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The originality of this publication has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

Cover Image: downloaded from https://pixabay.com, free for use.

ISBN 978-951-29-7772-7 (PRINT)
ISBN 978-951-29-7773-4 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Painosalama, Turku, Suomi 2019

‘Man, when you lose your laugh you lose your footing.’

- *McMurphy* in the novel *One Flew Over the Cuckoo's Nest* by Ken Kesey (1962)

To my Family

UNIVERSITY OF TURKU
Faculty of Medicine
Department of Psychiatry
Turku PET Centre
SANDRA MANNINEN: Endogenous opioid system and human sociability
Doctoral Dissertation, 64 pp.
Doctoral Programme in Clinical Research
September 2019

ABSTRACT

Social bonds have a profound impact on our everyday life. People differ in their capacity for establishing and maintain strong, intimate relationships. These differences are characterized as *attachment styles*. Particularly insecure attachment style may lead to developmental and psychiatric disorders, as well as to addictive behavior (Mikulincer & Shaver 2007), which cause large societal expenses due to treatment and sickness leaves. In addition to anti-nociception and reward, the endogenous opioid system has been proposed to regulate social bonding in mammals. That could explain the link between social distress and physical pain (Panksepp & Nelson 1997). However, the role of the endogenous opioid system in human social behavior remains poorly understood (Machin & Dunbar 2011).

The aim of this thesis was to investigate opioidergic basis of affiliative behavior with positron-emission tomography (PET). We approached the phenomenon from two perspectives: by 1) measuring how prosocial behavior affects endogenous opioid peptide release and 2) quantifying whether regional differences in opioid receptor availability explain differences in adult attachment styles. PET was used for quantifying endorphin release after social laughter manipulation (Study I) and levels of brain μ-opioid receptor (MOR) in baseline state (Study II). In the methodological part of the thesis (Study III), PET and MRI data were combined. Voxel-based morphometry (VBM) based indices of gray matter density (GMD) were correlated with tracer binding potentials (BP_{ND}).

Social laughter increased endorphin release in brain regions such as thalamus, caudate nucleus and putamen, in subcortical areas and in frontal cortices (Study I). Adult avoidant attachment style correlated negatively with brain MOR availability in thalamus, anterior, middle and posterior cingulate cortices, and medial and lateral prefrontal cortices (Study II). In study III, gray matter density (GMD and radiotracer binding (BP_{ND}) correlated positively in multiple brain areas, suggesting a link between brain's regional macrostructure and molecular functioning.

In sum, these results highlight the crucial role of endogenous opioid system in human prosocial functioning. Furthermore, they show that PET and anatomical MR provide complementary information regarding brains molecular organization, stressing the importance of fusion imaging for understanding brain basis of sociability.

KEYWORDS: Endogenous opioid system (EOS), Human sociability, Adult attachment styles, μ-opioid receptors (MOR), Positron-emission tomography (PET), Magnet resonance imaging (MRI).

TURUN YLIOPISTO
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Psykiatrian oppiaine
Turku PET keskus
SANDRA MANNINEN: Endogeeninen opioidiärjestelmä ja ihmisen sosiaalinen käyttäytyminen
Väitöskirja, 64 s.
Turun kliininen tohtoriohjelma
syyskuu 2019

TIIVISTELMÄ

Ihmisen käyttäytymiselle on tyypillistä muodostaa pitkäkestoisia ja monimuotoisia kiintymyssuhteita. Erot kiintymyskäyttäytymisessä voivat olla suuria, eikä ilmiön neurobiologista taustaa vielä täysin tunneta. Kiintymyssuhdehäiriöt liittyvät usein laajempiin mielenterveyden häiriöihin sekä mm. lisääntyneeseen päihdehakuisuuteen aikuisiällä (Egle et al. 2002). Fyysisen kivun kokemuksen sekä lievittymisen tiedetään olevan vahvasti opioidi-välitteistä. Opioidijärjestelmän ajatellaan evoluution myötä kehittyneen säätelemään fyysisen kivun lisäksi myös sosiaalista kanssakäymistä nisäkkäillä (Nelson & Panksepp 1998; Higham et al. 2011). Tämä voisi selittää yhteyden psyykkisen ja somaattisen kipukokemuksen välillä myös ihmisillä (Panksepp et al. 1978).

Tässä väitöskirjatyössä pyrittiin selvittämään aivojen opioidijärjestelmän merkitystä ihmisen sosiaalisen käyttäytymisen säätelijänä kahdella tavalla: 1) mittaamalla aivoissa vapautuvan endorfiinin määrää miellyttävän sosiaalisen tilanteen jälkeen sekä 2) vertailemalla aikuisen kiintymyssuhdetyypin yhteyttä aivojen μ-opioidireseptori-tasoihin (MOR) normaalitilassa. Menetelmänä käytettiin aivojen positroniemissiotomografiaa (PET) sekä [11C]karfentaniilia, jonka tiedetään sitoutuvan spesifisti MOR:iin keskushermostossa. Kolmannessa (3), metodologisessa osatyössä vertailtiin aivojen PET-dataa magneettikuvantamisella (MRI) saatuihin aivojen harmaan ja valkean aineen tiheyskarttoihin käyttäen vokselipohjaista menetelmää (VBM; voxel-based morphometry).

