Harnessing neuronal-glial interactions to prevent postanesthetic cognitive deficits

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Abstract

General anesthetics trigger memory deficits that persist long after the anesthetics have been eliminated, by increasing an extrasynaptic $GABA_A$ receptor-mediated tonic inhibitory current in the hippocampus. This increase in tonic current contributes to cognitive deficits associated with postoperative delirium, a debilitating disorder that affects 20% of elderly patients undergoing surgery. The α2-adrenergic receptor (α2R) agonist dexmedetomidine (DEX) reduces postoperative delirium in ICU patients. The mechanisms involved are unknown. Interestingly, DEX modulates astrocyte function to protect neurons in other injury models.

This thesis explores the mechanism of DEX-neuroprotection from anesthetic-induced increase in tonic current, using a cell culture model. My studies show that DEX stimulates astrocytic α2Rs to release neuroprotective factors, possibly including BDNF and EGF, which act on hippocampal neurons to prevent the anesthetic-induced increase in tonic current. These results present a potential mechanism by which DEX reduces anesthetic-induced cognitive deficits and identifies alternative drugs that may treat postoperative delirium.

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List of Contributions

Fariya Mostafa produced all the data and content of this thesis except those listed below. The data presented in Chapter 4, Figure 4.1B, was collected with the help of Dr. Irene Lecker. In addition, the data reported in Chapter 5, Figure 5.2B, was collected with the help of Ms. Kirusanthy Kaneshwaran.

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List of Abbreviations

α2R	α2-adrenergic receptor(s)
α5GABA$_A$R	α5-subunit containing GABA type A receptor(s)
δGABA$_A$R	δ-subunit containing GABA type A receptor(s)
ACM	Astrocyte-conditioned media
ANOVA	One-way analysis of variance
ATP	Adenosine triphosphate
AP5	2-amino-5-phosphonovarelate
BDNF	Brain-derived neurotrophic factor
BIC	Bicuculline
cAMP	Cyclic adenosine monophosphate
CNQX	Cyano-nitroquinoxaline-dione
Ca^{2+}	Calcium ion(s)
CNS	Central nervous system
CSF	Cerebrospinal fluid
Clo	Clonidine
CAM-ICU	Confusion Assessment Method for the Intensive Care Unit
DSM	Diagnostic and Statistical Manual for Mental Disorders
DEX	Dexmedetomidine
DMSO	Dimethyl sulfoxide
Etom	Etomidate
ECF	Extracellular fluid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor tyrosine kinase receptor(s)
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular-signal regulated kinase
GABA	γ-aminobutyric acid
GABA$_A$R	γ-aminobutyric acid type A receptor(s)

$GABA_BR$	γ-aminobutyric acid type B receptor(s)
HB-EGF	Heparin-binding epidermal-like growth factor
H_2O_2	Hydrogen peroxide
ICF	Intracellular fluid
ICU	Intensive care unit
I_1R	Imidazoline 1 receptor(s)
I_2R	Imidazoline 2 receptor(s)
I_3R	Imidazoline 3 receptor(s)
IPSCs	Inhibitory postsynaptic currents
JNK	c-Jun N-terminal kinase
IL-1β	Interleukin 1β
IL-6	Interleukin 6
K^+	Potassium ion(s)
MAO	Monoamine oxidase
mRNA	Messenger ribonucleic acid
PNS	Peripheral nervous system
POCD	Postoperative cognitive dysfunction
PKA	Protein kinase A
PKC	Protein kinase C
PI3K	Phosphatidylinositide 3 kinase
PBS	Phosphate buffer saline
RTK	Receptor tyrosine kinase
RVLM	Rostral ventrolateral medulla
RNA	Ribonucleic acid
TNFα	Tumour necrosis factor α
TrkB	Tropomyosin receptor kinase B
TTX	Tetrodotoxin
Yoh	Yohimbine

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1. Thesis Overview

1.1 General Overview

Postoperative delirium and postoperative cognitive dysfunction (POCD) are common disorders that are associated with poor long-term outcomes including loss of independence, higher mortality, and increased health care costs (Witlox et al., 2010; Steinmetz et al., 2009). The causes of these postoperative disorders are likely multifactorial; however, many studies suggest that general anesthetics are a major contributing factor (Inouye et al., 2014; Mo & Zimmerman, 2013; Zhang et al., 2012). The use of general anesthetics is unavoidable during surgery and unfortunately, no adequate strategies are available to treat postoperative delirium and POCD (Rudolph & Marcantonio, 2011; Leslie & Inouye, 2011). Thus, identifying a treatment that reduces the incidence of postoperative delirium and POCD is of great interest and importance (Sanders et al., 2011).

Dexmedetomidine or DEX is an α2-adrenergic receptor (α2R) agonist that is typically used as a sedative in the intensive care unit (ICU). The use of DEX during surgery has been reported to reduce the incidence of postoperative delirium in elderly and pediatric patients (Su et al., 2016; Reade et al., 2016; Maldonado et al., 2009; Pasin et al 2014; Zu et al., 2015; Kim et al., 2015).

Most clinical studies of the effects of DEX on postoperative delirium are conducted in the elderly (65+ year) since these patients are most vulnerable to developing delirium after surgery (Moller et al., 1998). A particularly important study of elderly patients investigated whether a prophylactic low DEX dose reduced the incidence of postoperative delirium after non-cardiac surgery (Su et al., 2016). In this double-blind, placebo-controlled study, the incidence of postoperative delirium was evaluated twice daily for the first 7 postoperative days. The incidence of postoperative delirium was significantly lower in the DEX-treated patients (32of 350 patients [9%]) than in the placebo-treated patients (79 of 350 patients [23%]). Furthermore, treatment with DEX had no noticeable adverse effects. These results strongly suggest that DEX can safely reduce the incidence of postoperative delirium in elderly patients undergoing non-cardiac surgery (Su et al., 2016). However, the mechanisms of DEX-mediated protection from delirium are unknown.

Interestingly, behavior studies in rodents have shown that pre-treatment with DEX reduces anesthetic-induced impairment in learning and memory in the Morris Water Maze spatial memory task (Qian et al., 2015; Si et al., 2016; Wang et al., 2016). Furthermore, *ex vivo* and *in vitro* studies, using colorimetric detection methods, have shown that DEX reduces anesthetic-induced neuroapoptosis in the hippocampus, cerebral cortex, and thalamus of rodents (Sanders et al., 2009). Little is known about the molecular mechanisms by which DEX protects from anesthetic-induced cognitive deficits and neurodegeneration.

Animal studies in an *in vitro* model of hypoxic-ischemia suggest that DEX targets both neurons and astrocytes (the primary support cells of the brain) to mediate neuroprotection. Furthermore, DEX stimulates the release neurotrophic factors and growth factors which protect neurons from ischemia and inflammation induced cell death (Rodriguez-Gonzalez, 2015; Peng et al., 2008; Yan et al., 2011). Although the source(s) of these factors are unknown, DEX has been shown to increase astrocytic expression of these neurotrophic and growth factors (Degos et al., 2013; Zhang et al., 2013). Taken together, these studies provide initial mechanistic insights into the neuroprotective function of DEX. Further *in vitro* studies are necessary to elucidate the molecular mechanisms of DEX-mediated neuroprotection.

Our lab is interested in understanding the mechanisms underlying DEX neuroprotection from anesthetic-induced cognitive deficits. Preclinical studies performed by us have demonstrated that general anesthetics cause memory deficits that persist for up to a week after the drugs have been eliminated (Zurek et al., 2014; Saab et al., 2010). We showed that general anesthetics trigger a persistent increase in inhibitory current generated by extrasynaptic γ-aminobutyric acid type A receptors ($GABA_ARs$), or tonic inhibitory current, in hippocampal neurons (Zurek et al., 2014). Furthermore, this persistent increase in tonic current causes memory impairments after exposure to an anesthetic (Zurek et al., 2014).

Our preliminary behavior studies in rodents show that co-treatment of DEX with an anesthetic reduces impairments in executive function in the puzzle box assay. Treatment with DEX before exposure to an anesthetic has also been shown to attenuate learning and memory deficits in rodents (Qian et al., 2015). Therefore, we were interested in investigating whether DEX attenuates

postanesthetic cognitive deficits by inhibiting the anesthetic-induced persistent increase in tonic current in hippocampal neurons.

This thesis will discuss my findings regarding the mechanism of DEX neuroprotection from anesthetic-induced tonic current which underlies postanesthetic cognitive deficits.

1.2 Hypothesis and specific aims

1.2.1 Hypothesis

I hypothesized that dexmedetomidine (DEX) acts on α2-adrenergic receptors (α2Rs) in astrocytes and stimulates the release of neuroprotective factor(s) that prevent an anesthetic-induced persistent increase in tonic GABA current in hippocampal neurons.

1.2.2 Specific aims

1. To determine whether dexmedetomidine (DEX) acts on α2-adrenergic receptors (α2Rs) in astrocytes to prevent an anesthetic-induced persistent increase in tonic current in hippocampal neurons.
2. To determine whether DEX stimulates the release of neuroprotective factors from astrocytes to prevent an anesthetic-induced persistent increase in tonic current in hippocampal neurons.

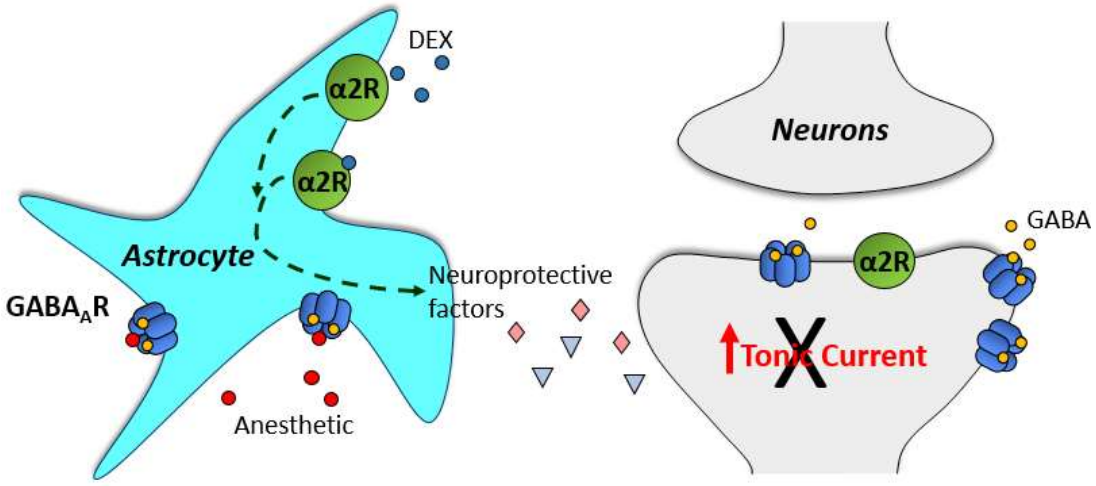


Figure 1.2: Pictorial depiction of the hypothesis. Activation of $GABA_A$Rs in astrocytes by an anesthetic causes persistent increase in tonic inhibitory $GABA_A$R-mediated current in hippocampal neurons. Co-treatment with dexmedetomidine (DEX) stimulates α2-adrenergic receptors (α2Rs) in astrocytes and stimulates the release of neuroprotective factors that prevent the anesthetic-induced persistent increase in tonic inhibitory current in hippocampal neurons.

1.3 Thesis Structure

Chapter 2 presents an overview of background information relevant to the hypotheses tested in this thesis. First, the role of anesthetics in the development of postoperative delirium and postoperative cognitive dysfunction (POCD) is discussed. Second, the evidence for $GABA_A$ receptor ($GABA_AR$)-mediated hippocampal tonic inhibition is presented as a major contributing factor in the development of anesthetic-induced memory deficits. Third, dexmedetomidine (DEX) is introduced as a treatment for postoperative delirium. Next, pertinent preclinical studies of DEX-mediated neurocognitive protection from anesthetics and other injury models are summarized. Finally, pharmacologic properties of DEX and other α2-adrenergic receptor (α2R) agonists are discussed, in order to provide an overview of the pharmacologic manipulations used in testing the hypotheses.

Chapter 3 provides elaborate descriptions of the drugs, materials, and techniques used to conduct the experiments reported in this thesis, in order to ensure reproducibility of the results. For all experiments, a reductionist *in vitro* model was used to study the anesthetic-induced hippocampal tonic current that is responsible for postanesthetic memory deficits. Particularly, the effect of anesthetic-induced tonic GABA current in neurons was measured following pre-treatment with various α2R ligands such as DEX.

Chapter 4 reports the results of the hypotheses tested in Aim 1. Specifically, the first studies show that DEX prevents the persistent increase in tonic current in hippocampal neurons from the anesthetics etomidate and sevoflurane. The next studies demonstrate that DEX action on astrocytes alone is necessary and sufficient to mediate DEX-reversal of tonic current in hippocampal neurons due to the anesthetic etomidate. Furthermore, DEX acts on astrocytic α2R to mediate this effect on hippocampal tonic current, in a dose-dependent manner. The final study of this chapter suggests that DEX neuroprotection from the etomidate-induced persistent increase in tonic current extends to other neurocognitive domains, including the cerebral cortex.

Chapter 5 presents the results of the hypotheses tested in Aim 2. The first studies demonstrate that DEX-treated cultured astrocytes release soluble factors into the astrocyte culture media, which prevents the etomidate-induced persistent increase in tonic current in hippocampal

neurons. Furthermore, heating the conditioned media from DEX-treated astrocyte cultures abolishes DEX-reversal of etomidate-induced tonic current, which suggests that the soluble factors are heat-sensitive proteins or peptides. Previous studies in other models, such as hypoxic-ischemic injury, have shown DEX to reduce neuronal death by stimulating the release of brain-derived neurotrophic factor (BDNF) and epidermal growth factor (EGF) (Rodriguez-Gonzalez, 2015; Zhang et al., 2013). Hence, the next studies in this chapter investigate whether BDNF or EGF contributed to DEX-mediated neuroprotection from anesthetic-induced tonic current. When hippocampal neurons are treated with either BDNF or EGF, etomidate fails to cause persistent increase in tonic current, which demonstrates that both BDNF and EGF mimic DEX effects. Furthermore, pharmacologic inhibition of either the BDNF neuronal TrkB receptor or the EGF tyrosine kinase receptor, prevents DEX-reversal of etomidate-induced persistent increase in tonic current. Collectively, these results suggest that BDNF and EGF may be two of the DEX-stimulated neuroprotective factors that help reverse etomidate-induced tonic current in hippocampal neurons.

Finally, Chapter 6 summarizes the main findings of this thesis and discusses how the data presented here compare to that in the existing literature. In addition, the implications for developing treatment strategies for postoperative delirium and POCD are discussed. Lastly, this chapter proposes future experiments that are necessary to understand the mechanisms of DEX-mediated neuroprotection from postoperative cognitive disorders as well as identify potential alternative treatments.

2. General Introduction

2.1 General anesthetics and postoperative cognitive disorders

Postoperative cognitive disorders refer to impairments in learning, memory, attention, and executive function that occur after surgery and anesthesia (Rudolph & Marcantonio, 2011). Although such disorders can affect patients of any age, they commonly occur in elderly patients who underwent major surgeries, as first described in studies by Bedford, 1955. For patients over 55 years, postoperative cognitive disorders are associated with serious long-term adverse consequences including loss of independence, reduced quality of life, and early death (Moller et al., 1998; Monk & Price, 2011). This thesis will focus on two major categories of postoperative cognitive disorders: postoperative delirium and postoperative cognitive dysfunction (POCD) (Denier & Silverstein, 2009).

2.1.1 Postoperative delirium

The Diagnostic and Statistical Manual of Medical Disorders (DSM), fourth edition, defines delirium as a 'disturbance of consciousness that is accompanied by a change in cognition that cannot be better accounted for by a pre-existing or evolving dementia'. Delirium is characterized by a reduced clarity of awareness of the environment, a fluctuating course of orientation, and an inability to focus, maintain, or shift attention. Hallucinations and inappropriate behaviors may also be observed during an episode of delirium (Inouye et al., 1990). Postoperative delirium has the same diagnosis and symptoms as delirium, except that it occurs in the postoperative period.

2.1.1.1 Patient outcomes

Postoperative delirium occurs in at least 20% of all patients ≥ 65 years who are hospitalized (Inouye et al., 2006). The development of postoperative delirium is strongly associated with loss of independence, an increased risk of morbidity and mortality, and increased healthcare costs (Rudolph & Marcantonio, 2011).

Patients who experience delirium after surgery have high mortality rates during hospital stay (4 - 17%) (Norkiene et al., 2007; Marcantonio et al., 1994). Furthermore, the mortality rates

of such patients remain elevated substantially for days, months, and even years after the incidence (Marcantanio et al., 2000; Robinson et al., 2009; Koster et al., 2009).

In addition, delirium-related complications significantly prolong the duration of hospital stay as well as increase the intensity of care required after surgery (Norkiene et al., 2007). Furthermore, postoperative delirium in elderly patients is associated with significantly higher rates of discharge to nursing homes (Witlox et al., 2010). In the USA alone, the total estimated costs attributable to postoperative delirium range from $38 to $152 billion per year (Leslie et al., 2008).

2.1.1.2 Incidence and risk factors

The incidence of postoperative delirium ranges from 9 – 87%, depending on the patient population, the degree of operative stress, and variations in the method of assessment (Monk & Price, 2011; Robinson & Eiseman, 2008; Rudolph & Marcantonio, 2011).

In older clinical studies, no standardized tests were used to assess the incidence of postoperative delirium (Alcover et al., 2013). However, more recent clinical studies utilize the Confusion Assessment Method for the ICU (CAM-ICU) and the Intensive Care Delirium Screening Checklist (ICDSC) to assess the incidence of postoperative delirium (Mo & Zimmermann, 2013).

Based on these standardized assessments, hip fracture patients have the highest incidence of postoperative delirium (35 – 65%) (Weed et al., 1995). This may be due to the urgent nature of the surgery and high comorbidity burden among hip fracture patients (Alcover et al., 2013). Other surgeries with high incidences of delirium include abdominal surgery (34 – 54%) and coronary artery bypass graft surgery (37 – 52%) (Roach et al., 1996; Maldonado et al., 2003). Overall, surgery alone may lead to postoperative delirium in 5 to 15% of patients (Sanders et al., 2011).

In addition, intra-operative factors are also thought to contribute to the development of delirium in the postoperative period (Rudolph & Marcantonio, 2011). For instance, the use of inhalational anesthetics and hypnotic or sedative agents are strongly associated with increased incidence of postoperative delirium (Xie et al., 2006; Marcantanio et al., 1994). Furthermore, the duration and depth of sedation (bispectral index (BIS) of approximately 50% for deep sedation;

BIS of ≥ 80% for light sedation) has also been shown to aggravate the incidence of postoperative delirium (Sieber et al., 2010; Guenter et al., 2011).

The major, nonoperative risk factor for postoperative delirium is pre-existing cognitive impairment, which has been demonstrated in patients whose cognitive performances were assessed operatively (Jones et al., 2010; Alcover et al., 2013). In addition, a history of psychiatric conditions, especially depression, or deficits in visual or auditory function may exacerbate the development of postoperative delirium (Greene et al., 2009; Smith et al, 2009; Kazmierski et al., 2006; Kazmierski et al., 2010; Rudolph & Marcantanio, 2011).

Additionally, abnormal preoperative laboratory values of plasma contents such as albumin, proinflammatory cytokines, glucose, and potassium are risk factors for postoperative delirium (Marcantonio et al., 1994). Abnormal plasma concentrations of blood chemicals are indicative of underlying severe disease or organ dysfunction, which are predisposing factors for postoperative delirium (Rudolph et al., 2009).

Finally, patients with multiple medical comorbidities are also at an increased risk for developing postoperative delirium. For instance, pre-existing cerebral vascular or neuronal damage, due to stroke or transient ischemia, is associated with postoperative delirium (Rudolph et al., 2009). As well, patients with a long history of alcohol abuse are also more vulnerable to developing delirium after surgery. (Marcantonio et al., 1994).

Taken together, it appears that multiple operation-related and independent risk factors are implicated in the precipitation of postoperative delirium.

2.1.2 Postoperative cognitive dysfunction (POCD)

Postoperative cognitive dysfunction or POCD refers to impairments in various neurocognitive functions including learning, memory, executive function, and attention after surgery (Monk & Price, 2011). POCD is not yet listed as a diagnosis in the Diagnostic and Statistical Manual of Mental Disorders (DSM) (Tsai et al., 2010). However, it is increasingly recognized as a common disorder after surgery, particularly in the elderly (≥ 65 years) population (Williams-Russo et al., 1995; Moller et al., 1998).

2.1.2.1 Patient outcomes

PCOD has long-term adverse consequences including impairments in daily functioning, premature retirement, and dependency on government economic assistance after hospital discharge (Phillips-Bute et al., 2006; Steinmetz et al., 2009). Furthermore, POCD at 3 months after surgery is associated with higher rates of mortality, even after controlling for age, sex, and cancer (Steinmetz et al., 2009).

Patients suffering from POCD are oriented and behave regularly. However, they experience a noticeable decline in cognitive function, ranging from mild memory loss to an inability to focus and concentrate, which significantly reduces their quality of life (Steinmetz et al., 2009; Alcover et al., 2013).

Deficits in learning and memory are particularly prevalent among patients with POCD (Steinmetz et al., 2009; Monk & Price, 2011). However, elderly patients often suffer from serious impairments in executive function that impede daily activities such as cooking, commuting, and managing medications (Steinmetz et al., 2009). As a result, elderly patients who develop POCD are often transferred to nursing homes following hospital discharge (Tsai et al., 2010).

2.1.2.2 Incidence and risk factors

Age is a strong preoperative risk factor for the development of POCD (Monk et al., 2008). According to the first prospective International Study of Postoperative Cognitive Dysfunction (ISPOCD1), 25% of elderly patients (> 60 years) met the criteria for POCD at hospital discharge (Moller et al., 1998). Furthermore, 10% of the elderly patients in the ISPOCD1 study had POCD 3 months after surgery (Moller et al., 1998). The incidence of POCD is expected to increase as the cohort of elderly grows and requires more surgical procedures (Tsai et al., 2010).

Another major risk factor for POCD is the type of surgery. Between 30 – 80% of cardiac surgery patients develop POCD at 1 week after surgery, whereas 10 – 60% of patients develop POCD 3 – 6 months after surgery (Steinmetz et al., 2009). In addition, the incidence of POCD is also high after major, non-cardiac surgeries (Alcover et al., 2013). For instance, 25.8% of major, non-cardiac surgery patients develop POCD 1 week after surgery (Moller et al., 1998).

Furthermore, 9.9% of the elderly non-cardiac surgery patients develop POCD 3 months after surgery (Moller et al., 1998).

As well, patients with preoperative cerebral infarct are at greater risk of developing POCD (Monk & Price, 2011). Pre-surgical MRI studies have indicated that patients with severe white matter lesions due to ischemia or small vessel vascular disease have a higher incidence of PCOD at 3 months after surgery (Lund et al., 2005).

Furthermore, patients with pre-existing cognitive deficits, as suggested by preoperative tests of memory and attention, are more likely to experience significant cognitive decline in the early postoperative period (Rasmussen et al., 2001).

Finally, intraoperative factors, such as proinflammatory cytokines, dehydration, and anesthetic agents are also thought to contribute to POCD (Alcover et al., 2013). For example, postoperative increases in serum proinflammatory cytokines, IL-6 and TNFα were observed in patients who displayed postoperative decline in cognitive performance (Beloosesky et al., 2007). As well, preclinical studies have demonstrated that clinical concentrations of inhalational anesthetics are neurotoxic to the aging brain (Eckenhoff et al., 2004; Qian et al., 2015). However, clinical studies are necessary to determine whether the use of general anesthetics is linked to an increase in the incidence of POCD (Monk & Price 2011).

Overall, multiple surgery-related as well as independent risk factors are associate with development of POCD, just as in postoperative delirium. Interestingly, POCD and postoperative delirium have multiple risk factors in common (e.g. surgery, preoperative cognitive function, and inflammation). This suggests that similar pathophysiologic mechanisms may be involved in the development of these disorders (Alcover et al., 2013).

2.1.3 Common pathophysiologic factors in postoperative delirium and POCD

The etiology of postoperative delirium and POCD are currently unknown (Mo & Zimmerman, 2013). However, at a molecular level, both these disorders culminate in neuronal destruction, impaired regeneration, and abnormal network function in the brain (Rudolph et al., 2008). Particularly, these neurodegeneration and abnormalities in neuronal network function occur

in brain regions responsible for learning, memory, attention, and executive function (Hanning et al., 2005).

Numerous clinical as well as preclinical studies have suggested that postoperative delirium and POCD have multiple, common pathophysiologic factors (Inouye et al., 2014; Abildstrom et al., 2000). This section will elaborate on the 4 factors that are most commonly implicated in the pathogenesis of postoperative delirium and POCD.

2.1.3.1 Genetic predisposition

Apolipoprotein E are plasma lipoproteins that redistribute and mobilize cholesterol, which is necessary for the repair and maintenance of myelin and neuronal membranes in the central nervous system. Genetic mutation in the apolipoprotein E allele, e4 (APO E4) has been shown to exacerbate neuronal injury and predispose patients to postoperative delirium and POCD.

For instance, the presence of one copy of APO E4 allele is significantly associated with a higher incidence of delirium in elderly patients on days 1 and 2 after a major, non-cardiac surgery (Leung et al., 2007). The association between the APO E4 allele and early postoperative delirium remained significant even after controlling for known demographic and clinical factors (Leung et al., 2007).

Furthermore, adult APO E4 carrier patients undergoing coronary artery bypass surgery have been shown to have a significantly higher incidence of early POCD (Lelis et al., 2006). Particularly, patients with a copy of the APO E4 allele had significantly worse performance in the Mini-Mental State Examination (MMSE) test compared to patients with the wild-type allele (Lelis et al., 2006).

2.1.3.2 Low cognitive reserve

Cognitive reserve refers to individual differences in cognitive processes or neural networks, pertaining to task performance, that allow some patients to cope better than others with brain damage (Stern, 2009). Therefore, patients with pre-existing brain damage and low cognitive reserve are more likely to develop postoperative cognitive impairments than patients with similar brain damage but higher cognitive reserve (Monk and Price, 2011).

Particularly, low cognitive reserve is a risk factor for PCOD and delirium in patients with pre-existing psychiatric disorders, preoperative cerebral infarctions, and low educational status (Staz, 1993; Jankowski et al., 2011).

For example, elderly hip-surgery patients with pre-existing psychiatric illness and low preoperative neurocognitive test scores were shown to have a higher predisposition to early postoperative delirium, compared to matched controls (Jankowski et al., 2011). The preoperative neurocognitive tests comprised of sensitive tests, such as verbal memory tasks, which detected subtle differences in cognitive function. However, there were no differences in clinically diagnosed cognitive function in any of the patients in this study. Hence, these results suggest that subtle preoperative impairments in neurocognitive function (low cognitive reserve) occurs in patients with a history of psychiatric illness and this deficit is a risk factor for postoperative delirium (Jankowski et al., 2011).

2.1.3.3 Inflammation

Circulating proinflammatory cytokines are elevated in patients after surgery (Rudolph et al., 2008). These cytokines can alter the integrity of the blood-brain barrier and increase systemic inflammation (Alcover et al., 2013). Numerous studies have suggested that an increase in systemic inflammation results in neuronal death and abnormal synaptic function (Qin et al., 2007; Cunnigham et al., 2009; Cottrell et al., 2012; Rudolph et al., 2008).

In addition, clinical studies have demonstrated that elevated plasma inflammatory cytokine concentrations are associated with a higher incidence of postoperative delirium and POCD (Cunnigham, 2011).

One such study demonstrated that preoperative plasma proinflammatory cytokines were significantly higher in elderly, knee-arthroplasty patients who developed POCD 1 week and 3 months after surgery (Zhu et al., 2016). Furthermore, patients pre-treated with an anti-inflammatory drug had significantly lower plasma proinflammatory cytokines (IL-6, IL-1β, and TNFα) and lower incidence of POCD at 1 week and 3 months after surgery (Zhu et al., 2016).

2.1.3.4 Anesthetic agents

General anesthetics have been shown to be neurotoxic to the developing and aging brain (Hudson & Hemmings, 2011). In neonatal animals, exposure to inhalational anesthetics has serious, long-lasting adverse effects on neurodevelopment, including neuroapoptosis and impaired neurogenesis. In older animals, exposure to general anesthetics lead to neurodegeneration which is characterized by cell death and neuroinflammation. Furthermore, anesthetic-induced neurodegeneration in elderly rodents has been associated with postanesthetic cognitive deficits (Qian et al., 2015).

The use of the anesthetics propofol and midazolam have been strongly associated with a higher incidence of postoperative delirium among patients in the intensive care unit (ICU) (Pasin et al., 2014). Furthermore, certain general anesthetics including propofol have been reported to exacerbate cognitive decline in these patients for up to 3 months after surgery (Royse et al., 2011).

Lastly, the depth of sedation and anesthesia with traditional anesthetic drugs (i.e. benzodiazepines) is strongly associated with a higher incidence of postoperative delirium and memory impairment after surgery (Guenter et al., 2011; Steinmetz et al., 2009). For example, cardiac surgery-patients who received a high-dose sedation with propofol had a significantly higher incidence of postoperative delirium than patients who received a low-dose propofol (Guenter et al., 2011). In addition, elderly, non-cardiac surgery patients who received deeper anesthesia with sevoflurane or propofol had a significantly higher incidence of POCD Steinmetz et al., 2010). Specifically, a significant decline in the speed of processing (assessed using ISPOCD1 neurocognitive test assays) was observed in these patients 1 week after surgery, compared to their performance before surgery (Steinmetz et al., 2010).

This thesis focuses on anesthetic-induced cognitive impairments in the postoperative period. Therefore, studies pertaining to general anesthetic-induced mechanisms of postoperative delirium and POCD will be the primary focus of subsequent sections.

2.1.4 Animal models of anesthetic-induced cognitive impairment

In order to understand the role of anesthesia in causing postoperative delirium and POCD, cognitive performances were studied in animals exposed to general anesthetics.

2.1.4.1 Learning and memory deficits after general anesthesia

A single exposure to an inhalational anesthetic (e.g. isoflurane) has been shown to impair performance in the Morris Water Maze spatial memory task in both adult and elderly mice. Particularly, the deficits in learning and memory function persistent for at least 2 weeks after treatment with the inhalational anesthetic isoflurane (Culley et al., 2004; Si et al., 2016).

The role of anesthetic-induced cognitive impairments has also been studied in neonatal and juvenile animals. In both rodents and non-human primates, exposure to an anesthetic in the early postnatal period leads to significant deficits in learning and memory that last for up to 3 months into adulthood (Brambrink et al., 2010; Slikker et al., 2007; Murphy et al., 2013).

Furthermore, a single exposure to propofol anesthesia *in utero* has been shown to cause learning and memory impairments in the young offspring. Specifically, juvenile rats exposed to propofol *in utero* made significantly more errors in the 8-Arm Radial Maze task, compared with rats that received control treatment (Wang et al., 2016).

Overall, general anesthetics lead to long-term learning and memory impairments in both young and elderly rodents.

2.1.4.2 Apoptotic pathways

Many studies have correlated anesthetic-induced memory deficits with neurotoxicity in rodents and non-human primates of all ages, although neonates are especially vulnerable (Sanders et al., 2009; Valentim et al., 2010; Brambrink et al., 2010).

Anesthetics significantly increase the expression of the apoptosis marker caspase-3 protein (Bekker et al., 2010; Slikker et al., 2007, Brambrink et al., 2010). As well, anesthetic exposure elevates the expressions of cyclin D1 and B-cell lymphoma 2 (Bcl-2) cell death proteins which are

responsible for the re-entry of neurons into cell cycle and consequent apoptosis (Liang et al., 2010; Walker et al, 2010; Bekker et al., 2010). Increased expression of these proteins is mainly responsible for neuroapoptosis in neonatal rodents. In addition, increased caspase activity has been shown to exacerbate neuronal death in adult and senile rodents (Fang et al., 2012; Komita et al., 2013; Istanphanous et al., 2011; Mawhinney et al., 2012; Lin et al., 2011).

Furthermore, anesthetic-induced increase in intracellular calcium concentrations results in caspase activation and subsequent neuroapoptosis. Particularly, some studies have demonstrated that chelation of calcium ions attenuates neuroapoptosis in cultured neurons (Zen et al., 2009; Stover et al., 2004). Other studies have reported that isoflurane anesthesia causes abnormal increases in cytosolic calcium by triggering the inositol-triphosphate (IP_3) pathway via the endoplasmic reticulum. Furthermore, pharmacologic inhibition of IP_3 reduces intracellular calcium and caspase activation, thereby reducing neuroapoptosis (Wei et al., 2008; Inan et al., 2010).

2.1.4.3 Mitochondrial dysfunction

Anesthetics have been shown to activate proapoptotic caspases by compromising the permeability of mitochondrial membranes. Specifically, isoflurane anesthesia increases the permeability of mitochondrial membranes which in turn increases cytochrome-c and triggers the activation of proapoptotic caspase-9 in neonatal rats (Jevtovic-Todorovic et al., 2012; Yon et al., 2005).

Structural abnormalities in mitochondrial membranes and cristae lead to an increase in autophagic vacuoles and lysosomes in the rodent brain which also contribute to neuronal death (Sachnez et al., 2011; Lunardi et al., 2010). As well, anesthetics stimulate the formation of reactive oxygen species (ROS), which further exacerbate neuroapoptosis (Boscolo et al., 2012).

2.1.4.4 Impaired synaptogenesis and neurogenesis

Exposure to anesthetics also hinders synaptogenesis and neurogenesis in the developing rodent brain. Anesthetic impairment of synapse generation is dependent on the animal's age and brain region. For instance, exposure to anesthesia on postnatal day 7 rats results in reduced synapse

formation in the hippocampus and loss of neuroglia, later in life (Lunardi et al., 2010). Whereas, a 30 minute exposure to inhalational anesthetics on postnatal day 16 leads to a reduction in the diameter of apical and dendritic spines in cortical pyramidal neurons (Briner et al., 2010). These anesthetic-induced abnormalities in synaptogenesis could disrupt the formation of functional neural networks in adulthood, thus increasing the likelihood of cognitive dysfunction.

