Molecular Adaptations in the Endogenous Opioid System in Human and Rodent Brain

MUHAMMAD ZUBAIR HUSSAIN
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Abstract

The aims of the thesis were to examine i) whether the endogenous opioid system (EOS) is lateralized in human brain areas involved in processing of emotions and pain; ii) whether EOS responses to unilateral brain injury depend on side of lesion, and iii) whether in human alcoholics, this system is involved in molecular adaptations in brain areas relevant for cognitive control of addictive behavior and habit formation.

The main findings were that (1) opioid peptides but not opioid receptors and classic neurotransmitters are markedly lateralized in the anterior cingulate cortex involved in processing of positive and negative emotions and affective component of pain. The region-specific lateralization of neuronal networks expressing opioid peptides may underlie in part lateralization of higher functions in the human brain including emotions and pain. (2) Analysis of the effects of traumatic brain injury (TBI) demonstrated predominant alteration of dynorphin levels in the hippocampus ipsilateral to the injury, while injury to the right hemisphere affected dynorphin levels in the striatum and frontal cortex to a greater extent than that to the left hemisphere. Thus, trauma reveals a lateralization in the mechanisms mediating the response of dynorphin expressing neuronal networks in the brain. These networks may differentially mediate effects of left or right brain injury on lateralized brain functions. (3) In human alcoholics, the enkephalin and dynorphin systems were found to be downregulated in the caudate nucleus and / or putamen that may underlie in part changes in goal directed behavior and formation of a compulsive habit in alcoholics. In contrast to downregulation in these areas, PDYN mRNA and dynorphins in dorsolateral prefrontal cortex, κ-opioid receptor mRNA in orbitofrontal cortex, and dynorphins in hippocampus were upregulated in alcoholics. Activation of the κ-opioid receptor by upregulated dynorphins may underlie in part neurocognitive dysfunctions relevant for addiction and disrupted inhibitory control.

We conclude that the EOS exhibits region-specific lateralization in human brain and brain-area specific lateralized response after unilateral TBI in mice; and that the EOS is involved in adaptive processes associated with specific aspects of alcohol dependence.

Keywords: Endogenous Opioid System, Dynorphins, Lateralization, Alcohol Dependence, Emotions, Traumatic Brain Injury, Anterior Cingulate Cortex, Dorsal Striatum

Muhammad Zubair Hussain, Uppsala University, Department of Pharmaceutical Biosciences, Box 591, SE-751 24 Uppsala, Sweden.

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To my Mother
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


All papers are written by first authors in association with co-authors. I have been involved in biochemical / molecular analysis as well as in manuscript writing in paper I, II and III. In paper IV, I have been involved in biochemical analysis.

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### Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>Asp</td>
<td>aspartate</td>
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<tr>
<td>BOLD</td>
<td>blood oxygen level dependent</td>
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<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<td>DA</td>
<td>dopamine</td>
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<td>dl-PFC</td>
<td>dorsolateral prefrontal cortex</td>
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<tr>
<td>DOR</td>
<td>delta-opioid receptor</td>
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<tr>
<td>Dyn A</td>
<td>dynorphin A</td>
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<td>Dyn B</td>
<td>dynorphin B</td>
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<td>EOS</td>
<td>endogenous opioid system</td>
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<tr>
<td>Glu</td>
<td>glutamate</td>
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<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<tr>
<td>KOR</td>
<td>kappa-opioid receptor</td>
</tr>
<tr>
<td>LER</td>
<td>Leu-enkephalin-Arg</td>
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<tr>
<td>LH</td>
<td>left hemisphere</td>
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<tr>
<td>MC</td>
<td>motor cortex</td>
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<tr>
<td>MOR</td>
<td>mu-opioid receptor</td>
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<tr>
<td>NA</td>
<td>noradrenaline</td>
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<tr>
<td>NAc</td>
<td>nucleus accumbens</td>
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<tr>
<td>NE</td>
<td>neoendorphin</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>Nor-BNI</td>
<td>norbinaltorphimine</td>
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<td>OFC</td>
<td>orbitofrontal cortex</td>
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<tr>
<td>OPRD 1</td>
<td>opioid receptor delta 1</td>
</tr>
<tr>
<td>OPRK 1</td>
<td>opioid receptor kappa 1</td>
</tr>
<tr>
<td>OPRM 1</td>
<td>opioid receptor mu 1</td>
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<tr>
<td>MEAP</td>
<td>Met-enkephalin-Arg-Phe</td>
</tr>
<tr>
<td>PAG</td>
<td>periaqueductal gray</td>
</tr>
<tr>
<td>PDYN</td>
<td>prodynorphin</td>
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<tr>
<td>PET</td>
<td>positron emission tomography</td>
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<tr>
<td>PENK</td>
<td>proenkephalin</td>
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<td>pgACC</td>
<td>pregenual anterior cingulate cortex</td>
</tr>
<tr>
<td>POMC</td>
<td>proopiomelanocortin</td>
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<tr>
<td>qRT-PCR</td>
<td>quantitative real-time polymerase chain reaction</td>
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</table>
RH  right hemisphere
RQI  RNA quality indicator
SD  standard deviation
SEM  standard error of mean
5-HT  serotonine
SN  substantia nigra
SNP  single nucleotide polymorphism
TBI  traumatic brain injury
TFA  trifluoroacetic acid
1. Introduction

1.1 The endogenous opioid system (EOS)

The opioid peptide precursors are encoded by three genes: proopiomelanocortin (POMC), proenkephalin (PENK), and prodynorphin (PDYN) that give rise to multiple opioid peptides including β-endorphin, Met- and Leu-enkephalins, dynorphins and neoendorphins (NE). The opioid peptides act through G-protein-coupled μ-, δ- and κ-opioid receptors (MOR, DOR and KOR). Dynorphins bind to the KOR with higher affinity than to the MOR and DOR, and therefore may function as endogenous ligands for the KOR in the central nervous system (CNS) (Chavkin et al., 1982; Smith and Lee, 1988; Zhang et al., 1998).

1.2 The EOS / dynorphins in emotions

The EOS is a critical neurotransmitter system regulating presentation and processing of emotions and pain. Endorphins and enkephalins are positive reinforcers and their interactions with MOR and DOR modulate positive emotional states (Bals-Kubik et al., 1993; Filliol et al., 2000; Leknes and Tracey, 2008). Activation of MOR and DOR can be counterbalanced through the stimulation of KOR by dynorphins, which can act as endogenous negative reinforcers and dysphorics. KOR agonists produce aversive effects in animals and dysphoria in humans and dynorphin upregulation may lead to anhedonia and depression (Knoll and Carlezon, 2010; Land et al., 2008; Narita et al., 2006; Schwarzer, 2009; Shippenberg, 2009). Opioid receptors are densely distributed in brain regions including the ACC and PFC that have a central role in the integration of emotionally salient stimuli and in processing the affective components of pain (Zubieta et al., 2001; Zubieta et al., 2003). Alterations in MOR in the ACC and other cortical regions may contribute to affective disorders including major depression and border line personality disorders (Kennedy et al., 2006; Pizzagalli, 2011; Prossin et al., 2010; Walter et al., 2009).
1.2.1 Lateralization in emotions

A fundamental property of the human brain is lateralization of higher functions. The left hemisphere (LH) is dominant for language, mathematical and logical reasoning; while the right hemisphere (RH) is specialized in shape recognition, spatial attention and musical and artistic functions (Bever and Chiarello, 2009; Craig, 2005; Dehaene et al., 1999; Gazzaniga, 2005; Gier et al., 2010; Joseph, 1988; Levy et al., 1999; Mesulam, 1999; Toga and Thompson, 2003). According to the “right hemisphere hypothesis”, emotions are predominantly processed in the RH. The left side of the face is emotionally more expressive (Sackeim et al., 1978). Similarly, the emotional intonation (prosody) of words is more easily recognized if stimuli are presented to the left ear (Erhan et al., 1998), and stimuli presented to the left visual field are perceived with greater emotion (Levine and Levy, 1986) and elicits greater autonomic response (Spence et al., 1996). Consistently, patients with frontal damage to the RH demonstrate deficits in both prosody (Ross and Mesulam, 1979) and in the recognition of emotional facial expressions (Mandal et al., 1999; Weddell, 1994). The alternative, “valence hypothesis” postulates that emotions are lateralized depending on their valence. The RH is dominant for negative emotions and pain, while the LH predominantly processes positive emotions (Davidson, 1992; Sackeim et al., 1982). Patients who suffered trauma to the left frontal lobe are more frequently depressed (Morris et al., 1996; Sackeim et al., 1982), while patients with right frontal damage show inappropriate signs of cheerfulness and mania (Starkstein et al., 1989). The valence hypothesis is supported by behavioral, neuroimaging and electrophysiological evidence (Carraquillo and Gereau, 2008; Ji and Neugebauer, 2009; Lugo et al., 2002; Symonds et al., 2006). Yet, a number of studies did not support the “right hemisphere” and “valence” hypotheses and instead proposed the region-specific functional lateralization of emotion processing (Beraha et al., 2012). Thus, lateralization of emotions may differ between prefrontal and subcortical areas (Demaree et al., 2005).