Tuloksena oli, että nauraminen läheisten ystävien kanssa lisäsi endorfiinien vapautumista (I) ja välttävä kiintymyssuhdetyyppi korreloi negatiivisesti vapaana olevien MOR:ien kanssa (II) sosio-emotionaalista käyttäytymistä ohjaavilla aivoalueilla. Lisäksi (III), harmaan aineen tiheys (GMD) korreloi positiivisesti merkkiaineiden sitoutumispotentiaalin (BP_{ND}) kanssa usealla aivoalueella. Tulokset I-II lisäävät tietoa opioidijärjestelmän keskeisestä roolista ihmisen sosiaalisen käyttäymisen säätelijänä. Lisäksi, osatyön III mukaan kaksi eri aivokuvantamisessa yleisesti käytettyä mittaria (BP_{ND} ja GMD) antavat toisiaan täydentävää tietoa aivojen molekyylirakenteesta, minkä vuoksi usean modaliteetin aivokuvantaminen on välttämätöntä sosiaalisen käyttäytymisen aivoperustan ymmärtämiseksi.

AVAINSANAT: Endogeeninen opioidijärjestelmä (EOS), Ihmisen sosiaalisuus, Aikuisen kiintymyssuhde, μ-opioidireseptori (MOR), [11C]karfentaniili, PET, MRI.

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Abbreviations

AAS	Adult attachment style
ACC	Anterior cingulate cortex
BMI	Body mass index
BOTSA	Brain opioid theory of social attachment
BP	Binding potential
BP_{ND}	Non-displaceable binding potential
DSM-IV	Diagnostic and statistical manual of Mental disorders, 4^{th} edition
ECR-R	the Experience in Close Relationships- Revised
EOS	Endogenous opioid system
FDR	False discovery rate
FWHM	Full witdth at half maximum
GM	Gray matter
GMD	Gray matter density
LOR	Line of response
MADAM	N,N-Dimethyl-2-(2-amino-4-methylphenylthio)benzylamine
MCC	Middle cingulate cortex
MDD	Major depressive disorder
MNI	Montreal Neurological Institute
MOR	μ-opioid receptor
MRI	Magnetic resonance imaging
MRP	Median root prior
mPFC	Medial prefrontal cortex
NAc	Nucleus accumbens
OFC	Orbitofrontal cortex
PCC	Posterior cingulate cortex
PET	Positron-emission tomography
RF	Radio frequency
ROI	Region of interest
SD	Standard deviation
SPM	Statistical parametric mapping
SRTM	Simplified reference tissue model

TAC	Time-activity curve
THA	Thalamus
T1/2	Half-life of a tracer
VBM	Voxel-based morphometry
VTA	Ventral tegmental area
vSTR	Ventral striatum
WM	White matter
WMD	White matter density

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Manninen S., Tuominen L., Dunbar R., Karjalainen T., Hirvonen J., Arponen E., Hari R., Jääskeläinen I.P., Sams M. and Nummenmaa L. Social laughter triggers endogenous opioid release in humans. The Journal of Neuroscience (2017); 37(25): 688-16.

II Nummenmaa L, Manninen S., Tuominen L., Hirvonen J., Kalliokoski K., Nuutila P., Jääskeläinen I.P., Hari R., Dunbar R. & Sams M. Adult attachment style is associated with cerebral u-opioid receptor availability in humans. Human Brain Mapping (2015); 36: 3621-3628.

III Manninen S., Karjalainen T., Tuominen L., Hietala J., Rinne J., Kaasinen V., Joutsa J., Nummenmaa L. (submitted for publication). Cerebral gray matter density is associated with neuroreceptor and neurotransporter availability.

1 Introduction

The need for others is a distinctive urge that characterises the essence of human being (Baumeister & Leary 1995). That drives us to seek conspecifics and to form new inter-individual bonds, which may deepen and become more important during a lifespan. Those bonds may persist when the relationship goes through hard times or when there is a long physical distance between the persons involved.