In addition, *in vivo* studies in neonatal rodents show that a single dose of an inhalational anesthetic can significantly reduce neurogenesis in the dentate gyri for several weeks (Zhu et al., 2010; Stratmann et al., 2010). Prolonged reduction in neurogenesis in the dentate gyrus likely contributes to impairments in hippocampus-dependent learning and memory in these rodents in adulthood. Collectively, the studies reported above provide a mechanism for anesthetic-induced neurotoxicity in the developing brain and help explain how anesthetic exposure during childhood can predispose patients to postoperative delirium and POCD later in life.

2.1.4.5 Neurodegeneration in the aging rodent brain

Although the same mechanisms of anesthetic-induced neuroapoptosis are present in adult rodent models, the degree of neuroapoptosis is less severe (Lin & Zuo et al., 2011; Pan et al., 2011; Hofacer et al., 2013). In the adult brain, anesthetics mainly decrease the number of new, maturing, and differentiating neurons (Lin & Zuo et al., 2011). In addition, anesthetic exposure in adulthood activates reactive astrocytes and triggers the release of proinflammatory cytokines which result in neuroinflammation (Erasso et al., 2013). Taken together, neuroapoptosis, neuroinflammation, and disruptions in neurogenesis impair brain regions involved in cognitive function and therefore contribute to anesthetic-induced cognitive impairment in adult rodents.

Another, major factor contributing to anesthetic-induced cognitive deficits is anesthetic potentiation of $GABA_A$ receptor ($GABA_AR$) activity (Zurek et al., 2014; Saab et al., 2010). But before I elaborate on the mechanism of anesthetic action on $GABA_AR$, an overview of GABAergic neurotransmission and $GABA_AR$ function will be provided.

2.2 GABA and $GABA_A$ receptor-mediated inhibition

2.2.1 GABA

γ-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the mammalian central nervous system (CNS) (McCormick., 1989). GABA is a small amino acid which exists as a zwitterion at physiological pH (7.4) (Rowley et al., 2012). The glutamic acid decarboxylase enzyme (GAD) synthesizes GABA by facilitating the removal of the two carboxyl (CO_2) groups from the amino acid glutamate. There are two isoforms of GAD (GAD67 and GAD65) that are encoded by different genes and distributed in distinct regions of the brain (Kaufman et al., 1991). GAD67 and its cofactor pyridoxal phosphate predominantly co-localize in the cytoplasm of GABAergic neurons where it produces GABA that is used for metabolic functions. In contrast, GAD65 is primarily bound to the neuronal-membrane of axon terminals and only 50% of GAD65 is active at a time. The main role of GAD65 is to produce GABA for vesicular neurotransmission at inhibitory synapses (Walls et al., 2010).

The main mechanism of GABA neurotransmission involves vesicular release at presynaptic terminals of GABAergic neurons. Specifically, vesicular GABA transporter (VGAT) is structurally and functionally coupled with GAD65 so that any GABA synthesized by GAD65 is packed into synaptic vesicles by VGAT (Jin et al., 2013). Next, the GABA vesicles dock at the plasma membrane and when an action potential depolarizes the neve terminal and activates voltage-gated calcium channels, these GABA vesicles fuse with the plasma membrane and are released into the synaptic cleft. Typically, synaptic concentrations of GABA, following vesicular release, is between 1.5 to 1.8 mM (Baberis et al, 2004). However, spillover of GABA also occurs in the extracellular space (Lerma et al., 1986). In addition, GABA release also occurs via non-vesicular methods (Yang et al., 2014). In neurons, this form of GABA release involves the activity of a reverse GAT transporter; whereas in astrocytes, all synthesized GABA is released via the bestrophin 1 (Best 1) channels (Lee et al., 2011). These non-vesicular sources of GABA primarily contribute to ambient GABA concentrations in the extracellular space. The ambient concentrations of GABA range from 0.2 to 0.8 μM (Tossman et al., 1986).

Although the ambient concentrations of GABA do not vary without external stimuli, GABA is constantly recycled and metabolized. Recycling of GABA primarily occurs via passive

diffusion and active reuptake by VGAT in neurons and GAT1 to GAT4 in both astrocytes and neurons (Zhou & Danbolt, 2013). Specifically, the driving force of these transporters (VGAT and GAT1 to 4) come from Na^+ moving down its concentration gradient (Scimemi, 2014). In addition, the expression profiles of GAT1 to 4 vary depending on the tissue. GAT1 and 3 are mostly expressed in the brain (Zhou & Danbolt, 2013). Furthermore, GAT1 is predominantly available in presynaptic axon terminals of neurons where it regulates presynaptic homeostasis as well as action potentials that trigger phasic and tonic $GABA_AR$-mediated inhibition in postsynaptic neurons (Melone et al., 2014). In contrast, GAT3 is mostly found at astrocytic processes in extrasynaptic areas where it regulates GABA concentrations in the extracellular space (Conti et al., 2004). Irrespective of the mode of transport and tissue type, all recycled GABA is converted to succinic semialdehyde by the enzyme GABA transaminase (GABA-T). The succinic semialdehyde from GABA catabolism next enters the tricarboxylic acid cycle (TCA) where it is either utilized as a source of metabolic energy or recycled in the synthesis of glutamate and GABA (Rowley et al., 2012).

2.2.2 Overview of GABA receptors

In the CNS, GABA mediates inhibitory neurotransmission by targeting specific receptors in postsynaptic neuronal membranes (Bianchi et al., 2009). There are two main types of GABA receptors: (1) $GABA_B$ receptors which are metabotropic and generate a slow, long-lasting response to GABA; and (2) $GABA_A$ receptors which are ionotropic and generate fast responses to GABA (Olsen & Sieghart, 2009).

2.2.2.1 $GABA_B$ receptors

$GABA_B$ receptors ($GABA_BRs$) are heterodimeric proteins consisting of two subunits, $GABA_{B1}$ and $GABA_{B2}$ (Bettler et al., 2004). Since $GABA_BRs$ are metabotropic receptors, they are coupled to G-proteins, exclusively, the $G_{i/o}$ family subtype. Signalling via G-protein coupled $GABA_BRs$ modulates the activities of ion channels and various enzymes present in the cell. When GABA or another ligand binds to the N-terminal of a $G_{i/o}$-coupled $GABA_BR$, it activates the $G_{i/o}$ heterotrimer which exchanges a GDP for a GTP and dissociates into $G\alpha_{i/o}$ and $G\beta_{i/o}$ subunits (Pinard et al., 2010). The signalling pathways of $G\alpha_{i/o}$ are not well-studied but there is some evidence that $G\alpha_{i/o}$ inhibits adenylyl cyclase and lowers the levels of cyclic adenylyl monophosphate (cAMP) in the

cell (Urwyler et al., 2001). In contrary, the signaling pathways of $G\beta_{i/o}$ are well known and they regulate the activities of adjacent ion channels. Particularly, $G\beta_{i/o}$ inhibits N, P, and Q-type of voltage-gated Ca^{2+} channels and activates G-protein gated inward rectifying K^+ (GIRK) channels. The GIRK channels in turn stimulate K^+ efflux and prevent Ca^{2+} influx which results in hyperpolarization of the neurons involved (Mintz & Bean, 1993). Since this thesis is concerned with the effect of anesthetics on $GABA_AR$-mediated tonic inhibitory current, $GABA_B$ receptors will not be discussed any further.

2.2.2.2 $GABA_A$ receptors

$GABA_A$ receptors ($GABA_ARs$) are ligand-gated ion channels that belong to the cysteine-loop superfamily of receptors, which also include nicotinic receptors (McDonald & Olsen 1994). $GABA_ARs$ can exist as homo or heteropentamer, where the heteropentamer is comprised of 5 subunits with a central pore which is permeable to Cl^- and HCO_3^- ions. All subunits of $GABA_ARs$ contain a hydrophilic extracellular N-terminal domain with the cysteine-loop, four transmembrane α helical regions (M1 to M4), and an intracellular C-terminal domain. $GABA_AR$ ligands bind to the N-terminal domain because it contains five extracellular binding sites at the interface between subunits (Sigel & Steinmann, 2012). Specifically, GABA molecules bind to two of the five binding pockets, at the junction between α and β subunits, whereas allosteric modulators of $GABA_ARs$ bind to other sites in the pentameric N-terminal (Olsen & Sieghart, 2009). In addition, there is a large intracellular loop between the M3 and M4 α helices in the transmembrane domain, which allows for subunit regulation via phosphorylation. Lastly, the M1 and M2 α helices line the channel pore of the receptor (Guan et al., 1996). Although all $GABA_AR$ subunits have these structural similarities, each $GABA_AR$ subunit has distinct pharmacological properties and expression profiles.

2.2.3 Subunit composition of $GABA_A$ receptors

Although all $GABA_A$ receptors ($GABA_ARs$) exist as heteropentamers, their subunit compositions have far more variability. So far, nineteen different subunits of $GABA_ARs$ have been cloned in the mammalian CNS (α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3); however, only some of the multiple possible combinations exist in nature. While some of these receptor subunits are found throughout the brain (α1, β1, β2, β3, and γ2), others are only expressed in certain specific regions of the brain (α2, α3,

α4, α5, α6, γ1, and δ) (Mody & Pearce, 2004; Olsen & Sieghart, 2009). Taken together, the above studies suggest that not all combinations of subunit pentamers are stable and that certain subunit expression patterns are necessary to mediate physiologic functions in distinct brain areas (Farrant & Nusser, 2005). Based on microscopy and immunocytochemical experiments, the most probable stoichiometric arrangement of the $GABA_AR$ pentamers include, two α subunits, two β subunits, and one γ subunit, ordered around the ion channel as γ-β-α-β-α (Farrant & Nusser, 2005). This arrangement ensures that there are two GABA-biding pockets per receptor, one at each of the β-α junctions. Furthermore, the most abundant receptor subtypes consist of α1, β2, and γ2 subunits. However, there are other common subunit assemblies of $GABA_AR$ including: α2β3γ2, α3β3γ2, α5β3γ2, α4βxγ2, and α6βxγ2 (Farrant & Nusser, 2005; Sigel & Steinmann, 2012). In addition, there are receptor assemblies that occur rarely. Examples would include receptors where the γ2 subunit is replaced by γ1, γ3, or δ (Olsen & Sieghart, 2009). Overall, the molecular diversity of $GABA_AR$ subunits directs their pharmacological properties, cell surface expression, and dynamic regulation. Hence, variations in the subunit properties govern diverse physiologic functions via $GABA_AR$-mediated inhibition.

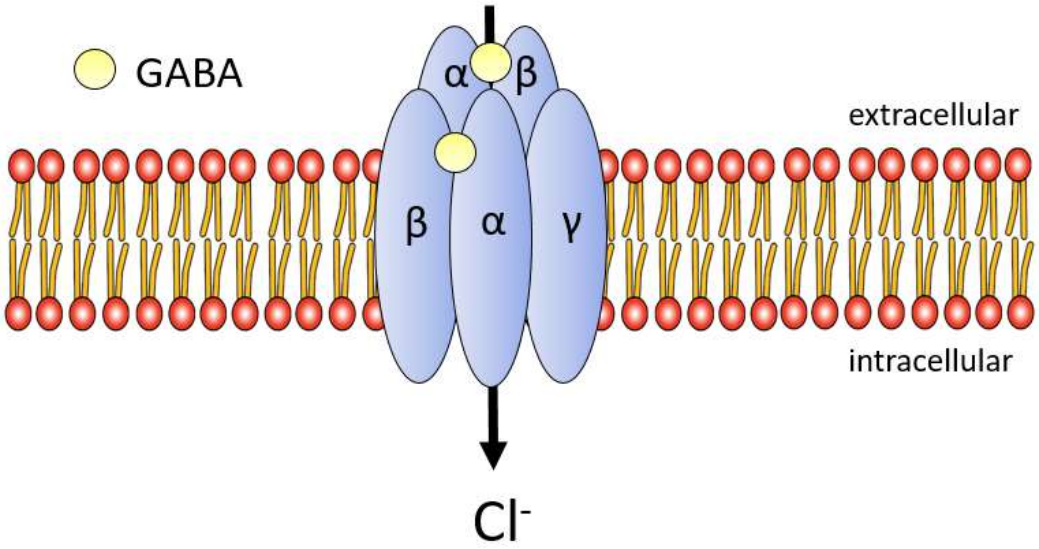


Figure 2.2: The heteropentameric $GABA_A$ receptor ($GABA_AR$). A single ionotropic $GABA_AR$ contains two GABA-biding sites, each located between the αβ junctions. The γ subunit contains the benzodiazepine-binding motif. The $GABA_AR$ allows Cl^- ions to pass through, when the heteropentamer is activated by two GABA molecules. In matured neurons, benzodiazepine-mediated activation of the $GABA_AR$ enhances Cl^- influx in neurons, resulting in hyperpolarization.

2.2.4 $GABA_A$ receptor-mediated current

$GABA_A$ receptors ($GABA_ARs$) and other ligand-gated ion channels generate a current by allowing ions to pass through the channel pore and across the cell membrane. This current can be measured using whole-cell electrophysiology, which is how the properties and functions of $GABA_AR$-mediated current in different brain regions are studied (Hille, 2001). Generally, ions channels can move cations into the cell or anions out of the cell to produce an inward current that increases the membrane potential and depolarizes the cell. Alternately, ion channels can move cations out of the cell or anions into the cell to produce an outward current that decreases the membrane potential and hyperpolarizes the cell. The movement of ions across a single ion channel is described by Ohm's law which states that the current (I) through an ion channel is directly proportional to the membrane potential or voltage (V). So for a particular membrane potential (V), an ion channel with a high conductance (g) will increase the current to a greater degree than an ion channel with low conductance (Hille, 2001). The equation for Ohm's law is given below, where I is the current in amperes, g is the conductance, and V is the voltage in watts:

$$I = gV$$

Furthermore, the direction of current flow (i.e. into or out of the cell membrane) is determined by the electrochemical gradient which provides the driving force for ion movement. In turn, the driving force for a specific ion to move across the membrane is determined by the difference between the cell membrane potential (V_m) and the equilibrium potential for that ion (E) (Hille, 2001). Hence, the driving force can be denoted as $V_m - E$.

The equilibrium potential of an ion depends entirely on the concentrations of that ion on either side of the cell membrane. At the equilibrium potential, the net movement of the ion across the cell membrane is zero. This means that at the equilibrium potential of an ion, $V_m = E$, and so there is no not driving force for the ion (Hille, 2001). Furthermore, the equilibrium potential of an ion such as a chloride ion, can be calculated using the Nernst equation:

$$E_{Cl^-} = \frac{RT}{zF} \ln \frac{[Cl^-]_o}{[Cl^-]_i}$$

In this case, E_{Cl^-} is the equilibrium or reversal potential for Cl^- ion, R is the thermodynamic gas constant, T is the temperature in Kelvin, z is the valence of the ion, which is -1 for Cl^- ion, F is the Faraday's constant (i.e., the amount of charge in coulombs per mole of ion), $[Cl^-]_o$ is the concentration of Cl^- outside the cell, and $[Cl^-]_i$ is the concentration of Cl^- inside the cell.

However, the cell membrane is permeable to many ions so the equilibrium (or reversal) potential, which is calculated by the Goldman-Hodgkin-Katz (GHK) equation, has to account of all the ions involved. Particularly, the mammalian nervous system is most permeable to K^+, Na^+, and Cl^- ions, so the equilibrium potential for a mammalian neuronal membrane is given by the GHK equation:

$$E = \frac{RT}{zF} \ln \left[\frac{P_K[K^+]_o + P_{Na}[Na^+]_o + P_{Cl}[Cl^-]_i}{P_K[K^+]_i + P_{Na}[Na^+]_i + P_{Cl}[Cl^-]_o} \right]$$

For the above equation, P_{ion} is the relative permeability of each ion, and all other symbols are the same as stated before (Hille, 2001).

$GABA_AR$s are permeable to two anions, Cl^- and HCO_3^-. Hence, only Cl^- and HCO_3^- ions contribute to the equilibrium potential for GABAergic neurotransmission (E_{GABA}). At baseline, $GABA_AR$s are 5 times more permeable for Cl^- ions compared to HCO_3^- ions. Therefore, E_{GABA} is significantly closer to the equilibrium potential for Cl^- than that of HCO_3^-. And since the equilibrium Cl^- potential (E_{Cl^-}) is determined by the intracellular chloride concentrations, E_{GABA}, like E_{Cl^-}, is dependent on factors that regulate intracellular Cl^- concentrations (Kandel et al., 2013).

The intracellular Cl^- concentrations are determined by Cl^- transport through the cation-chloride transporters that exist in the cell membranes. In immature neurons, Cl^- transport is governed by the sodium-potassium chloride cotransporter (NKCC1) which transports 1 Na^+, 1 K^+, and 2 Cl^- into the neuron. This leads to a greater concentration of Cl^- inside the cell, compared to outside. Hence, the Cl^- equilibrium potential is positive in immature neurons and as a result, activation of $GABA_AR$ in the immature brain leads to Cl^- efflux from the neuron and subsequent depolarization (Glykis et al., 2014). In contrast, neurons in the mature brain express other chloride transporters besides NKCC1, which results in $GABA_AR$-mediated hyperpolarization. Specifically, matured or maturing neurons express a significantly larger number of potassium-chloride

cotransporter (KCC2) compared to NKCC1 (Kaila et al., 2014). KCC2 transports K^+ and Cl^- ions out of the cell such that the relative concentration of Cl^- inside the cell is lower than outside the cell and the E_{Cl}^- is negative as a result. Therefore, activation of $GABA_A$Rs in mature neurons leads to Cl^- influx into the cell and subsequent hyperpolarization or shunting inhibition, depending on the resting membrane potential (Ben-Ari et al., 2012). Particularly, if the E_{GABA} is lower than the resting membrane potential (e.g., E_{GABA} in hippocampal neurons is between - 65 to - 70 mV), influx of Cl^- through $GABA_A$R will cause hyperpolarization. However, if E_{GABA} is equal to the resting membrane potential or between the resting membrane potential and membrane potential for action potential generation, Cl^- influx will shunt excitatory inputs (Farrant & Nusser, 2005). Specifically, shunting inhibition reduces the depolarizing effect of excitatory neurotransmission by decreasing the input resistance of the cell membrane, thereby reducing the amplitude of postsynaptic currents (Hartmann & Nothwang, 2015).

Interestingly, when $GABA_A$Rs are over-stimulated, Cl^- influx can further exceed Cl^- removal which may lead to temporary accumulation of Cl^- into the cell. This in turn disrupts the Cl^- gradient and the flux of HCO_3^- ions through $GABA_A$Rs becomes predominant. As a result, E_{GABA} shifts towards the more positive $E_{HCO_3}^-$. $GABA_A$R activation under these conditions, leads to an initial hyperpolarization, followed by a depolarization (Isomura et al, 2003). As well, $GABA_A$R activation in mature neurons is only depolarizing under pathological conditions where the function or expression of KCC2 is downregulated (Kaila et al., 2014). This thesis will only focus on $GABA_A$R activation in mature hippocampal and cortical neurons where regular Cl^- transport occurs through KCC2 and therefore $GABA_A$R activation results in inhibitory current.

Lastly, depending on the subunit composition and brain area, $GABA_A$Rs are either localized within the synapse which results in phasic, synaptic inhibitory current; or, $GABA_A$Rs are localized far away from the synapse (extrasynaptic $GABA_A$Rs) and their activation leads to tonic inhibitory current (Khale et al., 2008).

2.2.5 Synaptic $GABA_A$ receptors

Synaptic $GABA_A$ receptors ($GABA_ARs$) mediate phasic inhibition by conducting large, transient inhibitory postsynaptic currents (IPSCs) when GABA from the presynaptic terminal activates these receptors. IPSCs are generated by high concentrations of GABA (0.3 mM to 1 mM) and they last for less than a millisecond since GABA diffuses away from the synapse (Olsen & Sieghart, 2009; Mody & Pearce, 2004). Nonetheless, the magnitude and duration of IPSCs can vary depending on the quantity and subtypes of $GABA_ARs$ activated (Farrant & Nusser, 2005). More specifically, the number of $GABA_ARs$ varies from tens to hundreds of $GABA_ARs$ per synapse (Mody & Pearce, 2004). As well, several combinations of $GABA_AR$ subtypes exist in the postsynaptic region. However, certain subunit combinations such as α1, α2, α3, α6, β2, β3, and γ2 are more prevalent. Furthermore, electron microscopy and immunocytochemistry studies further suggest that γ2 subunits containing α1, α2, or α3 (e.g. $\alpha_1\beta_{2/3}\gamma_2$, $\alpha_2\beta_{2/3}\gamma_2$) are the most common combinations (Farrant & Nusser, 2005).

Besides the subunit composition and quantity of receptors, $GABA_AR$-mediated inhibition also depends on the vesicular mechanisms of GABA release (Mozryzmas et al., 2003). Although only two molecules of GABA are required to activate a $GABA_AR$, each vesicle at the presynaptic terminal contains thousands of GABA molecules (Farrant & Nusser, 2005). Hence, vesicular release can significantly raise synaptic GABA concentrations (from about 0.5μM to 1.8 mM) (Mozryzmas et al., 2003). While initial vesicular release of GABA activates postsynaptic $GABA_ARs$ from their closed to their open state, continued availability of GABA desensitizes the $GABA_AR$ channels. In addition, the subunit composition and phosphorylation state also affects receptor desensitization (Farrant & Nusser, 2005).

The specific biophysical properties of $GABA_ARs$ were determined by measuring two electrophysiological properties: (1) the concentration of GABA that causes a half-maximal response (EC_{50}), i.e. potency for GABA; and (2) the rate and duration of current desensitization in the continued presence of GABA (Farrant & Nusser, 2005). The potency of GABA at a given $GABA_AR$ is mostly dependent on the α subunit composition of the receptor (Farrant & Nusser., 2005). In general, synaptic $GABA_ARs$ have a low affinity for GABA and hence their EC_{50} concentrations are high. For instance, the EC_{50} of GABA for synaptic α1-3 and γ2-containing

$GABA_AR$ is between 120 to 160 μM (Karim et al., 2013). Like potency, the kinetics of $GABA_ARs$, especially their desensitization properties also depend on the specific subunit composition (Lavoie et al., 1997). For example, $GABA_ARs$ containing $\alpha_1\beta_x\gamma_x$ subunits desensitize more rapidly than $GABA_ARs$ containing α5 or α6 (Farrant & Nusser, 2005). Overall, synaptic $GABA_ARs$ require higher concentrations of GABA and rapid but prolonged desensitization compared to extrasynaptic $GABA_ARs$ (Farrant & Nusser, 2005).

2.2.6 Extrasynaptic $GABA_A$ receptors

Extrasynaptic $GABA_A$ receptors ($GABA_ARs$) conduct a baseline inhibitory current, or tonic current, in the central nervous system (Glykys & Mody, 2007). However, the magnitude of tonic current is quite small (0.5 pA to 40 pA) (Lee & Maguire, 2014). As a result, the tonic inhibitory current can only be measured when a $GABA_AR$ antagonist is applied to a neuron held or clamped at a specific membrane potential. $GABA_AR$-mediated tonic current is present in multiple areas of the brain including the hippocampus, cortex (layers I-V), and medulla (Lee et al., 2014). The GABA source for tonic inhibitory function is largely controversial. However, GABA release from spontaneous or action potential induced synaptic release, reverse activity of GABA transporters, and non-vesicular release from astrocytes, are all believed to contribute to ambient GABA levels (Lee et al., 2010; Song et al., 2013).

As mentioned previously, extrasynaptic $GABA_ARs$ have a high affinity for GABA, they are activated by low, ambient concentrations of GABA ranging from 10 nM to 1 μM (Farrant & Nusser, 2005). Hence, their EC_{50} values are much lower than that of synaptic $GABA_ARs$. For instance, the EC_{50} of extrasynaptic $GABA_ARs$ range from nanomolar ($\delta GABA_AR$) to 30 μM ($\alpha5GABA_AR$) (Karim et al., 2013). As well, the activation times for all extrasynaptic $GABA_ARs$ are much longer than that of synaptic $GABA_ARs$ since the extrasynaptic receptors desensitize much more slowly (Bianchi et al., 2002). Therefore, at any given time, extrasynaptic $GABA_ARs$ are likely to produce quantitatively more inhibition compared to synaptic $GABA_ARs$. Furthermore, like synaptic $GABA_ARs$, inhibitory function of extrasynaptic $GABA_ARs$ are also governed by their subunit composition and expression patterns (Lee & Maguire, 2014).

Unlike synaptic $GABA_AR$s, there are only a few possible subunit compositions for extrasynaptic $GABA_AR$s (Glykys & Mody, 2007). These heteropentameric receptors either contain the δ subunit (in association with α4, α6, or α1) instead of the γ subunit; or they contain an α5 subunit paired with β3 and γ2 (Farrant & Nusser, 2005). Amongst these subtypes, δ-subunit containing $GABA_AR$s ($\delta GABA_AR$s) are the most prevalent subtype and are found in multiple brain regions such as the dentate gyrus, cerebellum, and the pyramidal neurons (layers II/III) (Glykys & Mody, 2007). In the brain, the majority of tonic inhibition is mediated by $\alpha_4\delta$-containing $GABA_AR$s, with the exception of the cerebellum (mostly contains $\alpha 6\delta GABA_AR$s) and the dentate gyrus (mostly $\alpha_1\delta GABA_AR$s) (Chandra et al., 2006; Glykys & Mody, 2007). The other major subtype is the α5-subunit containing $GABA_AR$ ($\alpha 5GABA_AR$s) which are mainly responsible for tonic inhibition in the CA1 and CA3 pyramidal neurons of the hippocampus, layer V pyramidal neurons of the cortex, and olfactory cortical neurons (Loerbrich et al., 2006; Farrant & Nusser, 2005). Nearly 75% of the $\alpha 5GABA_AR$s are located extrasynaptically and they exist as the $\alpha_5\beta_3\gamma_2$ pentamer (Loerbrich et al., 2006). Furthermore, tonic inhibition by $\alpha 5GABA_AR$s in the CA1 and CA3 regions modulate memory function in the hippocampus (Caraiscos et al., 2004). Since this thesis is mainly concerned with tonic inhibition in the CA1 and CA3 regions of the hippocampus, $\alpha 5GABA_AR$s will be discussed further in the next section.

2.2.6.1 α5 $GABA_A$ receptors

α5-subunit containing $GABA_A$ receptors ($\alpha 5GABA_AR$s) are almost entirely responsible for tonic inhibition in the CA1 region of the hippocampus even though they comprise less than 5% of all $GABA_AR$s in the brain (Brickley & Mody, 2012). Compared to most $GABA_AR$s, $\alpha 5GABA_AR$s have a higher affinity for GABA and generate slowly desensitizing tonic inhibitory currents (Karim et al., 2013).

Numerous genetic studies in mice have shown that $\alpha 5GABA_AR$-mediated tonic inhibition is involved in hippocampus-dependent learning and memory function (Martin et al., 2010). Briefly, mice lacking $\alpha 5GABA_AR$s (*Gabra5*$^{-/-}$ mice) showed superior performance in various hippocampus-dependent learning and memory tasks (e.g. Morris water maze and trace fear conditioning tasks) compared to wild-type mice (Collinson et al., 2002; Yee et al., 2004; Martin et al., 2010).

In addition, pharmacologic inhibition of α5GABA$_A$Rs improves learning and memory function in wild-type mice in radial arm maze, Morris water maze, and fear conditioning assays (Koh et al., 2013; Martin et al., 2010). Importantly, these pharmacologic studies showed that α5GABA$_A$Rs are involved in both the acquisition and recall of hippocampus-dependent memory, since inhibiting these receptors before learning or during recall improved memory performance in rodents (Atack et al., 2006).

Some studies suggest that α5GABA$_A$Rs are implicated in the pathophysiology of developmental disorders such Down's syndrome and autism as well injury processes, such as inflammation and stroke (Martinez-Cue et al., 2013; Clarkson et al., 2010; Wang et al., 2012). Furthermore, published studies from our lab have demonstrated that α5GABA$_A$Rs play a role in anesthetic-induced cognitive deficits, which will be discussed in detail in the following section (Zurek et al., 2014).

2.3 Postanesthetic cognitive deficits and extrasynaptic GABA$_A$ receptors

Most anesthetics and sedatives mediate their clinical neurodepressive endpoints by increasing the activity of GABA$_A$Rs in the brain and spinal cord. Specifically, acute administration of an anesthetic results in amnesia (loss in memory and cognitive function), sedation (decreased motor activity and arousal), immobility, and hypnosis (unconsciousness) (Rudolph & Antkowaik, 2004). These endpoints are achieved by anesthetic-mediated inhibition in specific regions of the CNS. For example, anesthetic-induced GABA$_A$R activity in the hippocampus contributes to amnesia; whereas anesthetic-induced GABA$_A$R activity in the spinal cord results in immobility (Yamakura et al., 2001; Rudolph & Antkowaik, 2004).

It is widely assumed that the desired neurodepressive endpoints return to baseline once the anesthetic is eliminated from the body (McBain et al., 2015). However, preclinical studies by us and others have shown that while consciousness and motor function recovered soon after cessation of anesthesia, anesthetic-induced amnesia persisted for days after a single exposure to an anesthetic (Price et al., 2008; Monk et al., 2008; Saab et al., 2010; Zurek et al., 2014). We further discovered that anesthetic-induced persistent hyperactivity of extrasynaptic GABA$_A$Rs was responsible for lasting postanesthetic cognitive deficits in rodents (Zurek et al., 2014). The following section will

elaborate on the studies that demonstrate the role of extrasynaptic $GABA_ARs$ in the pathophysiology of cognitive deficits after anesthesia.

2.3.1 Extrasynaptic $\alpha5GABA_A$ receptors contribute to postanesthetic cognitive impairment

Since anesthetics target $GABA_ARs$ to mediate their clinical actions, we were interested in studying whether anesthetic action on $GABA_ARs$ also contributes to postanesthetic cognitive impairment. Our first studies explored the role of anesthetic action on cognitive performance in rodents. Specifically, adult mice were trained on the fear-conditioning task either 1 or 24 h after a single dose of the anesthetic isoflurane (Saab et al., 2010). Next, their short-term memory and long-term memory performances were assessed 30 minutes and 48 h after training, respectively. While both short-term and long-term memories were impaired 1 h after isoflurane treatment, only short-term memory showed significant impairment 24 h later. These data demonstrated that a single exposure to an anesthetic results in memory deficits. Interestingly, postanesthetic memory impairments in the fear-conditioning task were abolished when mice were pretreated with L-655,708, a specific inhibitor of α5-subunit containing $GABA_ARs$ or $\alpha5GABA_ARs$ (Saab et al., 2010). These results strongly implicated that anesthetic action on $\alpha5GABA_ARs$ played a role in postanesthetic cognitive deficits. Overall, these studies demonstrated that a single exposure to an anesthetic caused memory deficits, which could be prevented by pharmacologic blockade of $\alpha5GABA_ARs$.

Hence, we next asked whether the anesthetic effects on memory function were long-lasting. Particularly, adult mice were trained in the novel object recognition task either 24 h, 72 h, or 1 week after exposure to a sedative dose of the anesthetic etomidate (Zurek et al., 2014). The memory performances of the mice in the novel object task were tested 24 h after training. Surprisingly, a single sedative dose of etomidate resulted in memory loss for up to a week after exposure, suggesting that anesthetics cause long-term cognitive impairments.

The above studies stimulated us to investigate the mechanisms of anesthetic-induced memory deficits. Since memory function is dependent on synaptic plasticity in the hippocampus, we first studied whether anesthetics disrupted synaptic plasticity long after their elimination from the body. We observed that a sedative dose of the anesthetic etomidate *in vivo* significantly reduced

synaptic plasticity in the hippocampus for up to a week (Zurek et al., 2014). Furthermore, the same *in vivo* etomidate treatment (8 mg/kg) caused a persistent increase in extrasynaptic $GABA_AR$-mediated tonic current which also lasted for a week. However, there were no changes in postsynaptic $GABA_AR$-mediated current. Taken together, these results showed that anesthetics cause long-term changes in tonic (extrasynaptic $GABA_AR$-mediated) current in the hippocampus (Zurek et al., 2014). Therefore, the next outstanding question was whether this anesthetic-induced persistent increase in tonic current contributed to postanesthetic memory impairments.

To answer this question, we first explored the mechanism of anesthetic-induced persistent increase in tonic current in the hippocampus. Since extrasynaptic $\alpha5GABA_ARs$ are mainly responsible for tonic current in hippocampal regions involved in synaptic plasticity, we first studied the role of $\alpha5GABA_ARs$ on anesthetic-induced persistent tonic current. Specifically, mice were again injected with the sedative dose (8 mg/kg) of etomidate and hippocampal tonic current was measured from slices 24 h later (Zurek et al., 2014). But for some groups in this tonic current experiment, null mutant *Gabra5*$^{-/-}$ mice, lacking $\alpha5GABA_ARs$ were used. And we observed that while etomidate enhanced tonic current in wild-type mice, it failed to increase tonic current in the mice lacking $\alpha5GABA_ARs$ (Zurek et al., 2014). These results, demonstrated that etomidate targets $\alpha5GABA_ARs$ to cause persistent increase in tonic current in the hippocampus.

Hence, we next studied the mechanism by which etomidate causes long-term increase $\alpha5GABA_AR$-mediated tonic current in the hippocampus. The fact that a single etomidate treatment causes long-lasting increase in $\alpha5GABA_AR$-mediated tonic current suggested that etomidate increased the expression but not activity of $\alpha5GABA_ARs$. Therefore, we used a cell-surface biotinylation assay to probe whether etomidate treatment increased expression of $\alpha5GABA_ARs$ in mice hippocampal slices (Zurek et al., 2014). We observed that etomidate increased cell-surface expression of $\alpha5GABA_ARs$ for up to a week after treatment. However, etomidate did not increase the total protein expression of $\alpha5GABA_ARs$ in the hippocampus. Furthermore, the same cell-surface expression patterns were observed when mice were treated with the anesthetic isoflurane instead of etomidate (Zurek et al., 2014). Taken together, these results show that general anesthetics cause a persistent increase in hippocampal tonic current by increasing the cell-surface expression $\alpha5GABA_ARs$.

