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Effect of acute ethanol administration on the extracellular concentrations of the opioid peptides β-Endorphin, Met-Enkephalin and Dynorphin A_{1-8} at the level of ventral tegmental area in the rat.

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Abstract

There is experimental evidence suggesting that ethanol alters the activity of the endogenous opioid peptide systems in a dose and brain region dependent manner. These ethanol-induced alterations in opioid activity may influence the processes of ethanol reward and reinforcement. However, the precise nature of the link between ethanol-opioid interactions influencing reward and reinforcement is not clearly understood. Thus, it was the objective of the present study to investigate the response of the three major opioid peptide systems (endorphins, enkephalins, and dynorphins) to acute ethanol administration, at the level of the ventral tegmental area (VTA), a brain region important for drug, including ethanol, reinforcement. Using the *in vivo* microdialysis technique coupled with specific solid-phase radioimmunoassays for β-endorphin, met-enkephalin, and dynorphin $A_{1\text{-}8}$, changes of the extracellular concentration of theses peptides at the level of VTA were determined at distinct time points following the administration of 0.0 (saline), 0.8, 1.6, and 2.4 g ethanol/ kg B/Wt.. Results demonstrated a biphasic effect of ethanol on β-endorphin release with 1.6, but not 0.8 or 2.4, g ethanol /kg B.Wt. enhancing β-endorphin release. None of the ethanol doses used altered the extracellular levels of met-enkephalin, and dynorphin $A_{1\text{-}8}$ peptides. In conclusion, the present findings suggest that at the level of VTA interactions of β-endorphin with the μ and/or δ opioid receptors on GABA interneurons may contribute to the ethanol induced augmentation in the activity of the mesolimbic dopaminergic system, and influence ethanol reinforcement.

Résumé

Il existe une évidene expérimentale qui suggère que l'éthanol change l'activité du système d'opiacées endogènes selon le dose et la région du cerveau. Les altérations dans l'activité opioïde provoquée par l'éthanol peuvent influencer les processus de 'récompenses' et de 'renforcement' par l'ethanol. Cependant, la nature précise de l'influence entre l'interaction éthanol-opioïde et la 'récompense' et le 'renforcement' n'est pas entierment comprise. Donc, l'objectif de la presente étude etait d'investiger la réponse des 3 systèmes majeurs de peptides opiacées (endorphine, enkèphaline, dynorphine) à une seule administration d'éthanol au niveau du 'VTA', region importante du cerveau pour le 'renforcement' par la drogue incluant l'éthanol. En utilisant la technique microdialyse *in vivo* avec des 'radioimmunoassays' phase-solide spécifiques à des β-endorphine, met-enképhaline, et dynorphine A_{1-8}, des changements de la concentration extra-cellulaires de ces peptides au niveau du 'VTA' on été déterminés apres des intervalles distinctes suite à l'administration de 0.0 (saline), 0.8, 1.6, et 2.4 g éthanol/ kg de masse corporelle. Les resultats ont démontré un effet à double-phase de l'éthanol sur la libération de la β-endorphine avec une dose de 1.6 mais pas de 0.8 ou 2.4 g éthanol/ kg de masse corporelle augmentent la libération de la β-endorphine. Aucune des doses d'éthanol n'a altérée les niveaux extra-cellulaires de met-enképhaline et dynorphine A_{1-8}. En Conclusion, les résultats de cette étude suggèrent qu'au niveau 'VTA', les interactions de la β-endorphine avec les récepteurs opioïdes μ et/ou δ sur l'interneuron 'GABA' peuvent contribuer à l'augmentation de l'activité du système

dopamine mesolimbique provoquée par l'éthanol, et peuvent également influencer le 'renforcement' par l'éthanol.

Notes to the Reader

In the text that follows, the terms "alcohol" and "ethanol" are both used, and they both refer to ethyl alcohol (CH_3CH_2OH), a psychoactive drug found in alcoholic beverages.

Also, the terms "opioid" and "opiate" generally refer to endogenous and exogenous chemicals, respectively.

The studies included in this thesis have been presented at international conferences, and are to be submitted for publication in a peer-reviewed scientific journal.

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Statement of original scholarship

In this thesis, *in vivo* microdialysis studies on the effects of acute ethanol challenges, of various doses, on the activity of endogenous opioid peptides at the level of the rat ventral tegmental area are presented. Ethanol induced changes in the activity of distinct components of the endogenous opioid system in brain regions associated with the processes of reward and reinforcement, such as the ventral tegmental area, have been proposed to mediate, at least in part, ethanol's reinforcing effects. Several previously-reported studies have examined the effects of ethanol on opioidergic responses in various brain regions, however few have done so at the level of the ventral tegmental area, and those that have used post-mortem tissue at a single time point post-ethanol administration. Thus, I believe that the experiments described in this thesis constitute the first detailed time-course, dose-response study on the effects of ethanol on the release of opioid peptides at the level of VTA of the freely-moving rat, using the *in vivo* microdialysis technique. Results indicated that ethanol indeed may alter the release of β-endorphin at the level of VTA, supporting a role of β-endorphin in ethanol reinforcement.

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Despite having played the role of author in preparing this thesis, it is with some reluctance that my name alone has been printed on the title page. Indeed, seldom is the output of one individual truly theirs and theirs only. While actions of innumerable people have undoubtedly influenced the course of my studies, and this thesis, there are a few individuals, by sheer magnitude of their contributions, for whom an acknowledgement is appropriate and merited. Having come under their influence comprises an important reason why I tend to think of myself as a lucky person. They are as follows.

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Abbreviations

6-OHDA: 6-hydroxydopamine

AA/ANA: Alko-Alcohol/Alco-non-alcohol rat strain

ANOVA: Analysis of variance

BAC: Blood alcohol concentration

B.wt: Body weight

CPP: Conditioned place preference

DA: Dopamine

DAMGO: [D-Ala2,N-Me-Phe4-Gly5-ol]- enkephalin

DPDPE: [D-Pen2,D-Pen5]- enkephalin

EtOH: Ethanol

GABA: Gamma aminobutyric acid

HAD/ LAD: High/Low alcohol drinking rat strain

ID/OD: Inner/outer diameter RIA: Radioimmunoassay

ICV: Intracerebroventricular

IP: Intraperitoneal

P/NP: Preferring/non-preferring rat lines

POMC: Pro-opiomelanocortin

S.A. Specific activity

WSP/WSR: Withdrawal seizure prone/resistant mouse strain

CHAPTER 1

Literature Review and Objectives

1.1 The nature of alcohol

CH_3CH_2OH or ethyl alcohol, also referred to as ethanol or, more commonly, alcohol, is an organic compound prepared historically through the fermentation of sugars. While ethanol can now be synthetically prepared, its production by fermentation is still common, and it is produced for a wide range of uses including fuel, industrial solvents, and alcoholic food products. The production of ethanol through fermentation has been practiced throughout written history. Indeed, controlled fermentation has been dated as far back as 10 000 B.C., or earlier (Patrick, 1952). Despite the long tradition of fermentation, ethanol use has been proposed to have originated prior to the emergence of Homo Sapiens. Such deep roots are underscored by the profound presence of alcohol in human culture, and its physiological effects on individuals. It has even been suggested that the presence of low levels of ethanol in partially-fermented fruits may have represented a phenomenon significant in processes of natural selection dating back as far as 40 million years (Dudley, 2000). The sustained exposure of frugivores –including the ancestors of modern humans- to traces of ethanol has been posited to have promoted the evolution of a human propensity for ethanol in general, and a maladaptive vulnerability to alcohol abuse and dependence in certain individuals, in particular. Such vulnerability may be due in part to the availability of large amounts of ethanol in societies which produce alcoholic food products, compared to environments in which ethanol occurred independently of human invention - and in which a particular sensitivity to detecting, remembering, or conditioning to ethanol stimuli might be considered adaptive (Dudley, 2002). Although today alcohol is widely available, alcohol associated problems do not afflict every individual who consumes alcohol. Furthermore, despite the possibly deep

roots of the disorder of alcohol addiction in human history, and the possible evolutionary advantages of garnering a sensitivity to ethanol, alcoholism represents a significant problem in societies in which sugars are intentionally fermented for the production of alcoholic beverages.

1.2 Alcohol at the societal level

On a global level, alcohol may be the most prevalent drug of abuse, surpassed only by caffeine (Rajendram et al., 2006). Alcohol consumption is an accepted behaviour in many societies. A survey on drinking in the United States indicated that over 50% of adults consume alcohol, and about 9% meet the DSM-IV criteria for alcohol abuse or dependence (Grant, 1994). While low-to-moderate alcohol consumption may have some health benefits, notably through a possible protective effect against heart disease (Booyse and Parks, 2001), this meager benefit is offset by the comparatively staggering negative effects observed in heavy drinkers and alcohol-dependent individuals. A report from the World Health Organization (2002) indicated that in the year 2000 over 10% of the disease burden in established market economies was attributable to alcoholism, quantified by disability-adjusted life years, a measure factoring both the impact of disability and the premature loss of life. Worldwide, alcohol accounted for 4% of the total number of disability-adjusted life years. In addition to the disease burden attributable to alcoholism, there is a social cost of alcoholism that outpaces that of other common drugs of abuse (Rajendram 2006). Overall the cost of alcohol on Canadian society is in the excess of $7.5 billion annually (Single et al., 1996).

The magnitude of alcohol's impact on society dictates a need to investigate the factors promoting alcohol abuse and dependence. Indeed, various lines of investigation, ranging from social influences to molecular mechanisms associated with addictive processes may provide a better understanding of the biological and environmental factors associated with the development of alcoholism, may lead to the development of behavioural interventions and pharmacotherapy treatments, and may allow a better control of alcohol-related problems.

1.3 Genetics of alcoholism

There is experimental evidence indicating that alcoholism is a heritable disease. Investigations with experimental animals and studies of human populations (Hill, 2004; Koob, 2000; Cadoret et al., 1980) strongly support the heritability of alcohol use disorders. Indeed, parents or other relatives of individuals who exhibit alcohol use disorders are likely to have drug-related problems of their own, particularly alcohol-related problems (Marikangas et al., 1998). While studies of familial alcoholism are useful in demonstrating that alcoholism can be passed from parents to children, they do not necessarily delineate between the mechanisms of transmission of vulnerability, be they genetic or environmental.

Familial transmission of alcoholism is further supported by twin and adoption studies. Adoption studies permit the assessment of the contribution of a given genotype to a particular phenotype by controlling for environmental confounds. In the case of alcoholism, individuals whose biological parents were alcoholics, and were adopted by

non-alcoholic parents, present a higher incidence of alcoholism, regardless of the presence or absence of alcoholism in the adoptive environment (Cadoret et al., 1980).

Twin studies allow the assessment of the genetic contribution to a phenotype, whereby the concordance rate for a disorder is compared between sets of twins, either monozygotic or dizygotic, for a particular phenotype. Environmental confounds are controlled, because twin sets are presumed to experience similar environmental influences (Messas & Vallada Filho, 2004), although additional variation is introduced by specific environmental factors not shared within a twin pair (Prescott et al., 1999). Several such studies, comparing the concordance rates for monozygotic to those of dizygotic twins, have been carried out and have supported a strong genetic component to alcoholism, providing heritability estimates. Such approaches estimate the proportion of phenotypic variation in the expression of alcohol use disorders attributable to genotype, as opposed to environmental factors, to range from 50% to 60% in males and, comparably, from 40% to 60% in females (Hill, 2004).

Alchohol classification schemes, factoring differences in variables such as familial drinking, age of onset, and associated psychological and behavioural profiles, have distinguished different 'types' of alcoholism. A dichotomous typology, originating from Cloninger et al. (1981), classified alcoholism into two major types. Type I is heritable (familial type) and is characterized by an early onset and antisocial personality disorders. Type II is non-heritable (non-familial type) and is characterized by late onset,

and absence of antisocial personality disorder (Cloninger et al., 1981; Hesselbrock, 2006).

1.4 Biological markers of alcoholism

Indeed, there is an increased incidence of alcoholism in children of alcoholics, and these individuals provide an opportunity for the study of heritable biomarkers for alcoholism (Goodwin, 1984; Schuckit, 1988). The identification of biomarkers has been particularly fruitful in studies of familial alcoholism. The study of biomarkers for familial alcoholism may permit a greater understanding of genetic factors contributing to alcoholism per se, rather than looking at extraneous variables, such as common co-morbid pathologies that may precipitate, or result from, an accompanied alcohol problem.

Measurements of event-related potentials in offspring of alcoholics, young enough to have had minimal exposure to alcohol, display a diminished amplitude of the P300 component of event-related potentials (Hill et al., 1999), compared with offspring on non-alcoholic parents. Individuals with a positive family history of alcoholism are also reported to display reduced alpha power and heightened beta power in electroencephalograms, independent from measured personality traits (Finn & Justus, 1999). Using tests of musculo-skeletal balance, it was demonstrated that children of alcoholic parents exhibit greater body sway than children of non-alcoholic parents (Lipscomb et al., 1979). Other markers could be of biochemical nature such as enzymes, hormones, neurotransmitters and neuromodulators (Ratsma et al., 2002). Among the neurotransmitter systems associated with the processes of reward and reinforcement of

many drugs of abuse, including alcohol, is the endogenous opioid system (Di Chiara et al., 1996; Gianoulakis, 2004). Inherited differences in endogenous opioid system function may render individuals more or less susceptible to the rewarding effects of alcohol (Froehlich et al., 2000). These findings, and others like them, are indicative of latent markers of predisposition to alcoholism, and may offer insights into the genetic factors associated with the vulnerability to high alcohol consumption (Porjesz et al., 2005).

1.5 Endogenous opioid systems

The endogenous opioid system consists of opioid peptides and opioid receptors and is involved in various functions including nociception, thermoregulation, sleep and wakefulness, sexual behavior, eating and drinking, (O'Donohue, 1982; Olson et al., 1995).

Opioid Receptors:

Stereospecific opiate binding sites were demonstrated in 1973 (Pert & Snyder, 1973; Simon et al., 1973; Terenius, 1973). Currently, the consensus is that the opioid receptors are of three major types: the μ, δ, and κ receptors (Martin et al., 1976; Gillan & Kosterlitz, 1982; James & Goldstein, 1984), and each receptor type has been further characterized as displaying distinct subtypes (Charness, 1989). The opioid receptor types bear significant homology with one another, and are all seven transmembrane-spanning G-protein-coupled receptors (Mansour et al., 1995). Their activation produces neuron hyperpolarization, achieved through an increase in K^+ conductance by μ and δ receptors, and through a blocking of voltage-gated Ca^{2+} conductance by κ receptors (Simmonds,

1988; Su et al., 1998; Alvarez et al., 2002; Pacheco & Duarte, 2005). In the Rat, the μ opioid receptors are distributed throughout the brain, in regions that include neocortex, amygdala, hippocampus, striatum, hypothalamus, thalamus, interpeduncular nucleus, substantia nigra, ventral tegmental area, and colliculi (Mansour et al., 1988). The δ opioid receptors have been localized in cortical regions, amygdala, striatum, some thalamic and hypothalamic regions, substantia nigra, and nucleus tractus solitarius (Mansour et al., 1988). The κ opioid receptors are found in cortex, amygdala, striatum, thalamus, hypothalamus, and ventral tegmental area (Mansour, 1988).

Opioid Peptides:

The endogenous opioid peptides are of 3 distinct families, namely, the enkephalins, the endorphins, and the dynorphins (Hughes et al., 1975; Noda et al., 1982; Nakanishi et al., 1979; Bradbury et al., 1976; Li & Chung, 1976; Goldstein et al., 1979; Kakidani et al., 1982). Each family of opioid peptides has its own distinct precursor molecule from which the opioid peptides are derived.