1.2.2 Anterior cingulate cortex (ACC) in emotions

A distributed network of cortical regions including the prefrontal, insular and anterior cingulate (ACC) cortices is involved in the presentation and processing of emotional stimuli (Ansell et al., 2012; Corradi-Dell'Acqua et al., 2011; Craig, 2005; Fan et al., 2011; Lane et al., 1998). The ACC may function as a generator of physiological or behavioral responses, and may be involved in the top-down modulation of limbic and endocrine systems to regulate positive and negative emotions (Murphy et al., 2003; Phan et al., 2002; Vytal and Hamann, 2010). The ACC is a part of neural cortical network processing emotional-affective aspects of pain (Casey et al., 1997; Kulkarni et al., 2005; Wiech et al., 2006). The left ACC may predominantly
process positive emotions or happiness (Dolan et al., 1996; Phillips et al., 1998), whereas the right ACC negative emotions and pain (Brooks et al., 2002; Brügger et al., 2011; Hsieh et al., 1995; Hsieh et al., 1996; Kim et al., 2012; Symonds et al., 2006) that is consistent with the emotional lateralization model (Craig, 2005).

The pgACC, cytoarchitecturally and physiologically distinct subregion of the ACC is involved in processing of negative and positive emotions and affective component of pain and has anatomical connections to other core-emotional processing regions as amygdala, PAG and hypothalamus (An et al., 1998; Beckmann et al., 2009). Imaging studies using the selective μ-opioid receptor agonist [11C] carfentanyl, or non-selective opioid antagonist naloxone, identified a critical role of the EOS in control of these processes in the pgACC (Eippert et al., 2009; Kennedy et al., 2006; Wager et al., 2007). The pgACC is involved in processing and expression of positive (Lindgren et al., 2012; Vogt, 2005) and negative (Amemori and Graybiel, 2012; Etkin et al., 2011) emotions. The pgACC is the site where negative affect is associated with opioid receptor occupancy (Zubieta et al., 2003). The pgACC mediates the analgesic and placebo effects of opioids (Eippert et al., 2009; Petrovic et al., 2002). Opioid receptors are expressed at high levels in the pgACC (Vogt, 2005). Abnormalities in this area are associated with depression, schizophrenia, border-line personality and anxiety disorders (Fahim et al., 2005; Pizzagalli, 2011; Prossin et al., 2010; Walter et al., 2009), and autism spectrum disorder including Asperger syndrome (Oner et al., 2007). Thus, an aberrant neuronal activation of the pgACC may contribute to anhedonia in major depression (Walter et al., 2009). Many functional and structural changes in psychiatric patients were identified specifically in the right pgACC. They include a correlation between reductions in gray matter volume and self-conscious emotional reactivity in patients with dementia (Sturm et al., 2012); reduced gray matter volume in patients with border-line personality disorders (Soloff et al., 2008); hyperglutamatergic metabolism in subjects with autism spectral disorder (Bejjani et al., 2012); a higher N-acetylaspartate : choline ratio in Asperger syndrome (Oner et al., 2007); and abnormal BOLD responses in non-paranoid schizophrenia patients expressing fear (Williams et al., 2004). Thus, the right pgACC may contribute to emotional dysregulation in psychiatric disorders with negative emotional states.

Lateralization of positive and negative emotions and pain suggests that the neurotransmitter systems regulating these functions may be asymmetrically distributed between the LH and RH. By virtue of their ability to selectively mediate euphoria and dysphoria, as well as pleasure and pain, the EOS is an ideal candidate neurochemical system to subserve these lateralized brain functions. We hypothesize that the EOS could modulate lateralized processing of emotions in human brain. Therefore, it is important to investigate whether the euphoric and dysphoric components of EOS are asymmet-
rically distributed in human brain regions and if so, whether this asymmetri-
cal distribution of euphoric and dysphoric components of EOS is function-
ally associated with processing of positive and negative emotions.

1.3 The EOS / dynorphins in traumatic brain injury (TBI)

Several lines of evidence suggest a role of endogenous dynorphins in CNS
injury. Dynorphin A (Dyn A), dynorphin B (Dyn B), and α-neoendorphin (α-
NE), collectively known as dynorphins, are endogenous κ-opioid receptor
ligands. General and κ-receptor-selective opioid antagonists were reported to
be beneficial in experimental models of spinal cord injury and TBI, suggest-
ning that endogenous dynorphins contribute to secondary CNS injury
(Behrmann et al., 1993; Faden et al., 1987; McIntosh et al., 1987; Vink et al.,
1990). Furthermore, Dyn A, the most pathogenic dynorphin, may cause tis-
sue injury and cell death, and may exacerbate the clinical severity of traum-
atic injury to the head or spinal cord (Faden, 1990; Faden, 1996; Goody et
al., 2003; Hauser et al., 2005; Headrick et al., 1995; Hu et al., 1996;
McIntosh et al., 1994; Woods et al., 2006). Dyn A-induced tissue injury may
involve both opioid and non-opioid components (Adjan et al., 2007; Bakshi
et al., 1992; Faden, 1990; Hauser et al., 2005; Long et al., 1994; Woods et
al., 2006).

It has been demonstrated that mutations in the human prodynorphin gene
cause profound neurodegeneration in the cerebrum and cerebellum underly-
ing the spinocerebellar ataxia type 23, a dominantly inherited neurodegen-
erative disorder (Bakalkin et al., 2010). Three out of four mutations are lo-
cated in Dyn A. Generalized pathological changes including cerebral cortical
and subcortical atrophy, and agenesis of the corpus callosum, were charac-
teristics of patients carrying Dyn A mutations. The mutations apparently
enhance the pathogenic potential of Dyn A, as is evident from analysis of the
peptide’s ability to induce neuron death (Bakalkin et al., 2010). This fact,
along with the gross structural effects on brain morphology, indicate a fun-
damental role of wild-type Dyn A in modulating neuronal function and sur-
vival, and indirectly support the hypothesis that Dyn A has a pathogenic
function in TBI and spinal cord injury.

1.3.1 Lateralization in TBI

Several TBI effects in rodents and humans have been reported to be lateral-
ized (Heilman et al., 1978; Kerkhoff, 2001; Levin et al., 1995; Morrow et al.,
1981; Pavlovskaya et al., 2007; Pearlson and Robinson, 1981; Schaefer et
al., 2009). Previous studies have shown that region-specific differences in
norepinephrine turnover after somatosensory TBI were dependent on the side of injury in rats (Levine and Levy, 1986). These lateralized effects had both behavioral and biochemical correlates. Right but not left somatosensory lesions produced behavioral hyperactivity and bilaterally decreased cerebral and locus ceruleus norepinephrine concentrations (Pearlson and Robinson, 1981). Identical lesions of the left frontal cerebral cortex produce neither the hyperactivity nor a decrease in norepinephrine levels. In humans, left hemisphere damage produced deficits in controlling the trajectory of arm movement, but not in achieving final position. In contrast, the right hemisphere damaged group showed deficits in accuracy of final position, but not in the ability to coordinate complex movement of a limb with multiple joints (Schaefer et al., 2009). Furthermore, lateralization of the lesion was identified as the critical factor in emotional hyporeactivity in brain-injured patients; right hemisphere damaged patients were psychophysiological hypoaroused compared with left hemisphere damaged subjects (Heilman et al., 1978; Morrow et al., 1981).