Consequently, losing a significant other is a major stressor, which can cause depressive symptoms for an individual encountering a bereavement (Bowlby 1980). Put simply, social networks are essential for humans and other social species, and loneliness is a major risk factor for multiple adverse outcomes including shortening of life (Cacioppo et al. 2006; Holt-Lunstad et al. 2010). Affiliative behavior enables the bonding with others, but still, human adults differ remarkably from each other in their capability to seek and maintain social affairs. Such attachment styles develop early in life, and remain quite stable during one's life (Fraley 2002). Insecure early attachment is associated with mental health problems and drug abuse later in life (Egle et al. 2002). Over decades, researchers have tried to understand human affiliative behavior more profoundly and clarify differences in seeking and maintaining important social units, in addition to possible psychological consequences of broken meaningful bonds (Bowlby 1977; Crittenden & Landini 2011). Yet, the neurobiology underlying human attachment styles remains poorly understood.

The opioid system is a critical component of central pleasure and pain pathways (Leknes & Tracey 2008). On the other hand, *Social pleasure* is related to a successful and reproductive affiliative behavior among mammals (Insel 2003) – rewarding feeling discourages individuals from breaking the social bond. Receptor blocking studies in socially active non-human primates have established that endogenous opioid system is maintaining the social motivation towards other conspecifics (Fabre-Nys et al. 1982; Martel et al. 1995). Rodent-studies suggest that opioid system also supports *social pain* and distress processing during social isolation (Panksepp et al. 1980). Hence, theoretically a misuse of opioids could be explained as an attempt to exogenously maintain the lack of social comfort (Machin & Dunbar 2011).

Characterising the neurobiological mechanisms of normal social behavior could help to understand the bio-psychological consequences of unbalanced social bonding. The aim of this thesis was to unravel the neurochemical mechanisms, especially endogenous opioids, supporting social behavior and social attachment in human brain using *in vivo* PET imaging.

2 Review of the Literature

2.1 The Endogenous opioid system (EOS)

2.1.1 Endogenous opioids

Endogenous opioids are a group of endogenously produced peptides, that are protein-like products of pituitary glands (Smyth et al. 1981). Three major types of opioid peptides have been discovered in animals and in humans: endorphins, enkephalins and dynorphins. Their main receptor binding sites are MORs, DORs and KORs, respectively. The most recent discovery is orphanin FQ/nociceptin, with a sequence homology with aforementioned opioid peptides, but its function is poorly understood (Darland et al. 1998). The word *endorphin* is derived from words 'endogenous' and 'morphin' signifying *'morphine produced naturally in a body'*. Morphine has a long history in medicine, as it has been used for treating wide range of symptoms from cough and diarrhea to severe pain. Due to its high analgesic potency, morphine is still one of the most used pain-killers in acute, severe and chronic pain (Buschmann et al. 2002). Endorphins are affecting through opioid receptors in central nervous system (CNS) to cause effects for instance on respiratory functioning, gastrointestinal contraction, immune system reactivity, experience of pain, sedation and euphoria (Olson et al. 1995; Stefano et al. 2000; Zubieta et al. 2001). Endorphins are released from the pituitary gland after psychomotor actions, excercise, eating, maternal behavior and reward processing (Nummenmaa et al. 2018; Saanijoki et al. 2018; Tuulari et al. 2017). The field of opioid system research changed significantly after the discovery that endogenous (e.g. β-endorphin) and exogenous opioids (e.g. morphine) exert their systemic effects via same opioid receptors (Lord et al. 1977).

2.1.2 Opioid receptors

Three main subtypes of opioid receptors have been identified in humans: μ (MOR), κ (KOR), and δ (DOR). In addition to multitude of other functions opioid receptors mediate both rewarding and aversive sensations in mammals and humans (Le Merrer 2015). All opioid receptors are regulated by endogenous or exogeneus opioid

ligands, depending on a receptor subtype (Stefano et al. 2000). Endorphins mediate the rewarding and pleasurable sensations (i.a. euphoria) through MORs and partially through DORs, whereas dynorphins are related to opposite effects (pain, dysphoria and nausea) and are mediated through KORs (van Ree et al. 1999). MORs mediate also the effects of positive social interactions with different species (D'Amato & Pavone 2012). β-endorphin has a selective affinity to MORs (Taylor & Kaiser 1989), and together they play crucial role in mediating reward and pain pathways (Veening & Barendregt 2015).

Opioid receptors (ORs) are located in multiple sites in the human body spanning from the immune system, gastrointestinal tract to nervous tissue, depending on a cell subtype (Hiller & Fan 1996; Stefano et al. 2000). OR subtypes (μ,κ, and δ-receptors) (Le Merrer 2015) are located pre- and post-synaptically in neuronal tissue and are partially overlapping (Henriksen & Willoch 2008). In brain, ORs are mainly expressed in cortex, limbic system and brain stem. MORs are abundant in amygdala compared to other receptor subtypes (Le Merrer 2015). MORs are large proteins, belonging to vast transmembrane protein family called G-protein coupled receptors (GPCRs). G-proteins pass seven times through the surface of cellular membrane, and forward the signaling from the membrane to inner cell. Well known side effects of OR agonists include pupil constriction, sedation, euphoria, constipation (inhibits peristaltic moves in the guts), a deprivation of the respirational and circulatory system (bradycardia, lowered blood pressure), nausea and tolerance formation (in constant usage). Many of these side effects can be blocked or reduced by opioid antagonism (e.g. with naltrexone) (Chen et al. 2012; Pattinson 2008; Martin 1983).

