pm SEM.

Chapter 4: Discussion

Among all inhalational anesthetics isoflurane is most commonly used in preclinical studies. It is widely employed for its advantages including rapid control over anesthetic depth, rapid reversibility of anesthetic state by switching from one concentration to another and less depression of myocardial contractility and heart rate (Eger, 1981; Kroeger et al., 2013). *In vivo* experiments in pain and neuroscience research require monitoring the depth of anesthesia since subtle changes in anesthetic concentration may lead to inaccurate research outcomes. Stepwise increases in isoflurane dosage result in changes in EEG activities, including slow-wave EEG activities at low doses, which is replaced by burst-suppression events at higher doses. Burst-suppression events are followed by an isoelectric period (Kroeger et al., 2013). However, although these EEG trends have been well established, consistent dose related data demonstrating such effects of specific doses of isoflurane on EEG activities is lacking. Moreover, recent findings suggest that the isoelectric period may not be the final EEG signature at very high doses of anesthetics. Rather, increasing doses beyond the isoelectric period leads to the emergence of fast, large-amplitude, sharp (FLAS) waves like “Nu-complexes” in comatose human patient under antiepileptic medication as well as in cats during very deep anesthesia (Kroeger et al., 2013). This novel finding raises the question of whether this EEG characteristic is species-specific. The present study documents the concentration-dependent effects of isoflurane anesthesia on EEG activities and is the first to reveal the presence of FLAS-waves at very deep isoflurane anesthesia in rats. Furthermore, previous studies have reported that sleep restriction reduces the time required to lose consciousness following the induction of isoflurane anesthesia (Tung et al., 2002; Murphy et al., 2011), and that sleep and anesthesia share similar EEG characteristics. Thus, we tested the effect of sleep restriction on the dose-

response curve of the EEG activities produced by isoflurane. Findings from this study are discussed below.

4.1 Number of Bursts Reduces with Increasing Relative ISO%

We find the existence of significant variability among animals in terms of the ISO concentration (indicated by the vaporizer dial) that induces stable slow-wave activity. To normalize this inter-subject variability, the ISO% that exerted 20 min of continuous slow-wave activity just prior to the appearance of reliable burst-suppression activity was assigned as the baseline ISO% for each animal. Normalized ISO% values were reported in terms of 'relative ISO% to slow-wave', which was defined as the ISO% relative to each animal's baseline ISO%. In cohort I, the number of bursts gradually decreased as the depth of anesthesia increased (Figure 8), suggesting a dose-response relationship between the relative ISO% and the number of bursts. The number of bursts/min decreased significantly among relative ISO% values of 0.5, 1 and 1.5, and burst number was reduced to zero when anesthesia increased further to relative ISO% 2. These data are consistent with the other findings where a gradual increase in isoflurane concentration resulted in reduced number of burst-suppression events, which was followed by an isoelectric period (Kroeger et al., 2013). The present study quantified the burst events during the last 10 min of the 30 min EEG recording at each relative ISO% value. This data represents a more accurate way to monitor depth of ISO anesthesia using burst count rather than the vaporizer dial. Furthermore, since the ISO concentration that leads to the onset of EEG burst-suppression activities differs among animals, the 'relative ISO%' reliably normalizes the concentrations of ISO across all the animals for the purposes of reporting meaningful experimental results. This is

demonstrated by the consistency in the number of burst events that follow the same trend from relative ISO% 0.5 to relative ISO% 1.5 among all animals.