1.3.2 Brain regions affected by TBI

Along with effects on brain cortical areas, TBI causes atrophy and dysfunction of sub-cortical structures. Massive neuronal damage and cell loss occur in the cerebral cortex, hippocampus, and substantia nigra (SN) following experimental and clinical TBI (Adams et al., 1985; Anderson et al., 2005; Baldwin et al., 1997; Dietrich et al., 1994; Soares et al., 1995). Neuronal damage and degeneration in the hippocampus and thalamus correlate with the severity of post-traumatic motor dysfunction and cognitive deficits. Damage to the hippocampus may account for injury-related cognitive impairment (Bramlett et al., 1997; Colicos et al., 1996; Pierce et al., 1998). Striatal structures that participate in movement, emotional responses, and memory, undergo atrophic changes after TBI (Anderson et al., 1996; Shin et al., 2011). TBI also induces hypo-function of the striatal dopaminergic system (Shin et al., 2011).

Left and right brain damage may differentially affect higher brain functions. We propose that the left and right brain injury may differentially affect neurotransmitter systems involved in the regulation of lateralized processes in the brain. Specifically, we hypothesize that the endogenous dynorphins may be differentially sensitive to the left- and right-side TBI.

1.4 The EOS in alcohol dependence

Several lines of studies indicate a role of the EOS in alcohol dependence. Addictive substances including alcohol may induce molecular changes in the EOS which may underlie neuroplastic adaptations relevant for transition to
addiction (Koob and Volkow, 2010; Shippenberg et al., 2007; Wee and Koob, 2010). The EOS could be involved in cognitive control of substance addiction as a reward regulating system (Bencherif et al., 2004; Boettiger et al., 2009; Koob and Volkow, 2010; Love et al., 2009; Myrick et al., 2008; O'Malley et al., 2002). Alcohol consumption in animals has been shown to be altered by pharmacological and genetic manipulations with the opioid receptors (Altshuler et al., 1980; Cichelli and Lewis, 2002; Hall et al., 2001; Logrip et al., 2009; Myers et al., 1986; Shippenberg et al., 2007; Walker and Koob, 2008; Wee and Koob, 2010). The non-selective opioid antagonist naltrexone has been shown to reduce alcohol drinking and relapse rates in subgroups of alcoholics in clinical studies (Anton, 2008; O'Malley et al., 1992; O'Malley, 1996; O'Malley et al., 2002; Volpicelli et al., 1992). Several studies have associated polymorphisms in the OPRM1 (MOR) and OPRK1 (KOR) opioid receptor genes, and the PDYN gene with alcoholism (Bart et al., 2005; Edenberg et al., 2008; Oslin et al., 2003; Xuei et al., 2006; Xuei et al., 2007).

The EOS could modulate specific cognitive processes relevant for control of addictive behaviors including craving, decision-making and impulsivity, besides regulation of neurotransmission in reward circuits (Bencherif et al., 2004; Boettiger et al., 2009; Koob and Volkow, 2010; Love et al., 2009; Myrick et al., 2008; O'Malley et al., 2002). Effects of naltrexone in response to alcohol cues, and during decision-making are associated with the orbitofrontal cortex (OFC) in alcoholics (Boettiger et al., 2009). Alcohol craving and MOR binding are functionally related in the dl-PFC (Bencherif et al., 2004). The underlying processes modulating motivated behaviors, reward pursuing, and risk taking as well as the development of substance use disorders are relevant to functions of the EOS (Love et al., 2009). Neurocognitive processes controlling drug / alcohol seeking and taking behaviors are affected by alcohol. These effects of alcohol could be mediated in part through the EOS. Therefore, molecular dysregulation of this system may contribute to disruption of these processes which may ultimately lead to the alcohol dependence.

1.4.1 Animal studies
Several animal studies provide evidence for the involvement of EOS in alcohol dependence. Acute alcohol administration increases POMC and PENK mRNA and peptides in particular brain regions in rodents (Boyadjieva and Sarkar, 1997; Gianoulakis and Barcomb, 1987; Gianoulakis, 1990; Li et al., 1998). In vitro studies suggest that alcohol stimulates β-endorphin secretion from the hypothalamus (De Waele et al., 1992; de Waele and Gianoulakis, 1993; Gianoulakis, 1990; Keith et al., 1986). It has been demonstrated that opioid peptides may mediate the reinforcing effects of alcohol in acute alcohol exposure experiments (Koob et al., 1998). While the chronic alcohol
administration decreases POMC gene expression (Rasmussen et al., 2002), desensitize β-endorphin release (Boyadjieva and Sarkar, 1999; Boyadjieva and Sarkar, 1994; Pastorcic et al., 1994) and decrease MOR number and affinity (Yirmiya and Taylor, 1989). Therefore, the downregulation of the EOS by chronic alcohol administration could contribute to withdrawal and abstinence state, and promote alcohol consumption by negative reinforcement (Oswald and Wand, 2004; Seizinger et al., 1984).

Pharmacological experiments with opioid antagonists provide evidence for a role of the EOS in the regulation of alcohol consumption. Non-selective opioid antagonists i.e. naloxone, naltrexone and nalmefene, have been shown to decrease alcohol consumption in rodents (Altshuler et al., 1980; Froehlich et al., 1990; Hubbell et al., 1986; Kornet et al., 1991; Marfaing-Jallat et al., 1983; Myers et al., 1986; Samson and Doyle, 1985; Volpicelli et al., 1986; Weiss et al., 1990). MOR and DOR selective antagonists decrease alcohol self-administration in rodents under several experimental conditions (Ciccocioppo et al., 2002; Froehlich et al., 1991; Krishnan-Sarin et al., 1995). Experiments with selective opioid antagonists showed that both the β-endorphin and enkephalin systems are involved in the maintenance of alcohol consumption (Herz, 1997).

The role of EOS in alcohol intake is also strengthened by experiments with genetically modified animals. MOR knockout mice do not self-administer alcohol (Roberts et al., 2000), while DOR knockout mice showed a greater preference for alcohol and consumed more alcohol than their wild type counterparts (Roberts et al., 2001) which could be due to a compensatory increase in MOR activity in the absence of the DOR (Goody et al., 2002). Mice lacking β-endorphin peptide (with a targeted disruption of the POMC gene), a ligand for MOR, also drink less alcohol than their wild-type littermates (Grahame et al., 1998; Grisel et al., 1999).

1.4.2 Human studies (clinical trials and imaging studies)

American Food and Drug Administration in 1994, approved naltrexone, a nonselective opioid antagonist for the treatment of alcoholism. Naltrexone was advanced as one of the more promising pharmacological interventions for alcohol dependence (Litten, 1996), as results from naltrexone treatment trials showed fewer drinking days and lower rates of relapse (O'Malley et al., 1992; Volpicelli et al., 1992). This compound has been shown to reduce either the priming or rewarding effect of alcohol (Anton et al., 2004; Davidson et al., 1996; Davidson et al., 1999; Swift et al., 1994).

Naltrexone has been shown to exert beneficial effects on heavy drinking rates, particularly among those who are compliant with the medication (Anton et al., 1999; Chick et al., 2000; Monti et al., 2001). Naltrexone reduces alcohol consumption per drinking day (Anton et al., 1999; O'Malley et al., 1992) and craving (Volpicelli et al., 1992), and enhances resistance (re-
duce urge and impulse) to drink (Anton et al., 1999; Chick et al., 2000; Monti et al., 2001; Roberts et al., 1999). However, naltrexone did not show uniform treatment response; while some patients seem to benefit from this pharmacotherapy, others do not (Gastpar and Reymann, 2002; Krystal et al., 2001). It is not exactly known what opioid receptor subtypes are targeted by naltrexone in the brain of alcoholics.