2.1.3 Reward pathways

Survival in nature requires an individual to successfully resolve evolutionary challenges such as feeding, mating, taking care of offsprings and avoiding injuries. Specific neurobiological mechanisms help in monitoring these ancestral priorities in animals and humans. Reward pathways in CNS have evolved to promote security, reproduction and maintain homeostasis. They modulate motivation and elicitation of pleasurable signals upon reward consumption. The dopamine-containing neurons of the midbrain ventral tegmental area (VTA) and shell of nucleus accumbens (NAc) regions play a major role in reward modulation in brain. They function in tandem with prefrontal cortex, amygdala and brainstem (Berridge & Kringelbach 2015; Fields & Margolis 2015). Dopamine (Nutt et al. 2015) and opioid neurotransmitters (Contet et al. 2004) are intimately involved in reward processing in humans and other mammals. The rewarding neural effects of opiates (e.g. heroin) are thought to be based on the positive outcome of dopaminergic activation that opioids reinforce (by e.g. exiting the inhibitory secondary cells in VTA) (Johnson &

North 1992). Animal studies have shown the importance of dopamine in morphine-induced analgesia in laboratory mice (Hnasko et al. 2005). Other animal models suggest that opioids could also independently modulate the rewarding effects (without dopaminergic activation) in brain hedonic 'hot spots' (Berridge & Kringelbach 2015). Opioid receptors are expressed abundantly in the reward sites of brain including cortex, limbic system and brainstem (Le Merrer 2015). NAc regulates in particular the rewarding effects of sociability through MORs (Viviana Trezza, Ruth Damsteegt 2011). Other mesolimbic brain areas are involved in natural reinforcement, such as feeding, sexual behavior and mood regulation in animals and humans (Le Merrer 2015; Lutz et al. 2018). Brain mesocorticolimbic system is activated both by natural reinforcers as well as multiple drugs of abuse (Panksepp et al. 1978).

Of endogenous opiates, β-endorphins mediate the rewarding effects of mating, eating, drugs, exercise and safety signals through brain μ-opioid receptors (MORs) (Nummenmaa et al. 2018; Henriksen & Willoch 2008; Saanijoki et al. 2018; Karlsson et al. 2015). Exogenous opioids are well-known for their anxiety-alleviating effects (Colasanti et al. 2011). Direct (heroin, morphine) and indirect (nicotine, cannabinoids) MOR agonists harness the brain reward system (Contet et al. 2004), and may lead to disruption of EOS (Ross et al. 2005). Other types of addictive behavior, like compulsive overeating or alcoholism are also linked to altered function of mesolimbic MOR and dopamine systems (Karlsson et al. 2015; Weerts et al. 2011). A vast amount of evidence confirms that endogenous opioid system is activated also during positive (but also negative) basic emotions in humans (Nummenmaa & Tuominen 2018).

2.2 Social Attachment

Social reinforcement is vital for the survival of social species. In addition to modulation of sensory pleasure, reward circuits enhance both social bonding and attachment formation in animals and humans (Inagaki 2018). The term attachment is strongly related to *'the feeling of love'* – a familiar abstraction from poets and songs that has been hard to approach scientifically. After Sigmund Freud's psychoanalytic theories, British psychiatrist and psychoanalyst John Bolwby's *Attachment theory* (Bowlby 1980) delineated the basic parallels between human and animal attachment behavior: seeking for significant others and maintaining social ties with them, in addition to maternal attachment behavior (Bowlby 1977). On later half of 19th century, psychologist Mary Ainsworth continued Bolwby's work and refined the ideas for attachment formation between mother and child (see in Mikulincer & Shaver 2007).

Attachment typically involves mother-infant dyads or adults in romantic reproductive relationships. Both phenomena have been proposed to share common neurobiological and psychological mechanisms (Hazan & Shaver 1987; Panksepp 2006). Humans however tend to establish also other, non-sexual affiliative relationships with each other. That kind of attachment formation, which is directed clearly towards one individual - a friend - are more uncommon in the animal kingdom (Dunbar 2018). Most of the knowledge about human attachment variations comes from self-report-studies (Mikulincer & Shaver 2007), and the genetic vs. environmental influence on attachment formation is still discussed. However, attachment styles vary between human individuals in adulthood as well as in childhood (Fraley 2002), and the neurobiological explanation of those differences is yet unclear.