Interestingly, anesthetic-induced increase in cell-surface expression of α5GABA$_A$Rs lasts for a week, which is the same time-frame for which anesthetic-induced memory deficits are observed. This further suggested that α5GABA$_A$Rs play a critical role in anesthetic-induced memory deficits. Hence, we investigated whether inhibiting α5GABA$_A$R activity prevents anesthetic-induced memory deficits in adult mice. First, we pharmacologically inhibited α5GABA$_A$R activity and studied memory function after anesthesia. Specifically, wild-type adult mice were either treated with etomidate or vehicle controls (Zurek et al., 2014). Next, the mice were either treated with L655, 708 (α5GABA$_A$R inhibitor) or controls and trained on the novel object recognition assay. Finally, the memory performances of these mice were tested on the novel object recognition task 24 h, 72 h, and 1 week after exposure to etomidate. As before, mice treated with etomidate alone showed memory impairments for up to a week, whereas treatment with L655, 708 prevented etomidate-induced memory deficits (Zurek et al., 2014). These results suggested that anesthetic-induced α5GABA$_A$R hyperactivity caused postanesthetic memory deficits.

To confirm these observations, we investigated whether mice that were null mutant for α5GABA$_A$Rs (*Gabra5*$^{-/-}$) were immune to anesthetic-induced memory deficits. Particularly, *Gabra5*$^{-/-}$ mice were treated with a sedative dose of etomidate or vehicle control (Zurek et al., 2014). These mice were next trained for novel object recognition tasks 24 h, 72 h, and 1 week after etomidate exposure. The etomidate-treated *Gabra5*$^{-/-}$ mice performed as well as the control mice in the novel object recognition task, demonstrating that etomidate fails to cause memory impairment in mice lacking α5GABA$_A$Rs (Zurek et al., 2014). Altogether, these behavioral studies suggest that anesthetic-induced persistent increase in α5GABA$_A$R activity (and expression) leads to postanesthetic memory deficits.

Collectively, the studies described in this section provide strong evidence that anesthetic-induced hippocampal tonic current is central to the pathogenesis of postanesthetic memory deficits. While these behavior studies provided useful mechanistic insights, *in vitro* studies were necessary to probe the cellular and molecular pathways implicated in postanesthetic memory deficits. Hence, we performed studies using cell cultures to better understand the underlying molecular mechanisms, as discussed in the next section.

2.3.2 Role of astrocytes in anesthetic-induced hippocampal tonic current

To understand the molecular mechanisms of postanesthetic cognitive deficits, we studied anesthetic-induced tonic current in hippocampal neuron cultures.

We first investigated whether treatment with the anesthetic etomidate persistently increased tonic current in hippocampal neurons, just as in hippocampal slices. Surprisingly, treating pure hippocampal neurons with etomidate (1 h treatment) failed to increase tonic current in these neurons, 24 h later (Zurek et al., 2014). Although these results contradicted our *in vivo* observations, they suggested that etomidate targets non-neuronal (i.e. glial) cells in the hippocampus to increase neuronal tonic current.

There are two types of non-neuronal or glial cells: (1) microglial cells, which provide immunity to the surrounding neurons (Aloisi, 2001); and (2) astrocyte cells, which provide nourishment as well as regulate functions of nearby neurons (Sofroniew, 2005). Hence, we next explored whether anesthetics act on microglia or astrocytes to persistently increase tonic current in hippocampal neurons.

To study whether anesthetics act on microglia, microglia- hippocampal neuron cocultures were treated with etomidate for 1 h (Zurek et al., 2014). Tonic current from neurons in the microglia-neuron cocultures was measured 24 h later. As in pure neuron cultures, etomidate failed to increase neuronal tonic current in microglia-neuron cocultures. This suggested that etomidate did not target microglia to persistently increase tonic current in neurons.

We next studied whether etomidate increases neuronal tonic current in astrocyte-neuron cocultures. In this case, astrocyte and hippocampal neuron cocultures were treated with etomidate for 1 h and neuronal tonic current was measured 24 h later (Zurek et al., 2014). We discovered that unlike in pure neuron cultures or microglia-neuron cocultures, etomidate treatment persistent increased tonic current in the neurons in astrocyte-neuron cocultures. These results demonstrated that etomidate targets astrocytes to persistently increase tonic current in hippocampal neurons (Zurek et al., 2014).

Collectively, the above studies suggest that astrocytes are necessary for anesthetics to persistently increase tonic current in neurons. Therefore, we next investigated whether anesthetic action on astrocytes alone is sufficient to increase neuronal tonic current in the hippocampus. To address this *in vitro*, pure astrocyte cultures were treated with etomidate for 1 hour. And 2 h after incubation, the etomidate-treated astrocyte conditioned media (ACM) was transferred to pure hippocampal neuron cultures. Finally, tonic current from these neuron cultures were recorded 24 h later (Zurek et al., 2014). Interestingly, neurons treated with conditioned media from etomidate-treated astrocytes had persistent increase in tonic current, just like neurons in astrocyte-neuron cocultures (Zurek et al., 2014). Altogether, these results showed that astrocytes are both necessary and sufficient for etomidate to persistently increase tonic current in hippocampal neurons.

Since astrocytes are central to anesthetic-induced persistent increase in hippocampal tonic current, our next objective was to investigate the mechanism of anesthetic action on astrocytes. Astrocytes are known to express functional $GABA_AR$s (Araque et al., 2014). Furthermore, our unpublished studies show that application of the $GABA_AR$ antagonist, bicuculline, inhibits anesthetic-induced $GABA_AR$-current in astrocyte cultures and slices. This suggested that GABAergic anesthetics (i.e. most general anesthetics) target $GABA_AR$s in astrocytes to mediate their actions.

Particularly, activation of $GABA_AR$s in astrocytes depolarizes the astrocyte membrane, resulting in intracellular calcium influx (Angulo et al., 2008). This calcium influx triggers vesicular release of signaling molecules such as neurotransmitters, peptides, and proinflammatory cytokines from astrocytes (Araque et al., 2014). Furthermore, astrocytic release of signaling proteins, such as proinflammatory cytokines, has been shown to stimulate an increase in neuronal cell-surface expression of $GABA_AR$s (Perea et al., 2009). These studies suggest that anesthetic activation of astrocytic $GABA_AR$s may stimulate the release of signaling proteins which target neurons and cause persistent increase in neuronal tonic current. Indeed, our observation that anesthetic-treated ACM elicits a persistent increase in neuronal tonic current, suggests that anesthetic-triggered astrocytes release soluble factors into the medium. These evidence prompted us to study whether anesthetic-triggered astrocytic release of soluble factors is responsible for the increase in tonic current in hippocampal neurons.

To study the role of astrocytic soluble factors, pure astrocyte cultures were treated with etomidate as before (unpublished studies). But this time, the etomidate-treated ACM was heated to 99.9°C for 5 minutes before transferring to pure neuron cultures. The heat treatment was expected to denature signaling proteins which are suspected to increase the tonic current in neurons. Finally, tonic current from these neuron cultures were recorded 24 h later. As predicted, the heat and etomidate-treated ACM failed to increase tonic current, whereas the non-heated etomidate-ACM caused persistent increase in neuronal tonic current. These results demonstrated that etomidate triggers astrocytes to release heat-sensitive soluble factors which increase the tonic current in neurons. The fact that heat prevented the effects of the soluble factors indicated that these factors were signaling proteins.

Future studies will investigate the identities of the anesthetic-triggered soluble factors released by astrocytes. Past studies have reported that anesthetics enhance the release of proinflammatory cytokines such as interleukin 1β (IL-1β) in the hippocampus (Zhang et al., 2010). However, the source of IL-1β and other proinflammatory cytokines have not been identified (Zhang et al., 2010). Furthermore, our previous studies have shown that IL-1β increases the cell-surface expression of α5GABA$_A$Rs as well as tonic inhibition by α5GABA$_A$Rs in the hippocampus (Wang et al., 2012). Taken together, these studies suggest that anesthetic-triggered astrocytes may release proinflammatory cytokines like IL-1β, which increase α5GABA$_A$R-mediated tonic current in neurons.

Overall, these studies demonstrate that anesthetic action on astrocytic GABA$_A$Rs triggers the release of soluble factors which cause a persistent increase in tonic current in hippocampal neurons. Since this persistent increase in tonic current causes postanesthetic memory deficits, astrocytes likely play a key role in GABAergic anesthetic-induced cognitive deficits.

Unfortunately, most commonly used general anesthetics are GABAergic agents, i.e. they target GABA$_A$Rs to mediate their actions (Ben-Ari, 2012; Mo & Zimmermann, 2013). Furthermore, the use of GABAergic general anesthetics is strongly associated with higher incidences of postoperative cognitive disorders, as discussed previously in section 2.1.2. Since general anesthetics must be administered during surgery, an effective treatment is necessary to attenuate the development of postoperative cognitive disorders.

2.4 Potential treatment for postoperative cognitive disorders

There are no adequate treatments for postoperative cognitive disorders such as POCD and postoperative delirium (Silverstein et al., 2007). As discussed earlier, similar pathophysiologic factors are involved in the development of POCD and postoperative delirium (Rudolph & Marcantonio, 2011). Furthermore, postoperative delirium itself is a predisposing factor for POCD later in life (Krenk et al., 2010). Therefore, similar treatment strategies have been implemented to curb the development of POCD and postoperative delirium (Alcover et al., 2013).

POCD often has mild visible symptoms and surfaces weeks to months after surgery (Monk & Price, 2011). Therefore in many cases, no diagnoses or treatments for POCD are provided during the postoperative period. Nonetheless, certain inhalational anesthetics like desflurane and sevoflurane have been recommended for use over injectable anesthetics like propofol, to reduce the incidence and symptoms of POCD (Mandal et al., 2009). However, most common general anesthetics are GABAergic agents and therefore, they have been associated with POCD at varying rates (van Dijk et al., 2007; Mandal et al., 2009).

Acetylcholinesterase inhibitors are another treatment for postoperative cognitive deficits, as seen in POCD patients (Overshott et al., 2008; Rudolph & Marcantonio, 2011). Specifically, elective orthopedic surgery patients who received an acetylcholinesterase inhibitor had significantly better scores in cognitive performance tests compared to patients who did not receive the inhibitor. However, acetylcholinesterase inhibitors were not effective in improving postoperative cognitive outcomes in patients who underwent other forms of surgery (Sampson et al., 2007; Liptzin et al., 2005). Therefore, these inhibitors are also not an effective treatment for the POCD population at large.

Additionally, acetylcholinesterase inhibitors have also been explored as a treatment for postoperative delirium. A randomized, double-blind, placebo-controlled trial showed that the acetylcholinesterase inhibitor, donepezil, modestly reduced the incidence of postoperative delirium (Smapson et al., 2007). However, another similar clinical study with donepezil showed no difference in the incidence of postoperative delirium compared to the control group (Liptzin et al., 2005). Furthermore, a recent, randomized, double-blind clinical study on postoperative

delirium demonstrated that using the acetylcholinesterase inhibitor rivastigmine is associated with higher mortality rates (van Eijk et al., 2010). Future studies on acetylcholinesterase inhibitors as treatment for postoperative delirium have been discouraged by their lack of benefit and potential effect on mortality rates (Rudolph & Marcantonio, 2011).

To date, the most popular pharmacological intervention for postoperative delirium has been the typical antipsychotic haloperidol (Rudolph & Marcantonio, 2011). While treatment with haloperidol reduces the severity and duration of postoperative delirium, it does not reduce the incidence of postoperative delirium (Slor et al., 2011). Some studies suggest that haloperidol and other typical antipsychotics only attenuate the hyperactive symptoms of delirium (Rudolph & Marcantonio, 2011). Furthermore, the antipsychotics do not abolish the cognitive impairment and other latent symptoms associated with delirium (Marcantonio et al., 2011). In addition, the use of antipsychotics for treating delirium has been associated with a higher incidence of arrhythmia in patients (Battaglia, 2005). Therefore care must be taken before prescribing these drugs for cardiac patients (Pandharipande et al., 2007). Overall, antipsychotics such as haloperidol are effective in reducing symptoms of postoperative delirium but they do not treat delirium and may even exacerbate adverse heart conditions.

In contrast to the other treatments, the sedative dexmedetomidine (DEX) significantly reduces the incidence of postoperative delirium (Rudolph & Marcantonio, 2011). Furthermore, DEX reduces the duration of hospital stay, hospitalization costs, and does not seem to cause adverse side effects (Rikker et al., 2009; Pandharipande et al., 2007). Many studies suggest that sedation with DEX is associated with a significantly lower incidence of postoperative delirium, compared to traditional comparators or placebo (Pasin et al., 2014). The following paragraphs will discuss key studies that support the role of DEX in treating postoperative delirium.

One of the first successful studies suggesting the role of DEX in treating postoperative delirium was conducted in patients undergoing cardiac valve surgery. For all study patients (20 – 89 years), cardiac surgery was performed using standard anesthesia protocol after which, patients were randomized to one of three postoperative sedation protocols (Maldonado et al., 2009). Patients received postoperative sedation with either DEX, propofol, or midazolam. The study showed that the incidence of postoperative delirium was 3% in patients randomized to the DEX

group. However, the incidence of postoperative delirium was 50% in patients randomized to either the propofol or midazolam group (Maldonado et al., 2009). These results strongly suggest that sedation with DEX has a much better outcome for postoperative delirium after cardiac surgery, compared to traditional sedatives like midazolam and propofol.

Another important study explored the effects of DEX on the incidence, onset, and duration of postoperative delirium in cardiac surgery patients. A single-blinded, randomized controlled trial was conducted in patients over 60 years who were undergoing cardiac surgery (Djaiani et al., 2016). Any patients with a history of mental disorder, severe delirium or dementia were excluded from the study. Upon admission to the ICU, study patients were either treated with propofol or DEX sedation. The Confusion assessment method for the ICU (CAM-ICU) was used to assess delirium after discharge from ICU, at 12 h intervals, during the first 5 postoperative days. The CAM-ICU assessment showed that nearly twice as many patients in the propofol group (31.5%) suffered from delirium, compared to that in the DEX group (17.5%). Furthermore, the onset and duration of postoperative delirium was significantly higher in the propofol group compared to the DEX group (Djaiani et al., 2016). Overall, DEX treatment results in a lower incidence, later onset, and shorter duration of postoperative delirium in elderly cardiac surgery patients, compared to propofol treatment.

Furthermore, an international study reported the efficacy of DEX treatment on postoperative delirium in critically ill ICU patients (median age: 57 years) (Reade et al, 2016). In this case, DEX was added to the standard perioperative care in patients who were mechanically ventilated due to the severity of agitation and delirium. Either DEX (at physician-prescribed dose) or placebo was administered until no longer required or up to 7 days after treatment with these drugs. Compared to standard care, addition of DEX to standard perioperative care significantly accelerated recovery and reduced the time course for delirium, as measured by ventilator-free hours (Reade et al., 2016). Altogether, these results show that treatment with DEX is effective in reducing the duration of agitation and delirium in critically ill ICU patients.

Finally, a large-scale clinical investigation was conducted to observe the effectiveness of DEX treatment in elderly (≥ 65 years), non-cardiac surgery patients, residing in the ICU (Su et al., 2016). The study was a randomized, double-blind, placebo-controlled trial with 350 patients in the

DEX group and another 350 patients in the placebo group. The patients either received a low dose (0.1 μg/kg/h) of DEX or placebo (saline) on the day of surgery until 8 hours after postoperative day 1. Postoperative delirium was assessed using CAM-ICU, twice daily for 7 days post-surgery. The incidence of postoperative delirium was noticeably lower in patients who received DEX treatment (9%), compared to placebo treatment (23%) (Su et al., 2016). These promising results demonstrate that a low dose DEX treatment effectively reduces the incidence of postoperative delirium in ICU patients, who are most vulnerable to developing delirium. Overall, the studies discussed so far strongly indicate that DEX significantly reduces postoperative delirium in the elderly and adult patient populations.

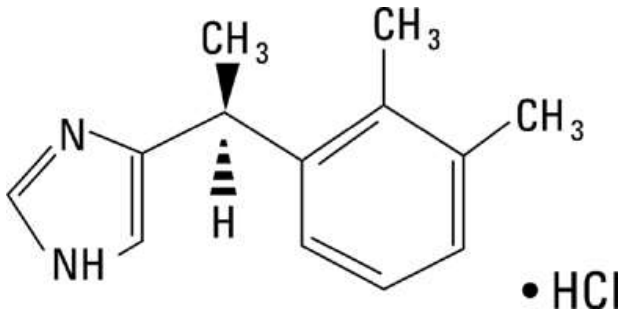
In addition to reducing postoperative delirium in adults, treatment with DEX alleviates the development of delirium in pediatric patients (Ibacache et al, 2004). Many separate studies have reported that compared to placebo treatment, sedation with DEX significantly reduces the incidence of delirium and agitation in pediatric patients in the ICU (Skukry et al., 2005; Hanafy et al., 2004; Mukhtar et al., 2006; Tobias & Berkenbosch, 2002; Tobias, 2007). Although ICU delirium resolves shortly in pediatric patients with no existing psychiatric conditions, an episode of delirium in childhood predisposes patients to future postoperative cognitive disorders (Tobias, 2007). Hence, these studies suggest that treatment with DEX helps reduce predisposition to postoperative cognitive disorders such as delirium and POCD, later in life.

Collectively, these clinical studies suggest DEX as a potential treatment for postoperative cognitive disorders for patients of all ages. Future preclinical and clinical studies are necessary to determine the effectiveness of DEX as a treatment strategy. Furthermore, the mechanisms of DEX-mediated protection from postoperative cognitive disorders are unknown. Since this thesis explores the molecular mechanisms of DEX protection after anesthesia, the properties DEX that help explain its mechanisms of action will be discussed next.

2.5 Dexmedetomidine

2.5.1 Structure

Dexmedetomidine or DEX is the dextrorotatory S-enantiomer of medetomidine (Yoo et al., 2015). The common trade name of DEX is Precedex and it is normally sold as dexmedetomidine hydrochloride powder (Yoo et al., 2015). DEX hydrochloride has a molecular weight of 236.74 g/mol and it readily dissolves in water (20 mg/mL) to give a clear, colorless solution (Rezende et al, 2015). As shown below, DEX hydrochloride is chemically expressed as 4[(S)-α,2,3-Trimethylbenzyl]imidazole monohydrochloride and has an empirical formula of $C_{13}H_{16}N_2$.HCl (Cortinez et al., 2015).



Dexmedetomidine hydrochloride

2.5.2 Overview of dexmedetomidine receptor targets

2.5.2.1 α1-adrenergic receptors

α1-adrenergic receptors (α1Rs) are G protein-coupled receptors that associate with the G_q heterotrimeric G-protein (Piascik & Perez, 2001). There are three homologous subtypes of α1Rs, $\alpha1_A$, $\alpha1_B$, and $\alpha1_D$ receptor subtypes (Minneman, 1988). Interaction of these receptors with catecholamines (e.g. norepinephrine) and exogenous α1R ligands (e.g. dexmedetomidine) triggers various physiologic responses in the central nervous system (CNS) and peripheral nervous system (PNS) (Piascik & Perez, 2001).

In the PNS, α1Rs are found in the smooth muscles of blood vessels of multiple organs where their primary function is to mediate vasoconstriction (Schmitz et al., 1981). Particularly,

α1Rs are present in blood vessels in the skin, kidneys, and sphincters of the gastrointestinal system (Schmitz et al., 1981). Hence, activation of the sympathetic nervous system results in decreased blood flow to these organs. This increases blood, oxygen, and nutrient supply to organs under exertion, such as the skeletal muscles during exercise (Piascik & Perez, 2001). α1Rs are also found in the smooth muscles of organs themselves. For example, α1Rs in the ureter regulate the passage of urine from the kidneys to the bladder, whereas α1Rs in the uterus modulate uterine contractions in pregnant women (Li et al., 2003).

In the CNS, α1Rs are located on postsynaptic neurons where they regulate the release of neurotransmitters (Piascik & Perez, 2001). Responses mediated by the α1Rs in the brain are usually stimulatory in nature. For instance, the α1Rs enhance the release of glutamate from layer V pyramidal terminals in the prefrontal cortex (Marken & Aghajanian, 1991). Besides increasing neurotransmitter release, α1Rs also potentiate the responses of excitatory neurotransmitters (e.g. glutamate and acetylcholine) in somatosensory areas of the brain (Mouradian et al, 1991). Furthermore, stimulation of α1Rs enhances excitability in subcortical brain regions including the reticular thalamic nucleus, geniculate nuclei, and spinal motor neurons (Piascik & Perez, 2001). Finally, activation of α1Rs in astrocyte (non-neuronal) cells in the hippocampus, increases calcium transients and possibly downstream signaling cascades (Kulik et al., 1999).

2.5.2.2 α2-adrenergic receptors

α2-adrenergic receptors (α2Rs) are G-protein coupled receptors that associate with the G_i heterotrimeric G-protein (Ruuskanen et al., 2002). Depending on the species, there are three or four different subtypes of α2Rs: $\alpha2_A$, $\alpha2_B$, $\alpha2_C$, and $\alpha2_D$. (Ruuskanen et al., 2002). Interaction of catecholamines or exogenous ligands (e.g. dexmedetomidine) with α2Rs regulate various autonomic processes in both the PNS and the CNS (Saunders et al., 1999).

One of the major functions of α2Rs is the regulation of neurotransmitters in the PNS and the CNS (Philip et al., 2002). The α2Rs in presynaptic terminals negatively regulate the release of their own neurotransmitters, particularly norepinephrine from presynaptic neurons (Phillip et al., 2002). Inhibition of norepinephrine supresses sympathetic activity in the brain and peripheral organs such as the heart. In addition, these receptors also inhibit the release of other

neurotransmitters in various organs. For instance, α2Rs in gastrointestinal motor neurons inhibit the release of acetylcholine, thereby inhibiting intestinal motility (Scheibner et al., 2002). Whereas, α2Rs in the basal ganglia inhibit dopamine release, modulating locomotion (Buchecler et al., 2002).

Another well-known function of α2Rs is in the regulation of blood pressure homeostasis in vascular smooth muscles, veins, and coronary arteries (Link et al, 1996; Phillip et al., 2002). Non-subtype-specific stimulation of α2Rs results in a biphasic response in blood pressure: First, there is a short period of vasoconstriction which is believed to be mediated by the $\alpha 2_B$ receptor subtype (Link et al., 1996). This is followed by a more prolonged period of hypotension (Phillip et al., 2002). The hypotension is a result of $\alpha 2_A$ receptor ($\alpha 2_A$R)-mediated inhibition of sympathetic activity as well as suppression of norepinephrine release from sympathetic nerves (MacMillan et al., 1996). Due to this $\alpha 2_A$R-mediated effect, α2R agonists have been used as antihypertensive medications in both humans and animals (Phillip et al., 2002).

In addition, activation of α2Rs mediates sedation, hypnosis, and analgesia (Phillip et al., 2002). Suppression of sympathetic neurotransmission and norepinephrine release by α2Rs in both the PNS and the CNS result in sedation (Kamabayashi & Maze, 2000; Giovannitti et al., 2015). Furthermore, α2R-mediated inhibition of norepinephrine release and neurotransmission in the locus coeruleus leads to hypnosis (Mizobe et al., 1996; Wang et al., 1996). On the other hand, activation of α2Rs in the dorsal horn of the spinal column inhibits the excitability of nociceptive neurons and suppresses the release of substance P (Jaakola et al., 1991; Fairbanks & Wilcox, 1999). These α2R actions in the dorsal horn reduces pain and mediates analgesia (Phillip et al., 2002). Due to these actions, α2R agonists are increasingly used as sedative, hypnotic, and analgesic drugs in anesthesia practice for both humans and animals (Giovannitti et al, 2015).

Finally, α2R agonists significantly reduce the requirement for anesthetics during the perioperative period (Phillip et al., 2002). This anesthetic-sparing function is primarily mediated by post-synaptic $\alpha 2_A$Rs (Lakhlani et al., 1997). Specifically, activation of inwardly rectifying K^+ channels and inhibition of voltage-gated Ca^{2+} channels by $\alpha 2_A$Rs causes hyperpolarization, resulting in depression of excitatory neurotransmission (Lakhlani et al., 1997). The reduction in

excitatory neurotransmission by $\alpha 2_A$Rs potentiates anesthetic-induced neurodepressive function, which in turn reduces anesthetic requirement (Lakhlani et al., 1997).

2.5.2.3 Imidazoline receptors

Imidazoline receptors only interact with chemicals containing an imidazoline structure (Head & Mayorov, 2006). There are three major classes of imidazoline receptors: I_1 imidazoline receptors (I_1Rs), imidazoline 2 receptors (I_2Rs), and imidazoline 3 receptors (I_3Rs) (Head & Mayorov, 2006). Each of these imidazoline receptor classes are found in distinct organs and mediate unique functions (Head & Mayorov, 2006).

I_1Rs are G-protein coupled receptors, primarily located in the rostral ventrolateral medulla (RVLM) of the brain (Regunathan & Reis, 1996). Activation of I_1Rs modulate basal and reflex control of sympathetic activity associated with cardiac function (Wenzel et al., 1998). Specifically, I_1R-mediated signaling (unidentified molecular pathways) inhibits tonic sympathoexcitatory reticulospinal vasomotor neurons in the RVLM. This reduces the sympathetic nerve activity related to cardiovascular function, resulting in a fall in arterial blood pressure and subsequent hypotension (Regunathan & Reis, 1996). As a result of their central hypotensive function, I_1R agonists are used to treat essential hypertension (Head & Mayorov, 2006).

Furthermore, preclinical studies suggest that sympathetic inhibition via I_1Rs indirectly inhibit excessive release of renin from the kidneys. The reduction in renin release decreases blood pressure in renal arteries, which in turn attenuates renal hypertension (Regunathan & Reis, 1996). Therefore, I_1R agonists (e.g. moxonidine, dexmedetomidine) are used as medications for essential and renal (secondary) hypertension (Head & Mayorov, 2006).

I_2Rs are non G-protein coupled receptors found on the mitochondrial membranes and monoamine oxidase (MAO) proteins of many tissues including the brain, carotid bodies, and pancreatic cells (Regunathan & Reis, 1996). In animal models, activation of I_2Rs regulates the release of catecholamines from sympathetic nerve endings in pulmonary arteries, aorta, and the heart (Head & Mayorov, 2006). Modulation of catecholamines contributes to the antidepressive function of I_2R agonists. Furthermore, I_2R-induced changes in central catecholamines also

modulate the activity of the hypothalamic-pituitary-adrenal (HPA) axis and neuroendocrine response to physiological stress (Finn et al., 2004). Collectively, I_2R stimulation seems to alleviate the effects of stress and depression.

Furthermore, I_2R agonists (e.g. agmatine, dexmedetomidine) mediate a number of physiologic functions that lead to neuroprotection (Head & Mayorov, 2006). Specifically, I_2R agonists increase glial fibrillary-acidic protein expression (GFAP) in astrocytes, inhibit nitric oxide production in the cerebral cortex, and reduce calcium overload in cells (Qiu & Zheng, 2006). These actions prevent neuronal degeneration and death following ischemic injury or glutamate-induced neurotoxicity (Khan et al., 1999; Head & Mayorov, 2006).

I_3Rs are a new class of imidazoline receptors found in pancreatic β cells (Head & Mayorov, 2006). These receptors modulate insulin secretion from pancreatic β cells via two mechanisms (Efendic et al., 2002). In one case, I_3Rs stimulate the closure of ATP-sensitive potassium (K_{ATP}) channels, which leads to depolarization, calcium influx, and finally, insulin release (Efendic et al., 2002). In addition, some I_3R agonists directly increase insulin secretion from β cells by increasing exocytosis. In this case, insulin secretion is dependent on glucose levels and does not involve K_{ATP} channels (Efanov et al., 2001). Future studies are necessary to determine whether I_3R agonists may be used as insulin secreting drugs in Type 2 diabetic patients (Head & Mayorov, 2006).

2.5.3 Biochemical properties

2.5.3.1 Pharmacodynamics

DEX is a highly potent and selective α2R agonist (Yoo et al., 2015). Specifically, the selectivity of DEX for α2Rs is 1620 times higher than that for α1Rs (Virtanen et al., 1989). Like many α2R agonists, DEX is an imidazole compound and it therefore interacts with imidazoline receptors to mediate some of its actions (Khan et al., 1999). Nonetheless, the selectivity of DEX for α2Rs is 32 times higher than that for the I_2Rs found in the brain (Ma et al., 2005). Overall, this selectivity profile suggests that most functions of DEX are mediated through α2Rs.

2.5.3.2 Pharmacokinetics

DEX has a rapid onset of action with a distribution half-life of 6 minutes (Khan et al., 1999). Since DEX is highly lipophilic, it is rapidly distributed in blood, with ~ 94% of DEX bound to serum albumin or α1-glycoprotein (Karol & Maze, 2000). However, DEX has a short duration of action, with an elimination half-life of 2 hours (Naaz & Ozair, 2014). These properties allow for efficient administration of DEX through infusions (Ma et al., 2005).

Furthermore, DEX undergoes nearly complete biotransformation with very little unmetabolized DEX excreted in urine (~ 95%) and feces (~ 4%) (Naaz & Ozair, 2014). Biotransformation of DEX involves direct glucuronidisation (the main pathway) and cytochrome P450-mediated metabolism (Phillip et al., 2002). DEX clearance is lower in individuals with impaired renal or hepatic function (Naaz & Ozair, 2014). However, the pharmacokinetic properties of DEX remain unaltered with age (Phillip et al., 2002).

2.5.4 Clinical applications

DEX mediates dose-dependent sedation, analgesia, anxiolysis, and sympatholysis (inhibition of sympathetic activity) which allow for its use in a number of clinical settings. (Maze et al., 2001). The clinical indication for which DEX is utilized directs its dose and route of administration in patients, as demonstrated in Table 2.5.

Route	Dose	Procedure	Endpoints	References
Intravenous	Loading dose: 1 μg/kg for 10 – 20 minutes. Maintenance infusion: 0.2 – 0.7 μg/kg/h	ICU sedation, procedural sedation	Sedation	Naaz & Ozair, 2014
Intramuscular injection	2.5 μg/kg	Premedication	Sedation, anxiolysis, analgesia, sympatholysis, hemodynamic stability	Naaz & Ozair, 2014
Spinal (or intrathecal)	0.1 – 0.2 μg/kg	Local anesthesia	Analgesia, sedation, hemodynamic stability	Ozair & Naaz, 2014, Kanazi et al., 2006
Epidural	1 – 2 μg/kg	Regional anesthesia	Analgesia, conscious sedation	Zhang et al., 2016
Buccal	1 – 2 μg/kg	Premedication (pediatric patients)	Sedation, anxiolysis, hemodynamic stability	Cimen et al., 2013
Intranasal	1 – 2 μg/kg	Premedication (pediatric patients)	Sedation, anxiolysis, hemodynamic stability	Cimen et al., 2013

Peripheral nerve block	1 – 2 μg/kg	Regional anesthesia	Analgesia, prolongs anesthetic-induced nerve blockade	Illfeld et al., 2010

Table 2.5: Clinical applications of DEX indicating procedure, dosage, and route of administration.

In addition to clinical procedure and desired endpoint, the dosage of DEX should be titrated to individual requirements and patient history. For instance, a lower dose (below standard range) of DEX is recommended for elderly patients with renal or hepatic impairment (Ozair & Naaz, 2014, Kaur & Singh, 2011). Furthermore, DEX use is not recommended for patients with diabetes or heart failure since they are more susceptible to DEX-induced hypotension (Afsani, 2010).

The most common clinical application of DEX is ICU sedation (Giovannitti et al., 2015). DEX sedation in the ICU is preferable because DEX maintains hemodynamic stability, oxygen availability, and reduces the use of opioids. Furthermore, DEX ensures faster recovery and shorter ICU stay in mechanically ventilated patients, compared to traditional sedatives like midazolam (Pandharipande et al., 2007). Hence, sedation with DEX allows earlier weaning from mechanical ventilators and reduces hospital costs (Riker et al., 2009). Due to these benefits, the longest duration of DEX infusion is approved for ICU sedation. However, the Food and Drug Administration (FDA) has limited DEX infusion to 24 h (Kaygusuz et al., 2008). This is because DEX usage beyond 24 h is associated with tolerance, tachyphylaxis (diminishing response to successive drug dose), and adverse reactions such as acute-respiratory distress syndrome (FDA, 2016).

Another common clinical use of DEX is as an anesthetic adjuvant during local anesthesia (El-Hannawy et al., 2009). DEX is particularly suited for this procedure due its rapid absorption into the cerebrospinal fluid, which results in rapid analgesia (El-Hannawy et al., 2009). In addition, DEX enhances local anesthetic-induced motor and sensory blockade, improving the quality of anesthesia (Memis et al., 2004).

DEX is increasingly being used for premedication before elective surgery in both pediatric and adult patients (Taittonen et al., 1997). DEX is favoured as a premedication due to its versatile use as an anxiolytic, analgesic, sedative, and sympatholytic agent. In addition, DEX use stabilizes hemodynamics and reduces oxygen consumption during the intra and postoperative periods (Ozair & Naaz, 2014).

Due to the hemodynamic stabilizing function, DEX is also used for sedation during cardiac and neurosurgery procedures (Wyjeysundera et al., 2003; Bekker & Sturaitis, 2005). Furthermore,

DEX prevents sudden increases in intracranial pressure during head pin insertion, intubation, and extubation during neurosurgery since DEX has minimal effects on arterial vasoconstriction. DEX-mediated maintenance of intracranial pressure reduces the risk for herniation of the brain substance (due to pressure gradients) and regional ischemia (due to reduced cerebral perfusion) (Bekker & Sturaitis, 2005).

Furthermore, DEX is recommended as a sedative for patients with obesity and sleep apnea (Naaz & Ozair, 2014). Unlike narcotics, DEX does not exacerbate respiratory depression in obese patients (Hofer et al., 2005). As well, DEX promotes natural sleep patterns which reduces sleep disturbances in patients with sleep apnea (Naaz & Ozair, 2014).

Lastly, intraoperative usage of DEX significantly reduces the requirements for anesthetics (by 80 – 90 %) and opioids (by 50 – 75%) (Venn et al., 2001; Naaz & Ozair, 2014). DEX-mediated reduction in anesthetic requirement decreases the risk for anesthetic-induced tachycardia and cognitive impairments (as discussed earlier in Section 2.3).