Our understanding of the role of the EOS in alcohol reinforcement and addictive behavior was increased by the use of neuroimaging studies. Positron emission tomography (PET) with 11C-labeled carfentanyl, a radioligand that binds specifically and reversibly to the MOR has shown association between EOS and alcohol craving (Henriksen and Willoch, 2008). Change in MOR availability has been suggested to be associated with increased alcohol craving and relapse in alcoholics (Mann et al., 2001). Lower MOR binding potential in dl-PFC, anterior frontal cortex and parietal cortex has been correlated with multiple behavioral measures, including high alcohol craving, mood, and withdrawal severity in alcoholics undergoing withdrawal (Bencherif et al., 2004). Therefore, a functional relationship between alcohol craving and MOR binding potential in dl-PFC, anterior frontal cortex and parietal cortex of alcoholics undergoing withdrawal, can be hypothesized. Thus, an important role can be attributed to the EOS, particularly MOR in alcohol dependence.

High impulsiveness and low deliberation scores were associated with significantly higher regional MOR concentrations and greater stress-induced EOS activation in a PET study. These effects were found in brain regions involved in motivational behaviors and drugs abuse i.e. dl-PFC, OFC, anterior cingulate, thalamus, NAc, and basolateral amygdala (Love et al., 2009). It can be argued that impulsivity and deliberation, behavioral facets relevant to motivational behaviors, the pursuit of reward, and risk taking, including the development of substance abuse, in human are related to the functions of the EOS.

In conclusion, neuroimaging studies has demonstrated that the EOS in dl-PFC, anterior frontal cortex and parietal cortex, OFC, ACC, thalamus, NAc, and basolateral amygdala, brain areas responsible for reinforcement, decision-making, motivation and impulse control is involved in the addicted behavior. Importantly, alterations in the EOS correlate with craving and relapse behavior (Bencherif et al., 2004; Love et al., 2009).

1.4.3 The dynorphin / kappa opioid system in alcohol dependence

Several studies support the hypothesis that dynorphin opioid peptides and KOR play a role in alcohol dependence (Shippenberg et al., 2007; Walker et al., 2011; Wee and Koob, 2010). Dynorphin levels are reported to be upregu-
lated in the brain regions associated with motivation and reward in rats after chronic alcohol intake (Lindholm et al., 2000). The strains of rats demonstrating high and low alcohol preference, exhibit different dynorphin levels. Lower levels of dynorphin are found in the NAc of alcohol preferring rats (Nylander et al., 1994).

The reduced escalated alcohol self-administration in dependent animals after intra-cerebroventricular administration of the selective KOR antagonist nor-BNI suggest that the dynorphin / KOR system functions are altered in alcohol dependence which could alleviate the negative emotional states associated with alcohol withdrawal and dependence (Walker and Koob, 2008). A modulatory role for dynorphins over alcohol intake in the dependent state is supported by this data. The activated dynorphin / KOR system may function to increase alcohol intake.

The analyses of limited studies with KOR deficient mice revealed a lower alcohol intake accompanied by a decreased consumption of saccharine in saccharine preference tests in KOR deficient mice (Kovacs et al., 2005). The mice lacking the Pdyn gene demonstrated lower alcohol preference and consumed lower amounts of alcohol in a two-bottle choice test (Blednov et al., 2006). This effect was observed only in female mice and is complicated to interpret due to strong reduction of saccharin preference in mice of both sexes, and the absence of differences between wild type and Pdyn knockout mice in several types of alcohol behavioral tests.

In summary, an important role of EOS in the development of alcohol dependence is highlighted by animal experiments. It can be concluded that MOR modulates the positive reinforcing effects of alcohol (Becker et al., 2002; Kim et al., 2000; Nylander et al., 1994; Roberts et al., 2001) while KOR activation counteract these effects (Le Merrer et al., 2009). Chronic alcohol intake may dysregulate the functioning of these two opposing opioid systems which could be a critical factor in the development and maintenance of alcohol dependence.

1.4.4 Prefrontal cortex (PFC)

The PFC plays important role in cognitive control such that it organizes thought and action according to internal goals. This function is based on reinforcement learning theory. The persistent activity observed in PFC reflects not only the working memory maintenance but other cognitive processes, including perceptual and reward-based decision-making. The PFC guides behavior in accordance with internal goals, reward prediction errors coded by mesocortical dopamine (DA) neurons and memories; the neural correlates of which may be persistent activity (Curtis and Lee, 2010; Miller and Cohen, 2001). The PFC is well positioned to coordinate a wide range of neural process, as it sends and receives projections from virtually all cortical sensory systems, motor systems, and many subcortical structures. Consis-
tently, it integrates reward prediction errors, memories and motor responses (Miller and Cohen, 2001). The PFC is critical when important behavior must be guided by internal states or intentions and the mappings between sensory inputs, thoughts, and actions either are weakly established relative to other existing ones or are rapidly changing. In the absence of a functional PFC, habitual response would predominate and, where those do not exist, behavior would be haphazard (Miller and Cohen, 2001) which is characteristic outcome of PFC damage.

Atrophy, morphological changes, alterations in glucose metabolism, gene expression in the PFC has been associated with alcohol dependence (Abernathy et al., 2010). Alcoholics perform worse on tasks dependent on PFC function e.g. reward evaluation (Noël et al., 2010). Evidences from diverse studies like human and animal behavioral work, brain imaging, electrophysiology, and molecular and cellular observations suggest that drug-induced changes in the PFC also critically regulate drug and alcohol addiction (Everitt and Robbins, 2005; Kalivas and Volkow, 2005; Kalivas, 2008). A mechanistic basis for such a role was provided in a study showing that acute ethanol affects persistent activity in vivo in the rat PFC in a manner influenced by mesocortical DA neurons in vitro in organotypic culture (Tu et al., 2007).

1.4.5 Dorsal striatum (putamen and caudate)

The dorsal striatum (DS) is involved in the mediation of learning mechanisms that allow brain to associate experience of reward with the stimulus that temporarily precede it (O'Doherty, 2004) or the attribution of incentive salience (wanting) to such stimuli (Berridge and Robinson, 1998). A key role of dorsolateral striatum and dorsomedial striatum in habit formation and control of goal directed actions has been proposed (Balleine and O'Doherty, 2010). Striatal ventral putamen has been associated with depression, anxiety, apathy and hypoactivity (Hasler et al., 2007; Mah et al., 2007).

There are several evidences pointing towards involvement of DS in addiction. Animal and human studies indicate that acute and chronic alcohol intakes stimulate DA release in the ventral and dorsal striatum (Melendez et al., 2003; Yoshimoto et al., 1992). Phasic DA stimulation of DS, may contribute more to habit learning and maintenance and ultimately to the automatic, compulsive alcohol seeking characteristics of end-stage alcohol dependence (Volkow et al., 2006). Rostroventral caudate and putamen (rvCP) may contribute to a variety of signs associated with ethanol withdrawal syndrome. Bilateral rvCP lesions significantly increase alcohol withdrawal severity (Chen and Buck, 2010). Reduced availability of dopamine D2 / D3 receptors in the ventral striatum and adjacent putamen of abstinent alcoholic subjects was associated with a high level of craving for alcohol and an increase in brain activation elicited by alcohol associated cues (Heinz et al.,
2004; Heinz et al., 2005). Altered function of the neuropeptide system (e.g. dynorphin) might have either a feedback effect on the originating DA system itself or cause downstream changes in pathways creating clinically important functional outcomes (Shippenberg et al., 2007). The neuropeptides have been shown to alter activity of DA pathways known to influence motor function, emotions and addictive behavior e.g. dynorphins (Reid et al., 1988; Steiner and Gerfen, 1998).
2. Hypotheses and aims of the study

Hypotheses

- The EOS may be lateralized in the brain and therefore differentially regulate neuronal networks that process lateralized higher brain functions to the left and right hemisphere. Changes in lateralization may contribute to pathological mechanisms of substance addiction, mood and stress disorders as well as unilateral traumatic brain injury and should be considered upon molecular analyses of human and animal brain.

- The EOS may undergo molecular adaptations in human alcoholics that may contribute to development of alcohol dependence.

Specific Aims

- To examine whether the EOS is lateralized in the human pregenual anterior cingulate cortex (pgACC) that processes positive and negative emotions and affective component of pain (Paper I).

- To evaluate whether dynorphins are involved in response to the unilateral TBI and whether this response depends on the side of injury (Paper II).

- To examine whether the EOS undergoes molecular adaptations in the dorsal striatum i.e. caudate and putamen, the circuits relevant for the goal directed actions and habit formation, in human alcoholics, and whether these adaptations are associated with PDYN promoter SNP rs1997794 in the brain of human alcoholics (Paper III).