Pro-enkephalin is the high-molecular weight precursor of the family of enkephalin peptides, including met-enkephalin, met-enkephalin-Arg6-Phe7, met-enkephlalin-Arg6-Gly7-Leu8, and leu-enkephalin. Enkephalinergic perikaria are considerably spread-out in the brain, including caudate-putamen, arcuate nucleus of the hypothalamus, hypothalamic areas, nucleus tractus solitarius, and raphe nucleus. Enkephalinergic terminals are found in mesencephalic regions, globus pallidus, and nucleus accumbens (Akil et al., 1984; Khacheturian et al., 1985). Enkephalins bind to both μ and δ opioid receptors, although

with 25 times greater affinity for δ than μ opioid receptors, and very weak affinity for κ receptors (Khachaturian et al., 1985).

Pro-opiomelanocortin (POMC) is the high-molecular weight precursor for the endorphin family of opioid peptides, including β-endorphin (1-31), β-endorphin (1-27), β-endorphin (1-26). Other peptides derived from POMC are α, β, and γ melanocyte stimulating hormones, adrenocorticotropic hormone, and corticotrophin-like intermediate lobe peptide. Further processing of β-endorphin (1-31) serves to reduce its binding potency, and associated functional consequences, including a loss of analgesia. N-terminal acetylated β-endorphin is devoid of receptor affinity, and may represent a means of inactivating β-endorphin (Akil et al., 1984; Deakin et al., 1980). Cells synthesizing endorphins are located mainly within the arcuate nucleus of the hypothalamus, and the nucleus tractus solitarius, as well as the anterior and neurointermediate lobes of the pituitary gland (Akil et al., 1984; Khachaturiuan et al., 1985). From the arcuate nucleus projections of endorphinergic neurons extend and innervate other brain regions, incuding the nucleus accumbens, amygdala, hippocampus, and midbrain (Khacheturian et al., 1985; Zakarian & Smyth, 1982). β-endorphin (1-31) binds with about equal affinity to both μ and δ opioid receptors, and presents no, or very low affinity for the κ opioid receptors (Khachaturian et al., 1985).

Prodynorphin is the high molecular weight precursor of the dynorphin family of opioid peptides, including dynorphin A (1-8, 1-17), dynorphin B (1-13, 14-29, 1-29), and α/β-neo-endorphins (Akil et al., 1984). Dynorphin-producing cells are widely distributed

in the brain, in regions including the hypothalamic areas, nucleus tractus solitarius, caudate-putamen, and periaqueductal gray (Akil et al., 1984). Dynorphins bind preferentially to κ opioid receptors (Khachaturian et al., 1985).

1.6 Opioid manipulations: behavioural effects

Numerous investigations on the involvement of endogenous opioid peptides and their receptors in motivational processes have used compounds that may act either as opioid receptor agonists or as antagonists. The role of these compounds in processes of reinforcement and motivation has been tested in numerous experimental paradigms. In a number of such studies, opioid receptors were stimulated by specific or non-specific opioid receptor agonists and the effect on the performance of animals in conditioned place preference (CPP), drug self-administration, and intracranial self-stimulation paradigms was determined. Results indicated that activation of distinct types of opioid receptors may mediate either rewarding or aversive motivational states (Van Ree et al., 1979; Mucha & Iverson, 1984; Bals-Kubic et al., 1989; Van Wofswinkel & Van Ree, 1985).

Studies examining the effects of specific and non-specific opioid receptor agonists in different experimental models of reward and reinforcement have elucidated some of the basic effects of opioid systems in the processes of reward and reinforcement. Using a self-administration paradigm, Van Ree et al. (1979) reported that Wistar rats will learn operant responses for intracerebroventricular (ICV) administration of heroin, a potent μ opioid receptor agonist, as well as of β-endorphin, an agonist having high affinity for

both μ and δ opioid receptors. Under the CPP paradigm, the reinforcing nature of μ and δ opioid receptor activation is further supported. Place preference can be conditioned by μ opioid receptor agonists such as morphine, the selective agonist [D-Ala2,N-Me-Phe4-Gly5-ol]- enkephalin (DAMGO), and β-endorphin, as well as by the δ opioid receptor agonist [D-Pen2,D-Pen5]- enkephalin (DPDPE), while opioid receptor antagonists that specifically target either μ or δ opioid receptors seem to reverse CPP (Mucha and Iverson, 1984; Bals-Kubic et al., 1990; Bals-Kubic et al., 1993). Thus, CPP and self-administration studies demonstrate that the activation of μ and δ opioid receptors can be reinforcing. On the contrary, agonists that stimulate κ opioid receptor activity have been shown to induce aversive states. Indeed, the selective κ opioid receptor agonists U50,488 and U 69593, and the dynorphin derivative E-2078 have been shown to induce conditioned place aversion (Mucha and Herz, 1985; Bals-Kubic et al., 1989; Bals-Kubic, 1993).

Other investigative approaches have employed the use of opioid receptor antagonists. In general, blocking the activity of the endogenous opioid receptor systems with either non-specific or receptor-type specific antagonists, the observed loss of CPP would suggest a tonically-active endogenous opioid activity, without which aversive states are produced. The effect of opioid receptor antoagonists on CPP may vary depending on the opioid receptor type targeted. Administration of the non-specific opioid receptor antagonist naloxone, through various routes, is reported to produce a conditioned place aversion in rats (Mucha and Iverson, 1984; Bals-Kubik et al., 1989). Furthermore, the μ opioid receptor-specific antagonist CTOP is found to produce conditioned place

aversion, and to abolish the CPP normally produced by β-endorphin. On the other hand, the δ and κ opioid receptor antagonists ICI 174,864 and nor-binaltorphimine, respectively, do not induce aversive effects, suggesting that the key action of non-specific opioid receptor antagonists in producing aversive effects is the blockade of tonic μ opioid receptor activity (Bals-Kubic et al., 1989; Bals-Kubic et al., 1990). Furthermore, studies using intra-VTA stimulating electrodes demonstrated that opioid receptor antagonists, such as naloxone, raised the reward threshold, which could be lowered by morphine administration (Van Wolfswinkel & Van Ree, 1985).

1.7 Effects of alcohol on the activity of the endogenous opioid systems

In addition to the positive and negative reinforcing effects resulting from various experimental manipulations of endogenous opioid neurotransmission, reports describing the effects of ethanol on endogenous opioid activity further establish the relevance and importance of opioid systems in the processes of alcohol reinforcement. Acute and chronic ethanol exposure has been shown to alter endogenous opioid activity in several ways, affecting opioid peptide biosynthesis and release, as well as receptor binding (Gerrits et al., 2003; Gianoulakis, 2004). Generally, the implication of endogenous opioids in self-administration of drugs of abuse is considered primarily, though not exclusively, to pertain to processes of reward or positive reinforcement, although their involvement in other processes - depending on the extent of the addictive state – such as negative reinforcement, may be equally important in motivating drug self-administration (Di Chiara et al., 1996; Gerrits et al., 2003). Indeed, the response of the endogenous

opioid system to ethanol tends to support its implication in the process of alcohol reward and reinforcement.

Investigations on the response of endogenous opioid systems to acute ethanol exposure have shown a stimulatory effect of ethanol. One approach has been to examine the effect of ethanol on the content of mRNAs coding for the high molecular weight precursors of the opioid peptides, in distinct regions of the rodent brain. Indeed, acute ethanol administration induces increases of POMC mRNA in the hypothalamus and of pro-enkephalin mRNA in nucleus accumbens, and hypothalamic regions, indicating increased biosynthesis of opioid peptides, and suggesting an ethanol-induced activation of opioid neurotransmission/neuromodulation (Morales et al., 2002; Rasmussen et al., 1998; Li et al., 1998; Pastorcic et al., 1994; Gianoulakis et al., 1994). In support of an ethanol-induced increased activity of the endogenous opioid system are reports demonstrating an increased release of opioid peptides in response to acute ethanol exposure (Oswald & Wand, 2004). Furthermore, there are several studies demonstrating that ethanol could produce dose-dependent increases of β-endorphin release from hypothalamic and pituitary tissues in *in vitro* systems (De Waele et al., 1994; De Waele et al., 1992; Gianoulakis, 1990; Gianoulakis et al., 1987; Keith et al., 1986). Additionally, increased release of β-endorphin following acute ethanol administration has been shown in the shell region of the nucleus accumbens (Olive et al., 2001; Marinelli et al., 2003), while increased β-endorphin content has been observed in post-mortem tissue of the VTA and accumbens, at 30 minutes post-ethanol administration (Rasmussen et al., 1998). Though there are fewer studies on the effects of ethanol on the other two families of

opioid peptides, the enkephalins and dynorphins, there are some reports demonstrating that acute ethanol administration increases the content of met-enkephalin in the rat striatum, hypothalamus, and midbrain (Schulz et al., 1980; Seizinger et al., 1983). Furthermore, *in vivo* microdialysis studies have shown a dose-dependent ethanol-induced increase in the release of met-enkephalin at the level of the rat nucleus accumbens, with low to moderate, but not high, ethanol concentrations increasing met-enkephalin release (Marinelli et al., 2005). On the other hand, acute exposure to high, but not low, ethanol concentrations increased the release of dynorphin peptides at the level of the nucleus accumbens (Marinelli et al., 2006).

Interestingly, while studies on the effects of acute (Marinelli et al., 2005; Marinelli et al., 2003; Olive et al., 2001) and of short chronic (Schulz et al., 1980; Angelogianni and Gianoulakis, 1993) ethanol exposure on β-endorphinergic and met-enkephalinergic systems demonstrate a stimulatory effect, prolonged chronic ethanol exposure suppresses the activity of the β-endorphin system as indicated by the decrease of hypothalamic and pituitary POMC mRNA content (Rasmussen et al., 2002; Scanlon et al., 1992; Winkler et al., 1995), associated with reduced levels of hypothalamic and plasma β-endorphin (Boyadjieva et al., 1994; Pastorcic et al., 1994 Winkler et al., 1995).

1.8 Animal models in alcoholism and the endogenous opioid system

Though there are a number of human studies investigating both the behavioural and biochemical effects of alcohol, the need for in-depth studies of the neurochemical effects of alcohol at the level of brain regions involved in the processes of reward and

reinforcement led to the development of several animal models exhibiting either preference or aversion towards ethanol solutions. Theses animal models were developed by selective inbreeding or out-breeding of either high-drinking males with high-drinking females to produce offspring with preference for alcohol solutions, or of low-drinking males with low-drinking females to produce offspring with no preference for alcohol solutions. Among the most studied animal models of alcoholism are the preferring (P) and non-preferring (NP) rats (Lumeng et al., 1982; Waller et al, 1983), the high alcohol drinking and (HAD) and low alcohol drinking (LAD) rats (Lumeng et al., 1986; Yoshimoto et al., 1992) and the Alko-Alcohol (AA) and Alko-Non-Alcohol (ANA) rats (Erickson, 1968; Sinclair et al, 1989). The P (Penn et al., 1978), HAD (Ritz et al., 1994) and AA (Sinclair et al., 1979) rats will lever-press for ethanol solutions, demonstrating the reinforcing properties of alcohol in these lines of rats. In addition, several inbred lines of mice have been shown to differ in alcohol consumption. Among these are the Withdrawal Seizure Prone (WSP) and Withdrawal Seizure Resistant (WSR) mice (Kosobud et al., 1998) and the C57BL/6 and DBA/2 mice (McLearn and Rogers, 1959). The WSP and C57BL/6 mice exhibit a preference for alcohol solutions, while the WSR and DBA/2 mice avoid consumption of alcohol solutions (Kosabud et al., 1988; McLearn and Rogers, 1959; George et al., 1991).

Studies using these selected lines of animals have uncovered differences between ethanol-preferring and non-preferring rodents, with regard to the activities of their respective opioid systems. Ethanol-preferring AA rats, in contrast to non-preferring ANA rats, display higher content of hypothalamic POMC mRNA (Marinelli et al., 2000;

Gianoulakis et al., 1992), and exhibit a greater β-endorphin release response to acute ethanol (De Waele et al., 1994). Studies using the C57BL/6 and DBA/2 mice demonstrated that the ethanol-preferring C57BL/6 mice, compared with the non-preferring DBA/2, display higher levels of spontaneous β-endorphin release from hypothalamic tissue *in vitro*, a greater increase of hypothalamic β-endorphin release in response to ethanol exposure, and greater hypothalamic content of POMC mRNA (De Waele et al., 1992; De Waele et al., 1993; De Waele et al., 1994; Jamensky et al., 1999). In addition, the amount of non-acetylated, opiate-active form of β-endorphin (1-31), released from the hypothalamus in response to ethanol exposure *in vitro* is higher in the AA than ANA rats (De Waele et al., 1992). The alcohol-preferring P rats, compared with the non-preferring NP rats, presented a more pronounced increase of pituitary POMC mRNA levels, in response to repeated ethanol challenges (Krishnan-Sarin et al., 1998).

1.9 Sensitivity of opioid systems to ethanol

Another line of inquiry implicating the endogenous opioid system in ethanol reward and reinforcement involves studies of the effects of manipulations of opioid activity on ethanol reward behaviours, such as drinking and place conditioning. These manipulations include the alteration of opioid receptor activities by administration of opioid agonist and antagonist drugs, as well as through the use of genetic 'knockout' animal models. Results from these studies suggest that the activation of distinct opioid receptor systems may be a crucial step in the sequence of neurological events producing ethanol self-administration by experimental animals (Koob et al., 1992; Gerrits et al., 2003).

An approach extensively used to delineate the role of the endogenous opioid systems in alcohol reinforcement is the use of opioid antagonists. In one report, monkeys pre-trained to self-administer ethanol showed a dose-dependent extinction pattern of operant responding for ethanol self-administration following treatment with the non-specific opioid receptor antagonist naloxone, indicating the inhibition or attenuation of the rewarding properties of ethanol (Altshuler et al., 1980). Similarly, systemic administration of either naloxone or naltrexone achieved an attenuation of operant responding for, and consumption of, ethanol in rats and mice (Hyytia and Sinclair, 1993; Stromberg et al., 1998; Middaugh et al., 1999; Reid et al., 1991; Hubbel et al., 1986; Samson & Doyle, 1983; Volpicelli et al., 1986; Froelich et al., 1990), and extinguished ethanol conditioned place preference (Cunningham et al., 1998). Intracerebral application of methylnaloxonium, a nonselective opioid antagonist, in the nucleus accumbens has been found to produce similar effects on ethanol drinking (Heyser et al., 1999). Likewise, local application of methylnaloxonium at the level of the VTA is reported to attenuate ethanol-induced CPP (Bechtholt & Cunningham, 2005). Indeed, key support of the findings from these studies is garnered from the similar effects of opioid antagonism on human alcohol consumption and craving (Volpicelli et al., 1992; O'Malley et al., 1992).

The question of specificity of the effects of opioid antagonists, in the attenuation of ethanol drinking could be a cause for concern. Regarding both their clinical applications, and their use in probing the involvement of opioid systems in reinforcement mechanisms, it is of interest that they indeed target ethanol-related behaviours specifically, but it has also been reported that naltrexone can diminish the expression of

CPP for sucrose, as well cocaine (Delamater et al., 2000; Gerrits et al., 1995). However, some overlap between the neural systems mediating drug reward, and those whose function relates to evolutionarily-adaptive, so-called natural rewards may exist (Herz, 1997; Di Chiara et al., 1996). Thus, the observation of some functional overlap between drug and natural rewards following treatments that disrupt one or the other, may be expected. This is certainly relevant to the endogenous opioids, which are involved in numerous reinforcement activities (Van Ree, 1996).