2.6 Mechanisms of neuroprotection by dexmedetomidine

2.6.1 Dexmedetomidine protection in animal models

Dexmedetomidine (DEX) has been shown to reduce neurodegeneration, cognitive deficits, and mortality rates in several studies using animal models. The following sub-sections will discuss some of the key studies of dexmedetomidine neuroprotection.

2.6.1.1 Dexmedetomidine reduces excitotoxicity-induced neurodegeneration

In the developing brain, exposure to hypoxic-ischemia or drugs, such as alcohol, can induce excessive activity of glutamate or other excitatory neurotransmitters (Laudenbach et al., 2002). The abnormal increase in excitatory neurotransmission results in excitotoxicity. Animal studies report that DEX protects the brain from excitotoxicity-induced neurodegeneration (Ma et al., 2005).

A recent study investigated the neuroprotective function of DEX against glutamate-induced excitotoxicity in rodents (Degos et al., 2013). Specifically, postnatal day 5 mice were

either treated with an intraperitoneal (i.p.) injection of DEX or vehicle control. An hour later, the mice were subjected to excitotoxicity via intracerebral injection of ibotenate (glutamate receptor agonist). Next, the mice brains were collected to either study cell survival or cerebral lesions (Degos et al, 2013).

To determine DEX effects on cell survival, primary cortical neuron and astrocyte cultures were prepared from the mice 3 h after ibotenate treatment (Degos et al., 2013). The cell viabilities of these cultures were quantified using a colorimetric assay. Neuron cultures from mice pre-treated with DEX had a significantly higher cell viability compared to that of mice pre-treated with saline. However, astrocyte cultures from mice pretreated with DEX had similar cell viabilities to mice pre-treated with saline. Taken together, these results demonstrate that DEX enhances survival of cortical neurons in neonatal rats exposed to glutamate agonist-induced excitotoxicity (Degos et al., 2013).

To assess DEX effects on cerebral lesions, the mice were sacrificed 5 days after ibotenate injection and sections of their cerebral cortices were visualized by microscopy (Degos et al., 2013). The area of ibotenate-induced lesions were significantly smaller in cerebral cortices of rats pre-treated with DEX, compared to that of saline treated rats. These results show that pre-treatment with DEX reduces ibotenate-induced cerebral cortical lesions in neonatal mice (Degos et al., 2013). In addition, these data align with previous studies in which pre-treatment with DEX reduced ibotenate-induced lesions in the hippocampus and white matter (Laudenbach et al., 2002; Kuhmonen et al., 1997).

Collectively, the studies here show that DEX protects the perinatal brain from glutamate-excitotoxicity-induced lesions and neuronal death. Excitotoxic damage to the cerebral cortex, white matter, and hippocampus during the perinatal period can impair cognitive functions later in life (Laudenbach et al., 2002). This suggests that treatment with DEX may reduce the risk of developing cognitive impairments after perinatal excitotoxicity (Sanders et al., 2011).

2.6.1.2 Dexmedetomidine attenuates anesthesia and surgery-induced cognitive deficits

Several behavior studies in rodents suggest that DEX attenuates impairments in learning and memory performance after anesthesia and surgery (Sanders et al., 2009; Qian et al., 2015).

One such study tested whether DEX reduces memory impairments in elderly mice (20 - 22 months) after surgery with isoflurane anesthesia (Qian et al., 2015). Specifically, spatial learning and memory performance of mice in the Y-maze task was assessed on days 1 and 3 after surgery. For both days, mice that underwent surgery with anesthesia had significantly worse spatial learning and memory performance compared to vehicle-treated mice without surgery. However, in mice that received DEX treatment before surgery, spatial learning and memory performances were similar to that of control mice (Qian et al., 2015). These results suggest that pre-treatment with DEX reverses learning and memory deficits after surgery with anesthesia.

A different study investigated the role of DEX in preventing long-term learning and memory deficits in elderly rodents after isoflurane anesthesia (Si et al, 2016). In this case, spatial learning and memory performance of mice in the Morris Water Maze task was tested 19 days after exposure to isoflurane anesthesia. Mice exposed to anesthesia alone had significantly worse learning and memory performance compared to vehicle-treated control mice. Furthermore, mice treated with DEX before anesthesia had significantly better performance than mice treated with anesthesia alone. These results demonstrate that DEX attenuates anesthetic-induced long-term learning and memory deficits in elderly rodents (Si et al., 2016).

Furthermore, DEX has also been shown to reduce postanesthetic memory impairments in rats exposed to isoflurane anesthesia in the early postnatal period (Sanders et al., 2009). Forty days after treatment with isoflurane, or saline, or isoflurane and DEX, hippocampus-dependent learning and memory performance was tested in these rats using the fear-conditioning behavioral assay. Rats treated with isoflurane anesthesia showed significantly worse performances in the fear conditioning task compared to that of rats treated with saline control (baseline). On the other hand, rats treated with isoflurane and DEX showed baseline performance in the fear conditioning task, similar to control rats. Taken together, this study demonstrates that DEX reverses anesthetic-

induced persistent deficits in hippocampus-dependent learning and memory in young rodents (Sanders et al., 2009).

Finally, co-treatment with DEX has been shown to restore learning and memory function in rats exposed to propofol anesthesia *in utero* (Li et al, 2015). Here, learning and memory performance of the rat offspring was tested using the 8-arm radial maze (8-ARM) task on postnatal days 28 to 35. Rats exposed to propofol anesthesia made significantly more errors in the 8-ARM task compared to rats exposed to propofol and DEX or saline control. Furthermore, rats exposed to propofol and DEX had similar error rates as rats exposed to saline control. This suggests that co-treatment with DEX reverses anesthetic-induced learning and memory impairments in rats exposed to anesthesia *in utero* (Li et al, 2015).

Collectively, the above studies demonstrate that pre and co-treatment with DEX significantly reduces anesthetic-induced learning and memory deficits in aged and young rodents. The role of DEX in reversing postanesthetic learning and memory impairment likely contribute to its mechanism of reducing the incidence of postoperative delirium (Rudolph & Marcantonio, 2011).

2.6.1.3 Dexmedetomidine reduces mortality and inflammatory response following systemic inflammation

Treatment with DEX has been shown to alleviate adverse effects of systemic inflammation in rodent models of endotoxemia and sepsis (Xiang et al., 2014; Zhang et al., 2015).

One important study investigated the role of DEX on mortality rates and inflammatory responses in adult rats exposed to endotoxin-shock (Taniguchi et al., 2004). Specifically, adult rats were injected with endotoxin from *Escherichia Coli* followed by an intravenous infusion of DEX or saline. However, control rats only received saline or DEX infusion without exposure to endotoxin (Taguchi et al., 2004).

Mortality rates, in the 8 h after endotoxin treatment, were significantly higher in endotoxin-treated rats (94%) compared to rats treated with endotoxin and DEX (44%) or controls (< 10%)

(Taniguchi et al., 2004). In addition, endotoxin-treated rats had significantly higher levels of the plasma proinflammatory cytokines TNFα and IL-6, compared to endotoxin and DEX-treated rats (Taniguchi et al., 2004). Taken together, these results demonstrate that DEX reduces systemic inflammation-induced mortality and plasma cytokine levels in adult rats exposed to endotoxemia.

Another key study investigated the effect of DEX treatment on inflammatory response in septic rodents (Zhang et al., 2015). Here, adult rats were subjected to cecal ligation and puncture (CLP) operation or sham operation followed by intravenous injection with DEX or no treatment (control). CLP was used to induce sepsis (infection resulting from organ damage) in the study rats. Proinflammatory cytokine levels in the plasma and bronchoalveolar lavage fluid (BALF) of the rats were measured using ELISA. Plasma and BALF concentrations of proinflammatory cytokines, TNFα and IL-6, were significantly lower in CLP rats that received DEX treatment, compared to CLP rats without DEX treatment. Furthermore, the proinflammatory cytokine levels were significantly higher in rats that underwent CLP operation compared to rats that underwent sham operation (Zhang et al., 2015). Overall, this study suggests that treatment with DEX reduces sepsis-induced excessive increase in proinflammatory cytokines in the plasma and lung BALF.

Sustained increases in the levels of proinflammatory cytokines can damage organs by triggering necrosis and apoptosis (Xiang et al., 2014). For instance, systemic inflammation can lead to neuroinflammation and apoptosis in the brain (Cunningham et al., 2011). Hence, treatment with DEX may help reduce inflammation-induced neurodegeneration and thus protect brain structure and function in septic patients (Cunningham et al., 2009).

2.6.2 Molecular mechanisms of dexmedetomidine neuroprotection

DEX has been shown to protect neurons from various types of injuries including hypoxic-ischemia, glutamate-induced excitotoxicity, and anesthetic-induced neurotoxicity (Ma et al., 2005; Bekker & Sturaitis). Studies using rodent models have provided an initial insight into the molecular mechanisms of DEX-mediated neuroprotection, as discussed in the following sub-sections (Degos et al., 2013).

2.6.2.1 Hypoxic-ischemic injury

Numerous studies using rodent models of hypoxic-ischemia have investigated the molecular mechanisms of DEX-induced neuroprotection (Ma et al., 2005). These studies have insinuated that modulation of neurotransmission, apoptotic pathways, and neuronal survival and differentiation by DEX help mediate its neuroprotective function (Ma et al., 2005).

One such study investigated whether DEX-induced reduction in circulating catecholamines contributes to its neuroprotective function in hypoxic-ischemia (Hoffman et al., 1993). Specifically, ventilated rats either received DEX or no treatment (control) before incomplete cerebral hypoxic-ischemia was induced using unilateral carotid artery ligation with hemorrhagic hypotension for 30 minutes.

Cerebral blood flow (CBF) during hypoxic-ischemia, as measured using optical laser Doppler, was significantly higher in rats pre-treated with DEX, compared to untreated control rats (Hoffman et al., 1993). Furthermore, radioenzymatic analysis of blood samples revealed that plasma catecholamine (norepinephrine and epinephrine) concentrations in DEX-treated rats were significantly lower than that in untreated controls. Finally, DEX-treated rats had significantly better neurologic outcomes (consciousness, walking, motor performance, and pain reflex) after hypoxic-ischemia, compared to untreated control rats. Overall, these data demonstrate that DEX-induced reduction in circulating catecholamines improves CBF during cerebral ischemia and helps improves neurologic outcomes after hypoxic-ischemic injury (Hoffman et al., 1993).

Another study investigated whether DEX-induced regulation of proapoptotic and antiapoptotic proteins contributed to its neuroprotection from hypoxic-ischemia (Engelhard et al., 2003). Here, ventilated rats either received DEX treatment or no treatment (control) before hypoxic-ischemia was induced for 30 minutes using unilateral carotid artery occlusion and hemorrhagic hypotension. Four hours later, the rat brains were removed to quantify the expression of proapoptotic and antiapoptotic proteins using immunofluorescence and Western blots. In addition, expression of these proteins were also analyzed in sham operated rats, which served as baseline control (Engelhard et al., 2003).

Relative to baseline, hypoxic-ischemia significantly increased expression of the proapoptotic protein Bax in rats that did not receive DEX treatment (Engelhard et al., 2003). Whereas, rats that received DEX treatment had similar expression of Bax as sham-operated controls. Furthermore, expressions of the antiapoptotic proteins Bcl-2 and Mdm2 were significantly higher in DEX-treated rats compared to that in untreated rats and sham-operated controls. Therefore, this study suggests that neuroprotection from hypoxic ischemia may partially result from DEX-induced decrease in proapoptotic proteins as well as increase in antiapoptotic proteins (Engelhard et al., 2003).

More recent studies have investigated the molecular pathways and cellular targets involved in DEX-neuroprotection from hypoxic-ischemic injury. One such study explored whether DEX mediates neuroprotection by modulating the expression of neurotrophic factors and proinflammatory cytokines, which are known to affect neuronal survival (Rodriguez-Gonzalez et al., 2016). In this study, primary cortical astrocyte-neuron cocultures from rats were either treated with DEX or control (no treatment). Next, the cocultures were subjected to oxygen glucose deprivation (OGD) to induced hypoxic-ischemia *in vitro*.

Twenty-four hours later, microphotographic visualization using Hoescht 33342 staining revealed that apoptotic cell death was significantly lower in DEX-treated cocultures than in the untreated cocultures (Rodriguez-Gonzalez et al., 2016). Immunoassay analysis showed that DEX-treated coculture media had significantly higher protein expression of the neurotrophic factor BDNF but lower expressions of the pro-inflammatory cytokines, IL-6 and TNFα. Neurotrophic factors are known to enhance neuronal survival, whereas proinflammatory cytokines are known to induce neuroapoptosis (Chao, 2003). Hence, this study suggests that DEX-induced upregulation of neurotrophic factors as well as downregulation of proinflammatory cytokines contribute to its neuroprotection from hypoxic-ischemia (Rodriguez-Gonzalez et al., 2016).

Another *in vitro* study investigated the mechanism by which DEX improves neuronal survival after hypoxia-induced oxidative damage (Zhang et al., 2013). In this case, primary cultures of cerebellar granule neurons from mice were exposed hydrogen peroxide (H_2O_2) to induced hypoxia-induced oxidative damage.

To investigate whether DEX targets neurons or astrocytes to mediate neuroprotection, either primary astrocyte cultures or neuron cultures were treated with DEX before neuronal exposure to H_2O_2 (Zhang et al., 2013). Direct treatment of DEX on H_2O_2 neurons failed to increase cell viability, as measured by methylthiazoletetrazolium (MTT) assay. However, application of conditioned media from DEX-treated astrocyte cultures significantly increased cell viability in the H_2O_2-exposed neurons. Furthermore, ELISA analysis of astrocyte conditioned media (ACM) revealed that heparin-bound epidermal growth factor (HB-EGF) was significantly higher in DEX-treated astrocytes, compared to that in controls (Zhang et al., 2013). Overall, these results indicate that DEX targets astrocytes to protect neurons from hypoxia-induced oxidative damage. Furthermore, DEX action on astrocytes triggers the release of the growth factor EGF which likely enhances neuronal survival (Zhang et al., 2013).

2.6.2.2 Glutamate-induced excitotoxicity

Glutamate or glutamate agonist (e.g. ibotenate) induced excitotoxicity is a major cause of neuronal cell death in the perinatal period (Degos et al., 2013). DEX has been shown to reduce glutamate-excitotoxicity induced lesions in the perinatal brain, as discussed in 2.6.1. This sub-section will elaborate on the mechanism by which DEX mediates neuroprotection from glutamate-induced excitotoxicity.

One key study investigated whether DEX targets α2-adrenergic receptors (α2RS) to reduce glutamate-excitotoxicity-induced brain lesion (Paris et al., 2006). Specifically, neonatal wild-type mice and mice lacking $\alpha2_A$ adrenergic receptor ($\alpha2_A$ KO) or $\alpha2_C$ adrenergic receptor subtypes ($\alpha2_C$ KO) were treated with DEX or vehicle control. Thirty minutes later, excitotoxicity was induced in all mice by intracerebral injection of the glutamate agonist ibotenate. Histopathologic examination of the cerebral cortex and white matter revealed significantly smaller lesion size in DEX-treated wild-type mice than in DEX-treated $\alpha2_A$ KO mice or vehicle-treated controls. However, lesion size in DEX-treated $\alpha2_C$ KO mice were similar to that in DEX-treated wild-type mice. Taken together, these results suggest that DEX acts on $\alpha2_A$ subtype of adrenergic receptors to mediate neuroprotection from glutamate-induced excitotoxicity (Paris et al., 2006).

Another important study explored the cell type and signalling pathway involved in DEX-induced protection from excitotoxic lesions in the cerebral cortex (Degos et al., 2013). Specifically, neonatal mice were exposed to one of the following treatments: DEX, vehicle (control), BDNF, or BDNF antibody. Thirty minutes later, all mice were subjected to glutamate-induced excitotoxicity via intracerebral injection of ibotenate (Degos et al., 2013).

Microscopic staining of cerebral cortical sections showed that mice pre-treated with DEX or BDNF had significantly smaller cortical lesion size, compared to vehicle-treated control mice (Degos et al., 2013). As well, cerebral cortical expression of BDNF, determined using BDNF antibody, was significantly higher in DEX-treated mice than in vehicle controls. Furthermore, astrocyte culture media from DEX-treated mice had a significantly higher BDNF protein expression, than that of astrocyte culture media from vehicle-treated mice. However, neuron culture media from DEX-treated mice did not display an increase in BDNF protein expression. Collectively, these results indicate that DEX protects the cerebral cortex from excitotoxic lesions by triggering an increase in the expression of BDNF. Furthermore, DEX like increases astrocytic expression of BDNF to mediate neuroprotection following glutamate-induced excitotoxicity (Degos et al., 2013).

2.6.2.3 Anesthetic-induced neurotoxicity

Many preclinical as well as some retrospective clinical studies suggest that exposure to general anesthesia can cause neurotoxicity and neurocognitive impairment in the developing and aging brain (Jevtovic-Todorovic et al., 2013). Studies using rodent models have demonstrated that treatment with DEX reduces neuroapoptosis and neuroinflammation following exposure to an anesthetic (Sanders et al., 2009; Qian et al., 2015).

One such study investigated whether DEX reduces proinflammatory cytokine and proapoptotic protein expressions in the brains of elderly rodents following surgery with anesthesia (Qian et al., 2015). Specifically, elderly mice either received DEX or no treatment (control) before surgery with isoflurane anesthesia. Hippocampal mRNA and protein expressions, measured using quantitative PCR and western blot respectively, of proinflammatory cytokines (IL-1β and TNFα) were significantly lower in mice-pretreated with DEX than in controls. Furthermore, hippocampal

expression of the proapoptotic caspase 3 and Bax, determined using western blot and immunocytochemistry, were significantly lower in DEX-treated mice than in controls (Qian et al., 2015). Collectively, these results suggest that DEX-induced reduction in hippocampal proinflammatory cytokines and proapoptotic proteins contribute to its neuroprotection from general anesthesia in the aging brain.

Another important study investigated whether treatment with DEX inhibited neuroapoptosis in neonatal rodents following exposure to an anesthetic (Sanders et al., 2009). Specifically, isolated brains from euthanized rats were stained immunohistochemically for caspase 3 to determine neuroapoptosis in the hippocampus, cerebral cortex, and thalamus. Rats that received DEX treatment during anesthesia had significantly lower neuroapoptosis in the hippocampus, cerebral cortex, and thalamus than rats that received saline control during anesthesia. However, DEX failed to reduce isoflurane-induced neuroapoptosis in the hippocampus, cortex, and thalamus of rats treated with an α2R antagonist (Sanders et al., 2009). Taken together, these results suggest that DEX acts through α2Rs to reduce anesthetic-induced neuroapoptosis in the developing brain.

The above studies provide an initial insight into the mechanism by which DEX reduces neuronal death following exposure to an anesthetic. However, little is known about the molecular mechanisms by which DEX reverses anesthetic-induced cognitive deficits in the perioperative period (Rappaport et al., 2015). Since DEX mediates most of its clinical functions and some of its neuroprotective functions via α2Rs, these receptors may also play a role in reversing postanesthetic cognitive deficits (Ozair & Naaz, 2014). Hence, the next section will provide an overview of the α2Rs, their signalling pathways, and cellular targets.

2.7 α2-adrenergic receptors: Primary target of dexmedetomidine

2.7.1 α2-adrenergic receptors in the brain

α2-adrenergic receptors (α2Rs) are 7 pass transmembrane proteins that span the lipid bilayer of cell membranes (Khan et al., 1999). These receptors interact with ligands to trigger a cascade of reactions that ultimately result in a physiologic response(s) (Giovannitti et al., 2015).

The α2R protein is composed of a long chain of amino acids that consist of hydrophobic and hydrophilic protein segments (Khan et al., 1999). The 7 hydrophobic segments are embedded in the cell membrane, whereas the hydrophilic segments loop in and out of the cell membrane. The hydrophobic segments are α helices comprised of 20 to 25 amino acids (Giovannitti et al., 2015).

Most species have 3 subtypes of α2Rs: $\alpha2_A$, $\alpha2_B$, and $\alpha2_C$ (Khan et al., 1999). However, humans express a fourth receptor subtype, $\alpha2_D$, which is found in certain brain cells including astrocytes (Ruuskanen et al., 2004). The α2R subtypes share a 70 to 75% homology in amino acid sequence in their α helical segments which span the cell membrane (Khan et al., 1999). Since these α helical segments determine selectivity for ligand interaction, all α2R subtypes bind to agonists or antagonists with similar affinities (Khan et al., 1999; Giovannitti et al., 2015). Furthermore, all subtypes of α2Rs are coupled to G-proteins at their cytoplasmic sides (Khan et al., 1999). Interaction with G-proteins allows α2Rs to trigger multiple signaling pathways that stimulate or inhibit physiological processes in target organs and systems (Matsui et al., 1989).

2.7.2 α2-adrenergic receptor-mediated signaling

G-proteins are ubiquitous transmembrane proteins that mediate signal transduction and affect various physiologic processes including cell survival, metabolism, and gene expression. Many receptors, such as the alpha adrenergic, adenosine (A1), and $GABA_B$ receptors, are coupled to G-proteins (Giovannitti et al., 2015).

All G-proteins exist as heterotrimers and consist of three polypeptide subunits, G_α, G_β, and G_γ (Giovannitti et al., 2015). In its inactive state, a G-protein coupled receptor (GPCR) is bound to its heterotrimeric complex (Trzaskowski et al., 2012). Upon binding a ligand, the GPCR undergoes a conformational change which is transmitted to the G_α subunit via interactions between

protein domains (Trzaskowski et al., 2012). The triggered G_α exchanges a guanosine triphosphate (GTP) molecule for a guanosine diphosphate (GDP) molecule. Binding to a GTP provides the activation energy for G_α subunit to dissociate from $G_{\beta\gamma}$ and the receptor (Giovannitti et al., 2015). Next, the dissociated G_α and $G_{\beta\gamma}$ interact with other intracellular proteins to trigger signaling cascades leading to physiologic events (Figure 2.7). The GPCR, on the other hand, binds to another heterotrimeric G-protein complex and awaits binding of additional GPCR ligands (Trzaskowski et al., 2012; Giovanniti et al., 2015).

α2Rs are coupled to pertussis toxin-sensitive G-proteins, G_o which does not affect adenylyl cyclase, and G_i which inhibits adenylyl cyclase (Khan et al., 1999). In most cells of the CNS, such as neurons and astrocytes, G_o regulates calcium translocation and phospholipase (PLC) enzyme activity (Khan et al., 2009). For example, α2R-G_o signalling in astrocytes regulates enzyme activities and metabolic reactions by increasing cytosolic calcium concentration $[Ca^{2+}]_i$ (Rosenbaum et al., 2009). On the other hand, G_i-mediated inhibition of adenylyl cyclase prevents the formation of the second messenger cyclic adenosine monophosphate (cAMP), which activates protein kinase A (PKA) (Chen et al., 2009). Hence, a reduction in cAMP inhibits PKA-induced signaling pathways. In addition, G_i proteins gating potassium ion channels hyperpolarize neurons by increasing K^+ efflux, thus reducing the rate of action potentials (Chen et al., 2009).

$G_{i/o}$ protein coupled α2Rs are found in both pre and post-synaptic terminals (Khan et al., 1999). Pre-synaptic α2Rs are found in sympathetic nerve endings and noradrenergic neuron groups where they inhibit the release of norepinephrine (Giovannitti et al., 2015). Postsynaptic α2Rs are found in various organs including the brain, liver, kidneys, and pancreas (Giovannitti et al., 2015; Khan et al., 1999). The target cell type and synaptic localization determine the specific physiologic responses mediated by α2R- $G_{i/o}$ protein signalling (Khan et al., 1999).

Studies in this thesis will utilize pharmacologic manipulation of hippocampal α2Rs to study the mechanism of dexmedetomidine (an α2R agonist)-mediated neuroprotection from general anesthetics. Therefore, the following sub-sections will elaborate on pharmacological properties of various α2R agonists and antagonists.

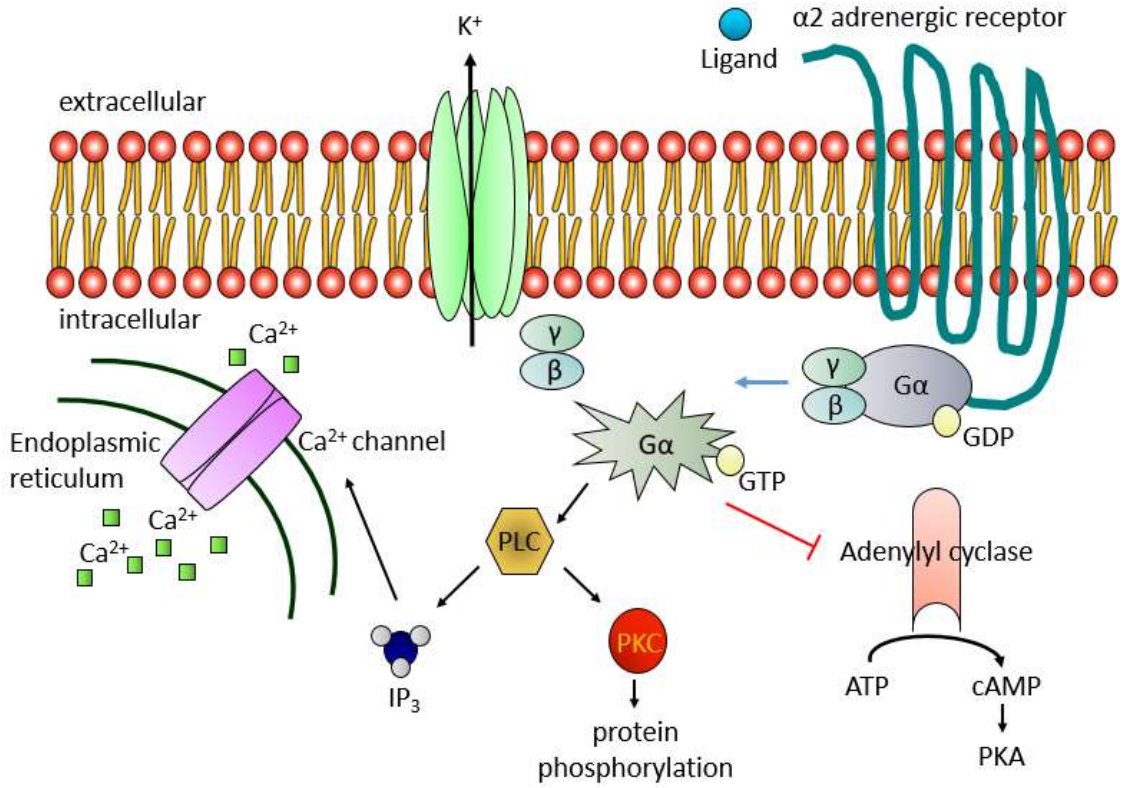


Figure 2.7: α2-adrenergic receptor and $G_{i/o}$ protein signaling. Stimulation of a G_i protein-coupled α2R by a ligand causes dissociation of Gα from the G-protein complex. The activated Gα and Gβγ interact with a number of intracellular proteins. For instance, the dissociated Gα and Gβγ inhibit adenylyl cyclase, supressing the production of cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA)-mediated singling. Simultaneously, they activate phospholipase C (PLC), which in turn, activates protein kinase C (PKC) and Ca^{2+}-dependent signalling pathways. In neurons, Gα also enhance the efflux of K^+ ions through G_i protein-coupled K^+ channel, which results in hyperpolarization.

2.7.3 Pharmacologic properties of common α2-adrenergic receptor ligands

2.7.3.1 Potencies of α2-adrenergic receptor agonists

Pharmacologic studies in rodents have quantified potencies of α2R agonists by measuring α2R-mediated responses and α2R ligand displacements in the presence of various α2R agonists (Virtanen et al., 1985; U'Prichard & Snyder, 1979).

For some studies investigating potencies, isolated mouse vas deferens were electrically stimulated to initiate twitch responses in the presence of various α2R agonists (Virtanen et al., 1985, Virtanen et al., 1988). Next, α2R agonist-mediated inhibition of twitch response was measured for various concentrations of the agonist. Graph plots of percentage inhibition of twitch response against agonist concentration were used to determine potencies of several α2R agonists, expressed as pD_2 values, as summarized in Table 2.7.1 (Virtanen et al., 1985).

In other studies, displacement of an α2R radioligand, with known potency, by other α2R agonists was used to determine the relative potencies of those agonists (Jarrott et al., 1980). For example, displacement of radiolabelled clonidine (known α2R ligand) from the rat cerebral cortex membrane was measured for various concentrations of a specific α2R agonist. For each α2R agonist studied, a displacement curve was plotted to calculate its potency for inhibiting clonidine binding to α2R in the cerebral cortex. In these studies, the potency values (not reported in the paper) were used to calculate the inhibition constants, k_i, which were reported, as summarized in Table 1 (Jarrott et al., 1980; U'Prichard & Snyder, 1979).

In an effort to compare the potencies of α2R agonists from various studies, I have calculated their EC_{50} values from their predetermined pD_2 and k_i values (Table 2).

The EC_{50} value was chosen to represent the potency of an α2R agonist since it is a common metric for quantifying the potency of agonists in general. In case of an α2R agonist, its EC_{50} is the molar concentration of agonist that causes 50% of its maximum effect via stimulation of α2Rs (Kenakin et al., 1990). However, for radioligand binding studies, the EC_{50} of an α2R agonist is the molar concentration of agonist which reduces the specific binding of an α2R radioligand by 50%.

The following equations were used to estimate the EC_{50} of α2R agonists from either their pD_2 or k_i values reported in the studies referenced in Table 1.

$$pD_2 = -\log_{10}EC_{50}$$

In this case, pD_2 refers to negative logarithm (10 base) of the EC_{50} of an α2R agonist.

In addition, the Cheng Prussoff equation was used to derive the potency of displacing the radioligand from the receptor (IC_{50}) using their inhibition constants, or k_i values, as shown below:

$$IC_{50} = k_i + \left(1 + \frac{[D]}{k_d}\right)$$

Here, [D] represents the concentration of the ligand (α2R agonist in this case) that displaces the radioligand, whereas k_d represents the dissociation constant of the radioligand.

α2R agonist	pD_2	k_i	EC_{50} (nM)	Model	Reference
Dexmedetomidine	9.0	n/a	1	Twitch response from isolated mouse vas deferens	Virtanen R. et al., 1988
Detomidine	8.8	n/a	1.58	"	Virtanen R. et al., 1985
Guanfacine	n/a	1.9	1.7	[^{3}H]Clonidine displacement in rat cerebral cortex membrane	Jarrott et al., 1980
Clonidine	8.5	n/a	3.16	Twitch response from isolated mouse vas deferens	Virtanen R. et al., 1988
-(-) Epinephrine	n/a	4	4.4	[^{3}H]Clonidine displacement in rat cerebral cortex membrane	U'Prichard & Snyder 1979
UK 14, 304	8.2	n/a	5.62	"	Virtanen R. et al., 1988
-(-) Norepinephrine	n/a	18.0	19.8	[^{3}H]Clonidine displacement in rat cerebral cortex membrane	U'Prichard & Snyder 1979
Xylazine	7.5	n/a	31.6	Twitch response from isolated mouse vas deferens	Virtanen et al., 1985

Table 2.7.3.1 A list of the potencies of common α2-adrenergic receptor (α2R) agonists presented in order of decreasing potency. The EC_{50} values have been estimated from the pD_2 or k_i values of the agonists derived from previous pharmacologic experiments. Dexmedetomidine appears to have a high potency compared to most other α2R agonists.

2.7.3.2 Efficacies of α2-adrenergic receptor agonists

The efficacy of an α2-adrenergic receptor (α2R) agonist is defined as the maximum α2R-mediated response that the agonist can produce, regardless of its dose or concentration (Jasper et al., 1998).

An immediate α2R-mediated response, following stimulation by an agonist, is the exchange of a guanosine diphosphate (GDP) molecule for a guanosine triphosphate (GTP) molecule (Khan et al., 1999). In isolated cells, this α2R agonist-induced response can be quantified by detecting radioactive GTP analogue, [^{35}S]GTPγS incorporation into cell membranes (Jasper et al., 1998).

Hence, studies using HEK 293 cells with recombinant α2Rs quantified α2R agonist-induced [^{35}S]GTPγS incorporation into cell membranes, in order to determine the efficacies of α2R agonists (Jasper et al., 1998). Specifically, liquid scintillation counting was used to detect [^{35}S]GTPγS incorporation in the presence of various concentrations of an α2R agonist. The maximum amount of [^{35}S]GTPγS incorporation by an α2R agonist, relative to that of the full α2R agonist norepinephrine, was determine to quantify its efficacy. Table 3 summarizes the efficacy values of common α2R agonists, as determined by [^{35}S]GTPγS incorporation studies (Jasper et al, 1998).

α2R Agonist	Efficacy for [^{35}S] GTPγS binding to cell membrane (relative to norepinephrine)
Epinephrine	1.10
Norepinephrine	1.00
UK-1434	0.90
Dexmedetomidine	0.82
Guanfacine	0.71
Xylazine	0.65
Clonidine	0.58

Table 2.7.3.2 Efficacies of common α2-adrenergic receptor (α2R) agonists to stimulate radiolabelled GTP binding in HEK 293 cell cultures, relative to that of the full agonist norepinephrine (adapted from Jasper et al., 1998). The endogenous α2R agonists (epinephrine and norepinephrine) have higher efficacies than synthesized agonists. Dexmedetomidine displays a higher efficacy for α2R –mediated GTP incorporation, compared to other synthesized α2R agonists.

2.7.3.3 Potencies of α2-adrenergic receptor antagonists

Radioligand displacement studies using the rat cerebral cortex or vas deferens cell membrane homogenates, have been used to determine the potencies of α2R antagonists (Jarrott et al., 1980). Particularly, the displacement of a potent α2R radioligand (e.g. [^{3}H]clonidine or [^{3}H]guanfacine) at various concentrations of an α2R antagonist was measured for all such studies (Jarrott et al., 1980; Tanaka et al., 1983; Brown et al., 1990; Pertovaara et al., 2005). A displacement curve (% radioligand displaced against agonist concentration) was plotted to compute the potency of the antagonist for inhibiting radioligand binding to α2R in the rat cell membrane. In these studies, the potency values (not reported in the paper) were used to calculate their inhibition constants for radioligand binding, k_i, as summarized in Table 4 (Jarrott et al., 1980; Tanaka et al., 1983; Pertovaara et al., 2005).