- To examine whether the EOS undergoes molecular adaptations in brain areas relevant for cognitive control of addictive behavior including the dl-PFC, OFC and hippocampus in human alcoholics (Paper IV).
3. Materials and Methods

3.1 Human samples / case selection

The postmortem human brain tissues were used for biochemical / molecular analyses. The tissues were obtained from two populations.

3.1.1 Swedish population

Brain tissues were collected at the department of Oncology and Pathology (Forensic Medicine), Karolinska Institute, Stockholm, Sweden, by qualified pathologists under the full ethical clearance from the Stockholm Ethical Review Board. The services of postmortem materials are handled by KI donatum, a core facility at Karolinska Institute. Informed written consent was obtained from the next of kin. All the subjects were of European descent. These tissues were collected from both male and female subjects. All the subjects have normal neuropathological examination and no known history of neuropsychiatric diseases. The detailed pathological and demographic characteristics are presented in supplementary Tables S1a and S1b (provided as supplementary material) in paper I, available online.

3.1.2 Australian population

Tissues were collected at the New South Wales Tissue Resource Centre (TRC), University of Sydney, Australia (Sheedy et al., 2008). Alcohol dependent subjects met criteria for Diagnostic and Statistical Manual for Mental Disorders, 4th edition (DSM–IV) and National Health and Medical Research Council / World Health Organization criteria, and consumed greater than 80 g of alcohol per day for the majority of their adult lives. Controls had either abstained from alcohol completely or were social drinkers who consumed less than 20 g of alcohol per day on average. Analysis included 24 controls and 26 chronic alcoholics for studies included in paper III and 14 controls and 14 alcoholics for studies included in paper IV. All subjects were males of the European descent. Control cases were matched to alcoholic cases by sex, age, race and postmortem interval. Cases with a history of polydrug abuse or with medical complications such as Wernicke–Korsakoff syndrome or alcoholic cases with concomitant diseases were excluded. Cases with a prolonged agonal life support or cases with a history of cerebral
infarction, head injury, or neurodegenerative disease (e.g. Alzheimer's disease) were also excluded. Samples were taken by qualified pathologists under full ethical clearance from the Sydney South West Area Health Service Human Ethics Committee. Informed written consent was obtained from the next of kin. The detailed demographic and pathological characteristics of tissues are presented in Tables S1a and S1b in supplementary material attached with manuscript for paper III; and Table S1 as supplementary material, available online for paper IV.

3.2 Animal studies

Wild type male mice (CF-1 mice, Taconic Farms Inc) were used for animal studies. All animal procedures were reviewed and approved by the institutional animal care and use committee at the University of Kentucky and carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize pain or discomfort.

3.3 Peptide radio-labelling

The peptides were labeled with radioactive Iodine ($^{125}$I) by chloramine-T method. Freshly prepared peptide solutions ($9.0 \times 10^{-5}$ M) were mixed with freshly labeled Iodine (Perkin Elmer, USA) in the presence of 0.2 M sodium phosphate buffer (pH = 7.4) and chloramine T (0.2 mg / mL) as an oxidizing agent. The reaction was run for appropriate time (40-60 seconds, depending on peptide) and then stopped by adding 15 % acetonitrile (ACN) with 0.04 % trifluoroacetic acid (TFA). The reaction mixture was injected immediately into column for reverse phase chromatography. The gradient was 15-40 % ACN in 0.04 % TFA. The purified radio-iodolabelled peptides were collected in collection tube, diluted with assay buffers and stored at –20 °C.

3.4 Preparation of peptide extract

The detailed procedure has been described elsewhere (Christensson-Nylander et al., 1985). Briefly, 1M hot acetic acid was added to finely powdered brain tissue and boiled at 95-100 °C for 5 minutes in a water bath and then cooled on ice. The extract was homogenized by sonication (Branson sonifier cell disruptor B15), centrifuged (Eppendorf 5417R) for 20 minutes at 14000 rpm. The supernatant was separated from pellets and applied to column containing 1 mL of Sephadex gel (Sephadex™ C-25, Amersham Biosciences, Uppsala, Sweden). The opioid peptides were eluted in a step-
wise fashion using buffers (mixtures of pyridine and formic acid) of increasing ionic strength. The fractions containing peptides were dried in a vacuum centrifuge (Speed Vac® Plus SC210A Savant) and stored at –20 °C for analysis of peptides.

3.5 Radioimmunoassay (RIA)

The procedure for RIA has been described elsewhere (Christensson-Nylander et al., 1985; Merg et al., 2006). Dyn A and Dyn B were assayed using buffer D (0.15 M NaCl, 0.02 % sodium azide, 0.1 % gelatin, 0.1 % Triton X-100 and 0.1 % bovine serum albumin in a 0.05 M sodium phosphate buffer). Samples were incubated with $^{125}$I-labelled peptides and primary antiserum of Dyn A with final dilution 1: 120,000 and primary antisera of Dyn B with final dilution 1: 350,000, overnight at +4 °C, and then with secondary antiserum (Sac-Cel, antisheep antirabbit antiserum) for 1 hour at +4 °C, centrifuged for 10 minutes (Beckman 4150 CS-15R) at 12000 rpm, and pellet was used for counting on automatic gamma-counter (1470 Wizard).

Leu-enkephaline-Arg$^6$ (LER) was assayed using gelatin buffer (0.15 M NaCl, 0.025 M EDTA, 0.1 % gelatin and 0.1 % bovine serum albumin in 0.05 M sodium phosphate buffer). Samples were incubated with $^{125}$I-labelled peptides and primary antiserum with final dilution 1: 35,000 overnight at +4 °C, and then incubated with 200 µL of charcoal suspension (500 mg charcoal, 50 mg dextran T-70 and 200 mL of 0.05 M sodium phosphate buffer) for 10 minutes at room temperature, centrifuged for 5 minutes at 12000 rpm and supernatant (300 µL) was used for counting. Met-enkephaline-Arg-Phe$^7$ (MEAP) was also assayed using gelatin buffer. MEAP antisera was used at a final dilution of 1:157,500. Samples subjected to MEAP assay were oxidised prior to the RIA procedure and the samples were incubated with antiserum for 24 hours before addition of labeled peptide and then incubated for further 24 hours. Charcoal suspension was added and incubated for 10 minutes at room temperature, centrifuged for 5 minutes at 12000 rpm and supernatant was used for counting.

Dyn A antiserum demonstrated 100 % molar cross-reactivity with Dyn A (9-17) and less than 0.1 % molar cross-reactivity with Dyn B, Dyn A (1-8), Dyn A (1-13), α-NE, Leu-enkephalin, LER and Met-enkephalin. Dyn B antiserum showed less than 0.01 % cross reactivity with Dyn A (1-13), Dyn A (1-8), Dyn A, Leu-enkephalin, LER, α-NE, neurotensin and Dyn A (9-17). Cross-reactivity with dynorphin 32 was 100 % and 1 % with Dyn B 29. LER antiserum showed < 0.1 % cross-reactivity with Dyn A, Dyn B and with Leu- and Met-enkephalin, 0.5 % with α-NE, 0.7 % with Dyn A (1-8), 1 % with MEAP, and 10 % with Met-enkephalin-Arg$^6$. MEAP antisera showed <
0.1 % cross-reactivity with Met-enkephalin, Met-enkephalin-Arg\(^6\), Met-enkephalin-Arg\(^6\)-Gly\(^7\)-Leu\(^8\), Leu-enkephalin and LER.

The peptide contents were calculated with the Prism program (GraphPad Software Inc, San Diego, CA, USA). The protein concentration in tissue pellet was determined by DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). The results are expressed either as femto moles per milligram of protein or per milligram of tissue.