4.2. Number of Fast, Large Amplitude, Sharp (FLAS) Waves Increased with Increasing Relative ISO%

As the anesthetic concentration was increased to relative ISO% 2, the number of burst events decreased and further increases in ISO concentration led to the appearance of fast, large-amplitude, sharp waves. The number of FLAS-waves gradually increased as the relative ISO% increased. This increase in FLAS-waves number was slow initially, however, with further increases in relative ISO% the number of FLAS-waves/min increased abruptly occurring at just below 1 Hz. Consistent with the findings by Kroger et al., (2013), the frequency of the FLAS-waves at relative ISO% 2, 2.5 and 3 measured 0.035 Hz, 0.046 Hz and 0.125 Hz respectively. Kroger et al., (2013), were the first to report the possible origin of these activities. They found that hippocampal field potential recording displayed higher amplitude FLAS-waves compared to other subcortical FLAS-waves. Moreover, FLAS-waves occurred first in the hippocampus whereas these cortically recorded FLAS-waves activities were delayed by at least 10 ms in other subcortical regions (thalamus, basal forebrain, brainstem) suggesting a central role for the hippocampus in generating FLAS-waves (Kroeger et al., 2013). Interestingly, although Kroeger and Amzica (2013) were the first to describe the potential origin of FLAS-waves, Ogawa and Shingu (1992) published earlier reports on the presence of similar high-amplitude EEG spike activities during very deep isoflurane (4.8%) and enflurane (3.6%) anesthesia in cats. Specifically, their findings reported on dose-related alterations of EEG activities (transition of EEG slow-wave activity to burst-suppression events followed by the large-amplitude spikes) using graded (lower to higher)

concentrations of isoflurane and enflurane. In 1994, Osawa et al., also reported EEG activities similar to the FLAS-waves/‘Nu-complexes’ using sevoflurane anesthesia in cats. Building on this, our study is the first to report the presence of FLAS-waves in rats. We find that increasing relative ISO% leads to increased FLAS-wave activity number suggesting a dose-related effect of this phenomenon at very deep anesthetized states. These fast, large-amplitude, sharp waves appear more frequently at very high concentrations of isoflurane (relative ISO% 3 and beyond). Our finding of the presence of FLAS-waves in rats following isoelectricity corroborates previous literature in cats (Ogawa et al., 1992; Osawa et al., 1994; Kroeger et al., 2013) and dogs (Joas et al., 1971), strongly suggests that this activity is not species-specific, and provides further evidence to update our current understanding of the nature of this novel brain activity.

4.3 Effect of 9 h Sleep Deprivation and 48 h Recovery on Mechanical and Thermal Pain Sensitivity

Sleep deprivation (8 h to 72 h) is known to produce hyperalgesia in rodents and humans (Onen et al., 2000; Schuh-Hofer et al., 2013; Wodarski et al., 2014). The increased pain sensitivity associated with hyperalgesia attenuates after 48 h of recovery in rodents (Steriade et al., 1993a; Bal et al., 1995; McCormick and Bal, 1997; Onen et al., 2000). In the present study, 9 h of sleep deprivation significantly increased the mechanical and thermal pain sensitivity in all four paws of the rats in cohorts II and III and recovery from sleep deprivation for 48 h led to complete recovery from allodynia and hyperalgesia in cohort III rats. These results (figure 12, 13, 14, and 15) served as a behavioral index for the sleep deprivation and indicated that 48 h of recovery following 9 h of sleep deprivation significantly reversed the mechanical allodynia and thermal hyperalgesia. Few studies to date have reported on the possible mechanism of action underlying

increased pain sensitivity after sleep-restriction. Ukponmwan et al., (1984) reported that continuous sleep is required for the maintenance of the analgesic action exerted by both endogenous and exogenous opioids. They observed that the anti-nociceptive effect produced by the opioids and monoamines were abolished following 96 h of REM sleep deprivation (Ukponmwan et al., 1984; Franks, 2008). Moreover, REM sleep-deprivation in female rats inhibited the synthesis of opioid proteins (Shapiro and Girdwood, 1981). Opioids and monoamines are the major neurotransmitters that are involved in the descending pain inhibition system (Basbaum and Fields, 1984; Franks, 2008). Another potential mechanism underlying the anti-nociceptive influence of sleep relates to the effect of sleep on serotonin levels. Studies reported decreased serotonin levels in the frontal and parietal brain regions in rats following 96 h of REM sleep disruption (Basbaum and Fields, 1984; Farooqui et al., 1996; Franks, 2008). Moreover, extracellular serotonin levels gradually declined in the frontal cortex and hippocampus during 8 h of sleep-deprivation in rats. Interestingly, Soja et al., (1993) observed suppression of sciatic nerve-evoked response during REM sleep compared to NREM sleep in cats (Soja et al., 1993). In a study by Onen et al., (2001), selective REM and NREM sleep deprivation resulted in decreased mechanical pain threshold in nine healthy human volunteers. The pain thresholds were increased following 40 h of total sleep recovery and NREM sleep recovery but not following REM sleep recovery. However, unlike in humans, REM sleep-deprivation (3 h - 96 h) in rodents resulted in significant decreases in mechanical pain thresholds, which reversed back to control level following recovery (Onen et al., 2000; Hakki Onen et al., 2001; Onen et al., 2001; Dametto et al., 2002; Tung and Mendelson, 2004). Thus, the experimental data in animals are not consistent with the human data following REM sleep-deprivation. Of note, due to procedural conveniences total and REM sleep deprivation were studied more commonly