2.2.1 Maternal attachment

Early attachment style reflects an infant's affiliative behavior towards the mother or other first caring figure (Hazan & Shaver 1987). Functional, nurturing dyadic bond between mother and child gives necessary resources for the offspring to survive. Secure attachment between mother and child effects positively on brain development, infant's mental health and emotion regulation (Schore 2001). When secure attachment cannot be established, an individual may become less socially active, have cognitive impairment and depressive or avoiding symptoms (rodents: Nelson & Panksepp 1998; humans: Cacioppo & Hawkley 2009; Cacioppo et al. 2011). In humans, inadequate social environment in early childhood may also contribute to the severity of major psychopathological symptoms in adulthood (Kirkpatrick 1997) and even a subsequent onset of personality disorders (Brennan & Shaver 1998). Hence, craving for early social attachment is a fundamental phenomenon in both animals and humans, which may vary due to both environmental and genetic factors (discussed further on p. 59).

Based on observational methods, human infant attachment can be classified as secure and insecure (avoidant or anxious) styles (Ainsworth et al. 1978). This is based on an objective experimental observation of how infant seeks the mother's attention, and how the infant reacts when the mother-figure is taken away or comes back. For example, avoidantly attached children don't show a strong emotional reaction when the mother leaves her alone with strangers. However, their autonomic nervous system activity is indicative for (strong) reactive emotion in the absence of it's overt manifestation. Other, non-observational methods were developed in late 1900s for quantifying infant and adult romantic attachment, for instance with self-report questionnaires about marriage, parenting and peer relationships (friends).

Those were clinically used as a basis of structured interview of adult attachment style (Bartholomew & Shaver 1998).

2.2.2 Adult attachment

Adult romantic attachment style (AAS) is assumed to share similar functional basis with a dyadic bond between a child and the caretaker, as attachment style is considered to stay quite stable during life (Fraley 2002; Hazan & Shaver 1987). Childhood attachment style predicts romantic and affiliative relationship satisfaction as well as relationship insecurity in adulthood (Hazan & Shaver 1987; Bowlby 1977). AAS in both romantic and non-sexual bonds denote individual's cabability to seek and maintain meaningful social units, and also other than sexual/romantic associates. Social skills and activity are important factors that affect individual's well-being through their lifespan. Conversely, asocial behavior and isolation are often part of the pathological process in many psychiatric conditions, such as social anxiety disorder, depression or even psychotic events (Teo et al. 2013; Kirkpatrick 1997). Insecure attachment strategies encourage repeated activation and suppression of negative emotions, which leads to continued reliance on distorted mental representations of self and others. Furthermore, those are well linked to physical and mental health problems (Mikulincer & Shaver 2007).

As well as infant-mother attachment, also adult attachment can be divided to secure and insecure. Insecure attachment can be further separated in two or more different dimensions, depending on the used model (e.g. Crittenden & Landini 2011). AAS can be psychometrically measured either by self-report questionnaires or by structural or semi-structural interviews. One widely used measure of AAS is a self-report questionnaire called the Experience in Close Relationships- Revised (ECR-R) (Fraley et al. 2000). The ECR-R divides attachment styles into two broad dimensions of insecurity: anxiety and avoidance. Alterations in both dimensions can lead to problems in affiliation formation. Avoidant attachment is associated with decreased trust to others, leading to lowered interpersonal trust and increased preference for staying alone. Anxiously attached persons in turn tend to worry about the partner braking the social bond (Bartholomew 1990).

Persons with avoidant attachment style have a lowered capability to feel pleasure in social situations, hence they prefer being alone and avoiding interpersonal exchange. Avoidant individuals tend to worry about their existing partner/attached figure to break the created social bond, but they see themselves beyond the support of others and have an emphasized self-reliance (Mikulincer & Shaver 2019). That results in a "segregated" mental system, as avoidantly attached persons are suppressing the emotional thoughts related to important relationships (Bowlby 1980). Anxiously attached persons are more prone to detect negative affective

emotional stimuli from the environment and in relationships, which means their negative affections are more easily aroused. Persons with anxious an attachment trait are interested in having meaningful social units, but in the same time they are worried about the negative consequences, that understandably complicates or narrows their personal social ties (Vrtička & Vuilleumier 2012).

Other questionnaires that can be used to determine subjects' capability to create and maintain meaningful social units are for instance: Adult attachment questionnaire (AAQ), Attachment Style Questionnaire (ASQ), Relationship Style Questionnaire (RSQ), Caregiving Questionnaire and the Adult Attachment Interview (AAI) (Mikulincer & Shaver 2007). They approach the question of an individual's affiliative capability from different point of views. For example, AAI is a semi-structural interview, where the main focus is on 'what' subject tells about his important life-events and 'how' she/he tells about it. AAI classifies adult attachment style in certain continuum (i.e. integrated – non-integrated) according to the used analyzing model (i.e. Crittenden's Dynamic-maturational model, DMM) (Crittenden & Landini 2011). Hence, AAI is not quantitatively sensitive, but good for clinical purposes (Zeanah et al. 2011). ECR-R was chosen as the attachment measure in Study II, because as a self-report it divides AAS in two main dimensions (avoidant and anxious) of insecure attachment, which may have distinct neurobiological bases.