In an effort to compare the potencies of α2R antagonists from various studies, I have calculated their IC_{50} values from the reported k_i values, as shown in Table 4.

The IC_{50} value was chosen to represent the potency of an α2R antagonist because it is a common metric for quantifying the potency of antagonists in general. The IC_{50} for an α2R antagonist, derived from a radioligand binding assay, is the concentration of the antagonist which reduces specific binding of an α2R radioligand by 50% ((Kenakin, 1981). Table 3 presents the IC_{50} values of α2R antagonists, derived from their k_i values using the Cheng Prussoff equation (1973).

The Cheng Prussoff equation represents the relationship between potency of displacing radioligand from receptor (IC_{50}) and the inhibition constant, k_i as shown below:

$$IC_{50} = k_i + \left(1 + \frac{[D]}{k_d}\right)$$

Here, [D] represents the concentration of the ligand (α2R antagonist in this case) that displaces the radioligand, whereas k_d represents the dissociation constant of the radioligand.

α2R antagonist	k_i	IC_{50} (nM)	Model	Reference
Atipamezole	1.6	2.24	[^{3}H]clonidine radioligand binding; rat vas deferens	Pertovaara A. et al. (2005). *CNS Drug Reviews.* 11: 273-88.
Phentolamine	5.5	8.33	[^{3}H]guanfacine radioligand binding; rat cerebral cortex	Jarrot B, William JL, & Summers RJ, (1980). *Eur J Pharmacol.* 66: 233-41
Yohimbine	180	273	[^{3}H]guanfacine radioligand binding; rat cerebral cortex	Jarrot B, William JL, & Summers RJ, (1980). *Eur J Pharmacol.* 66: 233-41
Rauwolscine	n/a	333	[^{3}H]clonidine radioligand binding; rat vas deferens	Tanaka T. et al. (1983). Japan J. Pharmacol. 33: 713-6.
Prazosin	26220	39700	[^{3}H]guanfacine radioligand binding; rat cerebral cortex	Jarrot B, William JL, & Summers RJ, (1980). *Eur J Pharmacol.* 66: 233-41

Table 2.7.3.3 A list of the potencies of common α2-adrenergic receptor (α2R) antagonists presented in order of decreasing potency. The IC_{50} values have been estimated from k_i values of the antagonists derived from previous pharmacologic experiments (Jarrott et al., 1980; Tanaka et al., 1983; Pertovaara et al., 2005).

2.7.3.4 Efficacies of α2-adrenergic receptor antagonists

The efficacy of an α2-adrenergic receptor (α2R) antagonist is defined as the maximum α2R-mediated response that the antagonist can inhibit, regardless of its dose or concentration (Tian et al., 1993).

As discussed in sub-section 2.7.3.2, α2R-mediated response can be quantified in isolated cell cultures by measuring radioactive GTP analogue, [^{35}S]GTPγS incorporation into cell membranes (Jasper et al., 1998). Therefore, a reduction in α2R-mediated [^{35}S]GTPγS incorporation in the presence of an α2R antagonist can be quantified to determine the efficacy of the α2R antagonist. This method has been used in many studies to determine the efficacies of α2R ligands.

In one such study, homogenized PC12 cell membranes, expressing mouse α2Rs, were subjected to liquid scintillation counting to detect [^{35}S]GTPγS incorporation in the presence of different α2R antagonists (Tian et al., 1993). In this case, [^{35}S]GTPγS incorporation into PC12 cell membranes was induced by 100 μM of epinephrine, a full agonist of α2Rs. The amount of bound [^{35}S]GTPγS in the presence of a particular concentration of an α2R antagonist was measured. The percentage of bound [^{35}S]GTPγS in the presence of an α2R antagonist provided a measure of the efficacy of the antagonist for inhibiting agonist action via α2R (Tian et al., 1993). Table 5 summarizes the efficacies of α2R antagonists derived using this method.

α2R Antagonist	% Efficacy for [^{35}S]GTPγS binding in the presence of 100 μM epinephrine
Yohimbine	60
Phentolamine	65
Idazoxan	67.5
Rauwolscine	72.5

Table 2.7.3.4 Efficacies of common α2-adrenergic receptor (α2R) antagonists to inhibit α2R-induced radiolabelled GTP incorporation into PC12 cell membranes, upon stimulation by the full α2R agonist epinephrine (adapted from Tian et al., 1993). Among the α2R antagonists studied using this model, the antagonist yohimbine has the highest efficacy for inhibiting α2R agonist-induced incorporation of [^{35}S]GTPγS into cell membranes.

3. General Materials and Methods

This section will elaborate on the experimental technique and general protocols used to test the hypotheses presented in this thesis. In addition, this chapter will provide detailed descriptions for the preparation of drugs used in all experiments. Lastly, an overview of statistical tests performed to analyse all data will be discussed here.

The primary objective of these studies was to investigate the mechanism of DEX protection from an anesthetic-induced persistent increase in neuronal tonic current using a mouse model. Since the effect of different pharmacologic interventions (e.g. treatment with an anesthetic and/or DEX) on tonic current was measured for all experiments, whole-cell voltage-clamp electrophysiology was used in all cases. Furthermore, all electrophysiological recordings were conducted *in vitro* because it is convenient for using pharmacologic probes to identify the mechanisms of DEX effects on tonic current.

To study DEX effects on the hippocampus, tonic current from primary hippocampal neurons were measured. Similarly, to investigate DEX effects in the cortex, tonic current from primary cortical neurons were measured.

Next, to establish whether DEX targets astrocytes or neurons to mediate its effects, either pure astrocyte cultures, astrocyte-neuron cocultures, or pure neuron cultures were treated with DEX or other drugs (as discussed in detail in Specific Methods, 4.2 and 5.2). However, the anesthetics were applied to either astrocyte cultures or astrocyte-neuron cocultures since anesthetics increase neuronal tonic current by targeting astrocytes, as discussed in Chapter 2.3.

In addition, different drugs were applied to pure astrocyte cultures, astrocyte-neuron cocultures and pure astrocyte and neuron cultures, depending on the specific experimental investigation. For most experiments, the $GABA_A$ receptors ($GABA_AR$)-selective, injectable anesthetic etomidate was used since tonic current is mediated by extrasynaptic $GABA_ARs$, as discussed in Chapter 2. However, the commonly used inhalational anesthetic sevoflurane was used for some experiments to determine whether DEX affects neuronal tonic current induced by various anesthetics. To study whether DEX targets α2-adrenergic receptors (α2Rs), different α2R agonists and antagonists were used for a number of experiments. As well, various

neurotrophic factors or growth factors and their inhibitors were used to investigate whether DEX releases factors that prevent the anesthetic-induced persistent increase in tonic current in neurons. The specific treatment protocols for each experiment will be described in detail in Chapters 4.2 and 5.2.

3.1 Study Approval

All experimental protocols involving animals were approved by the Animal Care Committee of the University of Toronto and performed according to guidelines from the Canadian Council on Animal Care. The specific methods have been described in detail to ensure reproducibility of the results reported in this thesis.

3.2 Whole-cell voltage-clamp recordings

For all whole-cell voltage clamp recordings reported here, the recording electrode (2 – 3 MΩ) was filled with intracellular solution containing: 140 mM CsCl, 11 mM EGTA, 2 mM TEA, 1 mM $CaCl_2$, 2 mM $MgCl_2$, and 4 mM Mg_2ATP. The pH of the intracellular solution was kept at 7.3 and the osmolarity was adjusted to between 290 – 295 mOsm. The pipette offset was compensated for when the recording electrode was in the bath which consisted of the extracellular solution containing 140 mM NaCl, 2 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 28 mM glucose, and 25 mM HEPES. The pH of the extracellular solution was maintained at 7.4 and the osmolarity was maintained between 322 – 328 mOsm.

To record from a given neuron, a tight gigaohm seal (≥ 1 GΩ) was formed on the cell membrane of the neuron. The pipette capacitance was compensated for before acquiring a whole-cell configuration, which was acquired by applying negative pressure to the recording electrode and rupturing the cell membrane of the neuron. Rupturing the cell membrane provided electrical access to the whole cell. This allowed us to control the intracellular environment by replacing the intracellular content with the artificial intracellular solution in the patch pipette. Furthermore, a 10 mV hyperpolarizing voltage step in Multiclamp software (Molecular Devices, Sunnyvale, California) was used to monitor the series resistance and all currents were sampled at a frequency of 5 kHz. All cells were voltage-clamped at -60 mV and automatic capacitance compensation was applied at from the Multiclamp software.

To measure the amplitude of tonic $GABA_AR$-current, exogenous GABA (0.5 μM) was applied to the extracellular solution at first. Next, the change in holding current was revealed during application of the $GABA_AR$-selective antagonist bicuculline (BIC, 20 μM). The 0.5 μM concentration of GABA is similar to the physiological concentrations of extracellular GABA observed *in vivo* (Bright & Smart, 2013).

3.3 Preparation of primary cell cultures

3.3.1 Primary neuron cultures

Primary cell cultures of hippocampal neurons were prepared from CD1 mice (Charles River, Montreal, Canada), as described in Zurek et al., 2014 with minor modifications. Briefly, fetal pups (embryonic day 18) were removed from time-pregnant mice euthanized by cervical dislocation. The hippocampi were extracted from each fetal pup and kept in an ice-cold culture dish. Next, the hippocampi were digested with 0.05% trypsin (Life Technologies, Grand Island, New York) for 20 min at 37°C and dissociated by mechanical titration using two Pasteur pipettes (tip diameter: 150 -200 μm). The dissociated neurons were then plated on 35 mm culture dishes coated with poly-D-lysine (Sigma-Aldrich, Canada) at a density of approximately 1×10^5 cells per dish plating medium (DMEM with 10% fetal bovine serum). The cell cultures were maintained in Dulbecco's Modified Eagle Medium or DMEM (Life Technologies, Grand Island, New York) supplemented with 10% fetal bovine serum (FBS). Two hours later, the cell culture media were changed to neurobasal medium supplemented with 2% B27 and 0.5 mM L-glutamate (Invitrogen, Canada). The neurons were cultured in an incubator set at 37°C with 95% humidified air and 5% CO_2. Neurobasal media in the cell cultures were replaced every 2-3 days. The hippocampal neurons were maintained under these culture conditions for 14 to 21 days prior to recording.

The same extraction and incubation protocols as above were used to harvest primary cultures of cortical neurons, with the exception that the cerebral cortices were extracted from fetal pups instead of the hippocampi.

3.3.2 Primary astrocyte cultures

Cerebral cortical astrocytes were prepared from newborn CD1 mice as previously described elsewhere (Wang et al., 2014; Qiao, et al, 2016), with minor modifications. Briefly, meninges-free cortices were cut into small cubes (<1mm^3), placed in modified Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen Corporation, CA, USA), and mechanically dissociated by vortex for 60 s. The resulting cell suspension was sieved through 40 µm filters (Millipore, Canada). The filtrated cells were plated in a flask consisting of 5 x10^5 cells per dish in DMEM supplemented with 10% FBS (Life Technologies, Grand Island, New York) for 14 days. The astrocyte cultures were incubated in a Napco incubator (Precision Scientific Inc., IL, USA) at 37°C with 95% humidified air and 5% CO_2. The cell cultures were fed twice weekly with DMEM and 10% FBS for the first two weeks and DMEM (7% FBS) for the following two weeks. The 4 week old cells were then dissociated with trypsin-EDTA (0.05%, Life Technologies, Grand Island, New York), and passaged three times to obtain an almost pure astrocyte culture. Next, the astrocytes were plated on 35 mm culture dishes at a density of 25,000 cells per dish. The astrocyte cultures were maintained in the Napco incubator as mentioned above until they became confluent.

3.3.3 Primary astrocyte-neuron cocultures

To coculture cortical astrocytes and hippocampal neurons, the 4 weeks old astrocyte cell-suspension from the flask (mentioned above) was placed over hippocampal neurons cultured at 14 days in neurobasal media. The astrocytes were monitored visually to ensure survival and confluence for the duration of the experiment.

For all studies reported in this thesis, data were acquired from at least three different dissections of brain tissue. In all cases, the $GABA_AR$ antagonist BIC was applied to the cell culture to reveal the amplitude of the change in holding current due tonic $GABA_AR$ activity in a given neuron. Figure 3.3 demonstrates an example of a raw trace of tonic current measured from a neuron.

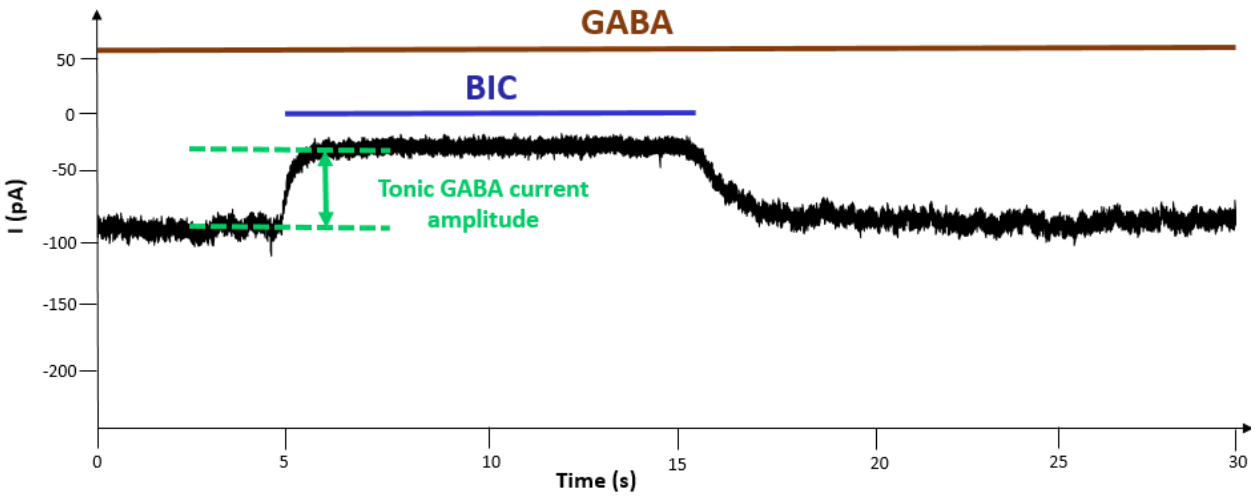


Figure 3.3: An example of a raw tonic GABA trace from a hippocampal neuron that is voltage-clamped at -60 mV. $GABA_AR$-mediated tonic current was measured for 30 s by continuously applying 0.5 μM GABA to the cell culture bath for the duration of the recording. The $GABA_AR$ antagonist, bicuculline (BIC, 20 μM) was applied for 10 s (between 5 s and 15 s, specifically) to reveal the amplitude of the change in holding current, which represents the $GABA_AR$-mediated tonic current amplitude. On average, the baseline holding current of a given neuron, in the presence of 0.5 μM GABA but no BIC, is around -90 pA. Application of BIC changes the holding current to a more positive value (e.g. to -20 pA, for a 70 pF control cell). The amplitude of the change in holding current, i.e. tonic GABA current, depends on the drug(s) that the cell culture is pre-treated with.

3.4 Drugs

All drugs were prepared fresh each week and stored in the refrigerator after use in order to minimize degradation and contamination.

3.4.1 Etomidate

Etomidate (Sigma-Aldrich, Canada) was first dissolved in 35% propylene glycol at 2 mg/mL. Next, the etomidate in propylene glycol was diluted with phosphate buffer saline, PBS, (Gibco, Thermo Fisher Scientific, Canada) to a stock concentration of 200 μM. In order to achieve an anesthetic dose *in vitro*, etomidate was applied at a concentration of 1 μM to the cell cultures.

For most experiments reported in this thesis, etomidate was the anesthetic of choice due to a number of reasons: (i) Etomidate is the most $GABA_AR$ selective anesthetic which suits the experiment model because tonic $GABA_AR$-mediated current is the primary signal measured here; (ii) Etomidate has rapid action and is easier to use in *in vitro* studies compared to volatile anesthetics.

3.4.2 Sevoflurane

Sevoflurane or SEVO (AbbVie Corporation, Saint Laurent, QC) was dissolved in freshly prepared extracellular solution to obtain a stock concentration of 11.8 mM. Specifically, one part SEVO was added to two parts extracellular solution and left to stand overnight in order to saturate the solution with SEVO. Cell cultures were treated with this stock solution of SEVO to achieve a final concentration of 266 μM.

3.4.3 Dexmedetomidine

Dexmedetomidine or DEX (Santa Cruz, USA) was dissolved in PBS (Gibco, Thermo Fisher Scientific, Canada) to prepare an initial stock concentration of 2 mM. The 2 mM stock was used in early screening experiments where a high dose of DEX (10 or 1 μM) was applied to cell cultures. But for most experiments reported in this thesis, the 2 mM stock was further diluted with PBS to obtain a stock concentration of 20 μM. Cell cultures were treated with this lower stock

concentration (20 μM) of DEX in order to mimic an *in vitro* clinically effective concentration of 100 nM.

3.4.4 Clonidine

Clonidine or Clo (Sigma Aldrich, Canada) was dissolved in PBS (Gibco, Thermo Fisher Scientific, Canada) to prepare an initial stock concentration of 20 mM. This stock was used for initial screening experiments where a high dose of Clo (100 μM) was applied to cell cultures. For subsequent experiments, the 20 mM stock was further diluted hundred times (0.2 mM). The 2 mM stock was used for experiments where the final concentrations of Clo in cell cultures were clinically relevant doses (either 1 μM).

3.4.5 Yohimbine

Yohimbine hydrochloride or Yoh (Sigma Aldrich, Canada) was dissolved in in PBS (Gibco, Thermo Fisher Scientific, Canada) to make an initial stock concentration of 1 mM. This stock solution of Yoh was applied to cell cultures at either a high dose (5 μM) for initial screening or a lower, more clinically relevant dose (1 μM).

3.4.6 Brain-derived neurotrophic factor (BDNF)

Brain-derived neurotrophic factor or BDNF (Alomone Labs, Jerusalem) was dissolved in PBS (Gibco, Thermo Fisher Scientific, Canada) to prepare an initial stock concentration of 20 μg/mL, which was further diluted with PBS to make a stock concentration of 40 ng/mL. The lower stock concentration was then applied to cell cultures to reach a final concentration of 200 pg/mL.

3.4.7 TrkB receptor inhibitor (ANA12)

The TrkB receptor antagonist, ANA12, is an inhibitor for the neuronal receptor of BDNF. ANA12 (Cedarlane, Canada) was reconstituted in PBS (Gibco, Thermo Fisher Scientific, Canada) to an initial stock concentration of 1 mg/mL. This stock solution was further diluted in PBS to obtain a lower stock concentration of 2 μg/mL. The 2 μg/mL stock was applied to cell cultures to achieve a final concentration of 100 ng/mL.

3.4.8 Epidermal growth factor (EGF)

Epidermal growth factor (EGF) (Sigma Aldrich) was reconstituted in 10 mM acetic acid (Sigma Aldrich, Canada) to make an initial stock concentration of 1 mg/mL. This stock was further diluted to 2 µg/mL. And the lower concentration stock (2 µg/mL) was applied to cell cultures at a final concentration of 10 ng/mL.

3.4.9 EGF tyrosine kinase inhibitor (Ag1478)

The EGF tyrosine kinase inhibitor receptor antagonist, Ag1478 prevents EGF-mediated signaling in neurons. Ag1478 (Sigma Aldrich, Canada) was reconstituted in one part dimethyl-sulfoxide (DMSO) and one part methanol to prepare an initial stock concentration of 20 mM. This stock concentration was diluted with distilled water to achieve a lower stock concentration of 0.2 mM. Finally, the 0.2 mM stock solution of Ag1478 was applied to cell cultures at a concentration of 1 µM.

3. 5 Statistical Analyses

All results are presented as mean ± standard error (SEM). Since three or more groups were compared at a time, a one-way analysis of variance (ANOVA) was applied to measure significance. If the ANOVA was statistically significant, then Tukey's Honestly Significant Difference was utilized for pairwise comparisons. All statistical analyses were performed using the GraphPad Prism software version 5.0 (GraphPad software, San Diego, California). For all studies, $p < 0.05$ was considered statistically significant.

4. Dexmedetomidine acts on astrocytic α2-adrenergic receptors to prevent anesthetic-induced persistent increase in neuronal tonic current

4.1 Introduction

The potent and selective α2-adrenergic receptor (α2R) agonist, dexmedetomidine (DEX), protects from anesthetic-induced neuroapoptosis and cognitive deficits in rodent models, as discussed in Section 2.6 (Sanders et al., 2009; Sanders et al., 2010; Virtanen et al., 1985; Qian et al., 2015). This section will summarize the key findings from previously published studies that drove the first hypothesis of this thesis.

Ex vivo studies in neonatal rodents show that co-treatment with DEX reduces anesthetic-induced neuroapoptosis in the hippocampus and cerebral cortex, which are cognitive domains responsible for memory and executive function, respectively (Sanders et al., 2009; Sanders et al., 2010). Furthermore, DEX dose-dependently reduced the anesthetic-induced neuroapoptosis and this neuroprotection was inhibited by pharmacologic inhibition of α2-adrenergic receptors (α2Rs) with an α2R antagonist (Sanders et al., 2009).

While the cellular targets of DEX-neuroprotection from anesthetics have not been determined, studies using other injury models have shown DEX to act on both neurons and astrocytes to mediate neuroprotection (Degos et al., 2013; Rodriguez-Gonzalez et al., 2015). Furthermore, recent *in vitro* studies suggest that DEX targets astrocytes to reduce glutamate-excitotoxicity-induced neuroapoptosis in the cerebral cortex of rodents, as discussed earlier in Section 2.6 (Degos et al., 2013).

Interestingly, previous studies from our lab have shown that astrocytes also mediate anesthetic-induced cognitive deficits in a rodent model (Zurek et al., 2014). Specifically, anesthetic action on astrocytes cause the persistent increase in $GABA_A$ receptor ($GABA_AR$)-mediated tonic current in hippocampal neurons that leads to postanesthetic memory loss (Zurek et al., 2014). As discussed previously, co-treatment with DEX has been shown to reverse postanesthetic memory deficits in rodents (Si et al., 2016; Qian et al., 2015). Collectively, these studies suggested that co-

treatment with DEX may also prevent the anesthetic-induced persistent increase in tonic current which is leads to postanesthetic memory deficits.

Therefore, on the basis of all evidence presented above, I tested whether:
DEX prevents anesthetic-induced persistent increase in tonic current in hippocampal neurons by targeting α2Rs in astrocytes.

4.2 Specific methods

All experiments in this chapter utilize whole-cell electrophysiological recordings of tonic current in neurons from primary cell cultures, as described in Section 3.2. In all cases, the amplitude of tonic current was revealed by the $GABA_A$ receptor antagonist bicuculline (20 μM), after exogenous GABA (0.5 μM) application to extracellular cell culture solution.

To investigate whether DEX can prevent an anesthetic-induced increase in tonic current astrocyte-neuron co-cultures were treated with dexmedetomidine (10 μM) and either the injectable anesthetic etomidate (Etom, 1 μM) or inhalational anesthetic sevoflurane (Sevo, 266 μM), or relevant controls for 1 h. Next, the co-culture media was replaced with fresh, drug free media and tonic current was recorded from neurons 24 h after the drug treatments.

For these initial experiments, the concentrations of etomidate and sevoflurane were chosen to mimic their anesthetic doses *in vitro*. However, a high *in vitro* dose concentration of DEX was used for these initial screening experiments.

It should be noted that for all remaining experiments in this chapter, anesthetic-induced tonic current was studied using the anesthetic etomidate because it is most selective for $GABA_AR$.

To test whether DEX acts on α2R to prevent an anesthetic-increase in tonic current, astrocyte-neuron cocultures were treated with etomidate (1 μM) and another potent α2R agonist clonidine (100 μM), in one case. After an hour, the culture media was replaced with fresh media and neuronal tonic current recorded 24 h later.

Next, to confirm that DEX effects on tonic current are mediated by α2R, the cocultures were treated with the common α2R antagonist yohimbine (5 μM). Thirty minutes later, those cocultures

were also treated with etomidate (1 μM) and DEX (10 μM) for an hour after which the media was replaced with fresh media. Finally, tonic current from these cocultures were recorded 24 h later.

All remaining experiments in this chapter use a lower, clinically relevant concentration of DEX since the previous experiments have established that DEX reduces anesthetic-increase in tonic current at high doses.

The following experiment investigated whether DEX targets astrocytes or neurons to reverse to anesthetic-induced increase in tonic current in neurons. Hence, for these studies, pure astrocyte cultures, and in some cases pure neuron cultures were pre-treated with the agents. This protocol was used for two reasons: (i) Previous studies from our lab demonstrated that anesthetics including etomidate target astrocytes to cause a persistent increase in tonic current (Zurek et al., 2014); and (ii) Published studies by others have suggested that DEX may target astrocytes to mediate some of its actions (Ma et al., 2005).

To test whether astrocytes are necessary for DEX effects on tonic current, pure astrocyte cultures were treated with etomidate (1 μM) and DEX (100 nM). After 1 h, the astrocyte culture media was replaced with fresh, drug-free media and the culture was incubated for 2 h. Next, conditioned media from the astrocytes (ACM) were transferred to pure neuron cultures and tonic current was recorded from these neurons 24 h later.

To test whether astrocytes were necessary for DEX reversal of anesthetic-induced tonic current, pure astrocyte cultures were treated with etomidate (1 μM); whereas pure neuron cultures were treated with DEX (100 nM). After an hour, both the astrocyte culture and hippocampal neuron culture media were replaced with fresh, drug free media. Two hours after incubation, the ACM was transferred onto the hippocampal neuron culture and the neuronal tonic current was recorded 24 h later.

To determine whether DEX acts on astrocytic α2R to prevent anesthetic-induced increase in tonic current, the next experiment tested whether another α2R clonidine action on astrocytes mimics DEX effects. In this case, pure astrocyte cultures were treated with etomidate (1 μM) and clonidine (Clo, 1 μM) for 1 h after which the culture media was replaced with drug-free media.

Two hours after incubation of astrocyte cultures, the ACM was transferred to pure neurons and tonic current from neurons were measured 24 h later.

Next, to confirm that DEX targets the α2R in astrocytes, pure astrocyte cultures were treated with yohimbine (Yoh, 1 μM) for 30 min after which, the cultures were also treated with etomidate (1 μM) and DEX (100 nM). One hour later, the astrocyte culture media was replaced with drug-free media. The ACM was transferred to hippocampal neuron cultures as described earlier and tonic current from the neurons measured 24 h later.

To determine the potency of DEX to inhibit etomidate-induced persistent increase in neuronal tonic current, pure astrocyte cultures were co-treated with etomidate (1 μM) and DEX at one of the following concentrations: 10 μM, 1 μM, 100 nM, 10 nM, 7.5 nM, 5 nM, 2.5 nM, 1 nM, or 0.1 nM). After an hour, astrocyte culture media were replaced with fresh, drug-free media and incubated for 2 h. ACM was transferred to pure hippocampal neuron cultures, as discussed earlier. Finally, tonic current from neurons were recorded 24 h later.

The numerical value of the potency, given by IC_{50} was determined using non-linear regression analysis to fit an inhibitory dose-response curve of log concentration of DEX (in nanomolar) against relative tonic current density (variable response). Here, the relative tonic current density was obtained from the ratio of tonic current density of etomidate and DEX-treated neurons over tonic current density of etomidate-treated neurons alone. It should be noted that, the tonic current density is given by the amplitude of tonic current observed (in picoamperes) divided by the size of the neuron, given by its capacitance in picofarads.

The specific equation used to plot the inhibitory dose-response curve is as follows:

$$Y = Bottom + (Top - Bottom) / (1 + 10 \wedge (\log IC_{50} - X)*Hillslope))$$

In this case, Y refers to the relative tonic current density (as discussed before). Top refers to the highest observed value of relative tonic current density, whereas bottom refers to the lowest observed value of relative tonic current density. X is the nanomolar concentration of DEX in a logarithmic scale (base 10). Lastly, Hillslope or the Hill coefficient refers to the largest absolute value of the slope of the curve.

The final study in this chapter investigated whether DEX can prevent anesthetic-induced tonic current in another neurocognitive domain of the brain, besides the hippocampus. To address this question, pure astrocyte cultures were treated with etomidate (1 μM) and dexmedetomidine (100 nM) or relevant controls. After 1 h after, the astrocyte culture media was replaced with fresh, drug-free media and the cultures were incubated for 2 h. Next, the ACM was transferred to pure cortical neuron cultures and tonic current from the cortical neurons were recorded 24 h later.

4.3 Results

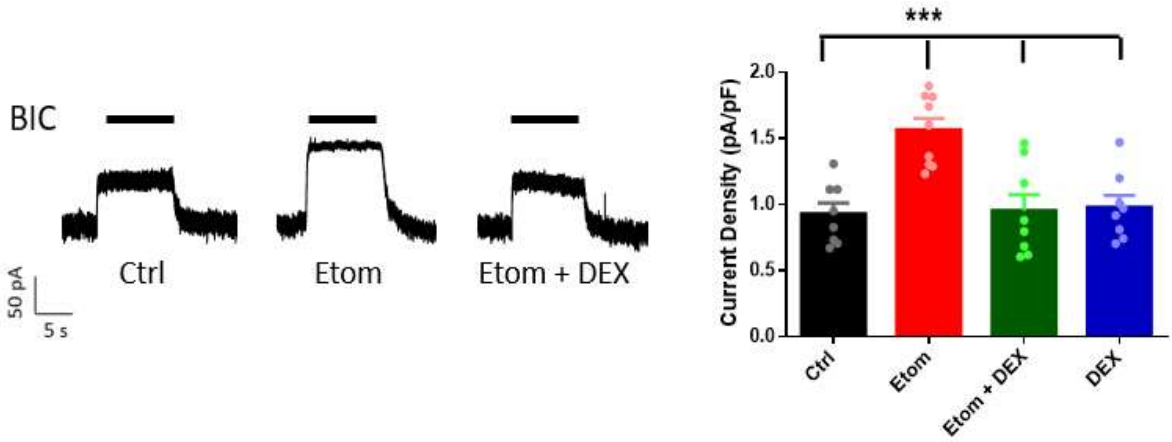
First, we investigated whether DEX can prevent a general anesthetic-induced persistent increase in tonic $GABA_AR$-mediated current in hippocampal neurons cocultured with astrocytes. To test this, we used two different general anesthetics: The injectable anesthetic etomidate was selected because it preferentially binds to $GABA_AR$; and the inhalational anesthetic sevoflurane was chosen because it is a commonly used during surgery in adults and children (Hill-Venning et al., 1997; Kodaka et al., 2004).

Treatment of astrocyte-neuron cocultures with etomidate alone increased tonic current (67.5 pA) to 150% of control (45 pA). Furthermore, co-treatment with DEX restored the tonic current to baseline levels (Figure 4B). Whereas, treatment of cocultures with DEX alone yielded tonic currents similar to that of baseline. These results demonstrate that DEX prevents etomidate-induced persistent increase in tonic current in hippocampal neurons, however, DEX itself does not alter tonic current amplitude in hippocampal neurons.

Neurons in cocultures treated with sevoflurane alone also displayed a significant and persistent increase in tonic current (125 pA; 250%), relative to control (50 pA) (Figure 4.1D). The tonic current in cocultured neurons treated with DEX and sevoflurane were similar to that of control cells. Altogether, these results show that DEX prevents sevoflurane-induced persist increase in tonic current.

A

B



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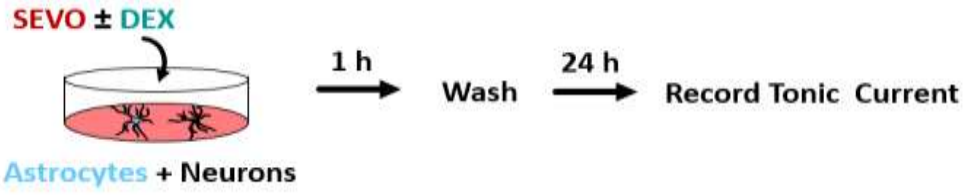

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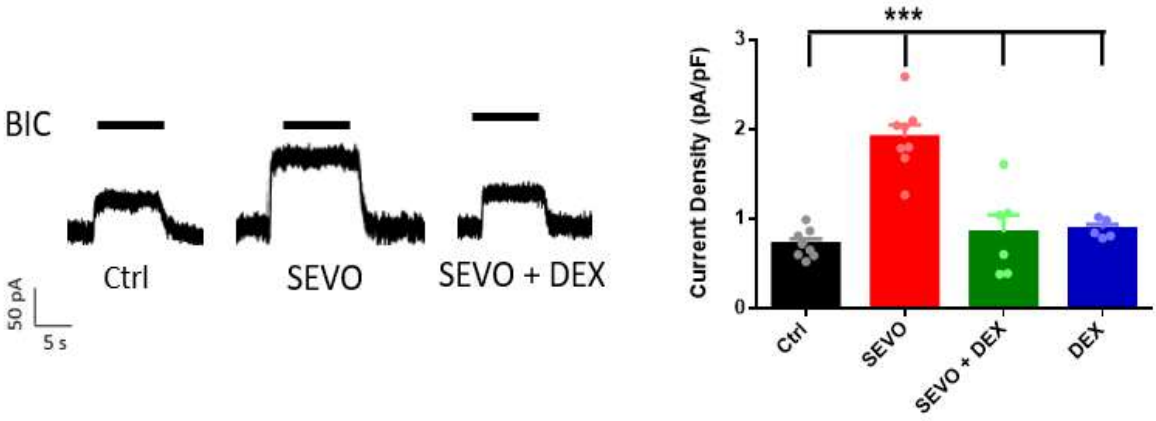

Figure 4.1: Dexmedetomidine reverses anesthetic-induced persistent increase in tonic current in hippocampal neurons cocultured with astrocytes. Neurons co-cultured with astrocytes from Swiss white mice were treated with drugs for 1 h. Twenty-four hours later, bicuculline (BIC, 20 μM) was applied to measure tonic current in neurons. **(A)** Astrocyte-neuron cocultures were treated with etomidate (Etom, 1 μM) + dexmedetomidine (DEX, 10 μM) or relevant controls. **(B)** Representative traces and bar graphs show that treatment with etomidate increases tonic current whereas co-treatment with DEX prevents etomidate-induced increase in tonic current ($n = 7\text{-}9$; *** $p < 0.001$). **(C)** Astrocyte-neuron cocultures were treated with sevoflurane (SEVO, 266 μM) + dexmedetomidine (DEX, 10 μM) or relevant controls for 1 h. **(D)** Representative traces and bar graphs show that treatment with sevoflurane increased the tonic current whereas co-treatment with DEX prevented the tonic current increase ($n = 6$; *** $p < 0.001$). All data are mean ± SEM.