than selective NREM sleep deprivation in animals (Tung and Mendelson, 2004; Lautenbacher et al., 2006).

4.4 Effects of Relative ISO% on the Number of Bursts (Control vs. Test vs. Recovery)

Similar to the control rats (cohort I) the number of bursts gradually decreased with increasing relative ISO% in the sleep-deprived (cohort II-Test) and recovered rats (cohort III), indicating consistency in our methodology. The results indicate that the dose-response relationship between bursts/min and relative ISO% followed the same pattern in the control, sleep-deprived and recovered rats. However, the number of bursts/min was significantly higher than controls in the sleep-deprived rats at relative ISO% 0.5, 1 and 1.5 values. The dose-response curve shifted to the right in the sleep-deprived rats, which then reversed back to the control level in the recovered rats. The result overall indicates that more ISO% is required in the sleep-deprived rats to produce an equivalent number of burst events that occurred at each relative ISO% (0.5, 1 and 1.5) value in control and recovered rats. Interestingly, Ching et al., (2012) reported that burst-suppression events during isoflurane anesthesia is associated with a reduced rate of metabolism in the brain. The authors suggested, reduced neural activity during general anesthesia leads to decreased cerebral metabolic rate of oxygen (CMRO) and cerebral blood flow (CBF), which in turn causes decreased production of ATP (Ching et al., 2012; Kroeger et al., 2013). In another study, propofol produced a dose-related decrease in CBF and CMRO in rabbits. The reduced CBF and CMRO was found to be associated with the marked EEG suppression (isoelectricity) (Ramani et al., 1992). Sleep deprivation on the other hand is known to increase the cerebral metabolic rate (CMR) in mice and humans (Wu et al., 1991; Vyazovskiy et al., 2008). It is therefore possible that

the increased burst number in the sleep-deprived rats found in this study is likewise associated with enhanced cerebral metabolism that is manifested by the rightward shift in the concentration-burst events curve. Future studies where *in vivo* cerebral metabolism is assessed in sleep-restricted brain vs. normal brain across varying concentrations of isoflurane (ISO%) would be useful to determine whether such an association exists. A third cohort (cohort III) of animals that were allowed to recover after 9 h of sleep deprivation showed a concentration dependent curve that was similar to the one observed in control (cohort I) group of animals, indicating a complete reversal of sleep deprivation. The sensory thresholds in the cohort III animals did not differ with the animals in the control group (cohort I), which further confirmed the recovery from sleep-deprivation.

4.5 Effects of Relative ISO% on the Fast, Large-Amplitude, Sharp (FLAS) Waves (Control vs. Test vs. Recovery)

Similar to the control (cohort I), the number of FLAS-waves increased with increasing relative ISO% values in the sleep-deprived (cohort-II-test) and recovered (cohort-III) rats. However, unlike burst events, mean number of FLAS-waves/min did not change significantly at ISO% 2, 2.5 and 3 values between control and sleep-deprived conditions. As the relative ISO% was increased, the FLAS-waves progressively increased both in number and amplitude in all 3 cohorts. However, the spike amplitude, latency-to-peak, fast spike duration, afterpotential amplitude and afterpotential duration were not significantly different among the three cohorts. Although statistical significance was not observed, the number of FLAS-waves/min at each relative ISO% value was higher in the sleep-deprived (cohort-II) vs. control (cohort-I) conditions. Hence, the trend in the dose-response curve obtained from FLAS-waves/min was similar to the curve that was

obtained from bursts/min in cohort I vs. cohort II. This may indicate that, additional subjects maybe necessary to observe a significant difference in the FLAS-waves/min at such deeper levels of ISO anesthesia following sleep deprivation.