Support for the specificity of the effects of opioid receptor antagonism on ethanol-reinforcement is offered through observations that naloxone attenuates ethanol self-administration without diminishing normal motor activity, or alcohol absorption (Sharpe & Samson, 2001; Linseman & Le, 1997; Morgan et al., 1989). Interestingly, μ opioid receptor antagonism does seem to reduce ethanol-induced locomotor activity (Pastor et al., 2005). Further, in rhesus monkeys trained to self-administer ethanol, treatment with naltrexone reduced drinking, even after a period of imposed abstinence, which typically prompts increased, 'compensatory drinking'. Indeed, this attenuation was achieved without affecting water intake, demonstrating an uncompromised capacity for consumptive activity on one hand, and a diminished motivational potential for ethanol on the other (Kornet et al., 1991). In general, it appears that administration of opioid receptor antagonists influences the processes of ethanol reward and reinforcement with a degree of specificity and without having significant adverse side effects.

1.10 The brain reward system and dopamine

The dopaminergic systems of the midbrain, including the substantia nigra and the VTA, are known to be essential to certain basic survival functions and are associated with a number of pathological conditions. Midbrain dopaminergic fibers, though relatively few in number, originate from the ventral tegmental area and substantia nigra, and innervate numerous other brain regions including frontal cortex, nucleus accumbens, and striatum, creating systems which exert a powerful influence on cognition, motivation, and behaviour (Oswald & Wand, 2004; Björklund and Lindvall, 1984). Bilateral lesions of the mesocorticolimbic dopaminergic system result in a profound state of behavioural unresponsiveness characterized by symptoms such as akinesia, catalepsy, sensory neglect, aphagia, and adipsia (Björklund and Lindvall, 1984; Gerrits & Van Ree, 1996).

The dopaminergic neurons of the ventral tegmental area are thought to play an important role in reward and have been shown to be implicated in the development of drug addiction (Wise, 1998). In fact, midbrain DA neurons may be thought of as a common factor underlying the brain's response to most addictive substances (Koob, 1992). Drugs such as cocaine, amphetamine, and methylphenidate act as dopamine agonists, serving as examples of psychoactive drugs which act directly on dopaminergic neurons and can lead to addiction. This is due, in part, to their binding to the presynaptic dopamine reuptake transporter and increasing extracellular concentrations of DA, resulting in a hyperactivation of postsynaptic neurons in the nucleus accumbens and ventral striatal domains of the caudate and putamen (Koob, 1992). The increased DA activity is thought to participate in the subjective rewarding effects of the drug, and

contribute to the syndrome of an addicted state. Other psychoactive drugs, such as ethanol, may also interact with mesolimbic reward systems, albeit in a less direct manner. Various neurochemical systems which exert control over dopaminergic neurons, including endogenous opioid systems, may provide mechanisms by which non-psychostimulant drugs engage dopaminergic systems.

1.11 Implication of endogenous opioid systems in the mechanisms of ethanol reinforcement

Considering the influence of opioids on reinforcement behaviours, the stimulatory effect of ethanol on endogenous opioids, and the alteration of ethanol reinforcement by opioid manipulations, questions are raised regarding the possible mechanisms by which opioids may affect reward and reinforcement. Experimental evidence indicates that the activity of endogenous opioid systems constitutes an important component of the neurological reward processes. Components of the endogenous opioid system (peptides and receptors) are located within brain regions associated with the processes of reward and reinforcement (Akil et al., 1984). Thus, it is of fundamental interest to understand how the endogenous opioid systems are involved in the mesolimbic dopaminergic mechanisms of reward and reinforcement.

1.12 The mesolimbic dopaminergic system and ethanol self-administration

As with other drugs of abuse, increased output of mesolimbic dopaminergic neurons, which originate in the ventral tegmental area and terminate in the nucleus accumbens, is associated with ethanol reinforcement (Weiss et al., 1993; Koob et al.,

1998; Wise 1998). Ethanol that is self-administered increases dopamine release at the level of the nucleus accumbens (Weiss et al., 1993; Weiss et al., 1996; Melendez et al., 2002; Blomqvist et al., 1997). The participation of dopaminergic neurons appears to be an important component of ethanol self-administration, and the VTA appears to be a key site at which their activity is modulated. The VTA itself is one brain region that will support self-infusion of ethanol, and more favorably in P, than NP rats (Rodd-Hendricks et al., 2000a; Gatto et al., 1994). Disruption of dopaminergic neurotransmission through the use of autoreceptor agonist drugs offers one way to assess the role of dopamine in ethanol reinforcement. Dopamine receptors can be expressed pre-synaptically and their stimulation by agonist drugs or dopamine itself, which causes hyperpolarization, distinguishes them as inhibitory-feedback autoreceptors (Rodd et al., 2004b). The microinjection of D2 and D3 dopamine receptor agonists at the level of the VTA exerts an inhibitory action on dopaminergic neuron activity, and attenuates ethanol intake by rats trained for ethanol self-administration (Hodge et al., 1993; Nowak et al, 2000; Congar et al., 2002; Rodd et al., 2004b; Rodd et al., 2005a).

Furthermore, postsynaptic dopamine receptor antagonists dose-dependently decrease ethanol self-administration (Samson and Hodge, 1993), and genetic knockout mice for the D1 receptor gene display a reduced preference for ethanol (El-Ghundi et al., 1998). Additionally, VTA dopaminergic activity is increased by drinking in mice that have been chronically-exposed to ethanol, particularly so in ethanol-preferring C57BL/6, compared to DBA/2 mice, implicating mechanisms that enhance the activity of mesolimbic dopamine in the processes of ethanol reward and reinforcement (Brodie

2002; Brodie and Appel, 2002; Brodie et al., 1999; Gessa et al., 1985). Thus, strain differences in the ethanol-induced increase of mesolimbic dopamine activity may contribute to the strain differences in voluntary ethanol consumption. In addition to the attenuation of alcohol self-administration behaviour, administration of naltrexone prior to the self-administration opportunity will also inhibit the ethanol-induced increase of dopamine release (Gonzales & Weiss, 1998; Benjamin et al., 1993; Koistinen et al., 2001). Furthermore, *in vitro* and *in vivo* sensitization of dopaminergic activity to repeated ethanol exposure can be attenuated by naltrexone (Brodie et al., 1990; Brodie et al., 1995).

1.13 Opioid mechanisms of ethanol reinforcement independent of mesolimbic dopamine

Interestingly, other investigations on the roles of dopamine and opioids in ethanol reinforcement, have used 6-OHDA lesions to selectively destroy VTA dopamine cells. In several of such studies, it was found that the destruction of these neurons does not affect maintenance, nor even acquisition of ethanol self-administration behaviours in rodents (Koistinen et al., 2001; Ikemoto et al.,1997; Rassnick et al., 1993). Furthermore, Koistinen et al. (2001) found that in 6-OHDA-lesioned animals, naltrexone could still attenuate drinking, indicating the presence of dopamine-independent opioid mechanisms of ethanol reinforcement. These findings contrast with studies employing drugs that induce acute deactivation of dopaminergic signaling, which typically disrupts initiation and maintenance of ethanol self-administration (Rodd et al., 2004b; Nowak et al., 2000). Furthermore, the finding that chronic disruption of dopamine neurotransmission fails to

attenuate ethanol self-administration does not necessarily detract from the importance of dopamine signaling in ethanol reinforcement. The differential effects of acute dopaminergic disruption and 6-OHDA lesions on ethanol self-administration have been explained by a functional adaptation hypothesis. Intrinsic to the methods of studies employing 6-OHDA is the tendency to allow experimental animals several days or weeks to recover from surgery before commencement of subsequent testing. Similar to studies of genetic 'knockouts' in which there is a possibility that developmental neuroadaptation may occur in response to, and to compensate for, a loss of function, lesion studies too, may result in functional compensations. In 6-OHDA lesion studies, the animals' capacity for ethanol reward may be restored following dopamine lesions, perhaps through greater involvement of dopamine-independent reinforcement mechanisms. Indeed, one point at which this may occur is at the level of opioid release in the nucleus accumbens which, similar to dopamine release in the nucleus accumbens, can decrease the activity of accumbal medium spiny neurons (Wise, 1998; Koistinen et al., 2001). Thus, an alteration of opioid peptide-receptor interactions offer one possible level at which functional changes may facilitate a compensation for 6-OHDA lesion (Cowen & Lawrence, 1999; Wise, 1998; Koistinen et al., 2001). Intriguingly, 6-OHDA lesions successfully disrupt self-administration of psychostimulants, whose mechanism of action is much more closely dependent on dopamine neurotransmission (Antoniou et al., 1998; Roberts & Koob, 1982), perhaps underscoring the complexity of ethanol reinforcement owing to its more ubiquitous effects throughout the brain. Chronic deactivation of dopaminergic activity by lesions serves to highlight non-dopaminergic processes mediating ethanol reinforcement, including actions of opioids outside of the VTA. Nevertheless,

considering the results of acute dopamine disruption studies using dopamine agonists and antagonists, the non-VTA sites of opioid action that naltrexone can disrupt, and the time allowed in lesion studies for functional adaptations to occur, the failure of 6-OHDA lesions to disrupt ethanol self-administration does not negate the possibility for an opioid role in mediating ethanol reinforcement through a mechanism involving the mesolimbic dopaminergic system (Ikemoto et al., 1997).

The activity, or more specifically the muting of the activity, of GABAergic interneurons or projection neurons that terminate in the VTA appears to comprise an important link in the VTA between endogenous opioid receptor activity, and dopaminergic activity. The activation of μ and possibly δ receptors on VTA GABA interneurons or projection neurons serves to hyperpolarize, and prevent the inhibitory influences of GABA neurotransmission on dopamine neurons (Wise, 1998; Johnson & North, 1992). In this way, inhibition of GABAergic neurons by opioid agonists like morphine produces increased firing of dopamine neurons at the shell, but not the core, of the nucleus accumbens, and this effect can be reversed by GABAb agonism (Gysling and Wang, 1983; Johnson and North, 1992; Kimberly et al., 2003). Thus, the activation of dopaminergic neurons induced by ethanol administration may be due, at least in part, to an opioid-mediated inhibition of GABAergic neurons at the level of the VTA, as depicted in figure 1.1.

1.14 Rational, proposed hypothesis and objectives.

There is experimental evidence supporting a role of the endogenous opioid system in ethanol reinforcement (Gerrits et al., 2003; Lê et al., 2001; Gianoulakis, 2004). Based on

many studies it has been proposed that the endogenous opioid system may mediate the reinforcing effects of alcohol by influencing the activity of the mesolimbic dopaminergic system as shown in figure 1.1. Thus it is proposed that reinforcing doses of ethanol (low to moderate doses) may increase the release of β-endorphin and enkephalin peptides and decrease or not alter the release of dynorphin peptides in brain regions associated with the processes of reward and reinforcement such as nucleus accumbens and VTA. On the other hand high doses of alcohol known to induce aversive effects (Koob, 1992) may stimulate the release of dynorphin peptides with no significant effect on the release of β-endorphin or enkephalin peptides. In support of this hypothesis *in vivo* microdialysis studies have shown that at the level of nucleus accumbens low to moderate doses of alcohol increase the release of β-endorphin and met-enkephalin but not of dynorphin A_{1-8} peptides, while high doses increase the release of dynorphin A_{1-8} but not of β-endorphin and met-enkephalin peptides (Marinelli et al., 2004; Marinelli et al., 2005; Olive 2001; Marinelli et al., 2006). At the level of VTA, interactions of endorphins and enkephalins with μ and δ opioid receptors on GABA interneurons may decrease the GABA inhibitory effect on the A10 dopamine neurons, increasing DA release in the nucleus accumbens, and may initiate the processes of reward and reinforcement. Interactions of dynorphins with κ opioid receptors on the A10 dopaminergic neurons may decrease dopamine release in the nucleus accumbens, produce aversive states, and may prevent reinforcement (Oswald & Wand, 2004). Considering the innervation of VTA by axons of endorphinergic neurons from the arcuate nucleus of the hypothalamus as well as the presence of met-enkephalin and dynorphin peptide-producing neurons and of μ, δ, and κ

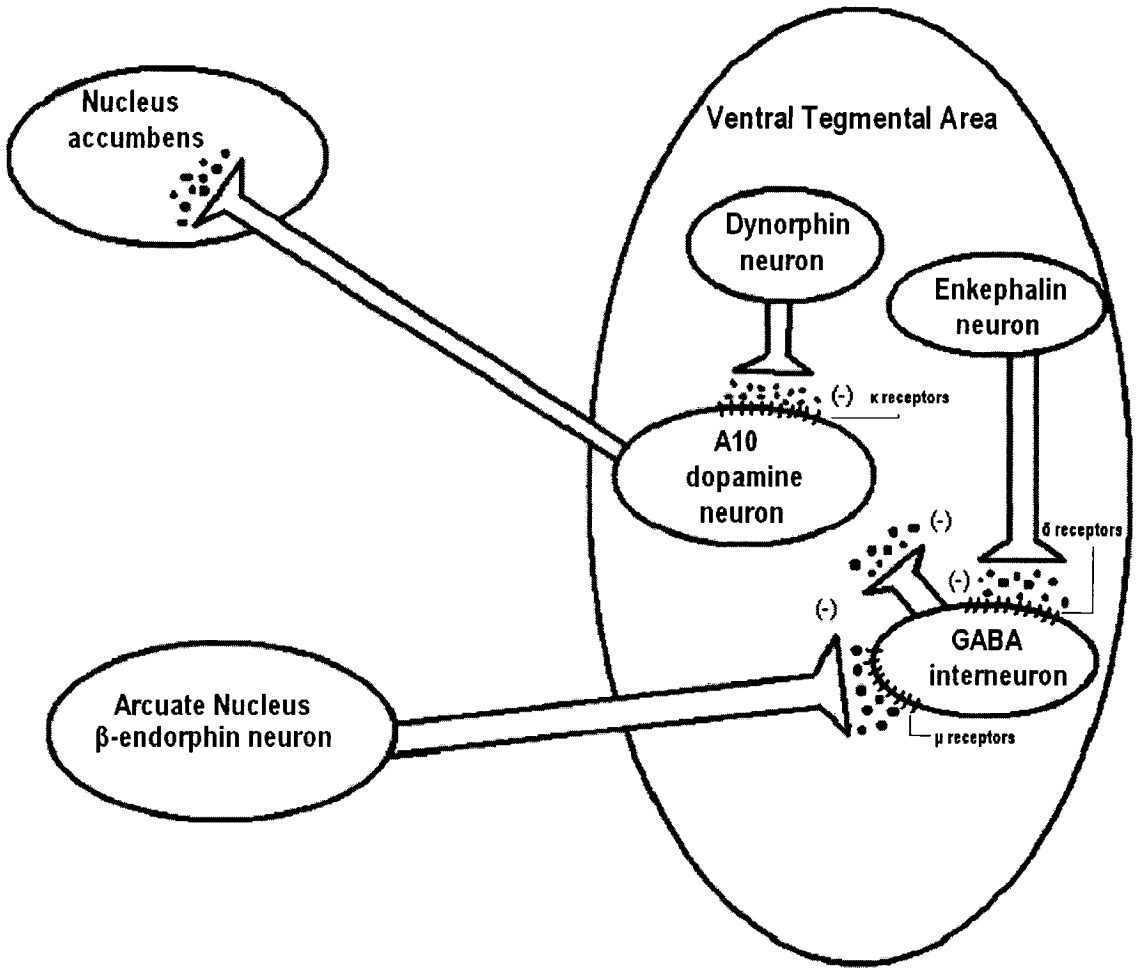

Figure 1.1 Schematic diagram illustrating endogenous opioid connections in VTA, including μ opioid receptor-mediated disinhibition of the A10 dopamine neurons from GABA by β-endorphin or met-enkephalin release, and κ receptor-mediated inhibition of the A10 dopamine neurons. (-) indicates inhibition.

opioid receptors at the level of the VTA (Khacheturian, 1985; Mansour et al., 1988), the proposed involvement of distinct components of the opioid system in the activation of the mesolimbic dopamine is possible. In support of such involvement, Gonzales and Weiss (1998) demonstrated that opioid antagonism using naloxone and naltrexone, at the level of the VTA, can inhibit the ethanol-induced increase of dopaminergic activity, and ethanol reinforcement behaviours. Indeed ethanol-induced increases of dopamine release at the level of nucleus accumbens could be mediated by the ethanol-induced activation of the opioid system at the level of the VTA. (Spanagel et al., 1992; Gonzales & Weiss, 1998; Marinelli et al., 2003). There is only a small number of studies examining the effects of manipulation of specific opioid receptor types, directly at the level of VTA, on the ethanol-induced increase of mesolimbic dopaminergic activity (Samson et al., 1997; Yim et al., 1998). Based on some experimental evidence and the hypothetical model described by figure 1.1, an inhibitory effect on the ethanol-induced increase of mesolimbic dopaminergic activity by either μ or δ opioid receptor antagonism at the level of the VTA is expected (Benjamin et al., 1993; Gonzales & Weiss, 1998; Hyytia and Kiianmaa, 2001).