Most of the clinical actions as well as some neuroprotective function of DEX are mediated by α2R (Giovannitti et al., 2015; Naaz & Ozair, 2014). Therefore, the next studies investigated whether DEX acts on α2R to prevent anesthetic-induced persistent increase in tonic current in hippocampal neurons.

To test whether α2Rs are involved, we tested whether another potent, but less selective α2R agonist clonidine mimicked DEX effects on tonic current. Etomidate alone significantly increased neuronal tonic current in astrocyte-neuron cocultures; whereas co-treatment with clonidine restored neuronal tonic current to baseline levels (Figure 4.2B). In addition, neurons in the cocultures treated with clonidine alone had similar tonic current densities as baseline. These data show that another the α2R agonist clonidine mimics DEX effects on tonic current in neurons cocultured with astrocytes.

To confirm that DEX acts through α2Rs, we studied whether pharmacologic inhibition of α2Rs by the α2R antagonist yohimbine blocked DEX reversal of anesthetic-induced increase in tonic current. When astrocyte-neuron cocultures were treated with yohimbine, etomidate, and DEX, tonic current in neurons remained elevated above that of control cells, similar to cocultures treated with etomidate alone (Figure 4.2D). However, neuronal tonic current in cocultures treated with yohimbine alone were similar to that of control cells. Hence, DEX fails to prevent etomidate-induced persistent increase in tonic current in the presence of the α2R antagonist, yohimbine. This confirms that DEX acts through α2Rs to reverse anesthetic-induced persistent increase in tonic current in hippocampal neurons.

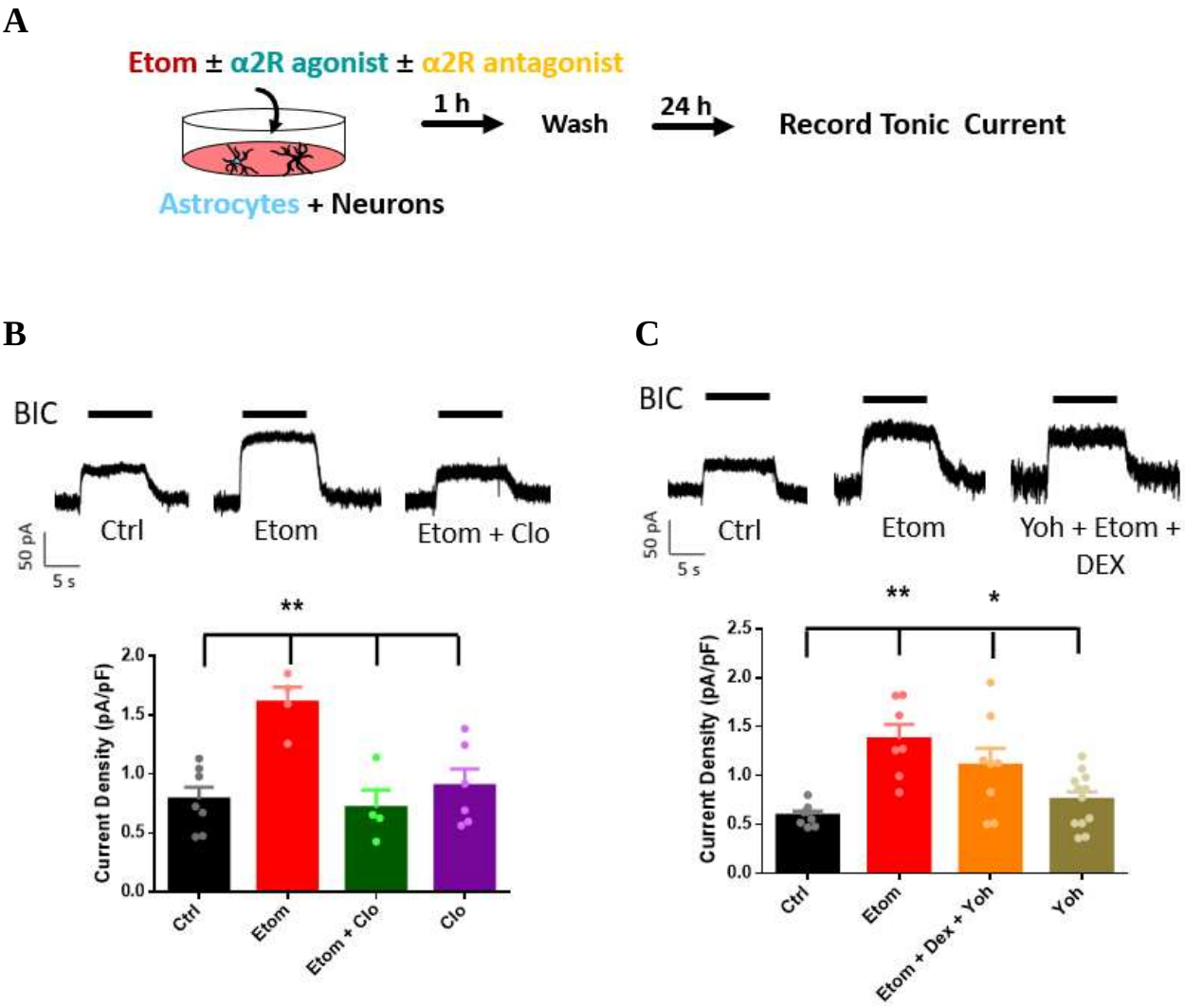

Figure 4.2: Dexmedetomidine acts on α2-adrenergic receptors (α2Rs) to prevent anesthetic-induced increase in tonic current in hippocampal neurons. **(A)** Neurons co-cultured with astrocytes from Swiss white mice were either treated with: etomidate (Etom, 1 μM) and the α2R agonist, clonidine (Clo, 100 μM) and relevant controls; or, etomidate (Etom, 1 μM), dexmedetomidine (DEX, 10 μM), and the α2R antagonist yohimbine (Yoh, 5 μM). For both treatment protocols, the cocultures were treated with the drugs for 1 h and tonic current was measured 24 h after treatment. **(B)** Representative traces and bar graphs show that clonidine prevents etomidate-induced increase in tonic current, thereby mimicking DEX effects (n = 6; ** $p < 0.01$). **(C)** Representative traces and bar graphs demonstrate that the α2R antagonist, yohimbine,

inhibits DEX reversal of etomidate-induced increase in tonic current (n = 9; * $p < 0.05$, ** $p < 0.01$). All data are mean ± SEM.

Our previous studies have established that general anesthetics, including etomidate, target astrocytes to mediate the persistent increase in tonic current in hippocampal neurons (Zurek et al., 2014). Since DEX prevents etomidate-increase in neuronal tonic current in astrocyte-neuron cocultures, the next outstanding question was whether DEX targets astrocytes or neurons to mediate this effect.

DEX is known to target neurons in the central nervous system (CNS) to mediate its clinical actions (Khan et al., 1999). However, DEX has also been shown to act on astrocytes to modulate neuronal survival and function, particularly in *in vitro* models of hypoxic-ischemic injury (Rodriguez-Gonzalez et al., 2015; Zhang et al., 2013).

Therefore, we first investigated whether DEX action on astrocytes alone could prevent the anesthetic-induced persistent increase in tonic current in hippocampal neurons. Hence, for these studies, astrocytes were treated with DEX and the anesthetic etomidate and conditioned media from astrocytes (ACM) were applied to hippocampal neurons before measurement of the tonic current. Hippocampal neurons that were treated with etomidate-treated ACM showed a persistent and significant increase in tonic current, compared to controls (Figure 4.3B). However, hippocampal neurons that received etomidate and DEX-treated ACM displayed tonic current similar to baseline. In addition, neurons that received DEX-treated ACM also had similar tonic current amplitude as control. Collectively, these results suggest that DEX action on astrocyte alone is sufficient to restore etomidate-induced persistent increase in tonic current to baseline levels.

The above results prompted us to investigate whether DEX action on astrocytes was necessary to prevent etomidate-induced persistent increase in neuronal tonic current. To test this, astrocyte cultures were treated with etomidate and hippocampal neurons were treated with DEX. Next, etomidate-treated ACM was applied to the DEX-treated hippocampal neuron cultures before the tonic current was recorded from the neurons. In neurons that received direct DEX treatment as well as etomidate-treated ACM, the tonic current remained elevated above baseline, similar to neurons that received etomidate-ACM alone. Hence, DEX fails to prevent etomidate-increase in tonic current when applied to neurons. Furthermore, neurons that received DEX treatment alone had tonic current amplitude similar to baseline. This indicated that DEX by itself does not alter the amplitude of tonic current in hippocampal neurons. Taken together, these data demonstrate that

astrocytes are necessary for DEX reversal of etomidate-induced persistent increase in tonic current in hippocampal neurons.

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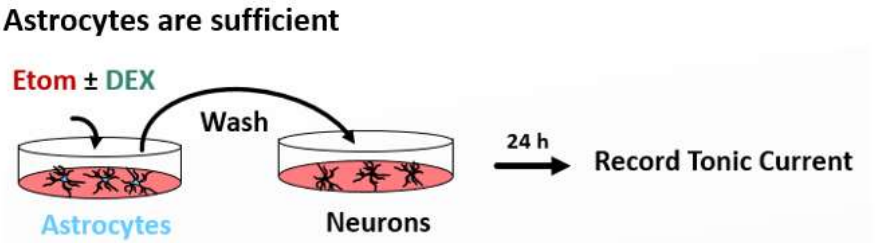

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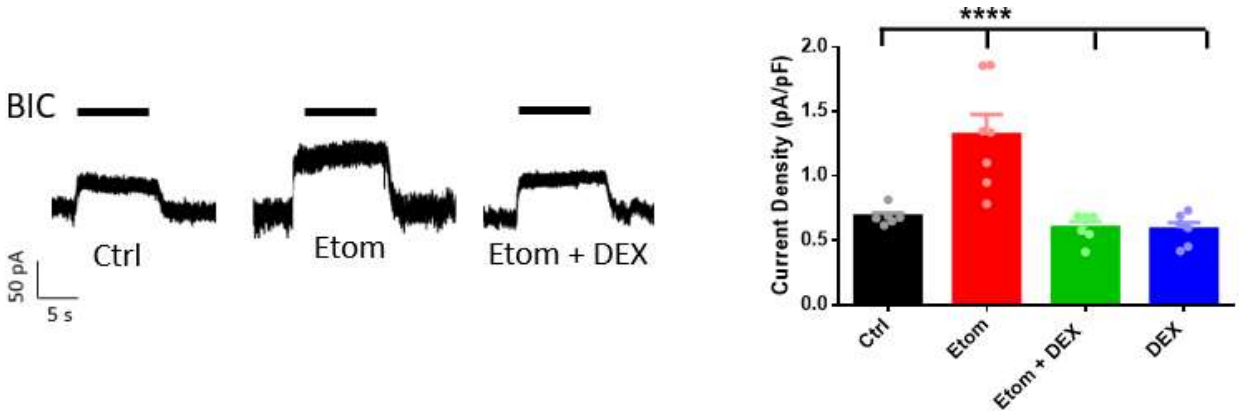

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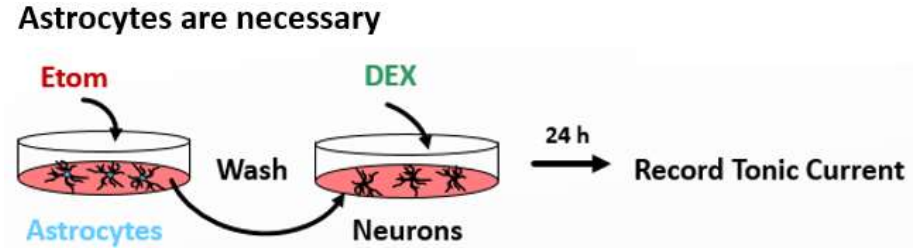

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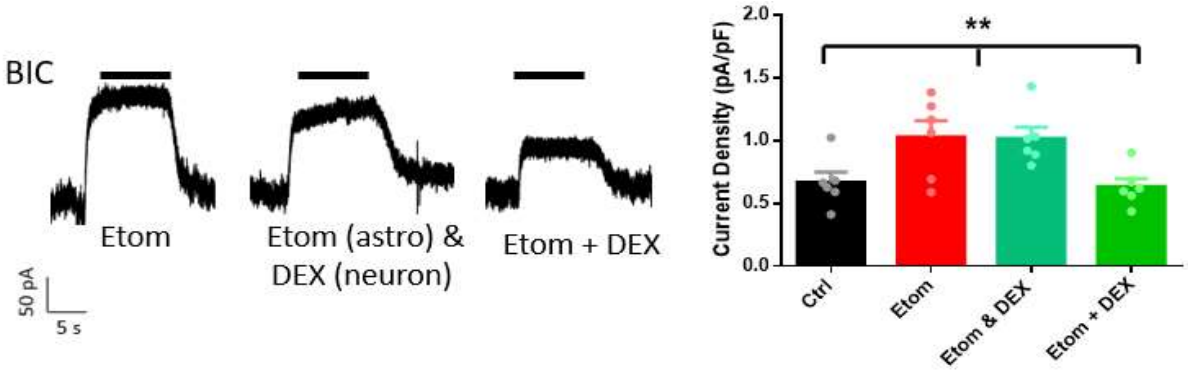

Figure 4.3: Astrocytes are sufficient and necessary for DEX-reversal of etomidate-induced persistent increase in tonic current. (A) Pure astrocyte cultures were treated with etomidate (Etom, 1 μM) and dexmedetomidine (DEX, 100 nM). An hour later, astrocyte conditioned medium (ACM) from drug-treated astrocytes were transferred to pure hippocampal neuron cultures. Tonic current hippocampal neurons, were revealed by application of bicuculline, BIC, 24 h later. **(B)** Representative traces and bar graphs show that etomidate action on astrocyte alone increased tonic current. Whereas, co-treatment with DEX on astrocyte culture was also sufficient to prevent the increase in neuronal tonic current. ($n = 6$-7; **** $p < 0.0001$). All data are expressed as mean ± SEM.

(C) Pure astrocyte cultures were treated with etomidate (Etom, 1 μM) and pure neuron cultures were treated with dexmedetomidine (DEX, 100 nM) or relevant controls for 1 h. As an additional control, pure astrocyte cultures were also treated with etomidate (Etom, 1 μM) and dexmedetomidine (DEX, 100 nM) for 1 h. Next, astrocyte conditioned medium (ACM) from drug-treated astrocytes were transferred to hippocampal pure neuron cultures and tonic current was recorded from the neuronal cultures 24 h later. **(D)** When neurons are treated with DEX instead of astrocytes, DEX fails to prevent the increase in tonic current, as depicted by the representative traces and bar graph. ($n = 6$, ** $p < 0.01$). All data are expressed as mean ± SEM.

Studies in this chapter have determined that DEX targets α2R as well as astrocytes to prevent anesthetic-induced persistent increase in tonic current in hippocampal neurons. Therefore, the next important question was whether DEX acts on astrocytic α2R to reverse the anesthetic-induced persistent increase in neuronal tonic current.

To address this question, we first tested whether another potent α2R agonist, clonidine, also targets astrocytes to prevent anesthetic-increase in tonic current in hippocampal neurons. In this case, astrocyte cultures were treated with etomidate and clonidine and ACM from these cultures were transferred to hippocampal neurons before tonic current was recorded from the neurons. The amplitudes of tonic current in neurons treated with etomidate and clonidine were similar to that of baseline; whereas, the amplitudes of tonic current in neurons treated with etomidate alone were significantly above baseline (Figure 4.4B). These results show that, like DEX, clonidine action on astrocytes is sufficient to prevent etomidate-induced persistent increase in tonic current in hippocampal neurons. Furthermore, these data imply the involvement of astrocytic α2R in DESX reversal of tonic current.

To confirm that DEX targets astrocytic α2R, we investigated whether pharmacologic inhibition of astrocytic α2R by the antagonist yohimbine prevents DEX effects on anesthetic-induced tonic current in neurons. Hence, astrocyte cultures were treated with the α2R antagonist yohimbine, etomidate, and DEX and ACM from these cultures were transferred to hippocampal neurons before tonic current was recorded. Hippocampal neurons co-treated with yohimbine had significantly higher tonic current amplitudes compared to that in neurons treated with etomidate and DEX only or baseline controls (Figure 4.4D). These results show that inhibition of α2R in astrocytes blocks DEX-reversal of etomidate-induced increase in neuronal tonic current. Hence, these studies confirm that DEX acts on astrocytic α2R to prevent anesthetic-induced persistent increase in tonic current in hippocampal neurons.

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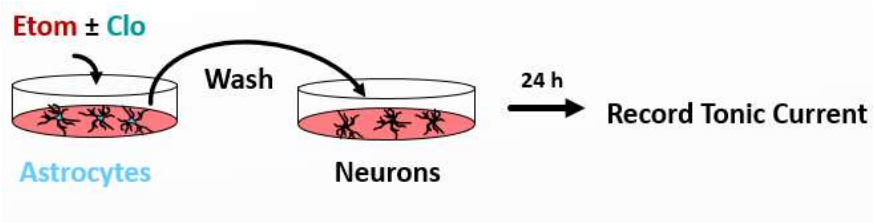

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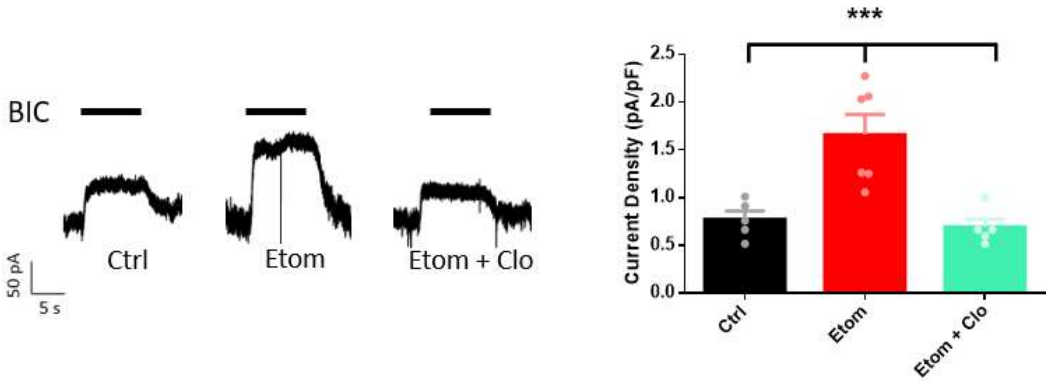

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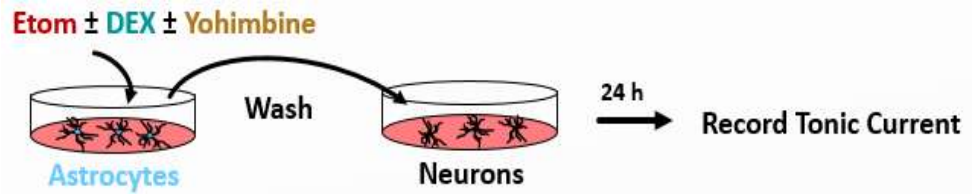

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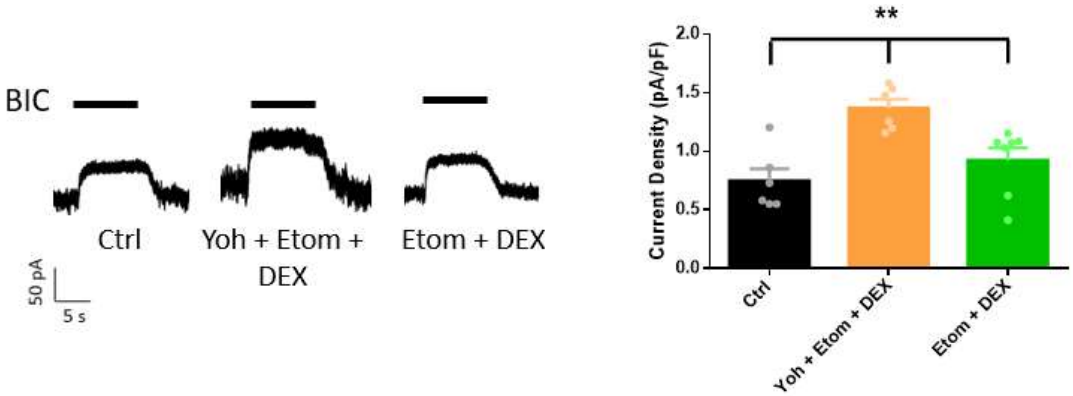


Figure 4.4: Dexmedetomidine acts on astrocytic α2-adrenergic receptors (α2Rs) to prevent anesthetic-induced persistent increase in tonic current in hippocampal neurons (A) To test whether another α2R agonist clonidine also targets astrocytes to mimic DEX effects, pure astrocyte cultures were treated with etomidate (1 μM) and clonidine (Clo, 1 μM). An hour later, astrocyte conditioned medium (ACM) were transferred to pure neuron cultures and tonic current from the neurons 24 h later. **(B)** Representative traces and bar graphs show that co-treatment of astrocytes with clonidine prevents etomidate-induced persistent increase in neuronal tonic current ($n = 5 - 6$; ** $p < 0.01$). All data are mean ± SEM.

(C) To confirm that DEX targets astrocytic α2R, pure astrocyte cultures were treated with etomidate (Etom, 1 μM), DEX (100 nM), and the α2R antagonist, yohimbine (Yoh, 1 μM) or relevant controls. One hour later, ACM were transferred to pure neuron cultures and tonic current from the neurons 24 h later. **(D)** In the presence of the α2R antagonist yohimbine, DEX fails to prevent etomidate-induced persistent increase in neuronal tonic current, as shown by the representative traces and bar graphs ($n = 6$; ** $p < 0.01$). All data are mean ± SEM.

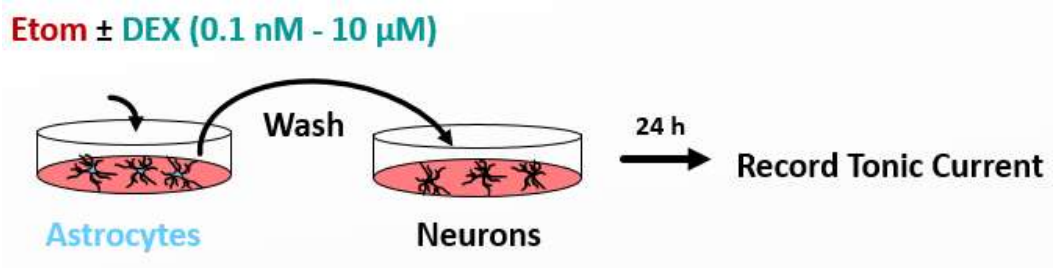
The results so far have established that DEX, via astrocytic α2R, prevents an anesthetic-induced persistent increase in tonic current in hippocampal neurons. Therefore, our next objective was to determine the potency of DEX to mediate this effect.

For these studies, the potency of DEX was quantified by measuring the concentration of DEX that is required to inhibit 50% of the etomidate-induced increase in tonic current in hippocampal neurons (i.e., IC_{50} of DEX). To achieve this aim, astrocyte cultures were treated with various concentrations of DEX but the same concentration of etomidate. As before, ACM from drug-treated astrocytes were transferred to hippocampal neuron cultures before tonic current was recorded from these neurons.

A wide range of concentrations of DEX (10 nM to 10 μM) completely reversed etomidate-induced increase in tonic current in hippocampal neurons (Figure 4.5B). DEX, at 5 and 7.5 nM partially inhibited etomidate-increase in neuronal tonic current. At 2.5 nM or lower concentrations of DEX, tonic current in neurons remained elevated just as in neurons treated with etomidate alone.

Next, the inhibitory dose-response curve of log concentration of DEX versus the amplitude of the tonic current density was constructed, as described in Section 4.2. The dose-response curve was used to calculate the potency or IC_{50} of DEX and the calculations were determined using the GraphPad 5.0 software (details specified in Specific Methods, Section 4.2). The potency (IC_{50}) of DEX to prevent etomidate-induced persistent increase in tonic current in hippocampal neurons was 4.6 nM, as shown in Figure 4.5C.

A



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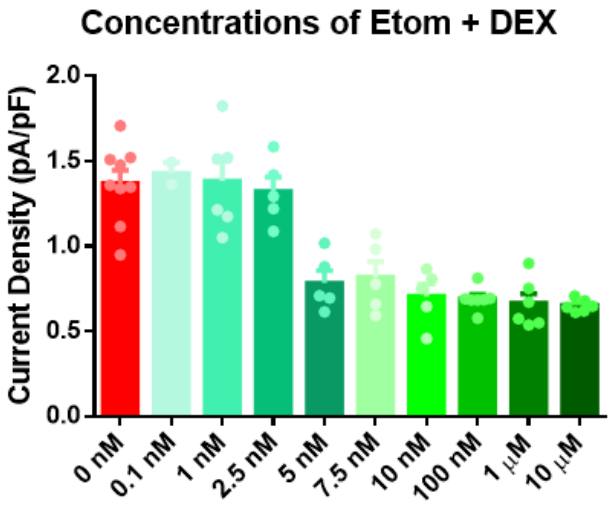

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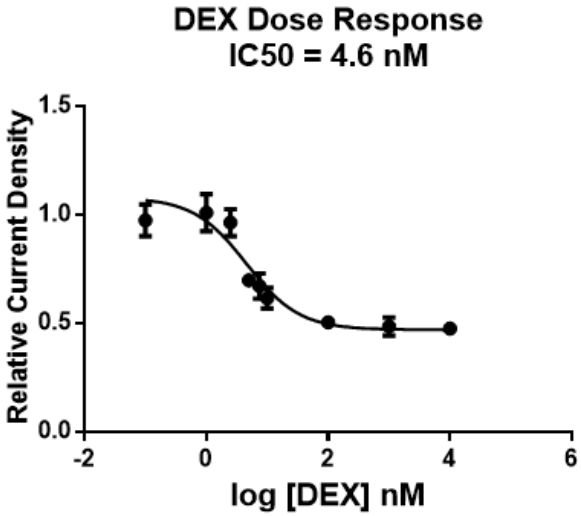

Figure 4.5: The half-maximal inhibitory concentration (IC_{50}) of DEX to prevent etomidate-induced increase in tonic current in hippocampal is 4.6 nM. (A) Pure astrocyte cultures were treated with etomidate (Etom, 1 µM) and dexmedetomidine (DEX, 1 nM - 10 µM) for 1 h. Next, astrocyte conditioned medium (ACM) from the drug-treated astrocytes were transferred to pure neuron cultures and tonic current from the neurons were recorded 24 h later. **(B)** Etomidate action on astrocyte alone increased the tonic current. Co-treatment with DEX inhibited etomidate-induced increase in neuronal tonic current from 10 µM down to 5 nM. However, lower concentrations of DEX (2.5, 1, and 0.1 nM) completely failed to inhibit etomidate-effect. (n = 5 - 7; ** $p < 0.01$). All data are expressed as ± SEM. **(C)** The dose-response curve of log concentration of DEX against relative tonic current density was plotted to determine the IC_{50} value.

Our previous studies have linked anesthetic-induced $GABA_AR$-tonic current to postanesthetic cognitive deficits. Hence, we were interested in studying the hippocampus and cerebral cortex, which are cognitive domains responsible for memory and executive function, respectively. So far, the studies in this chapter have investigated DEX reversal of anesthetic-induced increase in $GABA_AR$-tonic current in hippocampal neurons. However, $GABA_AR$ also mediate tonic current in other areas of the brain such as the cerebral cortex (Farrant & Nusser, 2005). Therefore, we investigated whether DEX prevents anesthetic-induced persistent increase in tonic current in neurons in the cerebral cortex.

As in previous studies, astrocyte cultures were treated with etomidate and DEX for an hour. However, for these experiments, drug-treated ACM was applied to primary cortical neuron cultures and tonic current was recorded from these cortical neurons 24 h later. Cortical neurons containing ACM from etomidate-treated astrocytes had significantly higher tonic current amplitudes, compared to baseline. However, cortical neurons with ACM from etomidate and DEX-treated astrocytes had tonic current amplitudes similar to baseline. In addition, cortical neurons with ACM from DEX-treated astrocytes also displayed tonic current similar to baseline control. Taken together, these results show that etomidate alone increases tonic current in cortical neurons. Whereas, co-treatment with DEX prevents etomidate-induced persistent increase in tonic current in cortical neurons.

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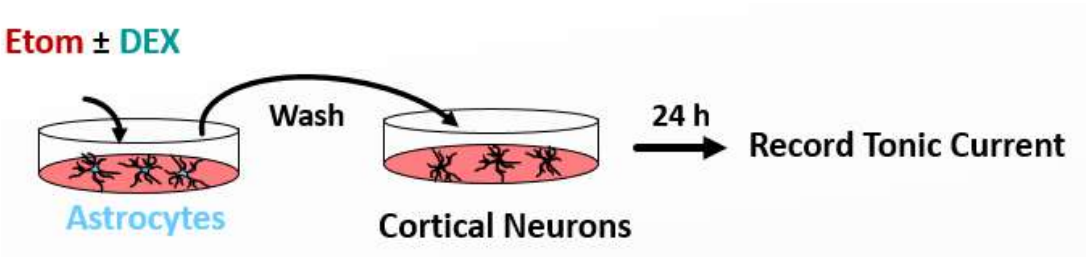

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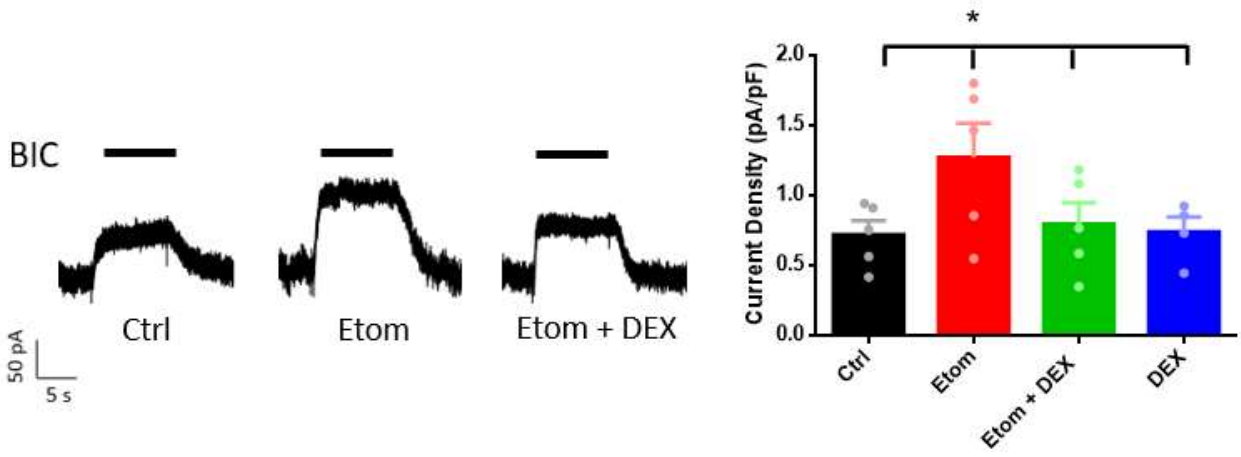


Figure 4.6: Dexmedetomidine reverses etomidate-induced persistent increase in tonic current in cortical neurons. **(A)** Pure astrocyte cultures were treated with etomidate (Etom, 1 μM) and dexmedetomidine (DEX, 100 nM) or relevant controls for 1 h. Next, astrocyte conditioned medium (ACM) from the drug-treated astrocytes were transferred to pure neuron cultures and tonic current was measured from the neurons 24 h later. **(B)** Representative traces and bar graphs show that treatment with etomidate alone caused significant increase in tonic current in cortical neurons. Furthermore, co-treatment with DEX prevented etomidate-induced increase in tonic current in cortical neurons ($n = 6$; $p < 0.05$). All data are mean ± SEM.

4.4 Discussion

Our studies are the first to show that DEX prevents an anesthetic-induced persistent increase in tonic inhibitory current in neurons in the hippocampus by targeting astrocytic α2-adrenergic receptors (α2Rs). Furthermore, DEX prevents the anesthetic etomidate-induced persistent increase in tonic current in hippocampal neurons with a high potency (4.6 nM), which is similar to its potency for α2R-mediated action (Virtanen et al., 1985). In addition, prevention of an anesthetic-induced persistent increase in tonic inhibitory current by DEX extends to other cognitive domains besides the hippocampus, such as the cerebral cortex. As discussed in Section 2.3, anesthetic-induced persistent increase in $GABA_AR$-mediated tonic current in the hippocampus causes postanesthetic memory impairments in rodents (Zurek et al., 2014). Therefore, these results suggest that DEX attenuates hippocampus-dependent and possibly, cerebral cortex-dependent cognitive impairment due to anesthetics, by inhibiting anesthetic-induced persistent increase in tonic $GABA_AR$-current in these cognitive domains.

Interestingly, patients who experience postoperative delirium display impairments in memory and executive function, which are cognitive functions, regulated by the hippocampus and cerebral cortex, respectively (Inouye, 2005; Monk & Price, 2011). Therefore, it is possible that DEX-reversal of tonic current in the hippocampus and cerebral cortex help reduce postoperative deficits in memory and executive function that are attributable to postoperative delirium. However, addressing this very important question is outside the scope of this thesis and so the studies here focus the mechanisms of DEX-mediated neuroprotection from anesthetics *in vitro*.

In neurons of the hippocampus, DEX prevents the increase in tonic $GABA_AR$-current mediated by the $GABA_AR$-selective, injectable anesthetic etomidate and the commonly used inhalational anesthetic sevoflurane (Krasowski & Harrison, 1999; Mandal et al., 2009). This suggests that DEX protects the hippocampus from various anesthetics that persistently increase tonic $GABA_AR$-current and ultimately cause postanesthetic memory deficits.