Defining neurobiological differences behind affiliative behavior in adults may not only improve the understanding of normal human behavior, but might also help diagnosing and treating psychopathologies that often involve social dysfunctions.

2.2.3 Behavioral parallels between social affection and addiction

Affection and attachment are social sensations that are similar to addictive states: humans tend to think of their loved ones a lot, especially in the beginning of a new, intimate relationship. Social attachment processes share similar neurobiological characteristics to substance use disorders (Insel 2003; Panksepp et al. 1980), and can further be classified into similar phases: 1) *the initiation* (beginning of a relationship or the first trial of a drug), 2) *maintenance* (using a drug or keeping touch with a person), 3) *withdrawal* (end of drug administration or braking a social bond) and 4) *relapse* phase (getting back to abuse with same drug or the missed person). It is known that insecure attachment style constitutes an independent risk factor for depending a substance use disorder (Mikulincer & Shaver 2007). Both natural reinforcers (e.g. food, sex) and exogenously administered drugs (e.g. heroin) recruit and modulate brain opioidergic reward pathways (Le Merrer 2015), especially MOR system (Contet et al. 2004). Similarity in behavioural patterns in social attachment

and substance use disorder leads to a presumption that they could also share similar neurobiological mechanisms.

Altered EOS is linked with antisocial behavior in humans, and antisocial personality may lead to opioid addiction (Ross et al. 2005). Also emotion recognition - a crucial social skill - is impaired in polysubstance abusers (Fernández-Serrano et al. 2010). That observation also links the socially relevant brain mechanisms to disrupted opioid neurotransmission. A compulsive usage of exogenous opioids results in to a tolerance (Koch & Hollt 2008), which may cause a permanent disruption of EOS and consequently increased asocial behavior (as more time and energy is needed to achieve a daily dose). Deficient social network may lead to increased risk of substance abuse and alcohol consumption as a self-treatment, especially in vulnerably attached adults (Brennan & Shaver 1995).

A current trend in treating opioid addiction is to find an individualised balance between social and pharmacological cures with mild opioid agonists (e.g. methadone or bubrenorphine), so that the sensation of euphoria triggered by opioidergic drugs can be diminished step by step (Contet et al. 2004; Kosten et al. 2003). Social support is a key factor in withdrawal success among drug users, alcoholics and cigarette smokers, whereas social stressors typically launch the relapse phase (Havassy et al. 1991). Thus, delineating the neurobiological mechanisms of attachment style and social behavior could give clues for effective treatment methods, increase understanding of patients with history of substance abuse and finally, why some people tend to fall more easily in continuous usage of drugs than others.

2.3 Neurobiology of attachment – what is known?

Although intensity of social activity varies a lot between different species, most social mammals (monogamous species) display some sort of affiliative behavior when growing from juvenile to adults (Carter 1998). Especially monogamous prairie voles (*Microtus ochrocaster*) provide a valuable animal model for studying affiliative behavior: they are highly social and establish exceptionally strong bond with a partner after mating (Beery et al. 2018). In socially active hetero-sexual prairie voles, different neuropeptide systems are involved in parturition, postpartum period, maternal caring habits, lactation, mating and maintaining adult social interaction (Carter 1998; Nelson & Panksepp 1998). There is a lack of direct evidence about neuronal mechanism in human social bonding, and most of the present knowledge about endocrine mechanisms in attachment formation comes from rodent and non-human primate studies (Carter 1998). The knowledge from animal-studies creates models of neuroendocrine basis of human sociability, as direct experimental work and pharmacological studies cannot be routinely done with humans (Machin & Dunbar 2011; Nelson & Panksepp 1998). The most studied peptides involved in

social events: oxytocin, vasopressin, dopamine and endogenous opioids, are shortly presented in the next chapter.

2.3.1 Non-opioid peptides in affiliative behavior

Oxytocin and vasopressin share similar molecular structures and both are generated in hypothalamus and released from the brain posterior pituitary gland (hypothalamus-pituitary-adrenal- axis; HPA). Oxytocin governs affiliative and sexual behavior in socially active species. For example, an oxytocin injection to intra-cerebroventricular space enhances partner preference in both male and female prairie voles (Carter 1992; Williams et al. 1994). In female mammals, oxytocin is related to reproductional events including vaginal stimulation, parturition, lactation and maternal behavior after delivery (see in: Nelson & Panksepp 1998; Pedersen 1997). Milk contains lots of oxytocin, and suckling forms a basis to a mother-infant relationship formation (Leake et al. 1981). Vasopressin is monitoring kidney functions, but it is also released from pituitary glands during sexual activity, labor and suckling parallel with oxytocin (Insel 2003; Seckl & Lightman 1987). Both vasopressin and oxytocin neuropeptides are proposed to modulate social memory in rats (Dantzer et al. 1987).