### 3.6 Gene expression analysis by quantitative real-time polymerase chain reaction (qRT-PCR)

#### 3.6.1 RNA isolation and quality evaluation

Total RNA was isolated using TRIzol Reagent (QIAGEN, Maryland, USA), treated with RNase-free DNase I on-column for 30 minutes at room temperature, and purified with RNeasy Lipid Tissue Mini kit (QIAGEN, Maryland, USA) and stored at -80 °C for further use. Total RNA was quantified using micro-spectrophotometry by Nanodrop® (Nanodrop Technologies, Inc., Wilmington, USA). RNA Quality Indicator (RQI) was measured using Bio-Rad Experion (Bio-Rad Laboratories, Hercules, CA) with Eukaryote Total RNA StdSens assay according to manufacturer's protocol. Briefly, RQI was measured in 6 µL total volume including 5 µL of loading buffer. RNA samples with RQI values above 5.0 are generally considered as suitable for qRT-PCR (Fleige and Pfaffl, 2006; Fleige et al., 2006).

#### 3.6.2 cDNA synthesis

The total RNA was reverse-transcribed, using cDNA iScript kit (Bio-Rad Laboratories, Hercules, CA, USA), in CFX96\(^\text{TM}\) Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's protocol. The reaction mixture included 500 ng of total RNA, 4 µL of 5x iScript reaction mixture, 1 µL of iScript reverse transcriptase with final volume up to 20 µL. The reaction mixture was pre-incubated at 25 ºC for 5 minutes, incubated at 42 ºC for 45 minutes, enzyme was inactivated by heating of the mixture at 85 ºC for 5 minutes, diluted three times and then stored at -20 ºC for further use. Two negative controls for reverse transcription were prepared under the same conditions without addition either of total RNA or reverse transcriptase.

#### 3.6.3 Primer selection

Most of the primers were chosen in different exons to avoid the occasional false positives caused by DNA contamination of the RNA preparations. The
specific primers were selected considering critical factors i.e. melting temperature (Tm: 60–65 ºC), GC content (40-60 %), and amplicon length (70-200 bp). The primers were taken from corresponding articles (referred in relevant papers), except PDYN which was designed by “Vector NTI advance 11” software.

3.6.4 qRT-PCR

qRT-PCR was performed on CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The reaction mixture contained cDNA (corresponding to 21 ng transcribed RNA), 5xHOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) and 2.5 pmol each of forward and reverse primers in a final reaction volume of 10 µL. Temperature conditions of the three steps qRT-PCR reaction were the following: initial denaturation at 95 ºC for 15 minutes followed by 40 cycles of amplification at 95 ºC for 15 seconds, annealing temperature for 61.6 ºC for 20 seconds and elongation at 72 ºC for 20 seconds. Melting curve analysis was performed for each run to confirm the specificity of amplification and lack of primer dimers. Negative controls i.e. non-template control, controls without reverse transcriptase, were also run. To ensure that the exact product was amplified in the reaction, all PCR-products were separated by electrophoresis on a 2 % agarose gel and sequenced in both direction. All PCR products were of the predicted size and sequence.

mRNA expression was calculated by relative quantification, normalized against the expression levels of the selected reference genes for each brain area, using CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The reference genes were selected from a panel of 10 housekeeping genes, using geNormPlus software (http://www.biogazelle.com) which is based on the principle that the expression ratio of two ideal reference genes should be identical in all samples, independent of the treatment, condition, or tissue type.

3.7 DNA purification and genotyping of SNP

DNA was purified from human brain samples using Wizard Genomic DNA Purification kit (Promega, Madison, USA). Genotyping of SNP rs1997794 located in PDYN promoter was performed by allelic discrimination using TaqMan SNP Genotyping Assay C_11670951_10 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocol. Polymerase chain reactions were set up in a total volume of 10 µL, including 1 × iTaq Universal Probes Supermix (Bio-Rad, Sunnyvale, CA, USA), 1 × TaqMan SNP Genotyping Assay (Applied Biosystems), and 10 ng of template DNA using the BioRad C1000 Thermal Cycler (CFX96 Real-Time
System) (Bio-Rad). After an initial denaturation step for 10 minutes at 95 °C, each cycle consisted of denaturation for 15 seconds at 95 °C and annealing and primer extension for 60 seconds at 62 °C for a total 40 cycles. The rs1997794 variant pattern was set up with DNA from positive controls previously genotyped (Taqi et al., 2011).

3.8 HPLC determination of neurotransmitters

3.8.1 Chemicals

All the chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) except methanol and tetrahydrofuran which were obtained from Merck (Darmstadt, Germany).

3.8.2 Tissue preparation

The brain tissue samples (20-30 mg) were mixed at a ratio of 1:10 (w/v) with 0.2 M perchloric acid including 100 µM EDTA-2Na. This mixture was homogenized at 0 °C in a glass-pestle micro-homogenizer. The homogenates were centrifuged at 12,000 g and 4 °C for 15 minutes, after a standing for 30 minutes on ice. The supernatants were carefully aspirated and mixed with 1 M Na-acetate buffer (pH 3) at a ratio 5:1 (v/v). This mixture was filtered through a 0.22 µm centrifugal filter at 12,000 g and 4 °C for 4 minutes. The filtrates were stored at -80 °C before HPLC analysis.

3.8.3 HPLC analysis

High-performance liquid chromatography (HPLC) with electrochemical detection (Kehr and Yoshitake, 2006) was used to determine the concentrations of noradrenaline (NA), DA and serotonin (5-HT). The HPLC system consist of a HTEC500 chromatograph (Eicom, Kyoto, Japan) and a CMA / 200 refrigerated microsampler (CMA Microdialysis, Stockholm, Sweden) equipped with a 20 µL loop and operating at +4 °C. The potential of the glassy carbon working electrode was +450 mV vs the Ag / AgCl reference electrode. The separation was achieved on a 200 x 2.0 mm (i.d.) Eicompak CAX column (Eicom) protected with a guard column CAX-GC2/20 (Eicom). The mobile phase consist of a mixture of methanol and 0.1 M phosphate buffer (pH 6.0), (30:70, v/v) containing 40 mM potassium chloride and 0.13 mM EDTA-2Na. The computerized data acquisition system Clarity (DataApex, Prague, The Czech Republic) was used for recording and integrating the chromatograms.

Gradient elution reversed-phase column liquid chromatography with fluorescence detection following precolumn derivatization with orthophptaldial-
dehydro / mercaptoethanol (OPA/MCE) reagent was used to determine the concentration of aspartate (Asp) and glutamate (Glu) in the brain samples. Briefly, the HPLC system consist of a gradient pump Spectra Physics SP8800 (Spectra Physics, USA), a CMA/260 degasser (CMA Microdialysis), a CMA / 280 Fluorescence detector (CMA Microdialysis) operating at excitation and emission wavelengths of 350 and 495 nm, respectively. 10 µL of each sample was mixed with 10 µL of the OPA/MCE reagent by use of a CMA / 200 refrigerated microsampler (CMA Microdialysis) equipped with a 20-μL loop and operating at +6 °C. Then, 10 µL of volume from this mixture was injected onto a HPLC column (60 x 4 mm i.d., Nucleosil 100 C18, 5µm; Knauer GmbH, Berlin, Germany), after a reaction time of 60 seconds. The mobile phase A consist of 0.03 M sodium acetate buffer (pH 6.95) containing 2.5 % (v/v) of methanol and 2 % (v/v) of tetrahydrofuran and pumped at a flow rate of 1 ml / minute. The amino acids were eluted by use of a linear gradient of methanol used as a mobile phase B and from 0-60 % at 4 minute to 28 minute. After that, the column was, regenerated with mobile phase A for 3 minutes. A computerized data acquisition system EZ Chrom data system (Scientific software Inc, CA, USA) was for recording and integrating the chromatograms (Kehr, 1998; Kehr and Yoshitake, 2006).

3.9 Statistical analyses

In paper I, the data was subjected to one-way multivariate analysis of variance (MANOVA) conducted on asymmetry index (AI) and two-way ANOVA on normalized levels with lateralization and sex as between factors, significant at alpha level p < 0.05. Univariate covariate analysis (ANCOVA) was applied when and where appropriate to check whether significance in MANOVA is confounded by covariates. The Spearman's correlations were calculated for all dependent variables within each of the four brain regions for the right and left hemispheres, significant at alpha level p < 0.01. The significance of difference between correlations was evaluated after applying Fisher's z-transformation. All the multiple comparisons were subjected to Bonferroni’s corrections.