Furthermore, our findings indicate that DEX acts on α2Rs to mediate neuroprotection from anesthetic-induced persistent increase tonic current. Specifically, DEX effects on tonic current are mimicked by another potent α2R agonist, clonidine (Giovannitti et al., 2015); whereas, DEX

effects are abolished by the α2R antagonist yohimbine. These results align with published studies that investigated the mechanism of DEX-neuroprotection from apoptosis in other models of neuronal injury, as discussed in Sections 2.6 and 4.1 (Sanders et al., 2009; Laudenbach et al., 2002; Zhang et al., 2013). Taken together, these results suggest that α2Rs are crucial for DEX-mediated neuroprotection in various forms of injury or impairment.

In addition, our results show that astrocytes are sufficient and necessary for DEX to mediate neuroprotection from anesthetic-induced persistent increase in tonic current. Specifically, treatment of hippocampal neurons with conditioned media from DEX-treated astrocyte cultures was sufficient to prevent anesthetic-induced persistent increase in tonic current. Furthermore, DEX failed to prevent anesthetic-increase in tonic current when neurons, but not astrocytes, were treated with DEX. Studies *in vitro* models of hypoxic-ischemic injury have also shown that conditioned media from DEX-treated astrocyte cultures improve neuronal survival after oxidative damage, as discussed previously in Section 2.6 (Zhang et al., 2013).

However, to the best of our knowledge, no other studies, except ours, have shown that DEX targets astrocytic α2Rs to mediate neuroprotection. In this case, pharmacologic inhibition of α2Rs in astrocyte cultures, by treatment with the α2R antagonist yohimbine, prevented DEX reversal of anesthetic-induced persistent increase in neuronal tonic current. Furthermore, conditioned media from astrocyte cultures treated with low, clinically relevant *in vitro* concentrations of DEX completely restored the anesthetic-induced increase in tonic current (dose-response curve, Figure 4.5). Interestingly, the potency or IC_{50} of DEX to inhibit anesthetic-effect on tonic current (4.6 nM) is close to the potency of DEX for α2Rs (1.0 nM) (Virtanen et al., 1988). Hence, at low, clinically relevant doses, DEX, via astrocytic α2Rs, prevents the anesthetic-induced persistent and harmful increase in tonic inhibitory current in hippocampal neurons. These results imply that a low, sedative dose of DEX may prevent the anesthetic-induced persistent increase in hippocampal tonic current which leads to postanesthetic memory deficits (Zurek et al., 2014).

In summary, these studies demonstrate that DEX, via astrocytic α2Rs, prevents the anesthetic-induced persistent increase in tonic current in hippocampal neurons that results in postanesthetic memory deficits. Additional studies are necessary to determine the molecular mechanisms by which DEX protects neurons from the anesthetic-induced persistent increase in tonic current.

5. Dexmedetomidine stimulates astrocytes to release neuroprotective factors that prevent anesthetic-induced persistent increase in neuronal tonic current

5.1 Introduction

Stimulation of astrocytic α2-adrenergic receptors (α2Rs) by dexmedetomidine (DEX) activates various $G_{i/o}$ protein-coupled signaling pathways. The activation of $G_{i/o}$ protein, in turn, may inhibit the c-Jun N-terminal kinase (JNK) pathway and reduce gene expression of proinflammatory cytokines (Chen et al., 2015; Zhang et al., 2015). Simultaneously, DEX-stimulated $G_{i/o}$ protein may activate the extracellular-signal regulated kinase (ERK) pathway and increase the expression of multiple genes including neurotrophic and growth factors in astrocytes (Peng et al., 2003; Degos et al., 2013). This suggests that DEX may stimulate astrocytic α2Rs to activate or inhibit multiple signaling pathways in parallel and affect various physiologic functions at the same time.

Increasing evidence suggests that DEX mediates neuroprotection by stimulating astrocytic α2Rs and either enhancing the gene and protein expressions of neurotrophic and growth factors, or suppressing the expressions of proinflammatory cytokines (Trendelenburg et al., 2005). Previous studies from our lab have suggested that the anesthetic etomidate releases harmful factors from astrocytes which mediate the persistent increase in neuronal tonic current. Therefore, the studies in this chapter investigate whether DEX reduces the release of etomidate-stimulated harmful factor(s) or promotes the release of neuroprotective factors from astrocytes.

Interestingly, unpublished results from our lab have shown that DEX enhances mRNA expressions of brain-derived neurotrophic factor (BDNF) and epidermal growth factor (EGF) in astrocytes. Further, conditioned media from DEX-treated astrocyte cultures had significantly higher concentrations of BDNF and EGF, compared to that of controls. Importantly, published studies in other injury models, including hypoxic-ischemic injury and glutamate-induced excitotoxicity, have demonstrated DEX-stimulated release of BDNF and EGF to protect hippocampal and cortical neurons from apoptosis (Paris et al., 2006; Dahmani et al., 2005).

For instance, one study demonstrated DEX to increase the release of BDNF from cortical astrocytes of neonatal mice exposed to excitotoxicity via intraperitoneal ibotenate (glutamatergic agonist) injection (Degos et al., 2013). Furthermore, inhibition of BDNF signaling, via BDNF antibody, prevented DEX-mediated reduction of cortical lesions in the mice subjected to excitotoxicity (Degos et al., 2013). Overall, these results indicate that BDNF plays a key role in DEX-mediated reduction of cerebral cortical lesions following glutamate-induced excitotoxicity.

In another study using rodent models, conditioned media from DEX-treated astrocytes increased viability of cerebellar neuron cultures exposed to hydrogen peroxide (H_2O_2), suggesting that DEX-treated media contained neuroprotective factors (Zhang et al., 2013). Indeed, ELISA analysis of the DEX-treated conditioned media revealed that DEX-stimulated astrocytes released heparin-bound epidermal growth factor (HB-EGF), which is known to promote neuronal survival and differentiation (Chao et al., 2003; Zhang et al., 2013). Furthermore, pharmacologic inhibition of EGF neuronal receptor abolished DEX-mediated reduction in H_2O_2-induced neuronal death (Zhang et al., 2013). Collectively, these results suggest that EGF is a key neuroprotective factor in DEX-mediated neuroprotection from H_2O_2-induced necrosis.

On the basis of the evidence presented above, I investigated whether:
DEX stimulates astrocytes to release neuroprotective factors, possibly including BDNF and EGF, which prevent anesthetic-induced persistent increase in tonic current in hippocampal neurons.

5.2 Specific methods

All experiments in this chapter utilized whole-cell electrophysiological recordings of tonic current from neurons in primary hippocampal neuron cultures, as described in Section 3.2. For all experiments, the amplitude of tonic current was revealed using the $GABA_AR$ antagonist bicuculline (20 μM), after exogenous GABA (0.5 μM) application to extracellular cell culture solution.

To investigate whether DEX stimulates astrocytes to release factors that help prevent anesthetic-induced persistent increase in neuronal tonic current, two separate experiments were performed:

For the first experiment, two separate astrocyte cultures were treated with relevant drugs: Specifically, one astrocyte culture was treated with etomidate (1 μM), whereas the other astrocyte culture was treated with DEX (100 nM) for 1 h. Next, conditioned media in both astrocyte cultures were replaced with fresh, drug-free media. Two hours after incubation, astrocyte-conditioned media (ACM) from both astrocyte cultures were transferred to a pure hippocampal neuron culture. Tonic current from neurons in the hippocampal culture were recorded 24 h later.

For the next experiment investigating DEX-induced release of factors from astrocytes, two separate astrocyte cultures were treated with drugs again, with one major exception. One astrocyte culture was treated with etomidate (Etom, 1 μM) and the other astrocyte culture was treated with DEX (100 nM) for 1 hour. Media from both astrocyte cultures were replaced with fresh, drug-free media, and incubated for 2 h as before. However, next, conditioned media from DEX-treated astrocyte culture was heated at 99.9°C for 5 min before media before transfer of conditioned media to pure hippocampal neuron cultures. Tonic current from neurons in the hippocampal neuron cultures was recorded 24 h later.

Only conditioned media from DEX-treated astrocyte cultures were heated in order to determine whether heat prevents the reversal of tonic current by these DEX-stimulated factors. If application of heat prevented the action of these factors on tonic current, then it would demonstrate that these factors are proteins. We investigated whether these factors were proteins because DEX has been shown to trigger the release of signalling proteins such as neurotrophic and growth factors from astrocytes (Li et al., 2008; Rodriguez-Gonzalez, 2015). Furthermore, the neurotrophic and growth have been shown to modulate various functions in neurons, including activity of ionotropic receptors (Grabenstatter et al., 2012).

Hence, we next investigated whether brain-derived neurotrophic factor (BDNF) and epidermal growth factor (EGF) were DEX-stimulated factors that help prevent anesthetic-induced persistent increase in tonic current. Specifically, BDNF and EGF were chosen because mechanistic studies in other models, e.g. hypoxic-ischemic injury, have shown these factors to contribute to DEX-mediated neuroprotection from apoptosis.

To determine whether BDNF and/or EGF are DEX-stimulated factors which protect neurons from persistent increase in tonic current, two different experiments were conducted, as discussed below.

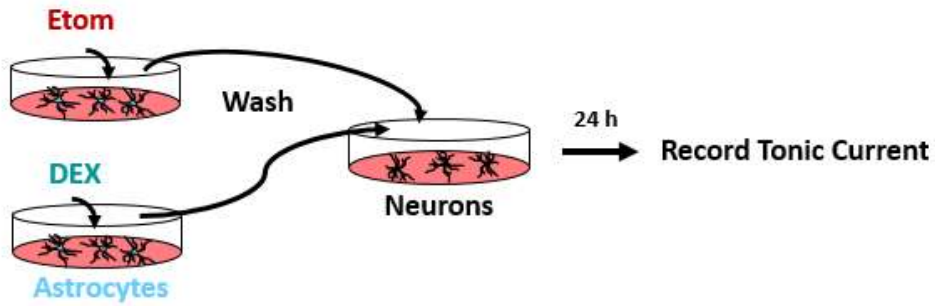
To investigate whether BDNF and EGF can mimic DEX-reversal of anesthetic-induced persistent increase in tonic current, first, astrocyte cultures were treated with etomidate (1 μM) for 1 h after, as before. Two hours after incubation, either BDNF (200 pg/mL) or EGF (10 ng/mL) was added to the etomidate-treated astrocyte cultures. Next, conditioned media from these cultures were transferred onto pure hippocampal neuron cultures. Tonic current from neurons in the hippocampal cultures were recorded 24 h later.

To confirm the role of these factors in DEX-reversal of tonic current, inhibitors of BDNF and EGF neuronal receptors were used. Specifically, astrocyte cultures were treated with etomidate (1 μM) and DEX (100 nM) for 1 h, after which the culture media was replaced as before. Next, pure hippocampal neuron cultures were treated with either the BDNF neuronal receptor antagonist ANA 12 (2 μg/mL); or, the EGF tyrosine kinase receptor inhibitor Ag1478 (1 μM). An hour later, conditioned media from the etomidate and DEX-treated astrocyte cultures were transferred onto the inhibitor-treated (either ANA12 or Ag1478) hippocampal neuron cultures. Tonic current from neurons in these hippocampal cultures were recorded 24 h later.

5.3 Results

As expected from previous studies, tonic current in hippocampal neurons that receive conditioned media from (etomidate + DEX)-treated astrocytes are similar to baseline (Figure 5.1B). Furthermore, hippocampal neurons that separately receive conditioned media from DEX-treated astrocytes as well as etomidate-treated astrocytes also display baseline levels of tonic current. Therefore, both co-treatment and separate treatment of DEX on neurons can prevent etomidate-induced persistent increase in tonic current. These data suggest that DEX stimulates astrocytes to release factor(s) that prevent anesthetic-induced persistent increase in tonic current in hippocampal neurons.

A



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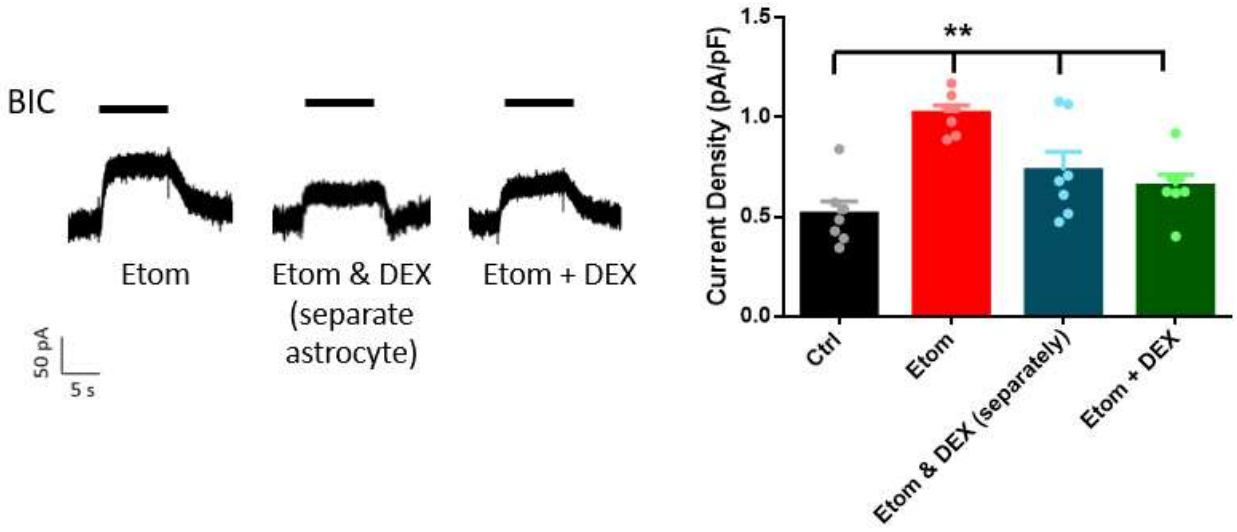

Figure 5.1: Dexmedetomidine stimulates astrocytes to release factor(s) which prevent anesthetic-induced persistent increase in neuronal tonic current. (A) Two pure astrocyte cultures were separately treated with drugs for an hour: one culture with etomidate (Etom, 1 μM) and the other culture with dexmedetomidine (DEX, 100 nM), or relevant controls. As a control (not shown in the diagram), pure astrocyte cultures were co-treated with etomidate (Etom, 1 μM) and dexmedetomidine (DEX, 100 nM) for 1 h. For all cultures, conditioned medium from drug-treated astrocytes were transferred to pure hippocampal neuron cultures and tonic current was recorded from neurons 24 h later. **(B)** DEX reverses etomidate-induced persistent increase in tonic current when treated separately from etomidate, just as in the co-treatment (Etom + DEX)

condition. This suggests that DEX triggers astrocytes to release factor(s) that prevent anesthetic-induced persistent increase in tonic current ($n = 7$, ** $p < 0.01$). All data are mean ± SEM.

DEX has been shown to trigger astrocytes to release proteins which are signalling molecules that trigger a series of downstream signalling cascades and modulate neuronal function (Li et al., 2008; Peng et al., 2003). Furthermore, DEX-stimulated release of such proteins from astrocytes has been shown to mediate neuroprotection in other models, such as hypoxic-ischemic injury (Zhang et al., 2013). Therefore, the next studies tested whether the factor(s) released by DEX-treated astrocytes, in our model, are proteins that protect neurons from anesthetic-induced persistent increase in tonic current.

Since proteins are deactivated by heat, we investigated whether DEX-treated astrocyte conditioned media fail to prevent anesthetic-increase in tonic current in hippocampal neurons, when exposed to heat (99.9°C for 5 min). Hippocampal neurons treated with heated conditioned media from DEX-treated astrocytes had significantly higher tonic current amplitudes, relative to baseline (Figure 5.2B). Whereas, hippocampal neurons with unheated conditioned media from DEX-treated astrocytes displayed tonic current amplitudes similar to baseline. Hence, these results show that the factors released by DEX stimulation on astrocytes are proteins.

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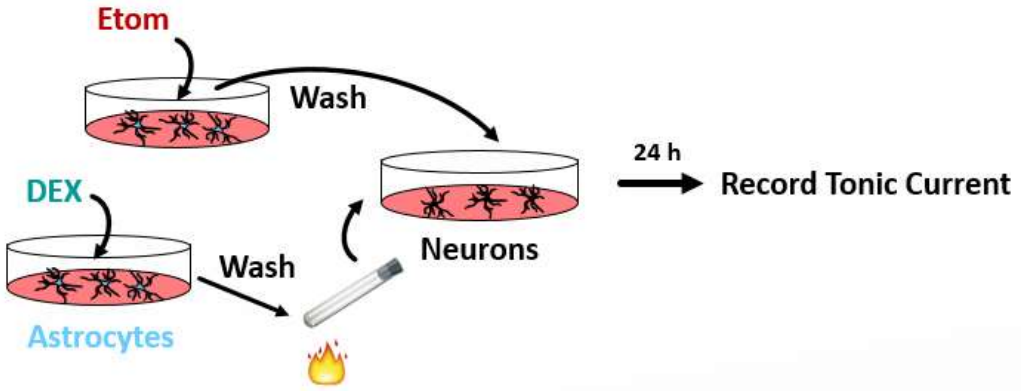

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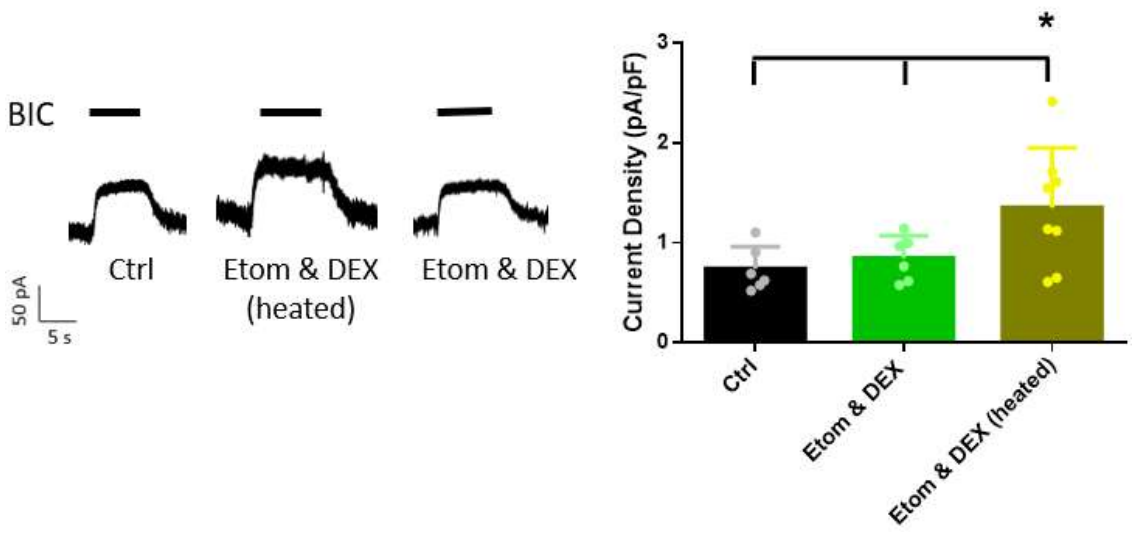

Figure 5.2: Neuroprotection from anesthetic-induced persistent increase in tonic current is abolished when dexmedetomidine-stimulated factors are deactivated by heat. (A) Two pure astrocyte cultures were separately treated with drugs; one culture with etomidate (Etom, 1 μM) and the other culture with dexmedetomidine (DEX, 100 nM), or controls for 1 h. For one experimental group, conditioned medium DEX-treated astrocyte cultures were heated at 99.9°C for 5 min before transferring conditioned media from DEX-treated and etomidate-treated astrocyte

cultures to pure neuron cultures. For the other experimental group (not shown in figure), astrocyte conditioned media from both DEX-treated cultures as well as etomidate-treated cultures were directly transferred to hippocampal neuron cultures without heating. For all groups, tonic current was recorded from neurons 24 h later. **(B)** DEX fails to prevent etomidate-induced persistent increase in tonic current in when conditioned media from DEX-treated astrocyte cultures are heated before transfer to hippocampal neuron cultures (* $p < 0.05$; $n = 6$-8 cells). All data are presented as mean ± SEM.

So far, the studies in this chapter have determined that DEX prevents anesthetic-induced persistent increase in tonic current by stimulating the release of neuroprotective factor(s), likely proteins, from astrocytes. Hence, we were interested in identifying the neuroprotective factor(s) involved in DEX-reversal of anesthetic-induced persistent increase in tonic current in hippocampal neurons.

In vitro studies in glutamate-induced excitotoxicity models have demonstrated that DEX reduces neuronal death by enhancing the release of BDNF from astrocytes (Degos et al., 2013). Furthermore, BDNF is a neuropeptide that has been shown to modulate various processes in neurons including regulation of ion channel activity (Han & Holtzman, 2000). Therefore, the next studies investigated whether BDNF contributes to DEX-mediated neuroprotection from anesthetic-induced persistent increase tonic current.

To determine whether BDNF provides neuroprotection from anesthetic-induced persistent increase in tonic current, BDNF was added to etomidate-treated astrocyte conditioned-media (ACM) before transfer of the ACM to hippocampal neurons. Tonic current in hippocampal neurons treated with BDNF and etomidate-ACM were similar to that in baseline controls (Figure 5.3B). Whereas, tonic current in neurons treated with etomidate-ACM alone were significantly higher than baseline levels. These results suggest that BDNF prevents etomidate-induced persistent increase in tonic current, mimicking DEX effects.

To confirm that activation of BDNF neuronal receptor contributes to DEX-mediated neuroprotection from tonic current, we investigated whether pharmacologic inhibition of BDNF neuronal receptor abolished DEX effects. Tonic current in hippocampal neurons treated with BDNF receptor antagonist, ANA12, as well as conditioned media from etomidate and DEX-treated astrocytes remained elevated above baseline (Figure 5.3C). Whereas, tonic current in hippocampal neurons treated with ANA12 alone were similar to that in controls. Hence, pharmacologic inhibition of BDNF receptors in neurons attenuates DEX-reversal of etomidate-induced persistent increase in neuronal tonic current in hippocampal neurons.

Collectively, the above studies show that BDNF likely contributes to DEX-mediated protection from anesthetic-induced persistent increase in tonic current in hippocampal neurons.

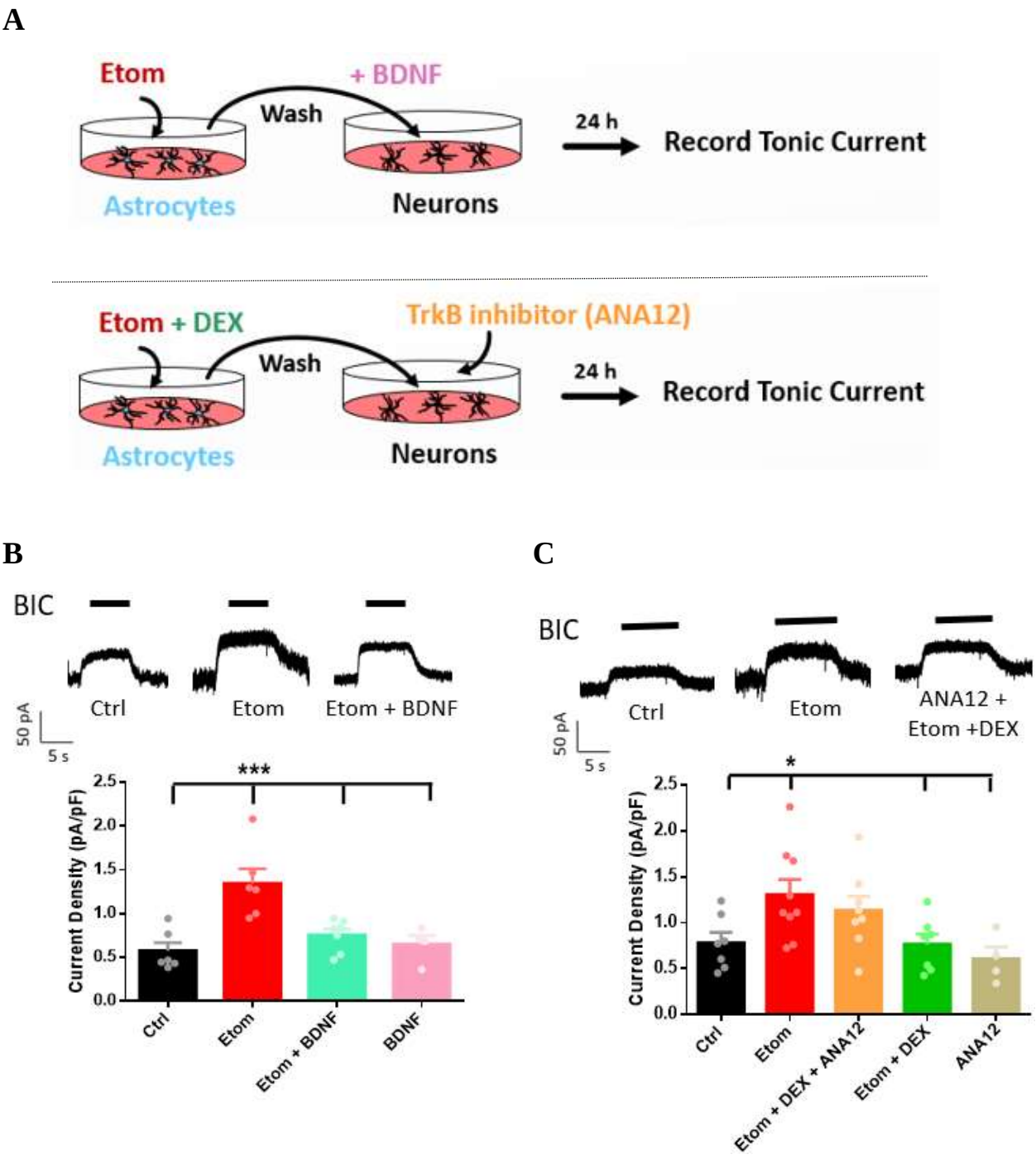

Figure 5.3: Brain-derived neurotrophic factor (BDNF) is a neuroprotective factor that contributes to the prevention of anesthetic-induced persistent increase in neuronal tonic current by dexmedetomidine. (A) Top panel: Pure astrocyte cultures were treated with etomidate (Etom, 1 μM) and BDNF (200 pg/mL) for 1 h. Bottom panel: Pure astrocyte cultures were treated

with etomidate (Etom, 1 μM) and dexmedetomidine (DEX, 100 nM) for 1 h; whereas pure hippocampal neuron cultures were treated with the BDNF neuronal receptor, i.e. TrkB receptor antagonist ANA12 (10 ng/mL). For both panels, conditioned medium from drug-treated astrocyte cultures were transferred to pure neuron cultures. Twenty-four hours later, tonic current was measured from neurons in the hippocampal neuron cultures. **(B)** BDNF prevents etomidate-induced persistent increase in neuronal tonic current, mimicking DEX effects (*** $p < 0.001$; $n = 5 – 7$ cells). **(C)** Pharmacologic blockade of BDNF signaling in neurons, by the TrkB receptor antagonist ANA12, attenuates DEX-reversal of etomidate-induced persistent increase in tonic current in hippocampal neurons (* $p < 0.05$; $n = 6$ cells Whereas, treatment of hippocampal neurons with ANA12 alone did not alter tonic current amplitudes. All data are presented as mean ± SEM.

In vitro studies in hypoxic-ischemic injury models have demonstrated that DEX stimulates astrocytic release of EGF, which helps enhance neuronal viability following hydrogen peroxide-induced hypoxia. Therefore, we next investigated whether EGF contributes to DEX-mediated neuroprotection from anesthetic-induced persistent increase tonic current.

To determine whether EGF provides neuroprotection from anesthetic-induced persistent increase in tonic current, EGF was added to etomidate-treated astrocyte conditioned-media (ACM) before transfer of the ACM to hippocampal neurons. Tonic current in hippocampal neurons treated with BDNF and etomidate-ACM were similar to that in baseline controls (Figure 5.4B). Whereas, tonic current in neurons treated with etomidate-ACM alone were significantly higher than baseline levels. These results suggest that EGF prevents etomidate-induced persistent increase in tonic current, mimicking DEX effects.

To confirm that activation of EGF neuronal receptor contributes to DEX-mediated neuroprotection from tonic current, we investigated whether pharmacologic inhibition of EGF neuronal receptor tyrosine kinase abolished DEX effects. Tonic current in hippocampal neurons treated with EGF receptor inhibitor, Ag1478, as well as conditioned media from etomidate and DEX-treated astrocytes remained elevated above baseline (Figure 5.4C). Whereas, tonic current in hippocampal neurons treated with Ag1478 alone were similar to that in controls. Hence, pharmacologic inhibition of EGF receptors in neurons attenuates DEX-reversal of etomidate-induced persistent increase in neuronal tonic current in hippocampal neurons.

Collectively, the above studies show that EGF likely contributes to DEX-mediated protection from anesthetic-induced persistent increase in tonic current in hippocampal neurons.

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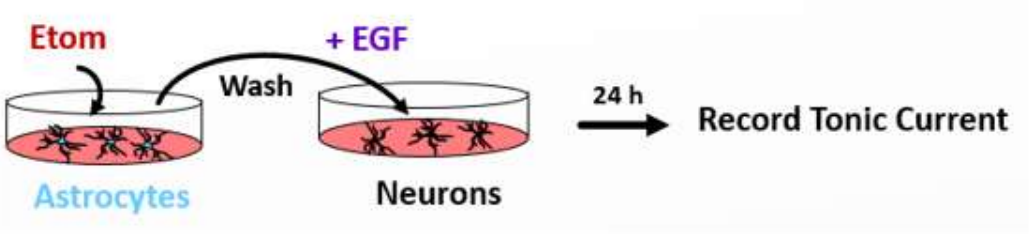

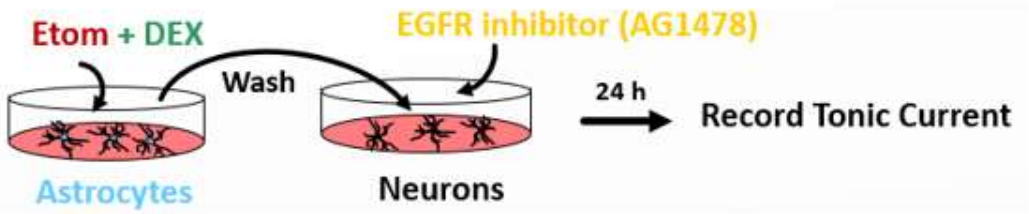

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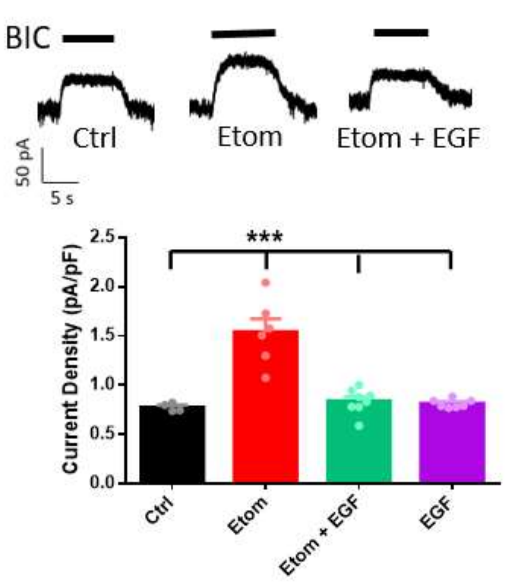

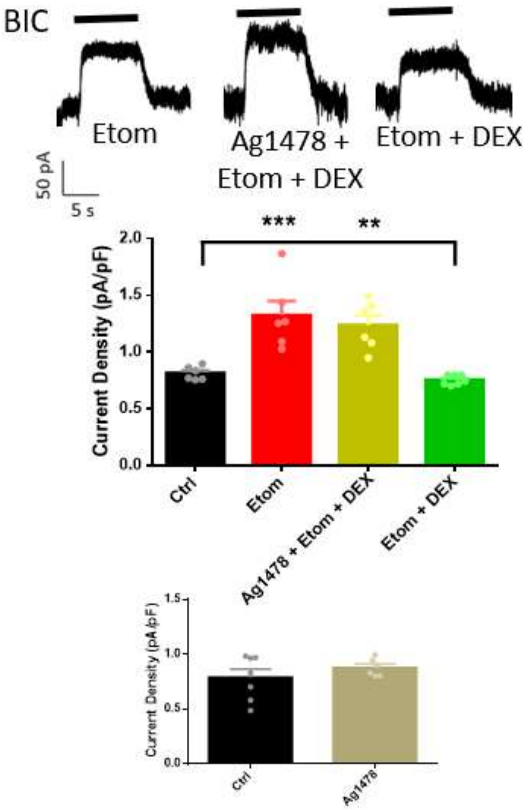

Figure 5.4: Epidermal growth factor (EGF) is a neuroprotective factor that contributes to the prevention of anesthetic-induced persistent increase in neuronal tonic current by dexmedetomidine. (A) Top panel: Pure astrocyte cultures were treated with etomidate (Etom, 1 μM) and EGF 10 ng/mL for 1 h. Bottom panel: Pure astrocyte cultures were treated with etomidate (Etom, 1 μM) and dexmedetomidine (DEX, 100 nM) for 1 h; whereas pure hippocampal neuron cultures were treated with the EGF receptor tyrosine kinase (EGFR) inhibitor Ag1478 (1 μM). For both panels, conditioned medium from drug-treated astrocytes cultures were transferred to pure hippocampal neuron cultures. Twenty-four hours later, tonic current was measured from neurons in the hippocampal neuron cultures. **(B)** EGF prevents etomidate-induced persistent increase in neuronal tonic current, mimicking DEX effects (*** $p < 0.001$; $n = 5$-8). **(C)** Pharmacologic blockade of EGF signaling in neurons, by the EGFR inhibitor Ag1478, attenuates DEX-reversal of etomidate-induced persistent increase in tonic current in hippocampal neurons (*** $p < 0.0001$; ** $p < 0.01$; $n = 6$ cells). Whereas, treatment of hippocampal neurons with the EFGR inhibitor Ag1478 alone did not alter tonic current amplitudes ($n = 6$-7 cells). All data are presented as mean ± SEM.