Currently, evidence indicates that alcohol may indeed increase VTA β-endorphinergic activity. One study investigating the β-endorphin content in the VTA following acute ethanol administration has provided some information on the ethanol-induced changes of β-endorphin release at the level of VTA (Rasmussen et al., 1998). In this study, it is reported that the administration by intragastric infusion of low-to-moderate doses of ethanol, producing blood ethanol concentrations (BAC) in the range of

80-150 mg ethanol/dl blood in Sprague-Dawley rats, produced an elevation in the tissue levels of β-endorphin in the VTA, at 30 minutes post-ethanol administration.

One aim of the current study was to add further clarity on the response of VTA β-endorphin to ethanol by addressing some limitations of the study by Rasmussen et al. (1998). One limitation of the study by Rasmussen et al. (1998) was the lack of information on the time course of the β-endorphin response to ethanol since it was assayed from post-mortem tissue, and only at one time point. In the current study, the use of *in vivo* microdialysis allows the sampling of dialysate samples for the estimation of β-endorphin at multiple time points prior to and post-ethanol treatment, permitting the monitoring of changes with time. Additionally, Rasmussen et al. (1998) provide an assessment of the overall tissue content of β-endorphin. While tissue content assessment can allow the observation of a change from basal values, it is difficult to discern whether changes reflect peptide release or whether they reflect a change in peptide biosynthesis and/or degredation. The *in vivo* microdialysis technique coupled with solid-phase radioimmunoassay used in the current study permit the assessment of extracellular levels of opioid peptides in VTA, thus allowing an estimate of peptide release. Finally, the current study expands on that of Rasmussen and colleagues (1998) by employing a greater range of ethanol doses, leading to BAC in the range of 60-300 mg ethanol/dl blood.

Previous studies demonstrated a transient reduction in the binding of ^{3}H-DAMGO (a specific μ opioid receptor agonist) to μ opioid receptors at the level of VTA, following

ethanol administration (Mendez et al., 2001). This reduced binding of ^{3}H-DAMGO may be attributed to an increase of β-endorphin release (an opioid peptide with a high affinity for μ opioid receptors) at the level of VTA in response to ethanol. However, met-enkephalin, though it has higher affinity for δ opioid receptors, may also bind to μ opioid receptors (Khachaturian et al., 1985). Therefore, the ethanol-induced reduction in the binding of ^{3}H-DAMGO to μ binding sites at the level of VTA may be attributed, at least in part, to an ethanol-induced increase in the release of met-enkephalin peptides in the VTA. While doubt may be cast on such a possibility by other findings, such as a reported decrease in VTA pro-enkephalin mRNA levels at 30 minutes following acute ethanol administration of a high dose of ethanol (2.5 g ethanol/kg B.Wt.) in Wistar rats (Mendez & Morales-Mulia, 2006), as well as lower VTA basal levels of pro-enkephalin mRNA in Sardinian ethanol-preferring rats, compared to non-preferring rats (Fadda et al., 1999), a direct measurement of the response of met-enkephalin, at the level of the VTA to various doses of ethanol has not yet been determined. Thus, a second aim of the current study was to estimate the changes in the extracellular concentration of met-enkephalin at the level of VTA in response to acute exposure to various doses of ethanol, using the *in vivo* microdialysis technique.

While there are few reports on the effects of acute ethanol on the activity of dynorphinergic systems at the level of the VTA, the presence of dynorphinergic terminals and κ opioid receptors in the VTA (Mansour et al. ,1988; Akil et al., 1984) and the responsiveness of mesolimbic activity to ethanol and kappa receptor manipulations (Gessa et al., 1985; Spanagel et al., 1992) allow for the possibility that acute ethanol may

alter dopaminergic activity via dynorphin peptides released at the level of the VTA and influence the process of ethanol reinforcement. Furthermore, comparisons of the endogenous opioid systems between ethanol-preferring and non-preferring mouse strains have detected differences in the content of dynorphin A_{1-8} in brain regions including the VTA, (Jamensky & Gianoulakis, 1997), suggesting that differences in ethanol preference could also be related to variations in dynorphinergic activity. Thus, since κ opioid receptor stimulation has been implicated in reducing mesolimbic dopaminergic activity, and possibly mediating aversive states (Koob, 1998), a third aim of the current studies is to investigate changes of dynorphin A_{1-8} release at the level of VTA following acute administration of various doses of ethanol using the *in vivo* microdialysis technique. Such information will allow the assessment of the impact of dynorphin peptides on ethanol reinforcement.

Thus, the main objective of the current studies was to investigate the hypothesis that acute ethanol exposure alters the release of the three endogenous opioid peptides at the level of the VTA in a dose-dependent manner. To investigate the changes over time in the release of β-endorphin, met-enkephalin, and dynorphin A_{1-8}, following acute administration of various doses of ethanol, the *in vivo* microdialysis technique coupled with specific solid phase radioimmunoassays were employed. Results from the proposed investigations will allow a better understanding of the response of the opioid peptides to various doses of ethanol and their possible involvement in ethanol reinforcement.

CHAPTER 2

Methods and Materials

2.1 Animals

Male Sprague-Dawley rats (Charles River, St-Constant, QC, Canada), weighing 280 – 350 g, were used. Rats were housed individually (to prevent gnawing of cannulae), and had access to food and water *ad libitum*. A period of at least one week was allowed for acclimatization prior to the initiation of experiments. Animals were kept in a temperature and humidity-controlled environment, on a 12-h light/dark cycle (lights on at 8:00 a.m.; off at 8:00 p.m.). Rats were treated in accordance with McGill University's Policy on the Handling and Treatment of Laboratory animals and the Canadian Council on Animal Care guidelines.

2.2 Surgery

Prior to surgical procedures, rats were anaesthetized with a cocktail comprised of ketamine (50 mg/kg), xylazine (5 mg/kg), and acepromazine (1 mg/kg). Guide cannulae (20 mm shaft length; 1mm O.D., S.P.E. Ltd., Concord, ON, Canada) were stereotaxically implanted at the coordinates, relative to bregma, of anteroposterior -0.6 mm, mediolateral +0.09 mm, dorsoventral -0.58 mm (from dura mater). Cannulae were anchored in place using 3 stainless steel screws and dental cement, and were blocked with obturators. A tether screw, to which microdialysis tethers would be connected, was also affixed to the dental cement. Animals were given 3-5 days to recover from surgery prior to habituation.

2.3 Habituation

Habituation and microdialysis were carried out in round, black, plastic testing cages measuring 31 (diameter) x 30 (height) cm. On three occasions, given on alternate days, rats were housed in the testing cages overnight. On the following day, rats received single intraperitoneal (IP) injections of sterile saline solution, to acclimatize them to the manipulations that they would experience during microdialysis testing. Rats remained untethered during habituation sessions.

2.4 Microdialysis

Following the third habituation session rats were weighed, obturators were removed, and microdialysis probes (20 mm shaft length, 0.6 O.D., 2 mm PES membrane, 15 kD cutoff; S.P.E.) were implanted under light isoflurane (Janssen, Toronto, ON, Canada) anaesthesia. A syringe pump (Harvard Apparatus, Mass., USA) was set to infuse artificial cerebrospinal fluid (aCSF: 124 mM sodium chloride, 3 mM potassium choride, 1 mM magnesium chloride-$6H_2O$, 0.5 mM sodium phosphate monobasic-H_2O, 5 mM Sodium phosphate dibasic, 1.3 mM calcium chloride-$2H_2O$, 0.2 mM L-ascorbic acid, 0.025% bovine serum albumin) overnight at a rate of 0.2 µl/min. PTFE tubing (0.56 mm I.D.; Cole-Parmer, Vernon Hills, Ill., USA) connected the pump to a dual-channel swivel (Instech, Plymouth Meeting, Pa., USA). The swivel was counter-balanced on an arm that swung laterally and vertically with the animals' movements. FEP tubing (0.12 mm I.D., CSC, Montreal, QC, Canada) ran to and from the probe through a stainless-steel spring tether that was connected to the tether srew. Dialysate was collected in 500 µl polypropylene vials immersed in ice. At 8:00 a.m. the following day, the pump rate was increased to 2 µl/min, and following a 2-hour period, dialysate collections at 30-minute

intervals commenced. At 12:00 p.m., rats were injected I.P. with 0.0, 0.8, 1.6, or 2.4 g ethanol/kg as a solution of 0, 5, 10, or 15 % (v/v), respectively, in 0.9% saline, ensuring volume-matched injections across dose groups. Dialysate samples were promptly frozen in CO_2 prior to storage at -70°C.

2.5 Choice of flow rates for microdialysis

The choice of the flow rate of 2.0 µl/min for the microdialysis studies was based on published reports from ours and other laboratories, on the recovery profiles of β-endorphin (Olive et al., 2001), enkephalin (Marinelli et al., 2004; Kendrick, 1990), and dynorphin (Marinelli et al. 2006), using probes having the same specifications as those used in the present investigations (20 mm shaft length, 0.6 O.D., 2 mm PES membrane, 15 kD cutoff; S.P.E.). Recovery profiles were estimated for each peptide using a range of flow rates from 0.5 to 3.0 µl/min. It was observed that, as expected, the lower the flow rate, the higher the percent recovery, but the lower the absolute recovery. The 2.0 µl/min flow rate used in the present investigations gave recovery rates of approximately 8%, 15%, and 10% for β-endorphin, enkephalin, and dynorphin, respectively.

2.6 Choice of alcohol doses

Doses of 0.8, 1.6, and 2.4 g ethanol/kg B.Wt., administered intraperitoneally as 5%, 10%, and 15% (v/v), respectively, were chosen because (a) these dose steps can encompass a broad range of doses, requiring fewer groups of animals than smaller dose-step gradations, such as 0.5 g ethanol/kg B.Wt. steps, (b) these doses have been used previously in our studies on the effect of acute ethanol exposure on the release of opioid

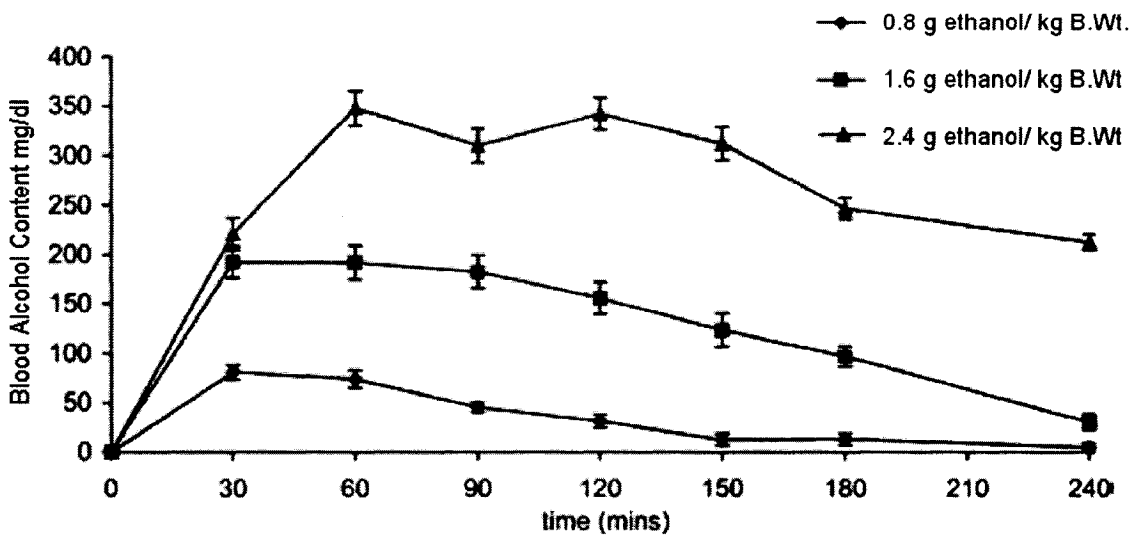

Figure 2.1 Blood alcohol concentration (BAC), measured in tail blood samples at distinct time points after the IP injections of 0.8, 1.6, and 2.4 g ethanol/kg B.Wt in Sprague-Dawley rats (With permission, from Marinelli et al., 2003. Psychopharmacology (Berlin) 169(1): 60-67).

peptides at the level of NACB (Marinelli et al., 2003; 2005; 2006), and would allow us to compare the ethanol-induced changes in the release of opioid peptides between the brain regions of NACB and VTA, and (c) the BACs achieved following administration of these doses of alcohol had been previously determined and were found to be significantly different from one another (figure 2.1) (Marinelli et al., 2003; 2005; 2006).

2.7 Histology

Following the microdialysis session the rats were euthanized with CO_2 and decapitated, and brains were removed and snap-frozen in isopentane, and stored at -70° C. Frozen brains were sectioned into 40 μm slices and mounted on gelatin-coated slides. Slides were Nissl-stained and inspected for accuracy of probe placement. Subjects in which the VTA was not accurately targeted were excluded from peptide content assessment.

2.8 Solid-phase radioimmunoassay for β-endorphin, met-enkephalin, and dynorphin A_{1-8}

Dialysate peptide concentrations were determined using a solid phase radioimmunoassay (after Maidment and Evans, 1991; Marinelli et al., 2003; 2005; 2006). Ninety-six removable-well microplates (Dynex Microlite 2, Chantilly, Va., USA) were filled with 0.8 μg protein A (Sigman, St Louius, Mo., USA) /100 μl 0.1 M sodium bicarbonate (pH approx. 8.4) and incubated for 24 h at 4°C. The next day, wells were emptied and rinsed with 200 μl buffer (0.15 M potassium phosphate dibasic, 0.2 mM L-ascorbic acid, 0.1% Tween 20, 0.1% gelatin, pH adjusted to 7.4 with 10 N hydrochloric

acid). A 50 µl aliquot of antiserum specific for β-endorphin (1:5000 dilution), met-enkephalin (1:5000 dilution), or dynorphin A1-8 (1:5000 dilution) was placed in each well and incubated for 24 h at 4°C. After a 24-hour incubation at 4°C wells were again rinsed with 200 µl buffer. 50 µl of appropriately diluted dialysate samples or standards (diluted in buffer) were added to wells, and again incubated for 24 hours at 4°C. Then, 50 µl of either iodinated β-endorphin (5000-6000 cpm/50 µl, specific activity (SA) 74 TBq/mmol; Amersham Pharmacia Biotech, Buckinghamshire, UK), iodinated met-enkephalin (5000-6000 cpm/50µl, SA = 1208-2078 Ci/mmol, Peninsula Laboratories, Inc.), or iodinated dynorphin A_{1-8} (5000-6000 cpm/50µl, SA = 1841-2037 Ci/mmol, Peninsula Laboratories Inc.) was added to each well, and incubated for 48 hours at 4°C. Following this incubation, wells were emptied and rinsed, and put into 5 ml polypropylene culture tubes and counted on a gamma-ray counter (Corbra II; Packard, Meriden, Conn, USA). The detection limit of these assays was 0.5 pg/tube, and the IC50 was 50 pg/tube for β-endorphin, 196 pg/tube for met-enkephalin, and 2 pg/tube for dynorphin A_{1-8}.