Corticosteroids (s.c. 'stress-hormones') are also produced and released to the circulation from the HPA-axis, especially during stressful life events. Increased concentration of circulating corticosteroids in circulation leads to an elevated blood pressure and other stress-related somatic symptoms in humans (Cornwell & Waite 2009). In animal models, stress (such as fear, isolation, separation etc.) increases plasma cortisol levels (Hennessy 1997; Crockford et al. 2008). Interaction with conspecifics reduced blood cortisol levels in socially naïve female rats (DeVries et al. 1995). In rats and guinea pigs, prenatal stress or treatment with stress hormones have effects on later hormonal levels and social behavior in adulthood (Carter 1998). Thus, stressful conditions induce HPA functioning, which presumably increases social activity and tendency to seek comfort as stress-alleviation from other conspecifics (Crockford et al. 2008).

Intense romantic feelings activate limbic brain regions involved in reward and motivation, VTA, that is also known to be dopamine-rich area (Aron 2005). Dopamine acts together with oxytocin and vasopressin by facilitating the same brain pathways that are needed to enhance maternal behavior (Pedersen 1997; Carter 1998). In adult voles, pup-exposure released dopamine in mother voles' NAc, and dopamine receptor agonism decreased the salience of pup-retrieval (Keer & Stern 1999). Furthermore, some rat and primate studies have showed that low social status increases self-administration of cocaine (dopaminergic agonist to D2/D3-receptors and dopamine transporters) (Jupp et al. 2016; Morgan et al. 2002). That provides

support for dopaminergic neuromodulation in social recessivity, and that latter seems to be a risk factor for developing drug dependence. Similar to endogenous opioids, dopamine seem to be crucial neurotransmitter in regulating *motivation* towards social activity (Insel 2003).

2.3.2 Endogenous opioids in affiliation formation

Also endogenous opioids are mediating sexual activity and motivation in social species (Argiolas & Melis 2013). The present knowledge about opioidergic activity in mating is not direct, but the indirect linkage of EOS and sexual activity is more evident instead (Paredes 2014). One aspect is that because sexual activity and sociability are pleasurable and rewarding states (Berridge & Kringelbach 2015), their hedonic properties could be governed by MOR system. MOR agonism enhances attractiveness towards opposite sex (Chelnokova et al. 2014), and morphine dependence lowers the levels of sex hormone concentrations in rodents and humans (Bodnar & Klein 2018). Both oxytocin and endogenous opioids are released during vaginal-cervical stimulation and suckling, which are the core actions of early affiliative bonding in social species (Keverne et al. 1997). The combination of progesterone and mild-opioid-treatment in late pregnancy inhibits maternal behavior after labor in rats (Cruz et al. 2015), suggesting maternal motivation partially been modulated by opioids.

Human infants recognize their mother's odor very soon after birth (Cernoch & Porter 2016), and securely attached mothers prefer their own offspring's body odors over other's (Croy et al. 2019). Neurogenesis of the olfactory bulb is opioid-dependent in rat pups (Santoyo-Zedillo et al. 2017), hence endogenous opioids are effecting on the development of important sensory pathway involved in early affective behavior. The smell of an important other also alleviates stress and increases comfort between securely attached adults (Granqvist et al. 2019).

When rat pups are separated from their mother, separation anxiety is usually indexed with elevated social distress vocalization. Similar behavioral reactions to separation from a mother have been found in other social mammals, like chickens (Bermant 1963), guinea pigs (Pettijohn 1979), monkeys (Seay & Harlow 1965) and human babies (Bell & Ainsworth 1972). Separation anxiety can be alleviated with exogenously administered opioid agonists (morphin injections) in rodents (Panksepp et al. 1978). On the other hand, a primate study showed that blocking MOR system with opioid antagonist (naloxone) enhanced offsprings' proximity seeking towards their mother (Martel et al. 1995). Those findings in the late 1900s form the basis of 'The brain opioid theory of social attachment' (BOTSA), that proposes proximity to important others being modulated by endogenous opioid system in central nervous system.

2.4 Opioidergic activity in social bonding

Social activity and surroundings may have shaped the outcome of human brain how they are today. Earlier evidence suggests that the relative size of neocortex (compared to whole brain volume) is linked with the sociability and the amount of daily social acts among some primates (Shultz & Dunbar 2010). For maintaining social bonds within the group, primates use multiple mechanisms including grooming, laughter and playing together (Dunbar 1991; Pellis et al. 2015). New social bonding mechanisms have emerged among humans during evolution and separation of *homo sapiens* from other great apes (Dunbar 1993). Increasing complexity of the human social networks might have led to need of "social processing power" and consequently caused the expansion of neocortex.