In paper II, the data were analysed first by two way analysis of variance (ANOVA) with treatment as a between subjects-factor and lateralization as a within subject-factor. The significant main effects of treatment were followed by Bonferroni's post-hoc analyses of the mean contents of peptide. One way ANOVA was used to better characterize any significant interactions observed in two-way ANOVA, followed by Bonferroni's post-hoc analyses.

In paper III, analyses were conducted in four general steps. First, data was subjected to one-way ANOVAs with group as between factor for each of dependent variable. Second, backward stepwise regression was used to re-
fine the model by determining the relative importance of each predictor and its statistical significance. Subjects with overly influential points were excluded from analysis. Third, analyses of covariance (one-way ANCOVA) with group as between factor to control for potential confounds was performed. Fourth, data was subjected to two-way ANCOVAs with group and genotype as between factors. ANCOVAs were followed by post hoc pairwise two-way Student’s \( t \)-tests on least squares means (between group and genotype factors, controlled for confounds) with Sidak correction for multiple comparisons. A p-value of 0.05 after Bonferroni's multiple testing corrections was accepted as statistically significant.

In paper IV, two-way ANOVA, followed by post hoc student's \( t \)-test, was used to analyze the data. Pearson’s correlations were calculated for various variables within two groups, with significance set at \( p < 0.05 \). However, in the absence of the data on the linear relationship between variables, Spearman's rank correlations were determined to characterize inter-area associations between parameters that significantly differed between controls and alcoholics.
4. Results and Discussion

4.1 Opioid system lateralization in human brain (paper I)

A fundamental property of the human brain is lateralization of higher functions. According to “the right hemisphere hypothesis”, emotions are predominantly processed in the RH. The alternative, “valence hypothesis” posulates that emotions are lateralized depending on their valence. The RH is dominant for negative emotions and pain, while the LH predominantly processes positive emotions. Yet, a number of studies proposed the region-specific functional lateralization of emotion processing. Lateralization of processing of positive and negative emotions and pain suggests an asymmetric distribution of the neurotransmitter systems regulating these functions between the left and right brain hemispheres. By virtue of their ability to selectively mediate euphoria, dysphoria and pain, the δ-, µ- and κ-opioid receptors and their endogenous ligands may subserve these lateralized functions.

The present study addressed this hypothesis by comparing the levels of six EOS genes and four opioid peptides in postmortem human specimens from symmetric areas of the left and right ACC, a key area for emotion and pain processing. LER and MEAP, the neuropeptide markers of PDYN and PENK, respectively, as well as Dyn A and Dyn B derived from PDYN, were analyzed. Dyn A and Dyn B function as KOR ligands; LER is potent DOR and MOR agonist, whereas MEAP may activate KOR and MOR. The neurotransmitters DA, 5-HT, NA, Glu and Asp, previously proposed to be lateralized in human brain were analyzed for comparison. Prompted by our preliminary evidence that opioid peptides displayed lateralization in the pgACC, we additionally assessed their levels in other brain regions involved in processing of emotions and pain, including the left and right dl-PFC and the caudate and putamen for comparison. The principal findings of the study are that (1) the opioid peptides LER and MEAP are markedly lateralized to the left and right pgACC, respectively (Fig. 1); and (2) Dyn B and LER strongly correlate in the left but not right pgACC. No substantial lateralization, greater than 20 % was evident in the levels of six EOS mRNAs and five neurotransmitters in the pgACC. No considerable asymmetry in the levels of four opioid peptides was detected in the dl-PFC and caudate, while Dyn B showed strong lateralization in the putamen (Fig. 2).
Figure 1. The asymmetry index (AI) of the opioid mRNAs, opioid peptides and neurotransmitters in the pgACC. The AI [(R-L)/(R+L)] is a measure of hemispheric differences which the -1.0 and +1.0 values indicate a complete left and right lateralization, respectively, and the 0.0 value symmetric distribution. Data bars represent means ± SD. Dashed lines at the -0.33 and +0.33AI values mark 2-fold differences between hemispheres. Significance in the MANOVA followed by ANCOVAs when appropriate: lateralization effect, *p < 0.05, **p < 0.01, ***p < 0.0001; sex effect, #p < 0.05.

LER has high binding affinity for both δ- and μ-opioid receptors. Endogenous μ- and δ-agonists are involved in the regulation of positive emotional states and pleasure. We hypothesize that the preferential activation of δ- and μ-opioid receptors by LER in the left pgACC has a role in the lateralization of positive emotions to the LH. MEAP binds to both κ- and μ-opioid receptors, but only weakly to δ-opioid receptors. The κ-system is critical for the modulation of negative emotional states and control of pain processing. Lateralization of MEAP, which may activate κ-opioid receptor, along with the differences between the LH and RH in the conversion of the κ-opioid receptor agonist Dyn B into δ- / μ-agonist LER may provide molecular basis for lateralization of negative emotions and pain to the right pgACC in the human brain. Difference between the two hemispheres in the Dyn B and LER correlation that is significant in the LH suggests complex regulation of conversion of Dyn B into shorter enkephalins in the RH compared to the LH.
The only peptidase that is involved in the left-right asymmetry and cleaves neuropeptides at the pairs of basic amino acid residues is the proprotein convertase PACE4. PACE4 is encoded by the PCSK6 gene. Our analysis revealed strong and significant negative correlation between Dyn B and PCSK6 in the right but not left pgACC. Thus PACE4 may be involved in conversion of Dyn B to enkephalins in the RH, where the variations in activity or expression of this enzyme may contribute to high inter-individual variability in Dyn B cleavage. These findings suggest that region specific lateralization of neuronal networks expressing opioid peptides underlies in part lateralization of higher functions including positive and negative emotions and pain in the human brain.

Figure 2. The AI of the opioid peptides in the dorsolateral prefrontal cortex (dl-PFC), caudate and putamen. The -1.0 and +1.0 values of the AI [(R-L)/(R+L)] indicate a complete left or right lateralization, respectively, and the 0.0 value symmetric distribution. Data bars represent means ± SD. Dashed lines at the -0.33 and +0.33 AI values mark 2-fold differences between hemispheres. Significance in MANOVAs followed by ANOVAs when appropriate: lateralization effect, *p < 0.05, **p < 0.01, ***p < 0.0001.
4.2 Lateralized response of Dyn A in unilateral TBI (paper II)

Traumatic brain injury (TBI) induces a cascade of primary and secondary events resulting in impairment of neuronal networks that eventually determines clinical outcome. TBI induces a broad range of short-term and long-term physical, behavioral, and cognitive impairments, depending on the severity of injury. The dynorphins, endogenous opioid peptides, have been implicated in secondary injury and neurodegeneration in rodent and human brain. Dyn A, the most pathogenic dynorphin, may cause tissue injury and cell death, and may exacerbate the clinical severity of traumatic injury to the head or spinal cord. Evidences also support for lateralized neurochemical and behavioral responses to unilateral brain injury in both rodents and humans.

To gain insight into the role of dynorphins in brain response to trauma, we analyzed short term (1 day) and long term (7 day) changes in Dyn A levels in the frontal cortex, hippocampus and striatum induced by unilateral left-side or right-side cortical TBI in mice. The effects of TBI were significantly different from those of sham surgery (Sham), while the sham surgery also produced noticeable effects. Both sham and TBI induced short-term changes and long-term changes in all three regions. Two types of responses were generally observed. In the hippocampus, Dyn A levels were predominantly altered ipsilateral to the injury (Fig. 3B, 4B). In the striatum and frontal cortex, injury to the RH affected Dyn A levels to a greater extent than that seen in the LH. The R-TBI but not L-TBI produced Dyn A changes in the striatum (Fig. 3C, 4C) and frontal cortex (Fig. 3A, 4A) at 7 days after injury. Effects of the R-side injury were similar in the two hemispheres. In naive animals, Dyn A was symmetrically distributed between the two hemispheres.