2.9 Specificity of antisera

The antibody used for β-endorphin detection was specific for the C-terminal of β-endorphin and recognized proopiomelanocortin, β-lipotropin, and both acetylated and non-acetylated forms of β-endorphin 1-31, 1-27, and 1-26. This antibody didn't recognize adrenocorticotropic hormone, alpha-melanotropin, or β -lipotropin fragments 1-65, 62-67, and 80-84 (Gianoulakis and Gupta, 1986). Met-enkephalin antiserum (Peninsula Laboratories, Inc., San Carlos, CA) product specifications indicate that the antibody

presents cross-reactivity with Met-enkephalin (100%), Leu-enkephalin (2.8%), Met-enkephalin-Arg-Phe (0.1%), and β-endorphin (0.1%), and no cross-reactivity with Met-enkephalin-Arg-Gly-Leu, dynorphin A1-17, adrenocorticotropic hormone, or endothelin-1. Dynorphin A1-8 antiserum (Peninsula Laboratories Inc, San Carlos, CA), displays cross-reactivity with dynorphin A1-8 (100%), large dynorphin, dynorphin A, dynorphin A1-13, and dynorphin A1-9 (less than 0.01%), and no cross-reactivity with dynorphin A1-7, dynorphin A1-6, dynorphin A6-17, dynorphin B, β-endorphin, Met-enkephalin, alpha-neoendorphin, and leu-enkephalin-arg.

2.10 Data analysis

Basal dialysate concentrations of each peptide assayed (β-endorphin, met-enkephalin, or dynorphin A_{1-8}) were analyzed for the four ethanol dose groups using one-way, independent samples ANOVAs. The effect of ethanol on the extracellular levels of each peptide was estimated as a percent change from the four baseline measurements. The data of the percent change from baseline for each peptide were analyzed using a mixed 2-way ANOVA with ethanol dose as the independent variable and time as the repeated variable. Analysis of main effects and interactions was done with the Tukey post hoc test and simple ANOVAs. Significance was regarded at $p < 0.05$ level.

CHAPTER 3

Results

3.1 Behavioural responses to various doses of ethanol

In the present investigation animals were injected IP with, three doses of ethanol and a volume-matched saline control. Among the behavioural responses observed following ethanol administration was higher activity shortly after administration of the lower doses of ethanol (0.8 g ethanol/kg B.Wt.), and behavioural depression and hypnosis following higher doses of ethanol (1.6 and 2.4 g ethanol/kg B.Wt.). Blood alcohol concentrations (BAC) were estimated from the tail blood at various time points following the IP injection of ethanol using different groups of rats from those used in microdialysis experiments and have been previously reported (Marinelli et al., 2003; 2005; 2006), as shown in figure 2.1.

3.2 Probe location

Figure 3.1 shows tracks left by microdialysis probes, identified in 40 μm Nissl stained coronal sections, which were plotted on a stereotaxic map of the VTA -6.3, -6.04, and -5.8 mm from bregma. The animals in which the VTA was considered to be accurately targeted were included in peptide content assessment and data analysis.

3.3 Concentration of β-endorphin in the dialysates under basal conditions

Mean basal values of β-endorphin, illustrated in figure 3.2, were assessed for each ethanol dose group, using the four 30 minute dialysate collections taken prior to ethanol or saline administration. Basal β-endorphin values were statistically analyzed using a one-way, independent variables ANOVA and revealed no significant difference in basal β-endorphin levels between the four ethanol dose groups ($P>0.05$).

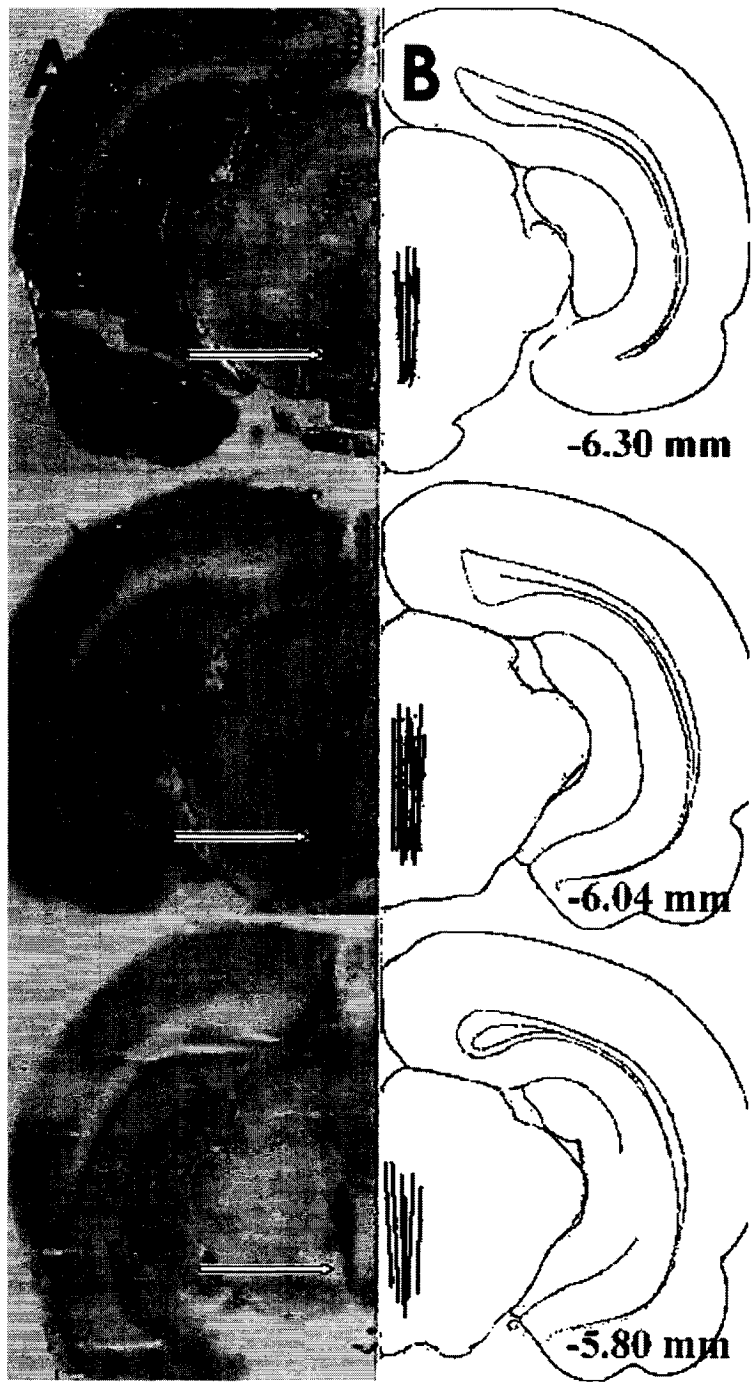

Figure 3.1 A: Representative photographs of the VTA. Arrows indicate probe location. B: Diagrams of coronal sections indicating the position (in mm) posterior from bregma. Lines indicate tracings of approximate probe locations in the brains of the subjects used for peptide content analysis.

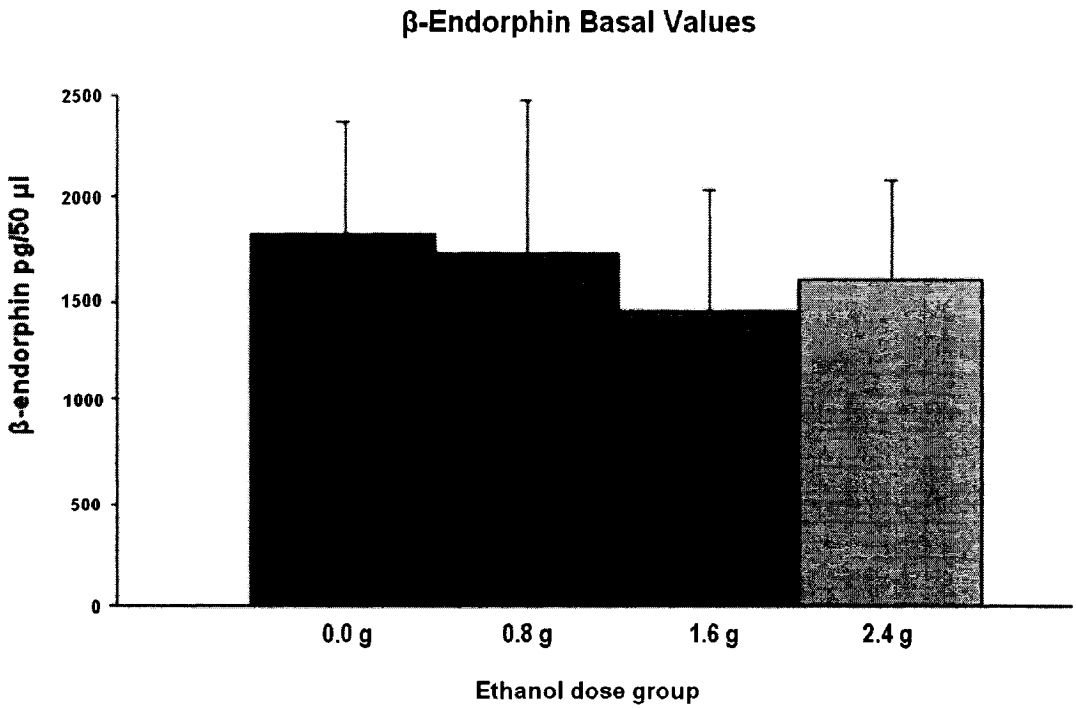

Figure 3.2 Content of β-endorphin in the dialysate samples collected at the level of VTA, under basal conditions for each ethanol dose group. For each animal the mean β-endorphin content in the 4 baseline collections of the dialysate was used. The number of animals in each group were 8, 8, 9, and 10 for the 0.0, 0.8, 1.6, and 2.4 g ethanol/kg B.Wt. dose groups, respectively. One way ANOVA indicated no significant difference for the basal β-endorphin levels among the 4 dose groups ($p > .05$).

3.4 Effect of various concentrations of ethanol on the release of β-endorphin at the level of the VTA

Figures 3.3 A, B, and C present the changes in the dialysate β-endorphin content, following IP injection of 0.0, 0.8, 1.6, or 2.4 g ethanol/kg B.Wt., expressed as a percentage of the basal values. The mixed, two-way ANOVA, in which treatment was the non-repeated measure, and collection time points were the repeated measure demonstrated a significant main effect of dose [$F(3, 30)=5.72$, $P=0.0032$], and of collection time [$F(8, 240)=7.93$, $P<0.0001$], and no significant interaction between dose and time ($P=0.154$). Tukeys honestly significant difference post-hoc tests revealed that β-endorphin release was significantly greater in the 1.6 g ethanol/kg B.Wt. dose group than in the saline control, the 0.8, and the 2.4 g ethanol/kg B.Wt. groups. Thus, animals receiving 1.6 g ethanol/kg B.Wt. presented significantly greater β-endorphin release than those receiving saline ($P<0.05$). β-endorphin release in the dose groups receiving 0.8 or 2.4 g ethanol/kg B.Wt. did not significantly differ from the saline group ($P>0.05$) or each other. A significant main effect of time was also found [$F(8, 240)=7.93$, $P<0.0001$]. Tukey's post-hoc testing revealed significant increases from basal values in all eight dialysate collections following the IP administration of 1.6 g ethanol/kg B.wt ($P<0.0001$). No significant differences from basal values were observed following all other treatments ($P>0.05$).

3.5 Concentration of met-enkephalin in the dialysates under basal conditions

Mean basal values of met-enkephalin, (figure 3.4), were assessed for each ethanol dose group using the four 30 minute dialysate collections taken prior to ethanol or saline

Figure 3.3 Direct comparison of the effect of 0.8 (A), 1.6 (B), and 2.4 (C) g ethanol/kg B.wt with the effect of saline (0.0 g ethanol/kg B.Wt.) on dialysate β-endorphin levels in VTA, expressed as percentage of basal levels. Basal levels were estimated as the mean of the β-endorphin content in the first four 30-minute dialysate collections preceding the IP injection of the saline or alcohol solution, and were considered as 100%. Arrow indicates the time of IP injection of saline or ethanol. n indicates the number of animals for each dose group. Error bars denote SEM. * denotes significant difference of the corresponding time point from the basal values ($p < 0.05$). + indicates significant difference from the saline treated group at the same time point ($p < 0.05$).

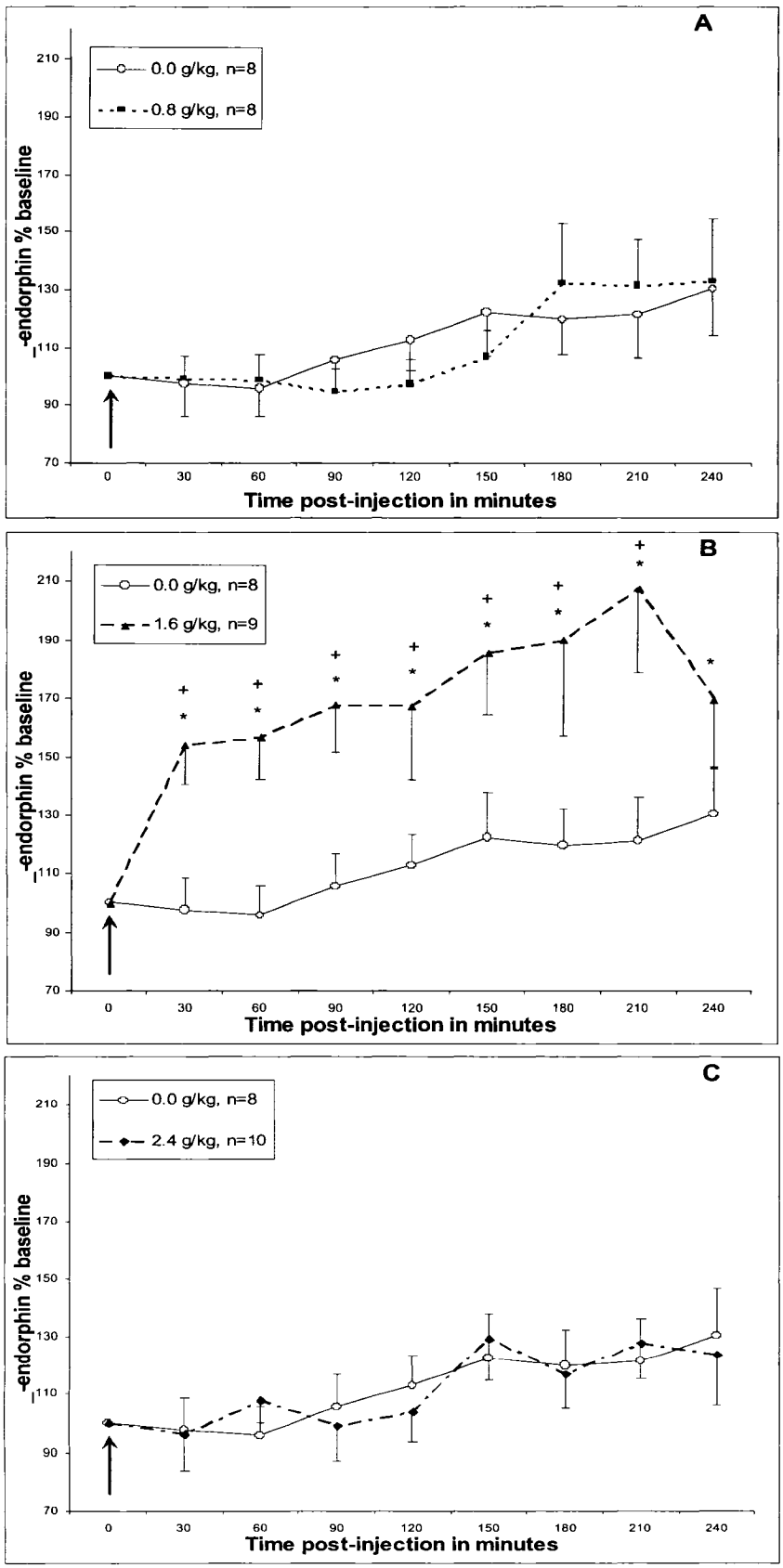
A
0.0 g/kg, n=8
0.8 g/kg, n=8
-endorphin % baseline
Time post-injection in minutes
B
0.0 g/kg, n=8
1.6 g/kg, n=9
-endorphin % baseline
Time post-injection in minutes
C
0.0 g/kg, n=8
2.4 g/kg, n=10
-endorphin % baseline
Time post-injection in minutes

Figure 3.3

administration. Basal met-enkephalin values were statistically analyzed using a one-way, independent variables ANOVA. Analysis revealed no significant difference in basal levels between the four ethanol dose groups ($P>0.05$).