4.6 Future Directions and Limitations

The findings of this thesis suggest a plethora of research outcomes. Several studies have indicated that sleep-restriction enhances cerebral metabolism (Wu et al., 1991; Vyazovskiy et al., 2008), whereas deep isoflurane anesthesia with accompanying EEG burst-suppression activity leads to reduced cerebral metabolism (Ching et al., 2012). Future investigations quantifying the effect of isoflurane on cerebral metabolism in conjunction with increasing ISO% in sleep-deprived vs. control rats *in vivo* would be useful to confirm weather the increased burst event number leads to sleep restriction-induced increased cerebral metabolism. In clinical settings this is important since individuals who may have inadequate sleep the night prior to a major (elective) surgery may differ in the level of anesthetic depth compared to those who had sufficient sleep. Hence, collecting information on patient sleep pattern from the previous night(s) may be beneficial for optimizing the anesthetic concentration accordingly. Sevoflurane and propofol are another group of anesthetics that are often used in conjunction with hypnotics and muscle relaxants during surgery. Future studies with sevoflurane, propofol and other routine drugs that are used in clinical settings would provide new insights into whether these general classes of GABAergic anesthetics have similar effects on burst-suppression activities.

Another beneficial area of investigation would be to determine whether neuropathic pain has any effect on the dose-related curve obtained in this study. Studies can be

conducted by assessing the anesthetic plane as a function of bursts/min in control vs. test conditions where neuropathic pain is induced. The present study showed a dose-response relationship of ISO on burst-suppression events in male rats. Previous studies have shown that women required a lower dose of propofol for the loss of consciousness (Kodaka et al., 2005) and they recover from anesthesia faster than male patients (Gan et al., 1999). Thus, it would be beneficial to study whether there are changes in the pattern of the dose-response curve in the female rats.

The study by Kroeger et al., (2013) suggested that the fast, large-amplitude, spikes (“Nu-complexes”) were generated from the hippocampus at very deep ISO anesthesia (Kroeger et al., 2013). As a classic animal model system of behavioral, neurophysiological, cellular, and molecular studies, rats are ideal for additional study directed at elucidating the underlying mechanisms of this relatively novel and poorly understood phenomenon. In addition, since mice are best suited to study involvement of genes in mammals, studies should be conducted to confirm whether these activities are present in mice as well.

Another venue of fruitful investigation would be to investigate brain functional activity using neuroimaging techniques such as PET and fMRI during the presence of FLAS vs. burst-suppression events under ISO anesthesia. These studies would determine whether cerebral metabolism and cerebral blood flow are altered during very deep anesthesia. Several inhalational, barbiturates, and propofol anesthetics are well known for their neuroprotective effects when administered following cerebral ischemic injury (Kawaguchi et al., 2005). Thus, prolonged anesthesia accompanied by the EEG burst-suppression state vs. FLAS-waves state may prove to be neuroprotective in nature during conditions like cerebral ischemia. Moreover, it is yet to be determined whether the

presence of FLAS-waves in EEG during anesthesia has post-operative cognitive deficits or any other behavioral impairment in young vs. older population. These studies can be conducted using rats as an animal model since they are well suited for behavioral and cognitive studies (Culley et al., 2003; 2004). The findings by Kroeger et al., (2013) indicates that the hippocampus might be the origin of FLAS-waves. This further suggests that FLAS-waves may have potential role in memory. Indeed, marked suppression of long-term-depression was found to occur in the hippocampal slice harvested from the brain of rats exposed to 3 h of ISO when FLAS-waves were present (Tadavarty et al., 2015).