Our results suggest that trauma may reveal a lateralization in the mechanism mediating the response of Dyn A-expressing neuronal networks in the brain. These networks may differentially mediate effects of left and right brain injury on lateralized brain functions.
Figure 3. Impact of left and right sham operation (L-SO and R-SO), or cortical traumatic brain injury (L-TBI and R-TBI) on Dyn A levels in the frontal cortex, hippocampus and striatum at 24 hours and 7 days after the operation. Dyn A levels are shown as mean values for the left and right hemispheres [(L+R)/2]). ANOVA testing (9 levels) was followed by Bonferroni's post-hoc analyses (*p < 0.05, **p < 0.01, ***p < 0.001). Data bars represent means ± SD.
Figure 4. Impact of L-Sham (L-SO), R-Sham (R-SO), L-TBI or R-TBI on Dyn A levels in the frontal cortex, hippocampus and striatum, analyzed separately in the ipsilateral and contralateral regions at 24 hours and 7 days after surgery. ANOVA (9 levels) was followed by Bonferroni's post-hoc analyses (*p < 0.05, **p < 0.01, ***p < 0.001). Data bars represent means ± SD.
4.3 PDYN downregulation in the dorsal striatum of human alcoholics (paper III)

The endogenous opioid peptides dynorphins and enkephalins and their receptors have a critical role in drug and alcohol dependence. Animal studies propose that drug / alcohol induced changes in PDYN and PENK expression underlie neuroplastic adaptations critical for addiction. These changes may be brain-area specific, and may differentially contribute to specific stages of an addiction cycle.

We compared the levels of PDYN and PENK mRNAs (qRT-PCR), and dynorphins and enkephalins (RIA) in the caudate nucleus and putamen between alcoholics and control subjects. We also evaluated whether PDYN promoter variant rs1997794 associated with alcoholism affects PDYN expression. For the caudate nucleus, one-way ANCOVAs revealed effect of alcoholism on PDYN mRNA and MEAP. Post hoc $t$-test showed downregulation of PDYN (1.45-fold; $p = 0.024$) and MEAP (1.47-fold; $p = 0.022$) in alcoholics (Fig. 5a). Two-way ANCOVAs with group (controls vs. alcoholics) and PDYN genotype (CC and CT genotypes vs. TT genotype; subjects with the C, high risk genotype were pooled) as between factors revealed a significant main effects of alcoholism for PDYN, Dyn A and LER, and a trend for Dyn B in the putamen (Fig. 5b). For the combined CC and CT genotypes post hoc $t$-test showed downregulation of PDYN (2.0-fold; $p = 0.048$) and LER (2.2-fold; $p = 0.02$) in alcoholics.

Striatal subregions may be differentially involved in addiction cycle. The caudate nucleus participates in control of goal-directed actions, and thus may influence goal-directed alcohol seeking. The putamen has key roles in habit formation, and may participate in the development of habitual alcohol use. Downregulation of opioid peptides developed over the course of heavy alcohol drinking and withdrawal may underlie in part changes in goal-directed behavior and formation of a compulsive habit in alcoholics.
Figure 5. Relative levels of PDYN mRNA and PENK derived Met-enkephalin-Arg-Phe (MEAP) in the caudate nucleus; and those of PDYN mRNA and Dyn A, Dyn B and Leu-enkephalin-Arg (LER) separately for subjects carrying CC + CT allele, or the risk TT allele of PDYN promoter SNP rs1997794 in the putamen in controls and alcoholics. Mean values in the control groups were taken as 1; data is shown as normalized means ± SEM.
4.4 Upregulation of kappa opioid system in neurocognitive brain areas in human alcoholics (paper IV)

The EOS plays a critical role in addictive processes. Molecular dysregulations in this system may be specific for different stages of addiction cycle and neurocircuitries involved, and therefore may differentially contribute to the initiation and maintenance of addiction.

We analyzed the expression of EOS genes including OPRM1, OPRD1 and OPRK1 opioid receptor and POMC, PENK, and PDYN opioid peptide precursor genes in the dl-PFC (Brodmann area 9), hippocampus (dentate gyrus), and OFC (Brodmann area 47), areas involved in cognitive control of impulsivity and the reoccupation/anticipation stage of addiction cycle, in human alcohol dependent and control subjects. The motor cortex (MC; Brodmann area 4), not involved in alcohol dependence was included as control for regional specificity. POMC expressed at low levels was excluded from further analysis. PDYN and OPRK1 mRNAs in the dl-PFC and OFC, respectively, were significantly higher in alcoholics compared to controls. No significant differences in expression of other four genes in these two brain regions and all five genes in the hippocampus and MC were evident between the two groups.

We also examine whether dynorphins A and B were altered in alcoholics by RIA. The levels of dynorphins A and B were significantly increased in the dl-PFC and the hippocampus in alcoholics (Fig. 6). No significant differences were found in the MC. Analysis of Spearman rank correlations identified significant (p < 0.05) correlations between PDYN mRNA and dynorphins (all subjects: PDYN mRNA - Dyn B in the dl-PFC (r = 0.54) and MC (r = 0.61); alcoholics: PDYN mRNA - Dyn B (r = 0.73) in dl-PFC). Dynorphins acting through KOR may control release of glutamate and other neurotransmitters in the cortical areas and hippocampus. Cycles of alcohol consumption and withdrawal may dysregulate hippocampal and cortical neurotransmission by targeting the dynorphin/KOR system and that may induce a shift to a new, pathological neurotransmission pattern.

The present findings suggest that long-term heavy alcohol consumption leads to activation of the selective EOS component, the dynorphin/KOR system in discrete brain loci. This may contribute to neurocognitive dysfunctions relevant for craving and disrupted inhibitory control.
Figure 6. The EOS including PDYN mRNA and dynorphins in the dl-PFC (a), OPRK1 mRNA in the OFC (b) and dynorphins in the hippocampus (c) were upregulated in human alcoholics. Levels in alcoholics (n = 14) are presented as the means ± SEM in relative units for EOS mRNAs and dynorphins A and B. *p < 0.05; **p < 0.01; ***p < 0.001.
5. Conclusions

The findings of this study support the hypotheses on the lateralization of EOS in the brain and changes in lateralization induced by unilateral brain injury; and on the brain area- and addiction stage-specific molecular adaptations in the EOS in human alcoholics.

- Analysis of human pregenual anterior cingulate cortex (pgACC) that processes positive and negative emotions and affective component of pain demonstrated lateralization of the EOS in this brain area. Leu-enkephalin-Arg and Met-enkephalin-Arg-Phe, preferential \( \delta \)-/\( \mu \)- and \( \kappa \)-/\( \mu \)-opioid agonists demonstrated marked lateralization to the left and right ACC, respectively. Dyn B strongly correlated with Leu-enkephalin-Arg in the left but not right ACC suggesting different mechanisms of conversion of this \( \kappa \)-opioid agonist to \( \delta \)-/\( \mu \)-opioid ligand in the two hemispheres; in the right ACC Dyn B may be cleaved by PACE4, a proprotein convertase regulating left-right asymmetry formation. These findings suggest that region-specific lateralization of neuronal networks expressing opioid peptides underlies in part lateralization of higher functions including positive and negative emotions and pain in the human brain.

- Evaluation of events induced by TBI identified two types of EOS responses. In the hippocampus, Dyn A levels were predominantly altered ipsilaterally to the injury. In the striatum and frontal cortex, injury to the right hemisphere affected Dyn A levels to a greater extent than that in the left hemisphere. Thus, trauma reveals a lateralization in the mechanism mediating the response of Dyn A-expressing neuronal networks in the brain. These networks may differentially mediate effects of left and right brain injury on lateralized brain functions.

- Investigation of the EOS in the brain of human alcoholics demonstrated that \( PDYN \) mRNA and Met-enkephalin-Arg-Phe, a marker of PENK were downregulated in the caudate nucleus, while \( PDYN \) mRNA and Leu-enkephalin-Arg, a marker of PDYN were decreased in the putamen of alcoholics carrying high risk 1997794 C
allele. Downregulation of opioid peptides developed over the course of heavy alcohol drinking and withdrawal may underlie in part changes in goal-directed behavior and formation of a compulsive habit in alcoholics.

- The dynorphin / KOR system including PDYN mRNA and dynorphins in the dl-PFC, dynorphins in the hippocampus, and OPRK1 mRNA in the OFC, was found to be upregulated in human alcoholics. Activation of the κ-opioid receptor by upregulated dynorphins in alcoholics may underlie in part neurocognitive dysfunctions relevant for addiction and disrupted inhibitory control. Our findings support the hypothesis that EOS dysregulation in specific neurocircuitries differentially contribute to different aspects of alcohol addiction.
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