3.6 Effect of various concentrations of ethanol on the release of met-enkephalin at the level of the VTA

Figures 3.5 A, B, and C represent the changes in the dialysate met-enkephalin content following IP injections of 0.0, 0.8, 1.6, 2.4 g ethanol/kg B.Wt, expressed as a percentage of the basal values. The mixed, two-way ANOVA, with treatment the non-repeated measure, and collection time points the repeated measure, demonstrated no significant main effect of dose [$F(3, 29)=0.25$, $P=0.8610$], a significant main effect of collection time point [$F(8, 232)=4.38$, $P<0.0001$], and no significant interaction between dose and time ($P=0.9$). Post hoc testing, using Tukey's honestly significant difference test, revealed a general increase at 150, 180, 210, and 240 minutes compared with baseline ($P<0.05$), although further Tukey's tests did not reveal significant differences, in any of the treatment groups, between the met-enkephalin content at the various time points and the basal values ($P>0.05$).

3.7 Concentration of dynorphin A_{1-8} in the dialysates under basal conditions

The mean basal values of dynorphin A_{1-8}, as shown in figure 3.6, were assessed for each ethanol dose group using the four 30 minute dialysate collections taken prior to ethanol or saline administration. Basal dynorphin A_{1-8} values were statistically analyzed using a one-

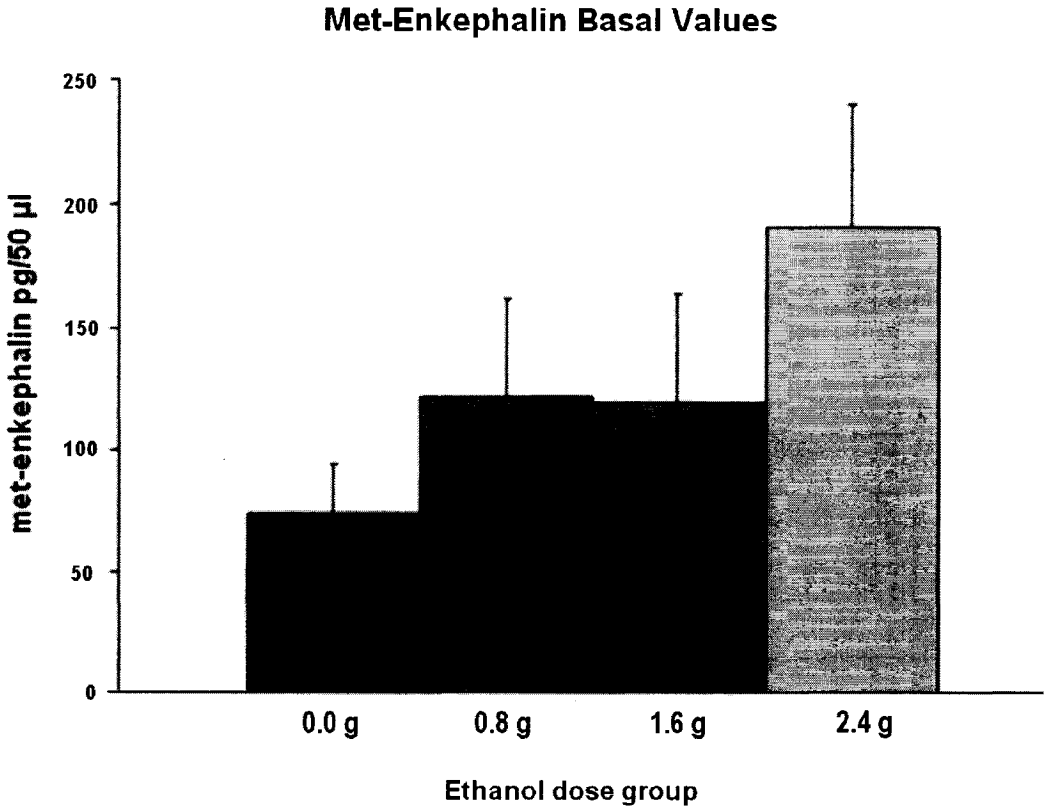

Figure 3.4 Content of met-enkephalin in dialysate samples collected at the level of VTA, under basal conditions for each ethanol dose group. For each animal the mean met-enkephalin content in the 4 baseline collections of the dialysate was used. The number of animals were 7, 8, 9, and 9 for the 0.0, 0.8, 1.6, and 2.4 g ethanol/kg B.Wt. dose groups, respectively. One way ANOVA indicated no significant difference for the basal met-enkephalin levels among the 4 dose groups ($p > .05$).

Figure 3.5 Direct comparison of the effect of 0.8 (A), 1.6 (B), and 2.4 (C) g ethanol/kg B.wt with the effect of saline (0.0 g ethanol/kg B.Wt.) on dialysate met-enkephalin levels in VTA, expressed as percentage of basal levels. Basal levels were estimated as the mean of the met-enkephalin content in the initial four 30-minute dialysate collections preceding the IP injection of saline or ethanol, and were considered as 100%. Arrow indicates the time of IP injection of saline or ethanol administration. Error bars denote SEM. n indicates the number of animals for each dose group.

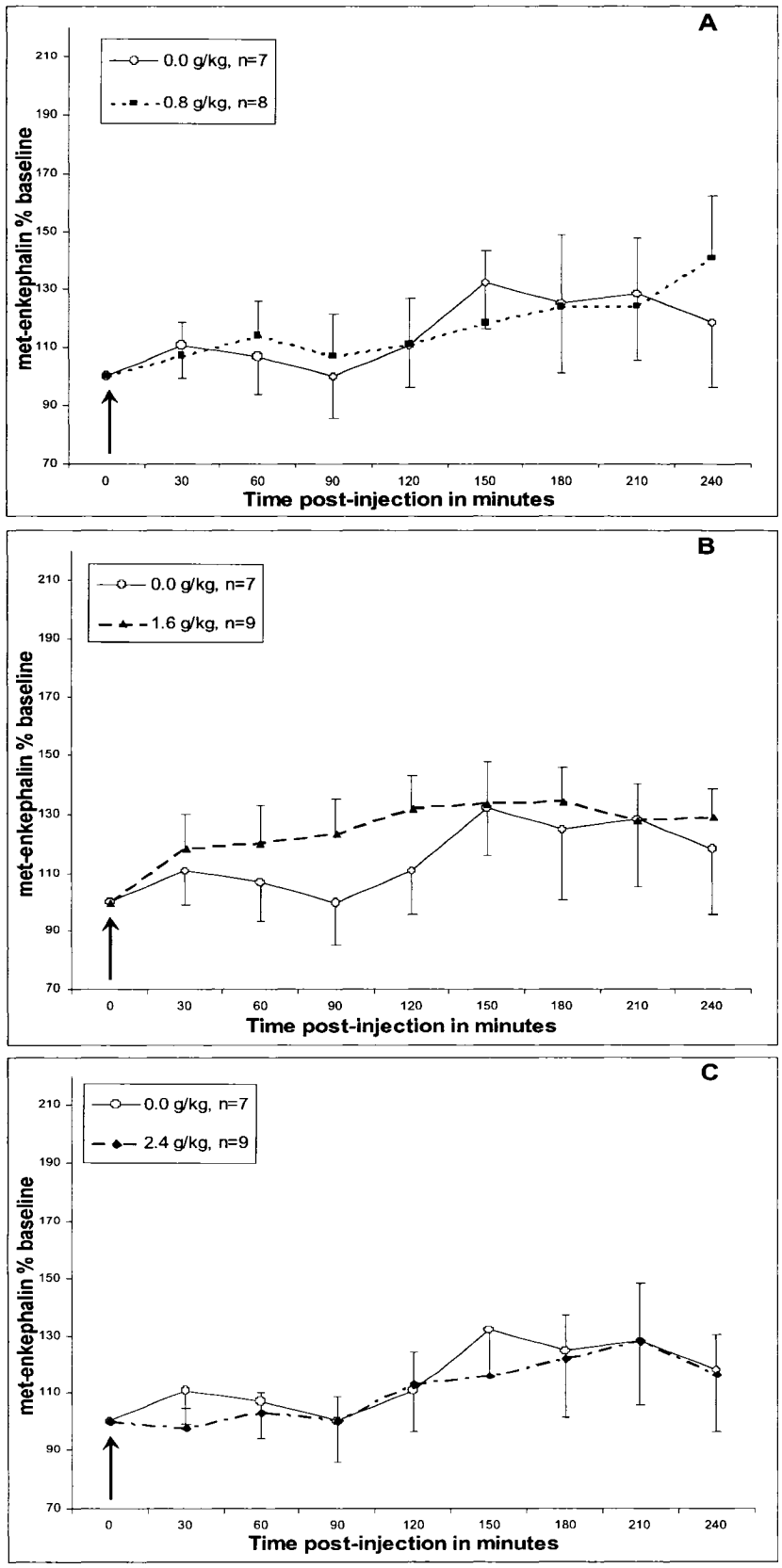
A
0.0 g/kg, n=7
0.8 g/kg, n=8
met-enkephalin % baseline
210
190
170
150
130
110
90
70
0
30
60
90
120
150
180
210
240
Time post-injection in minutes
B
0.0 g/kg, n=7
1.6 g/kg, n=9
met-enkephalin % baseline
Time post-injection in minutes
C
0.0 g/kg, n=7
2.4 g/kg, n=9
met-enkephalin % baseline
Time post-injection in minutes

Figure 3.5

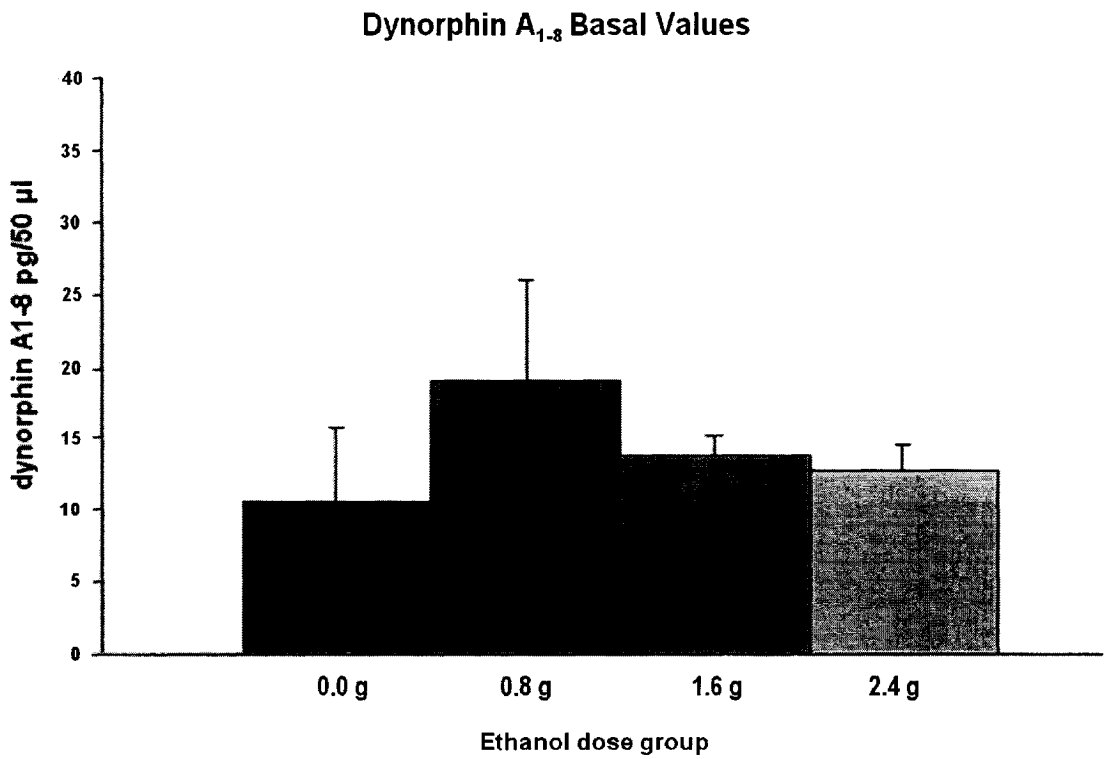

Figure 3.6 Content of dynorphin A_{1-8} in dialysate samples collected at the level of VTA, under basal conditions for each ethanol dose group. For each animal the mean dynorphin A_{1-8} content in the initial 4 baseline collections of the dialysate was used. The number of animals were 5, 8, 6, and 7 for the 0.0, 0.8, 1.6, and 2.4 g ethanol/kg B.Wt. dose groups, respectively. One way ANOVA indicated no significant difference for the basal dynorphin A_{1-8} levels among the 4 dose groups ($p > .05$).

way, independent variables ANOVA. The analysis indicated no significant difference in the basal levels of dynorphin A_{1-8} between the four dose groups ($P>0.05$).

3.8 Effect of various concentrations of ethanol on the release of dynorphin A_{1-8} at the level of the VTA

Figures 3.7 A, B, and C represent the changes in the dialysate dynorphin A_{1-8} content following IP injection of 0.0, 0.8, 1.6, or 2.4 g ethanol/kg B.Wt, expressed as a percentage of basal values. The mixed, two-way ANOVA, with treatment the non-repeated measure, and collection time points the repeated measure, indicated no significant main effect of ethanol dose [$F(3, 33)=0.39$, $P=0.7632$], a significant main effect of collection time points [$F(8, 242)=1.91$, $P=0.039$], and no significant interaction between dose and collection time points ($P=0.836$). Post hoc analysis using the Tukey's test did not reveal significant differences between the dynorphin A_{1-8} content at the various time points and the basal values ($P>0.05$).

Figure 3.7 Direct comparison of the effect of 0.8 (A), 1.6 (B), and 2.4 (C) g ethanol/kg B.wt with the effect of saline (0.0 g ethanol/kg B.Wt.) on dialysate dynorphin A_{1-8} levels in VTA, expressed as percentage of basal levels. Basal levels were estimated as the mean of the dynorphin A_{1-8} content in the initial four 30-minute dialysate collections preceding the IP injection of saline or ethanol, and were considered as 100%. Arrow indicates the time of ethanol administration. n indicates the number of animals for each dose group. Error bars denote S.E.M.