A key limitation of this study is that three different cohorts were used for the dose-response curve obtained from varying concentrations of ISO. As an alternative, the same animals with chronically implanted cortical EEG electrodes can be used as their own control, test and recovery groups. However, chronic implantations have additional caveats associated with unwanted side effects resulting from unsuccessful recovery from surgical implantation processes and long-term health complications. In addition, whereas using the same animals as their own control and test might eliminate some variability among animals, prolonged and repeated exposure of deep ISO anesthesia may also cause cognitive impairment (Culley et al., 2004). To exclude effects related to the age of animals, for this study, we consistently used 400-480g rats that were between 10-12 weeks of age. Thus, it is important to interpret the results of this study within this context, and it may be prudent to determine whether this dose-response curve obtained is similar in younger vs. older animals.

4.7 Significance

The present study is the first known dose-related description of the effect of isoflurane concentration on EEG burst-suppression events in rats. This study provides a basis for performing future *in vivo* neuropharmacological studies keeping the anesthetic plane constant across animals using EEG bursts/min as an indicator. This would minimize inherent error in control vs. test conditions due to variability in the calibration status of the vaporizers that are used to administer anesthetic gas as well as variability from different effects of ISO concentration on functional anesthetic depth in different animals. Moreover, an approximate number of burst-suppression events can be used at the ED₅₀ or ED₉₀ of an anesthetic agent as a useful target to achieve a desired anesthetic plane in *in vivo* neuropharmacology experiments. The increase in the burst number at relative ISO% values in the sleep-deprived rats compared to control and recovered rats provides an insight into how neurophysiological alterations after sleep deprivation affects EEG burst-suppression events during ISO anesthesia. The data in the present study provided a simplified way to monitor the depth of ISO anesthesia using the number of bursts per minute at each relative ISO% values. In the present study, the change in EEG burst-suppression events at 'relative ISO% to slow-wave activity' allowed detection of the true physiological depth of ISO anesthesia rather than relying on the vaporizer dial, which may not provide sufficiently accurate measure of actual anesthetic depth, rather than the nominal anesthetic output. This is an important distinction, since during surgery, functional anesthetic depth, rather than the nominal anesthetic output, determines the level of pain and unconsciousness.

Lastly, we also describe the presence of FLAS - waves in rats and open an avenue to explore the underlying mechanism of these activities and their roles in ISO anesthesia in

a well-established model for pharmacological, behavioral, and electrophysiological studies.

4.8 Summary and Conclusion

Major findings of this study include:

1. EEG slow-wave activity occurred at a relatively low concentration of ISO that fragmented into burst-suppression events as the concentration was increased to relative ISO% 0.5. Further increases in ISO concentrations resulted in an isoelectric period, which was followed by the appearance of fast, large-amplitude, spikes at relative ISO% 2. This trend was observed in all three cohorts.
2. The present study documented the ISO anesthetic plane by quantifying group mean bursts/min at varying ISO concentrations. Following EEG slow-wave activity, the number of bursts/min gradually decreased as the depth of ISO anesthesia was increased. Thus, a dose-dependent effect of burst events was observed. Similarly, the group mean duration of bursts decreased and the suppression duration gradually increased as the ISO concentrations were increased.
3. The fast, large-amplitude sharp waves increased in number in a dose-related manner and reached a maximum as the depth of ISO anesthesia increased up to relative ISO% 3.
4. 9 h of sleep-deprivation resulted in decreased sensory threshold associated with mechanical allodynia and thermal hyperalgesia. Sensory thresholds recovered back to normal following 48 h of recovery period, which provided behavioral confirmation of recovery from sleep-deprivation.

5. The burst events were significantly higher in number at relative ISO% 0.5, 1 and 1.5 values in cohort II (test-sleep-deprived) vs. cohort I (control). Following 48 h of recovery the number of bursts/min reversed back to control level in cohort III. Thus, the dose-response curve obtained in cohort II shifted to the right compared to the cohort III and I.
6. The fast, large-amplitude, spikes were increased in number at relative ISO% 2, 2.5, and 3 values in cohort II as compared to cohort I. However, the number did not alter significantly in cohort II vs. cohort I.
7. The data in the present study demonstrated a complete overview of the EEG activities at the low ISO concentration exhibiting slow-wave activity, followed by the burst-suppression events and finally up to the highest ISO concentration which resulted in fast, large-amplitude, sharp waves.

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