5.4 Discussion

These studies are the first to demonstrate that DEX prevents anesthetic-induced tonic inhibitory current in hippocampal neurons by triggering astrocytic release of neuroprotective factors, which are likely signalling proteins. Furthermore, signalling via BDNF and EGF neuronal receptors contribute to DEX-mediated neuroprotection from the anesthetic-induced persistent increase in tonic current. Collectively, these results suggest that DEX-stimulated neuroprotective factors, potentially including BDNF and EGF, act on hippocampal neurons to prevent the anesthetic-induced persistent tonic current which causes postanesthetic memory deficits.

Particularly, the anesthetic-induced persistent increase in 'memory blocking' tonic current in hippocampal neurons is abolished by the neuroprotective factors released from DEX-triggered astrocytes. Specifically, Figure 5.1 demonstrated that DEX-treated ACM counteracted the effects of etomidate-treated ACM on hippocampal neurons, thus preventing the persistent increase in neuronal tonic current. Interestingly, our previous studies have shown that anesthetics target astrocytes to mediate the persistent increase in tonic current in hippocampal neurons (Zurek et al., 2014). Therefore, the results reported here suggest that can DEX protect hippocampal neurons from anesthetic-induced persistent increase in tonic current without directly inhibiting the anesthetic-action on astrocytes.

The data here further suggest that the neuroprotective factors, released from DEX-triggered astrocytes, are signalling proteins. Specifically, we observed that the neuroprotective factors in DEX-treated astrocyte conditioned-media (ACM) fail to prevent persistent increase in neuronal tonic current when the ACM is briefly exposed to heat. This demonstrated that the neuroprotective factors are deactivated by heat. Since proteins are known to be deactivated by heat, these results indicate that the neuroprotective factors are proteins (Park et al., 2000). Furthermore, numerous studies have shown astrocytes to release neuropeptides and neurotransmitters that target neurons and regulate various processes in neurons, including ion channel expression and function (Araque et al., 2001; Gasser & Hatten, 1990). Taken together, these studies reinforce the notion that DEX-triggered astrocytes release signaling proteins which act on neurons to prevent the anesthetic-induced persistent increase in neuronal tonic current.

As discussed in Section 5.3, BDNF and EGF mimic DEX-effects on tonic current and inhibition of BDNF and EGF neuronal receptor-mediated signalling prevents DEX-reversal of tonic current. Although these results do not show that DEX-treated astrocytes release BDNF and EGF, they strongly suggest the involvement of BDNF and EGF in DEX-neuroprotection from tonic current. Furthermore, our unpublished biochemistry studies (Section 6.1) utilized the same treatment protocol as the electrophysiological studies and demonstrated that DEX-treated astrocyte culture media have significantly higher concentrations of BDNF and EGF. Collectively, these results indicate that DEX triggered-astrocytes release BDNF and EGF, and that BDNF and EGF act on their respective neuronal receptors to prevent the anesthetic-induced increase in neuronal tonic current.

Additional studies are necessary to determine whether these neuroprotective factors target multiple downstream signaling pathways or a common pathway to mediate DEX-reversal of tonic current. Interestingly, activation of the extracellular signal-regulated kinase (ERK) signaling pathway, by TrkB or BDNF neuronal receptor, has been demonstrated to regulate cell-surface expression of $GABA_A$ receptors ($GABA_ARs$) (Roberts, 2006; Grabenstatter et al., 2012; Porcher et al., 2011). Particularly, one study reported that treatment of rat hippocampal neuron cultures with BDNF decreased cell-surface expression of certain synaptic α2, β3, and γ2-subtypes of $GABA_ARs$, as detected by immunoreactivity levels (Brunig et al., 2001). Although the study did not investigate whether extrasynaptic $GABA_ARs$ were downregulated, β3 and γ2 subunits are known to associate with extrasynaptic α5 subunit-containing $GABA_ARs$ in the hippocampus (Farrant & Nusser, 2005). Therefore, it is possible that BDNF, and perhaps other neuroprotective factors, downregulate the expression of extrasynaptic $GABA_ARs$, which are responsible for mediating tonic inhibitory current in the hippocampus.

In conclusion, these data show that DEX stimulates astrocytic release of neuroprotective factors, possibly including BDNF and EGF; Furthermore, the DEX-stimulated neuroprotective factors prevent the anesthetic-induced persistent increase in hippocampal tonic current. Future experiments will confirm whether: (1) BDNF and EGF are the only DEX-stimulated neuroprotective factors; and (2) These neuroprotective factors target downstream ERK-signaling

pathway and reduce the cell-surface-expression of extrasynaptic $GABA_AR$s to prevent the anesthetic-induced persistent increase in tonic current.

6. General Discussion

6.1 Summary

The primary objective of this thesis was to determine whether dexmedetomidine (DEX) mediates neuroprotection from anesthetics by preventing the persistent increase in tonic inhibitory current in hippocampal neurons that causes postanesthetic memory deficits.

The results show that DEX stimulates astrocytic α2-adrenergic receptors (α2Rs) to release heat-sensitive neuroprotective factors that act on neurons to prevent an anesthetic-induced persistent increase in tonic current in hippocampal neurons.

Specifically, DEX prevents the persistent increase in tonic current in hippocampal neurons 24 h after exposure to the GABAergic anesthetics etomidate and sevoflurane. Notably, DEX prevents the anesthetic etomidate-induced persistent increase in tonic current in hippocampal neurons with a high potency (4.6 nM), which is similar to its potency for α2R-mediated action (Virtanen et al., 1985). Another α2R agonist, clonidine, mimics DEX-effects on tonic current; whereas, an α2R antagonist, yohimbine, inhibits DEX-reversal of etomidate-induced persistent increase in neuronal tonic current. Furthermore, astrocytes are necessary and sufficient for DEX-mediated reversal of etomidate-induced persistent increase in tonic current in hippocampal neurons. In addition, pharmacologic inhibition of astrocytic α2Rs, by the α2R antagonist yohimbine, abolishes DEX-reversal of etomidate-induced persistent increase in neuronal tonic current. Overall, these results demonstrate that DEX, via astrocytic α2Rs, prevents etomidate-induced persistent increase in tonic current in hippocampal neurons.

Furthermore, DEX action on astrocytic α2Rs stimulates the release of heat-sensitive neuroprotective factors, which are likely signalling proteins. Brain-derived neurotrophic factor (BDNF) and epidermal growth factor (EGF) can also prevent etomidate-induced persistent increase in tonic current in hippocampal neurons, mimicking DEX effects. In addition, pharmacologic inhibition of either the BDNF neuronal receptor (TrkB) or the EGF neuronal tyrosine kinase receptor (EGFR), prevents DEX-reversal of etomidate-induced persistent increase in tonic current. Collectively, these results suggest that DEX-stimulated neuroprotective factors,

possibly including BDNF and EGF, act on hippocampal neurons to prevent the anesthetic-induced persistent increase in tonic current which causes postanesthetic memory deficits.

The significance of these findings are two-fold. First, these data present a potential mechanism by which DEX reverses memory deficits, in rodent models, after general anesthesia and surgery (Qian et al., 2015; Si et al., 2016). Second, understanding the mechanism of DEX-mediated neuroprotection from anesthetics will help identify alternative treatment for patients who are contraindicated for DEX (Venn et al., 1999; U.S. Food and Drug Administration, FDA).

6.2 Discussion

These studies are in line with previous studies investigating the mechanism of DEX-mediated neuroprotection from anesthetics, as discussed previously, in Sections 4.4 and 2.6 (Sanders et al., 2009; Sanders et al., 2010; Li et al, 2013).

To the best of our knowledge, all previous mechanistic studies have focused on the role of DEX in preventing anesthetic-induced neuroapoptosis in neonatal and adult rodents. Hence, our studies are the first to demonstrate that DEX prevents anesthetic-induced persistent increase in tonic inhibitory current in matured hippocampal neurons from rodents. Interestingly, previous studies by our lab have established that anesthetic-induced persistent increase in hippocampal tonic current causes postanesthetic deficits in retrograde memory. Furthermore, preclinical studies by others have shown that DEX reverses hippocampus-dependent spatial memory impairment in rodents after surgery with general anesthesia and anesthesia alone (Qian et al., 2015; Si et al., 2016; Sanders et al., 2009). Hence, our studies offer a mechanism by which DEX prevents postanesthetic impairments in retrograde and spatial memory, which are common in patients with POCD and postoperative delirium (Monk & Price, 2011).

Importantly, recent clinical studies have reported that DEX reduces the incidence of postoperative delirium, a cognitive disorder in which general anesthetics are believed to play a major contributing role (Su et al., 2016; Pasin et al., 2014; Reade et al., 2016). In this case, our results demonstrate a neuroprotective mechanism which contributes to DEX-mediated reduction in the incidence of postoperative delirium. Taken together, these initial studies suggest DEX as a

potential, safe treatment for postoperative cognitive disorders including postoperative delirium and POCD.

Hence, our findings will encourage future clinical as well as preclinical studies to determine whether DEX can be repurposed as a safe and effective treatment for postoperative delirium and POCD.

6.2.1 Dexmedetomidine: A non-GABAergic, neuroprotective anesthetic drug

Our finding that DEX prevents anesthetic-induced persistent increase in tonic inhibitory current in hippocampal neurons is the first evidence to suggest that DEX inhibits anesthetic-induced neurodepressive effects. Prior to this study, the sedative and analgesic drug, DEX, has only been shown to potentiate anesthetic-induced neurodepression in the central nervous system (CNS) (Phillip et al., 2002). For instance, DEX has been used as an anesthetic adjunct to prolong the sensory and motor blockade after spinal anesthesia and awake craniotomy, where DEX enhances the effects of the anesthetic sevoflurane. (Kanazi et al., 2006). Furthermore, DEX dramatically reduces the perioperative requirement for isoflurane anesthesia during abdominal surgery (Aho et al, 1991). These functions of DEX would suggest that, like other anesthetic drugs, DEX enhances tonic inhibition (Nelson et al., 2002).

However, DEX is a non-GABAergic sedative whose clinical functions are mediated by α2Rs in the CNS and the PNS (Shehabi et al., 2010; Kaur & Singh, 2011). Hence, unlike GABAergic anesthetics like etomidate, DEX does not target $GABA_A$ receptors ($GABA_ARs$) to mediate neurodepression. Instead, DEX acts on α2Rs to stimulate inward-rectifying K^+ channels and inhibit Ca^{2+} channels which hyperpolarizes neuronal membranes, and suppresses excitatory neurotransmission (Lakhlani et al., 1997). These studies explain why treatment with DEX does not cause acute or persistent increase in tonic inhibitory current, as shown by our previous studies (Zurek et al., 2014).

Furthermore, our results that DEX, at clinically relevant concentrations, activates astrocytic α2Rs has important implications for DEX-mediated regulation of anesthetic action. First, stimulation of G-protein-coupled α2Rs is known to trigger various intracellular signaling cascades and result in a wide range of cellular responses including changes in cell metabolism and protein

expression (Bekker et al., 2005; Engelhard et al., 2002). For instance, DEX-stimulation of astrocytic α2Rs has previously been shown to increase the expression of BDNF protein (Degos et al., 2013).

Second, astrocytes are known to modulate function and survival of neighbouring neurons by releasing signaling molecules such as BNDF and EGF (Nedergaard et al., 2003; Araque et al., 2001). Therefore, DEX-stimulated increase in BDNF and EGF release by astrocytes can regulate neuronal function, as suggested by our studies where BDNF and EGF reversed anesthetic-increase in tonic current in hippocampal neurons. Collectively, these studies strongly suggest that treatment with a sedative dose of DEX during surgery mediates sedation as well as neuroprotection from anesthetics.

6.2.2 Parallel pathways of dexmedetomidine-mediated neuroprotection from anesthetics

6.2.2.1 Downregulation of proinflammatory cytokines by dexmedetomidine

Our previous studies have demonstrated that the anesthetic etomidate triggers astrocytes to release factors that act on neurons to cause the persistent increase in tonic current in hippocampal neurons (Zurek et al., 2014). Furthermore, our preliminary studies suggest that the etomidate-triggered factors include the proinflammatory cytokine interleukin 1β (IL-1β), which acts on its neuronal IL-1 receptors to increase the cell-surface expression of α5-$GABA_A$A receptors (α5$GABA_A$Rs) in hippocampal neurons. Since α5$GABA_A$Rs mediate tonic inhibitory current in hippocampal CA1 and CA3 regions, increase in cell-surface expression of these receptors leads to persistent increase in tonic current (Glykys et al., 2008). Indeed, we have previously demonstrated that treatment of adult rodents with IL-1β increases cell-surface expression of α5$GABA_A$Rs (Wang et al., 2012). In addition, co-treatment of IL-1β with etomidate enhances etomidate-induced increase in tonic current in hippocampal slices (Wang et al., 2012). Taken together, these data suggest that IL-1β, and likely other proinflammatory cytokines, are key to etomidate-induced persistent increase in tonic current, which underlies postanesthetic memory impairments.

Interestingly, my results in Chapter 5 show that DEX-triggered release of neuroprotective factors from astrocytes is sufficient to prevent etomidate-induced persistent increase in tonic current in hippocampal neurons. Hence, altogether, our studies suggest that DEX counteracts the effects of IL-1β and other factors released by etomidate-triggered astrocytes.

However, studies by others have demonstrated that DEX reduces the expression of proinflammatory cytokines in the hippocampus of elderly rodents exposed to surgery with anesthesia (Qian et al., 2015). Specifically, pre-treatment with DEX significantly reduces hippocampal mRNA and protein expressions of proinflammatory cytokines IL-1β and tumour-necrosis factor α (TNFα) (Qian et al., 2015). Since this model involves surgery with anesthesia, it is possible that surgery exacerbates the expression of proinflammatory cytokines which are thought to mediate persistent increase in tonic current. However, anesthetics alone have been shown to increase proinflammatory cytokines, including IL-1β and IL-6, in the hippocampus and cerebral cortex of adult and neonatal rodents (Cao et al., 2012; Lei et al., 2012).

Collectively, the above studies suggest that DEX-mediates neuroprotection from anesthetic-induced tonic current via two parallel pathways: DEX inhibits anesthetic-induced expression of proinflammatory cytokines and simultaneously, releases neuroprotective factors, which prevent the anesthetic-induced persistent increase in tonic current in hippocampal neurons.

6.2.2.2 Suppression of apoptotic pathways

DEX has been shown to reduce anesthetic-induced neuroapoptosis and suppress apoptotic pathways in the hippocampus and cerebral cortex of both adult and neonatal rodents (Qian et al., 2015; Sanders et al., 2009). Specifically, pre-treatment with DEX before exposure to isoflurane anesthesia significantly reduced the mRNA and protein expressions of proapoptotic proteins Bax and caspase-3; and simultaneously, increased the expression of the cell survival protein Bcl-2 (Qian et al., 2015; Sanders et al., 2009; Sanders et al., 2010).

To the best of our knowledge, no studies have investigated anesthetic-induced tonic current in the developing brain. However, it seems that in the matured hippocampus and cerebral cortex, anesthetic-induced apoptosis, neuroinflammation, and persistent increase in neuronal tonic current all contribute to anesthetic-induced neurotoxicity (Hudson & Hemmings, 2011; Zurek et al., 2014;

Erasso et al., 2013). Therefore, it is plausible that DEX simultaneously supresses apoptotic pathways, inflammation, and persistent increase in neuronal tonic current to protect from anesthetic-induced cognitive deficits.

6.3 Implications for treating postoperative delirium and POCD

Our studies on the role of DEX in preventing anesthetic-induced persistent increase in hippocampal tonic current provide insights into the mechanism and therapeutic potential of DEX. Not only do these findings identify a mechanism contributing to DEX-mediated protection from postoperative delirium; they also propose alternative strategies for the treatment of postoperative cognitive disorders in general. Hence, these studies will prompt future clinical and preclinical studies to determine whether DEX can be repurposed as a treatment or preventative strategy for postoperative cognitive disorders.

6.3.1 Dexmedetomidine protects from common pathophysiologic factors in postoperative delirium and POCD

Anesthetic drugs and perioperative systemic inflammation are two major, common pathophysiologic factors that predispose patients to postoperative delirium and POCD, as discussed in Section 2.1.3 (Monk & Price, 2011; Mo & Zimmerman, 2013). Since recent studies have suggested the role of DEX in treating postoperative cognitive disorders, this sub-section briefly reviews how DEX reduces the risks associated with these pathophysiologic factors.

6.3.1.1 Dexmedetomidine reduces the requirement for anesthetic drugs

The use of DEX during surgery has been shown to result in a dramatic reduction of the requirement for GABAergic general anesthetics and sedatives (Khan et al., 1999; Maze et al., 1988). For instance, sedation with DEX during awake craniotomy decreases the required dose of sevoflurane by 50%. (Bekker et al., 2001). By reducing the dose, DEX reduces the allosteric actions of these GABAergic drugs that is responsible anesthetic-induce neurotoxicity in the brain (Mo & Zimmerman, 2013). An example of such allosteric effect is etomidate's action on $GABA_A$

receptors ($GABA_ARs$) in astrocytes that result in persistent increase in tonic inhibitory current in hippocampal neurons, as demonstrated by our studies (Zurek et al., 2014).

Furthermore, DEX potentiates the sedative, hypnotic, and analgesic effects of anesthetics without targeting $GABA_ARs$. As discussed previously, the use of GABAergic anesthetics has been strongly associated with the incidence of postoperative delirium (Pandharipande et al., 2006; Pasin et al., 2014). Although DEX by itself does not produce adequate neurodepression, the significant reduction in GABAergic anesthetic dose during surgery reduces the risk of incurring postoperative delirium (Sanders & Maze, 2001; Naaz & Ozair, 2014).

Lastly, DEX does not inhibit cholinergic neurotransmission unlike anesthetic drugs used for premedication (Mo & Zimmerman, 2013). Particularly in elderly patients, premedication with anticholinergic drugs such as lorazepam has been associated with a higher incidence of postoperative delirium (van Gool et al., 2010). Although the mechanisms are unknown, the cholinergic neurotransmitter system modulates cognitive processes including learning, memory, and attention. Hence, disruption in cholinergic neurotransmission by anticholinergic agents contribute to impairments in memory and awareness observed in delirious patients (Zaal et al., 2012).

Collectively, the above evidence suggests that the anesthetic-sparing action of DEX plays a key role in its neuroprotection from postoperative cognitive disorders.

6.3.1.2 Dexmedetomidine decreases systemic inflammation

Systemic inflammation has been strongly associated with a higher incidence for POCD as well as postoperative delirium, as discussed previously.

DEX has been shown to reduce systemic inflammation in rodent models (see Section 2.6 for details) as well as patients after surgery (Taniguchi et al., 2004; Pandharipande et al., 2010). More importantly, sedation with DEX has been shown to improve outcomes for delirium, recovery, and survival after surgery in septic patients (Pandharipande et al., 2010). Specifically, adult intensive care unit (ICU) patients were randomized to either DEX or lorazepam-based sedation protocol for 5 days and delirium was assessed daily for the first 12 postoperative days. Septic patients who

received DEX-sedation had significantly 3.2 more delirium and mechanical ventilation free days compared to septic patients who received lorazepam-sedation. In addition, the risk of mortality at 28 days, as indicated by the hazards ratio, was 70% lower in patients in who received DEX-sedation, than in patients who received lorazepam (Pandharipande et al., 2010). Taken together, these results suggest that DEX significantly improves the outcomes of postoperative delirium and associated comorbidities following systemic inflammation.

6.3.2 Alternative treatments for postoperative cognitive disorders

Our findings suggest that DEX, via astrocytic α2Rs, triggers the release of neuroprotective factors that prevent the anesthetic-increase tonic current, which contributes to DEX-mediated protection of postanesthetic cognitive deficits. In addition, this mechanism implies that other α2R agonists as well as the DEX-stimulated neuroprotective factors may also mimic DEX-mediated neuroprotection from anesthetics. This sub-section will summarize the alternative treatment strategies that have been implicated by the mechanism of DEX-mediated neuroprotection suggested in this thesis.

6.3.2.1 Clonidine and other α2-adrenegic receptor agonists

The results in Chapter 4 demonstrate that another α2R agonist, clonidine, also targets astrocytes to prevent anesthetic-induced persistent increase in tonic current in hippocampal neurons, just as DEX. These findings suggest the possibility that clonidine, and possibly other α2R agonists may also prevent postanesthetic memory deficits by reversing anesthetic-induced persistent increase in hippocampal tonic current (Qian et al., 2015; Si et al., 2016). Hence, our results implicate that like DEX, other α2R agonists may also reduce the incidence of postoperative delirium (Su et al., 2016; Reade et al., 2016).

Interestingly, clonidine and DEX are both used as sedatives in the ICU (Giovannitti et al., 2015; Kamibayashi & Maze, 2000). Therefore, if clinical trials demonstrate positive outcomes, both DEX and clonidine may be rapidly adopted as treatment strategies for postoperative cognitive disorders.

However, potent α2R agonists such as DEX and clonidine present a higher risk of bradycardia and hypotension in elderly and diabetic patients. (Gertler et al., 2001; Kamibayashi & Maze, 2000; Ma et al, 2005). In such cases, other, less potent α2R agonists, such as xylazine, may be used to treat postoperative cognitive disorders, provided that their use is safe and effective (Ma et al., 2005; Virtanen et al., 1985). Future clinical and preclinical studies are necessary to establish whether α2R agonists in general can be used to reverse or treat postoperative delirium and POCD.

6.3.3.2 BDNF

Studies reported in this thesis have shown that DEX-stimulated neuroprotective factors, which do not target α2Rs, can mimic DEX-mediated neuroprotection from anesthetics. Furthermore, pharmacologic inhibition of neuronal receptors of the potential neuroprotective factors, BDNF and EGF, attenuates DEX-reversal of tonic current in hippocampal neurons. Since co-treatment with DEX has been shown to reverse postoperative cognitive deficits in rodent models, it is likely that co-treatment with BDNF or EGF would have similar protective effects (Qian et al., 2015). Collectively, these results suggest that signalling pathways downstream of BDNF and EGF neuronal receptors are involved in DEX-mediated reversal or attenuation of memory deficits associated with POCD and postoperative delirium.

Interestingly, a double blind phase II clinical trial in Amyotrophic Lateral Sclerosis (ALS) patients demonstrated that treatment with BDNF is safe and alleviates the sensory and motor symptoms associated with ALS (Ochs et al., 2000). In this case, intrathecal infusion of recombinant methionyl human BDNF reduced ALS symptoms in patients between the ages of 21 – 65 years (Ochs et al., 2000). Although these clinical findings are not related to our model of neuroprotection, these studies suggest that treatment with recombinant BDNF is safe in human patients.

Collectively, the above studies suggest that like DEX, treatment with an effective and safe dose of BDNF may prevent the development of postoperative cognitive disorders, including postoperative delirium. If this is the case, then BDNF would be the recommended treatment for patients contraindicated for DEX. For instance, treatment with BDNF would be particularly useful for patients who are particularly vulnerable to α2R agonist-mediated bradycardia. Future studies

in animals and human patients are necessary to determine whether DEX-stimulated neuroprotective factors, such as BDNF, can safely reverse or treat postoperative delirium and POCD.

6.4 Future Directions

This thesis presents the first evidence for the role of DEX in preventing anesthetic-induced persistent increase in tonic current in hippocampal neurons. Particularly, the studies demonstrate that DEX stimulates astrocytic α2Rs to release heat-sensitive neuroprotective factors that act on neurons to prevent the anesthetic-induced persistent increase in tonic current. While these results advance our understanding of DEX-mediated neuroprotection from anesthetics, several questions remain unanswered and must be addressed in future studies.

6.4.1 Does dexmedetomidine-reversal of tonic current reduce postanesthetic cognitive deficits?

These studies show that DEX action on astrocytic α2Rs prevents the anesthetic-induced persistent tonic current, which underlies postanesthetic cognitive deficits. Furthermore, behavior studies by our lab demonstrate that co-treating mice with DEX reduces anesthetic-induced deficits in cognitive performance, as suggested by the puzzle-box assay. However, our studies do not show whether DEX-reversal of anesthetic-induced tonic current is responsible for the reduction in postanesthetic cognitive deficits in these mice.

To address this question, mutant mouse colonies that are a conditional knockout for astrocytic α2Rs should be constructed (Ladehsmaki et al., 2002). Behavior studies using the mutant and wild-type mice should be performed next to determine whether DEX fails to reduce postanesthetic cognitive deficits in conditional α2R knockout mice. If so, then we can deduce that DEX-reversal of anesthetic-induced persistent increase in tonic current causes DEX-mediated neuroprotection from postanesthetic cognitive deficits.

6.4.2 Does dexmedetomidine directly prevent anesthetic action on astrocytes?

Our results demonstrate that DEX-triggered astrocytic release of neuroprotective factors is sufficient to prevent anesthetic-induced persistent increase in tonic current in hippocampal neurons. However, these studies do not address whether DEX also directly inhibits anesthetic action on astrocytes.

Interestingly, preliminary studies from our lab have shown that treatment with the anesthetic etomidate increases the concentration of the proinflammatory cytokine IL-1β in astrocyte culture media. Furthermore, we have previously demonstrated that IL-1β enhances $GABA_AR$-mediated tonic current in hippocampal neurons, as discussed in Section 2.3 (Wang et al., 2012). Finally, studies in other models of injury, such as hypoxic-ischemia, have reported that DEX mediates neuroprotection by suppressing the protein expression of a number of proinflammatory cytokines in astrocytes (Rodriguez-Gonzalez et al., 2016; Zhang et al., 2013).

Hence, the next outstanding question is whether DEX-mediated reduction in proinflammatory cytokines contributes to DEX-reversal of anesthetic-induced persistent increase in tonic current in hippocampal neurons. To determine whether DEX reduces protein expression of IL-1β, and possibly other proinflammatory cytokines, high-throughput techniques such as mass spectrometry or flow cytometry should be utilized. Once the proinflammatory cytokines are identified, *in vitro* electrophysiological studies can be undertaken to confirm whether those proinflammatory cytokines enhance anesthetic-induced persistent increase in tonic current in hippocampal neurons.

6.4.3 Are BDNF and EGF DEX-stimulated neuroprotective factors and do they target a common signaling pathway to mediate neuroprotection?

The results reported in Chapter 5 demonstrate that BDNF and EGF-receptor mediated signaling in neurons is crucial for DEX-neuroprotection from tonic current, which poses two outstanding questions: (1) Is DEX-stimulated release of BDNF and EGF necessary for DEX-mediated neuroprotection from anesthetic-induced cognitive deficits?; and (2) Do BDNF and EGF target common signaling pathways to mediate DEX-reversal of tonic current?

To determine whether BDNF and EGF are necessary for DEX-mediated neuroprotection, first, conditioned media for the anesthetic and DEX-treated astrocyte cultures should be screened using mass spectrometry or flow cytometry. Significantly elevated concentrations of BDNF and EGF in (anesthetic + DEX)-treated astrocytes, compared to that in anesthetic-treated astrocytes, would confirm that DEX triggers the release of these factors. Along with the studies reported in Chapter 5, these positive results would establish BDNF and EGF as key factors in DEX-reversal of anesthetic-induced increase in tonic current. If that is the case, the next studies should investigate whether BDNF and EGF are responsible for DEX-reversal of postanesthetic memory deficits. To test this, cerebrospinal fluid (CSF) from DEX-co-treated mice could be sampled and analyzed using interdigitated microelectrode (IME) biosensors containing BDNF or EGF antibodies (Yoo et al., 2016). Specifically, CSF microdialysates could be collected from (anesthetic + DEX)-treated mice with baseline performance in the novel object recognition memory task, and compared to that of anesthetic-treated mice with impaired performance. If DEX-co-treated mice have significantly higher IME concentrations of BDNF and EGF compared to anesthetic-treated mice, it would demonstrate that BDNF and EGF are crucial for DEX-reversal of postanesthetic memory deficits.

Next, to determine whether BDNF and EGF reduce extrasynaptic $GABA_AR$ expression in the hippocampus by activating the ERK signalling pathway, a few biochemical and electrophysiology experiments are necessary. First, immunohistochemistry experiments should be conducted in hippocampal slices from adult rodents. If co-treatment with DEX reduces extrasynaptic α5-subunit-containing $GABA_ARs$ ($\alpha5GABA_ARs$), relative to anesthetic alone, then it can be deduced that DEX downregulates anesthetic-induced increase in the cell-surface expression of $\alpha5GABA_ARs$.

If so, the next experiment would be to determine whether pharmacologic inhibition of ERK phosphorylation prevents DEX-mediated downregulation of $\alpha5GABA_ARs$. In this case, the same immunohistochemistry protocol can be performed, except that an ERK inhibitor (e.g. PD98059) would be co-injected with DEX and an anesthetic, such as etomidate. If DEX fails to reverse etomidate-induced increase in $\alpha5GABA_AR$ expression in the presence of the ERK inhibitor, then it would suggest ERK as the common downstream signaling pathway.

Additionally, electrophysiological experiments in hippocampal neuron cultures should be conducted to confirm that pharmacologic inhibition of ERK signaling in neurons prevents DEX reversal of etomidate-induced persistent increase in tonic current. Evidence for DEX-reversal of tonic current via ERK signaling in neurons would then confirm that neuroprotective factors, including BDNF and EGF, target a common downstream signaling pathway in hippocampal neurons.

6.4.4 BDNF: An alternative treatment strategy

The use of DEX or other α2R agonists to treat postoperative cognitive disorders is not safe for certain patient populations. Particularly, DEX and α2R agonists can mediate severe bradycardia in patients with congestive heart failure or ventricular dysfunction. Hence, an alternative treatment for postoperative cognitive disorders needs to be identified for these patients.

Our studies have identified BDNF as a key factor contributing to DEX-mediated neuroprotection from anesthetics. Therefore, it is possible that BDNF may also revere postanesthetic cognitive deficits as well as contribute to the reduction in postoperative cognitive disorder. Since BDNF does not target α2Rs and cause bradycardia, it may present a safe treatment strategy for patients contraindicated for DEX (Chao et al., 2003; Naaz & Ozair, 2014).

To determine whether BDNF may be used as an alternative treatment, first, preclinical studies are necessary to investigate whether BDNF, like DEX, reverses postanesthetic cognitive deficits. Hence, cognitive behavioral assays, such as the Morris Water Maze assays, should be performed in rodents exposed to BDNF and an anesthetic, to identify whether BDNF reduces postanesthetic cognitive deficits (Qian et al., 2015).

Next, to confirm whether BDNF is a key factor in DEX-reversal of postanesthetic cognitive deficits should be confirmed with additional behaviour studies. In this case, cognitive performance assays should be performed in rodents exposed to a BDNF neuronal receptor inhibitor, such as ANA12, along with DEX and an anesthetic (Cazorla et al., 2011). If pharmacologic inhibition of BDNF receptor-mediated signaling prevents DEX-reversal of postanesthetic cognitive deficits, then it would confirm BNDF is a key factor in DEX-mediated neuroprotection.

If the preclinical studies are successful, clinical studies will determine whether treatment with human recombinant BDNF is safe and effective for the treatment of postoperative cognitive disorders (Ochs et al., 2000).

6.3 Conclusions

In conclusion, dexmedetomidine (DEX) prevents an anesthetic-induced persistent increase in tonic current in hippocampal neurons. Specifically, DEX stimulates astrocytic α2-adrenergic receptors (α2Rs) to release neuroprotective factors, potentially including brain-derived neurotrophic factor (BDNF) and epidermal growth factor (EGF). BDNF and EGF, in turn, act on their respective neuronal receptors to prevent the anesthetic-induced persistent increase in tonic current in hippocampal neurons.

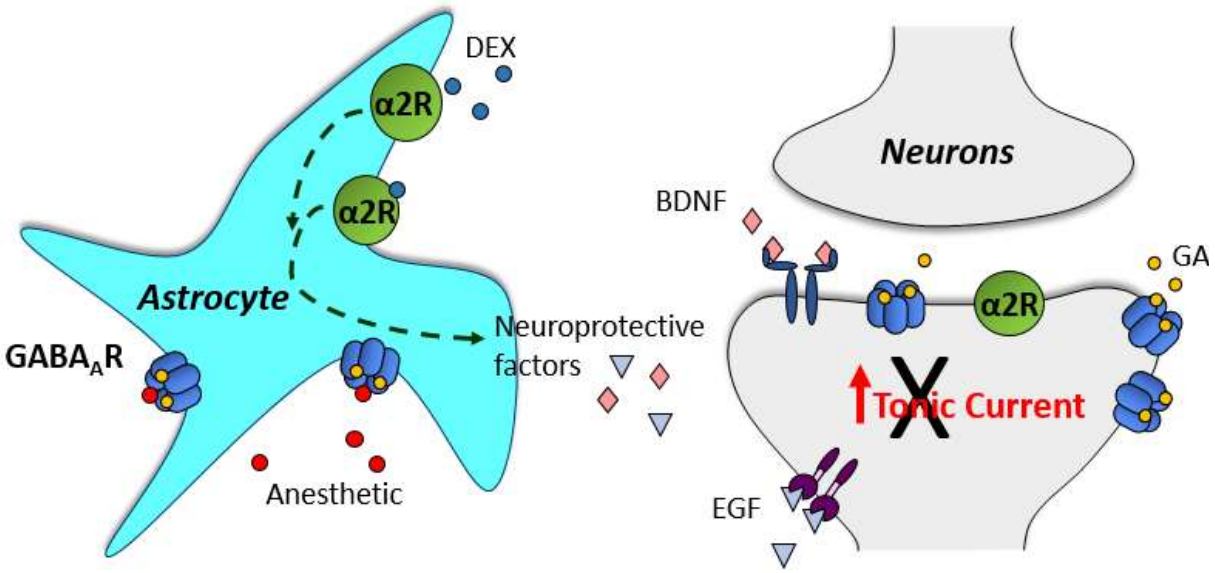

Figure 6.4: Pictorial summary of the main conclusions. Dexmedetomidine (DEX) stimulates the α2-adrenergic receptors (α2Rs) in astrocytes and triggers the release of neuroprotective factors, which potentially include brain-derived neurotrophic factor (BDNF) and epidermal growth factor (EGF). BDNF and EGF likely act on their respective receptors in hippocampal neurons and prevents the anesthetic-induced persistent increase in tonic current in these neurons.

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