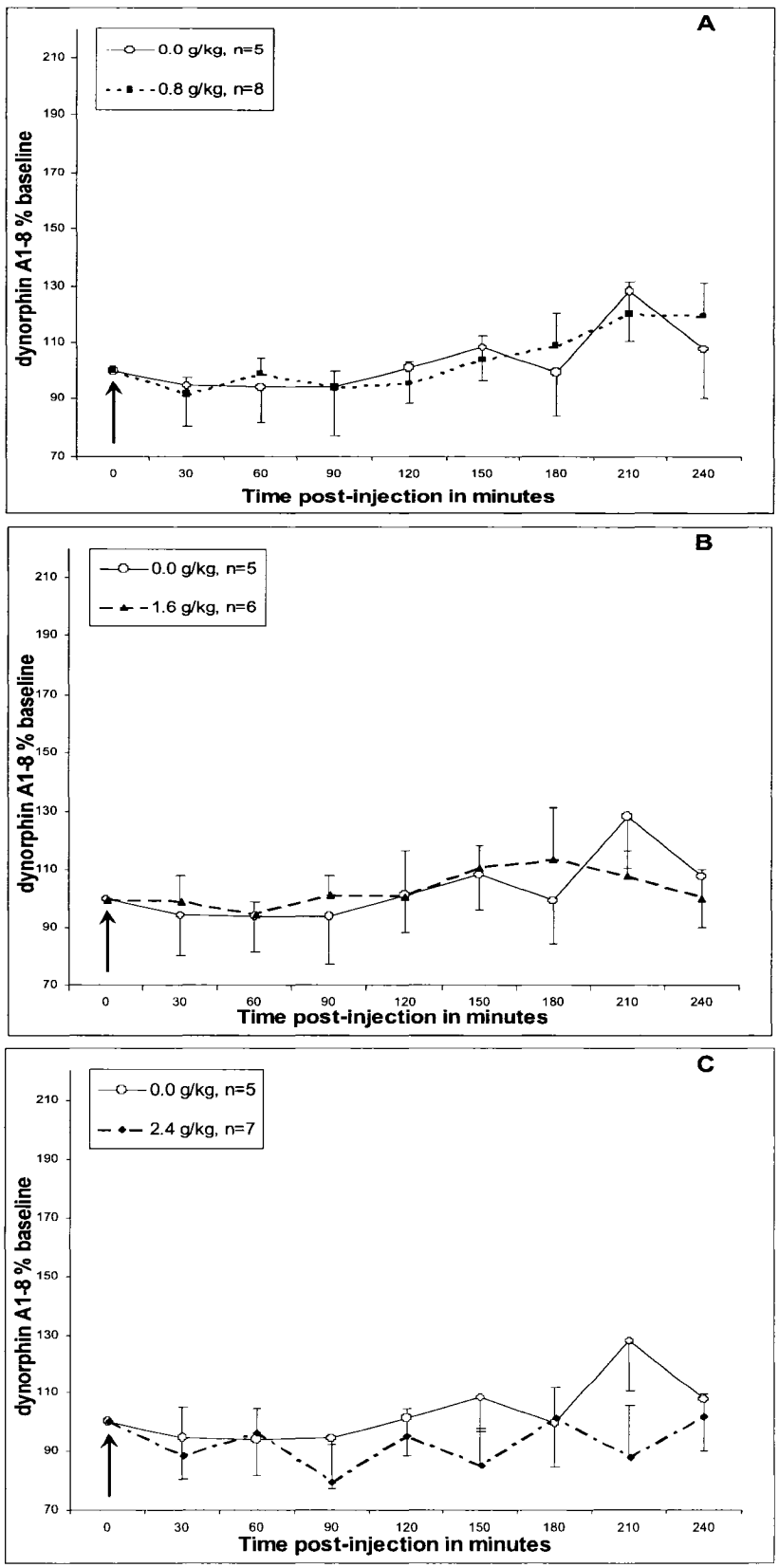
A
0.0 g/kg, n=5
0.8 g/kg, n=8
dynorphin A1-8 % baseline
210
190
170
150
130
110
90
70
0
30
60
90
120
150
180
210
240
Time post-injection in minutes
B
0.0 g/kg, n=5
1.6 g/kg, n=6
dynorphin A1-8 % baseline
Time post-injection in minutes
C
0.0 g/kg, n=5
2.4 g/kg, n=7
dynorphin A1-8 % baseline
Time post-injection in minutes

Figure 3.7

CHAPTER 4

Discussion

4.1 Major findings and their significance

Using the *in vivo* microdialysis technique the response of the three major opioid peptide systems (endorphins, enkephalins, and dynorphins) to various doses of ethanol at the level of the VTA was investigated in Sprague-Dawley rats. Among the major findings of the present investigations were (a) the biphasic effect of ethanol on the release of β-endorphin with moderate (1.6 g ethanol /kg B.Wt.) but not low (0.8 g ethanol /kg B.Wt.) or high (2.4 g ethanol /kg B.Wt.) doses of ethanol inducing a significant increase of β-endorphin release, and (b) the lack of a significant effect of ethanol, at the doses tested, on the release of enkephalin and dynorphin peptides. These observations suggest that at the level of the VTA, β-endorphin may be the opioid peptide which, through its interactions with μ or possibly δ opioid receptors on GABA interneurons inhibits GABA neurotransmission leading to disinhibition of the A10 mesolimbic dopaminergic neurons and increased release of dopamine at the level of the nucleus accumbens. Therefore, this ethanol-induced stimulation of β-endorphin release at the level of the VTA may play a significant role in mediating some of the reinforcing effects of ethanol.

4.2 Ethanol does not alter the release of enkephalin and dynorphin peptides at the level of the VTA

While acute ethanol administration induced an alteration of β-endorphin release, the levels of met-enkephalin and dynorphin A_{1-8} were, contrary to the proposed hypothesis, not affected. The anatomical positioning of opioid systems within the VTA, including the presence of met-enkephalin and dynorphin A_{1-8}, as well as δ, μ, and κ opioid receptors (Khacheturian et al., 1985; Akil et al., 1984) suggests their possible role

in modulating the effects of reinforcing agents. Furthermore, the sensitivity of the reinforcing effects of ethanol, and dopaminergic responsiveness to δ antagonism and κ agonism suggest that met-enkephalin and dynorphin A_{1-8} may be involved in ethanol reinforcement. Indeed, manipulations of δ and κ opioid receptor systems have been shown to influence the activity of neurotransmitter systems in the VTA (Xuei et al., 2006; Lindholme et al., 2001; Holter et al., 2000; McBride et al., 1999; Matsuzawa et al., 1999; Bals-Kubik et al., 1993; Spanagel et al., 1992; Hakan & Henriksen, 1989). Similar to past reports, a rising trend of met-enkephalin levels towards the end of the experimental session was observed. Circadian variations of met-enkephalin levels in regions including CNS areas has been reported elsewhere, although the controls necessary to determine this possibility were not in place in the current study (Kurumaji et al., 1988; Dumont et al., 1991).

This lack of effect of ethanol on the dynorphin A_{1-8} and met-enkephalin opioid peptide systems at the level of VTA could indicate either that these systems are not sensitive to ethanol or that due to methodological limitations the effects of ethanol could not be detected. Among the methodological limitations could be the sensitivity of the microdialysis coupled with RIA technique to detect small changes in the extracellular concentrations of these peptides. The fact that dialysate dynorphin A_{1-8} concentrations in the VTA were relatively low may suggest that any subtle changes in dynorphin A_{1-8} release could have been too small to be detected by the *in vivo* microdialysis coupled with RIA techniques. Techniques with higher sensitivity may be needed to detect the subtle effects of ethanol on the VTA dynorphin system. As was the case for dynorphin

A_{1-8}, no alteration was observed in met-enkephaline release following administration of each of the doses of ethanol. In addition, previous studies at the level of nucleus accumbens (Marinelli et al., 2003; 2005; 2006) demonstrated a dose-dependent biphasic effect of ethanol on the release of enkephalin, dynorphin A_{1-8}, and β-endorphin peptides with low to medium doses, but not high doses increasing the release of enkephalin and β-endorphin, while high but not low doses of ethanol increased the release of dynorphin peptides. Therefore, since large dose steps were utilized in the present study it is possible that administration of additional ethanol dose steps, between those used, could have altered the release of met-enkephalin, while doses higher than 2.4 g ethanol/kg B.Wt. could have altered the release of dynorphin peptides. Furthermore, since dialysate samples were collected at 30-minute intervals, the temporal resolution was limited, and one can not rule out the possibility of an early transient and short-lasting change in the rate of met-enkephalin and/or dynorphin A_{1-8} release in response to one or more of the ethanol doses tested.

4.3 Biphasic effect of ethanol on the activity of the β-endorphin system

The increased release of β-endorphin in response to moderate doses of ethanol at the level of VTA observed in the present investigations is in agreement with previous reports demonstrating increased content of β-endorphin peptides in tissue extracts of the VTA and nucleus accumbens at 30 minutes following intragastric ethanol administration (Rasmussen et al., 1998). Both of these regions receive endorphinergic innervation from POMC-producing cells of the hypothalamic arcuate nucleus (Khacheturian et al., 1985). Short chronic treatments with ethanol have been shown to increase (de Waele &

Gianoulakis, 1994; Angelogianni & Gianoulakis, 1993; Krishnan-Sarin et al., 1998), while prolonged chronic treatments with ethanol have been shown to decrease the hypothalamic content of POMC mRNA (Chen et al., 2004; Rasmussen et al., 2002). Furthermore, studies by Rasmussen et al. (1998) demonstrated that acute intragastric administration of ethanol, sufficient to produce BACs of 120-150 mg/dl, increased the hypothalamic content of POMC mRNA. However, intragastric administration of lower doses of ethanol sufficient to produce BACs in the range of 40-110 mg/dl did not significantly alter the content of hypothalamic POMC mRNA. In the present studies IP administration of 1.6 g ethanol/kg B.Wt. produced BACS of about 180 mg/dl at 30 minutes and 140 mg/dl at 180 minutes post-ethanol administration. Therefore the dose of 1.6 g ethanol/kg B.Wt. may be associated not only with increased release of β-endorphin in VTA but also increased hypothalamic POMC mRNA content suggesting increased synthesis of POMC peptides in the hypothalamus. In the present investigations the ethanol-induced increase in the extracellular concentration of β-endorphin at the level of VTA was maintained for the entire experimental session. A gradual decrease towards basal values was observed only at 240 minutes post-injection. It is tempting then to speculate that the long-lasting increase in the release of β-endorphin at the level of VTA observed in the present studies is associated with an ethanol-induced increase of synthesis and post-translational processing of hypothalamic POMC (Rasmussen et a., 1998). On the other hand, the IP administration of 0.8 g ethanol/ kg B.Wt. that produces BACs lower than 100 mg/dl is associated with neither increased content of hypothalamic POMC mRNA (Rasmussen et al., 1998) nor increased β-endorphin release at the level of VTA (present investigations).

Among the findings of the present studies was that the 2.4 g ethanol /kg B.Wt. dose of alcohol failed to enhance the release of β-endorphin in the VTA. Considering the significant and long-lasting increase of β-endorphin release in response to the 1.6 g ethanol /kg B.Wt. dose, this lack of response to the 2.4 g ethanol/kg B.Wt. dose is difficult to explain. The IP administration of 2.4 g ethanol /kg B.Wt. produces BACs of about 210 mg/dl, 350 mg/dl, 260 mg/dl, and 250 mg/dl at 30, 60, 180, and 240 minutes post-injection, respectively. Although, high concentrations of ethanol may increase the release of neurotransmitter systems that exert an inhibitory effect on β-endorphin release, an initial transient increase of β-endorphin release was anticipated during the early period post-ethanol administration, at time points that BAC was 150 mg/dl or lower. However, such a transient increase was not observed in the present investigations. Indeed, with the exception of two rats that presented a small increase of approximately 10-15% in β-endorphin release following administration of the 2.4 g ethanol/kg B.Wt. dose, most of the 10 rats included in the data analysis displayed a lack of change of β-endorphin release from the basal values. This lack of response of VTA β-endorphin to high concentrations of ethanol could be due to the fact that the concentration of ethanol in the blood increased rapidly, reaching 210 mg/dl within the first 30 minutes, while there are reports indicating that the ethanol concentration in the brain increases even more rapidly than in the blood (Lumeng et al., 1982; Nurmi et al., 1994)). Thus, if there was an early transient increase of β-endorphin release it would last for a very short period and it would be difficult to be detected with the temporal resolution in the present study that utilized 30-minute collections of dialysate samples. Thus, collection of dialysates at more frequent intervals,

such as 10-15 minute intervals, may indeed demonstrate a transient increase of β-endorphin release in response to the 2.4 g ethanol/kg B.Wt.ethanol dose.

Although the studies by Rasmussen et al. (1998) did not investigate the effect of BACs higher than 150 mg/dl on the content of hypothalamic POMC mRNA, there are previous studies reporting a biphasic effect of ethanol on the *in vitro* release of hypothalamic β-endorphin. Thus, studies using Sprague Dawley, AA and ANA rats, as well as C57BL/6 and DBA/2 mice demonstrated that in *in vitro* preparations of intact hypothalamus moderate concentrations of ethanol (20 mM) induced a more pronounced increase of β-endorphin release, than higher concentrations (30-40 mM), while when the concentration of ethanol in the incubation medium was increased to 60 mM it did not alter the β-endorphin release from the basal levels (de Waele et al., 1994; 1993). Using *in vivo* microdialaysis, a biphasic, dose-dependent effect of ethanol on the release of β-endorphin and enkephalin peptides was also observed in the rat nucleus accumbens (Marinelli et al., 2003; 2005; 2006), providing further support for the presence of a similar biphasic effect of ethanol on β-endorphin release at the level of VTA.

4.4 Evidence for a biphasic effect of ethanol on nucleus accumbens dopamine release

The current study demonstrated that at the level of VTA, ethanol produced a biphasic response of β-endorphin, with significant elevations of the extracellular levels of β-endorphin following administration of a moderate dose (1.6 g ethanol/kg B.Wt.) and no significant responses at lower (0.8 g ethanol/kg B.Wt.) and higher (2.4 g ethanol/kg B.Wt.) doses of ethanol, producing an inverted 'U'-shaped dose-response curve. Similar

biphasic responses to ethanol have been observed for other neurotransmitter systems, including the midbrain dopaminergic system. Indeed, dopaminergic activity has been reported to be increased by doses of ethanol comparable to the 1.6 g ethanol/kg B.Wt. dose used in the present studies, while higher or lower doses produced either smaller, or had no, effect on dopaminergic activity. (Mocsary & Bradberry, 1996). Nevertheless, it must be noted that while biphasic effects of ethanol on dopaminergic activity are commonly reported, the particular doses that do, or do not elicit responses are inconsistent and vary among investigations. For instance, several reports indicate that dopaminergic activity becomes augmented following doses both lower and higher than the 1.6 g ethanol/kg B.Wt. dose (Blanchard et al., 1993; Marinelli et al., 2003; Di Chiara & Imperato, 1985; Tizabi et al., 2002; Yan, 1999; Gessa et al., 1985). While these differences may in some cases be ascribed to variations of administration routes, and species/strains used, these differences may also indicate that the VTA β-endorphin is but one of many neurotransmitter systems that may mediate ethanol's effects on the mesolimbic dopaminergic system. Furthermore, it should be noted that investigations on the functional significance of the effects of ethanol on the endogenous opioid system were beyond the objectives of the present study. The ethanol induced increase in β-endorphin release at the level of VTA, observed in the present study, is in agreement with published reports indicating that the disinhibition of dopaminergic neurons by ethanol is mediated, at least in part, by ethanol induced changes in the activity of the endogenous opioid system (Gerrits et al., 2003; Johnson & North, 1992; Di Chiara & Imperato) However, studies specifically designed to elucidate the functional significance of the ethanol induced increase of β-endorphin release at the level of VTA should be performed.

4.5 Significance of the ethanol-induced increase in VTA β-endorphin

The current study provides data that enhance the understanding of the effects of ethanol on distinct components of the endogenous opioid systems at the level of the VTA, a brain region associated with the processes of reward and reinforcement. A large body of evidence demonstrates an interaction of ethanol with endogenous opioid systems at the level of VTA, and supports their involvement in the process of ethanol reinforcement (Koob, 2000; Gerrits et al., 2003; Gianoulakis, 2004). While a range of studies have provided support for the capacity of opioid receptors in the VTA to produce alterations in brain responses which have implications for reinforcement (Van Wolfswinkel & Van Ree, 1985; Bechtholt & Cunningham, 2005), the present investigation represents one of only a few studies on the activity of endogenous opioid peptides at the level of the VTA following acute ethanol administration. While the current investigation allowed for a detailed time-course, dose-response study on the activity of opioid peptides at the level of VTA following ethanol administration, the study is still limited in its temporal resolution, and higher temporal resolutions are needed. Nonetheless the current findings appear to provide support for a role of β-endorphin in alcohol reinforcement, at the level of the VTA.

The notion that ethanol-induced A10 dopaminergic neuronal activity may be modulated by opioid receptors in the VTA, is supported by the present observation that enhanced VTA β-endorphin release indeed occurs following administration of alcohol. This finding is in agreement with reports on the stimulatory effect of ethanol on dopaminergic activity (Gessa et al., 1985; Weiss et al., 1993; Marinelli et al., 2003), as

well, on the mechanisms proposed to account for the involvement of opioid systems in the ethanol induced augmentation of the activity of the mesolimbic dopaminergic neurons at the level of VTA. Furthermore, the rapid commencement of the increase in β-endorphin release fits well with the ethanol-induced increase in dopaminergic activity and release, while the dose that increased the release of VTA β-endorphin is one which has previously been shown to stimulate dopamine release in nucleus accumbens (Yim et al., 2000; Marinelli et al., 2003). However, there are some apparent discrepancies, between β-endorphin release observed here and dopaminergic responses to alcohol reported previously including differences in the timing and duration of release (Yim et al., 2000; Kohl et al., 1998), indicating that other neurotransmitter systems, in addition to VTA β-endorphin may be contributing to the ethanol-induced increase in dopamine release at the level of nucleus accumbens.

4.6 Time course of VTA β-endorphin release in response to acute ethanol administration

A notable feature of the increase in the extracellular levels of β-endorphin at the level of the VTA observed following a dose of 1.6 g ethanol/kg B.Wt. ethanol, in addition to the fast commencement, was the tendency for β-endorphin levels to remain elevated above baseline for most of the duration of the experimental session. Indeed, β-endorphin levels began to show signs of returning towards basal values only in the last dialysate sample, collected between 210-240 minutes after ethanol administration. In contrast with this protracted release of β-endorphin, studies on the nucleus accumbens dopamine release induced by acute ethanol administration of doses comparable to those

used in the present studies demonstrated a more rapid cessation of the enhanced dopamine response, typically within approximately 1 to 2 hours post-ethanol (Yim et al., 2000; Kohl et al., 1998). Following acute ethanol administration of doses similar to those used in the current study, BACs can remain elevated for several hours post-ethanol. Indeed, BACs persist long after nucleus accumbens dopamine levels return to normal. Similarly, the current study found that, β-endorphin levels roughly mirrored blood ethanol levels, which also remain elevated long after dopaminergic responses typically cease. Thus, despite the fact that the ethanol-induced increase in nucleus accumbens dopaminergic activity appears to be partly mediated by the ethanol-induced increase of β-endorphin release, with time other factors become involved, leading to an attenuation of the enhanced dopaminergic activity. This phenomenon is generally known as an acute tolerance of the dopaminergic system to ethanol. There are a number of reports of acute tolerance to the ethanol effects on various neurotransmitters or behavioural responses (Yim et al., 2000; Le and Kalant, 1992; Waller et al., 1983). While it is well established that enhanced VTA μ opioid receptor activity is mediating, at least in part, the nucleus accumbens increase in dopamine activity following acute ethanol administration (Rodd et al., 2004b), the current study suggests that the activation of VTA μ opioid receptors by the elevated extracellular levels of β-endorphin persists long after the dopaminergic activity has decreased towards basal values (Yim et al., 2000), suggesting a temporal dissociation between the ethanol induced increase of VTA β-endorphin and mesolimbic dopaminergic activities. This lack of temporal correspondence between β-endorphin elevations found here, and dopaminergic augmentations reported elsewhere (Yim et al., 2000), does not necessarily detract support for a causal link between VTA β-

endorphinergic activity on the one hand, and dopaminergic activity on the other, even if the opioid influence is but a modulatory one. The neurophysiological events responsible for the acute tolerance of the dopamine response to ethanol are not clear. The finding that β-endorphin levels seem to follow blood alcohol levels would rule out an acute attenuation of β-endorphin release at the level of the VTA as the cause of the acute tolerance of dopaminergic activity to ethanol. Indeed, the time profile of ethanol-induced dopaminergic activation is influenced by a number of stimulatory mechanisms, including a modulatory role of VTA β-endorphin, an excitatory glutamatergic input, and an inhibitory input mediated by dopaminergic autoreceptors or GABAergic interneurons (Kohl et al., 1998; Rahman and McBride, 2001). The nature of glutamatergic inputs to the mesolimbic dopaminergic system presents an increased complexity since glutamatergic terminals are reported to be modulated by presynaptic opioid-mediated inhibition, and add another dimension to the modulation of dopaminergic neurons by endogenous opioids (Chergui et al., 1993; Stobbs et al., 2004; Margolis et al., 2005).

4.7 Conclusions and future directions

The current study adds support to the involvement of endogenous opioids in the reinforcing effects of ethanol. Nevertheless, it also suggests that further avenues of inquiry are needed to clarify their role. Because the large dose steps used in the current study produced a change in the extracellular levels of β-endorphin that was confined to one dose group, further investigations are needed to assess the effects of ethanol dose steps in between those used here. Additionally, in the present investigations the temporal resolution was limited to 30-minute dialysate collection intervals. Since β-endorphin

elevations had taken place within the first 30-minute time point, microdialysis utilizing more frequent collection intervals (10-15 minutes) could provide more accurate information on the time course of β-endorphin elevations in response to ethanol.

Acute administration of a single dose of ethanol, while arguably pharmacologically active, and instructive regarding the neurophysiology of reinforcement, is nonetheless insufficient to induce a state of addiction. Therefore, in future studies the effect of chronic ethanol administration on the activity of the VTA opioid peptide systems and particularly β-endorphin, could provide important information on their role in ethanol addiction. Such studies should be performed after the development of stable voluntary drinking by the animals. In addition, the use of alcohol-preferring and non-preferring lines of animals, such as the AA and ANA lines of rats, which have been shown to present differences in the activity of distinct components of the endogenous opioid system (Gianoulakis, 2004), may provide important information.

Since β-endorphin binds with high affinity to μ receptors, the lack of changes in met-enkephalin levels following alcohol administration suggests that the effects of μ receptor activation on mesolimbic activity (Hyytia, 1993) are attributable to β-endorphin actions, the release of which was increased in response to ethanol (present study). The lack of response of dynorphin A_{1-8} to ethanol administration, may appear contrary to expectations, considering the capacity of κ receptor manipulations to alter dopaminergic activity. It seems probable that the doses of ethanol used in the current study were not sufficiently high to produce the aversive states typically associated with activation of κ

receptors. Additionally, dynorphin release in the nucleus accumbens was reported only after a high dose of 3.2 g ethanol/kg B.Wt. (Marinelli et al., 2006), so the highest dose of 2.4 g ethanol/kg B.Wt. used here may have been insufficient to induce a response. Thus, future studies examining a greater range of doses may provide a better insight into the contribution of dynorphin systems in mediating ethanol responses. Similarly, examination of dynorphin activity using a chronic ethanol paradigm might prove fruitful, considering the attenuation of mesolimbic dopaminergic activity in response to stimulation of κ opioid receptors, as well as the alterations of ethanol reward thresholds associated with κ opioid receptor stimulation (Koob, 1998).

In conclusion, the current investigations demonstrated that at the level of VTA systemic administration of ethanol induced a prolonged, dose-dependent increase of β-endorphin release, but did not significantly alter the release of either met-enkephalin or dynorphin A_{1-8} peptides. This ethanol induced increase of β-endorphin release in the VTA may play a significant role in the ethanol induced stimulation of the mesolimbic dopaminergic system and the initiation of the processes of ethanol reward and reinforcement.

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NUCLEAR SUBSTANCES AND RADIATION DEVICES LICENCE | PERMIS PORTANT SUR LES SUBSTANCES NUCLÉAIRES ET LES APPAREILS À RAYONNEMENT

Licence Number / Numéro de permis

I) LICENSEE

Pursuant to section 24(2) of the Nuclear Safety and Control Act, this licence is issued to:

Hôpital Douglas/
Douglas Hospital
6875 LaSalle Boulevard
Verdun, QC
H4H 1R3
Canada

hereinafter «the licensee».

II) PERIOD

This licence is valid from: May 1 2002 to April 30 2007.

III) LICENSED ACTIVITIES

This licence authorizes the licensee to possess, transfer, import, export, use and store the nuclear substances and the prescribed equipment listed in section IV) of this licence.

This licence is issued for: laboratory studies: 10 or more laboratories where radioisotopes are used or handled (836)

IV) NUCLEAR SUBSTANCES AND PRESCRIBED EQUIPMENT

ITEM	NUCLEAR SUBSTANCE	UNSEALED SOURCE MAXIMUM QUANTITY	SEALED SOURCE MAXIMUM QUANTITY	EQUIPMENT MAKE AND MODEL
1	Hydrogen 3	4 GBq	n/a	n/a
2	Carbon 14	1 GBq	n/a	n/a
3	Iodine 125	1500 MBq	n/a	n/a
4	Calcium 45	40 MBq	n/a	n/a
5	Phosphorus 32	400 MBq	n/a	n/a
6	Sulfur 35	1 GBq	n/a	n/a
7	Phosphorus 33	1 GBq	n/a	n/a
8	Cesium 137	n/a	1480 kBq	Beckman LS (series)
9	Radium 226	n/a	370 kBq	Canberra-Packard 4000 series

The total quantity of an unsealed nuclear substance in possession shall not exceed the corresponding listed unsealed source maximum quantity. The total quantity of nuclear substance per sealed source shall not exceed its corresponding listed sealed source maximum quantity. Sealed sources shall only be used in the corresponding listed equipment.

V) LOCATION(S) OF LICENSED ACTIVITIES

used or stored at:

Lehmann and Frank B.Common Pavilion
6875 LaSalle Boulevard
Verdun, QC

VI) CONDITIONS

1. Prohibition of Human Use
This licence does not authorize the use of nuclear substances in or on human beings.
(2696-0)

2. Area Classification
The licensee shall classify each room, area or enclosure where more

than one exemption quantity of an unsealed nuclear substance is used at a single time as:

(a) basic-level if the quantity does not exceed 5 ALI,
(b) intermediate-level if the quantity used does not exceed 50 ALI,
(c) high-level if the quantity does not exceed 500 ALI,
(d) containment-level if the quantity exceeds 500 ALI; or
(e) special purpose if approved in writing by the Commission or a person authorized by the Commission.

Except for the basic-level classification, the licensee shall not use unsealed nuclear substances in these rooms, areas or enclosures without written approval of the Commission or a person authorized by the Commission.
(2108-1)

3. Laboratory Lists
The licensee shall maintain a list of all areas, rooms and enclosures in which more than one exemption quantity of a nuclear substance is used or stored.
(2569-1)

4. Laboratory Procedures
The licensee shall post and keep posted, in a readily visible location in areas, rooms or enclosures where nuclear substances are handled, a radioisotope safety poster approved by the Commission or a person authorized by the Commission, which corresponds to the classification of the area, room or enclosure.
(2570-1)

5. Thyroid Monitoring
Every person who
(a) uses at a single time a quantity of volatile iodine-125 or iodine-131 exceeding;
(i) 5 MBq in an open room;
(ii) 50 MBq in a fume hood;
(iii) 500 MBq in a glove box;
(iv) any other quantity in other containment approved in writing by the Commission or a person authorized by the Commission; or
(b) is involved in a spill of greater than 5 MBq of volatile iodine-125 or iodine-131;
(c) or on whom iodine-125 or iodine-131 external contamination is detected; and shall, undergo thyroid screening within five days following the exposure to iodine-125 or iodine-131.
(2046-7)

6. Thyroid Screening
Screening for internal iodine-125 and iodine-131 shall be performed using:
(a) a direct measurement of the thyroid with an instrument that can detect 1 kBq of iodine-125 or iodine-131; or
(b) a bioassay procedure approved by the Commission or a person authorized by the Commission.
(2600-1)

7. Thyroid Bioassay
If thyroid screening detects more than 10 kBq of iodine-125 or iodine-131 in the thyroid, the licensee shall immediately make a preliminary report to the Commission or a person authorized by the Commission and have bioassay performed within 24 hours by a person licensed by the Commission to provide internal dosimetry.

(2601-4)

8. Extremity Dosimetry
The licensee shall ensure that any person who handles a container which contains more than 50 MBq of phosphorus 32, strontium 89, yttrium 90, samarium 153 or rhenium 186 wears a ring dosimeter. The dosimeters must be supplied and read by a dosimetry service licensed by the Commission.
(2578-0)

9. Contamination Criteria

The licensee shall ensure that for nuclear substances listed in the licence application guide table titled "Classification of Radionuclides";
(a) non-fixed contamination in all areas, rooms or enclosures where unsealed nuclear substances are used or stored does not exceed:
(i) 3 becquerels per square centimetre for all Class A radionuclides;
(ii) 30 becquerels per square centimetre for all Class B radionuclides; or
(iii) 300 becquerels per square centimetre for all Class C radionuclides;
averaged over an area not exceeding 100 square centimetres; and
(b) non-fixed contamination in all other areas does not exceed:
(i) 0.3 becquerels per square centimetre for all Class A radionuclides;
(ii) 3 becquerels per square centimetre for all Class B radionuclides; or
(iii) 30 becquerels per square centimetre for all Class C radionuclides;
averaged over an area not exceeding 100 square centimetres.
(2642-2)

10. Decommissioning
The licensee shall ensure that prior to decommissioning any area, room or enclosure where the licensed activity has been conducted;
(a) the non-fixed contamination for nuclear substances listed in the licence application guide table titled "Classification of Radionuclides" does not exceed:
(i) 0.3 becquerels per square centimetre for all Class A radionuclides;
(ii) 3 becquerels per square centimetre for all Class B radionuclides; and
(iii) 30 becquerels per square centimetre for all Class C radionuclides;
averaged over an area not exceeding 100 square centimetres;
(b) the release of any area, room or enclosure containing fixed contamination, is approved in writing by the Commission or person authorized by the Commission;
(c) all nuclear substances and radiation devices have been transferred in accordance with the conditions of this licence; and
(d) all radiation warning signs have been removed or defaced.
(2571-2)

11. Storage
The licensee shall:
(a) ensure that when in storage radioactive nuclear substances or radiation devices are accessible only to persons authorized by the licensee;
(b) ensure that the dose rate at any occupied location outside the storage area, room or enclosure resulting from the substances or devices in storage does not exceed 2.5 microSv/h; and
(c) have measures in place to ensure that the dose limits in the Radiation Protection Regulations are not exceeded as a result of the substances or devices in storage.
(2575-0)

12. Disposal (Laboratories)
When disposing of unsealed nuclear substances to municipal garbage or sewer systems, the licensee shall ensure that the following limits are not exceeded:

COLUMN 1	COLUMN 2(a)	COLUMN 3(b)
-	LIMITS	LIMITS
Nuclear	solids to	liquids(water
Substance	municipal	soluble)to
-	garbage	municipal
-	system	sewer system
-	(quantity	(quantity per
-	per kilogram)	year)
Carbon 14	3.7 MBq	10 000 MBq
Chromium 51	3.7 MBq	100 MBq