Opioid receptor antagonism (naltrexone) increases grooming and grooming solicitations in socially active *Rhesus macaques* and *Talapoin monkeys* (Graves et al. 2002; Keverne et al. 1989). In the same studies, opioid agonism (for example morphine) decreases sociability and grooming calls. In addition, Keverne's group (1989) established that grooming after social isolation increased the amount of β-endorphin in cerebrospinal fluid in Talapoin monkeys (Keverne et al. 1989). In humans, opioid antagonism reduces warmth and pleasurable sensations in social actions, and morphine or internally released opioid peptides increase the feeling of social connectivity (Inagaki 2018).

Humans, as well as some social non-human primates, use touching to mediate positive affection. Allowed touching-areas in receiver's body are strongly depended on the relationship between the toucher and the touched one (Suvilehto et al. 2015). A soothing touch may tighten the original bond between individuals (Von Mohr et al. 2017), and it also alters EOS activity in human brain as measured with PET (Nummenmaa et al. 2016). However, grooming is time-consuming and can be done properly only between two or three individuals at a time. Hence, during evolution new, time-saving 'grooming-mechanisms' were needed to maintain group cohesiveness in larger populations (Dunbar 2012). Humans use a variety of group activities such as vocalization, gossiping, laughing, dancing, playing music and singing together for maintaining existing bonds. All of them are linked with a tightened sense of belonging, elevated pain perception, better co-operation and improved prosocial skills in humans (Bowling et al. 2013; Dunbar 1993; Dunbar et al. 2012; Dobek et al. 2014; Schellenberg et al. 2015; Slater et al. 2018; Tarr et al. 2015; Wu et al. 2016). Furthermore, part of these means are evidently linked with EOS alterations.

Laughter is a powerful communicative element of human social interaction. It is also highly contagious in groups (Provine 1992). Primitive vocalizations such as laughing occur quickly, do not need physical contact to be recognized by others and can effect on multiple conspecifics at the same time. Thus, laughter helps to keep

other conspecifics near even when they are not within reach. Some other mammals also use laughter-like communication, but it's only studied more among social primates. *Macaca Sylvanus, Barbary macaque* and chimpanzees, use laughter-like vocalization with similar range and acoustic parameters than human laughter (open mouth, relaxed breathing with sound 'ahh', 'ahh', 'ahh') during tickling and to signal playfulness towards other conspecifics (Van Hooff 1972; Vettin & Todt 2005). Because laughter can enhance positive affect and rewarding sensation among laughers and listeners (Neuhoff & Schaefer 2002), it is a candidate mechanism for maintaining group cohesion, allowing increasing of social group size among social primates (Dunbar 2012). Laughing and language are thought to maintain positive social actions also in humans (Preuschoft 2010). Endogenous opioids (especially B-endorphin) are potent analgesics, and measuring pain tolerance can be considered as a proxy of MOR-activity (Johnson & Dunbar 2016). Behavioral studies with healthy volunteers have denoted that soothing social laughter elevates pain threshold in humans (Dunbar et al. 2012), and additionally, pain threshold predicts the size of human social network among adults (Johnson & Dunbar 2016). That speaks out strongly for social laughter being mediated by MORs and endogenous opioids.

2.4.1 opioids and social pain

Opioid system is involved in acute and chronic analgesia, and opioid receptors are expressed in the central descending pain pathway (Stefano et al. 2000). Sustained pain induces EO release in numerous cortical and subcortical brain regions in humans (Zubieta et al. 2001). In rodents morphine injection alleviates pain, even with stronger effect in the proximity of kins (D'Amato 1998). This accords with the proposed linkage between attachment-related comfort and pain management in animals (Panksepp et al. 1997). Genetically, the MOR-coding gene OPRM1 A118G polymorphism is related to lowered MOR availability in pain regulation, personality traits (neuroticism) and therapeutic expectations of pharmaceutical pain treatment (so called *placebo effect*) in humans (Peciña et al. 2015). Dorsal anterior cingulate cortex (dACC) and anterior insula (AI) mediate the aversive feeling and negative affection during somatic pain, and the same regions are involved in depression (Eisenberger & Lieberman 2003). AI and dACC are also activated in episodic social rejection (Kross et al. 2011).

The expression '*feels painful*' is often heard in bereavement, when a person has lost a significant other (Bowlby 1980). Also a psychosocial trauma alters opioidergic tonus in socially relevant brain areas (Liberzon et al. 2007). In addition to comforting effects of sociability, some social acts may also be harmful to an individual, for example when the social bond breaks, or when a person is excluded from the group by (trusted) others. This kind of adverse social experiences have shown to alter brain

EOS in humans (Hsu et al. 2013; Hsu et al. 2015). Sensitivity to social rejection may vary between individuals, and was linked to MOR-gene A118G polymorphism in human fMRI-study (Way et al. 2009). In addition to a genetic predisposition, feeling of loneliness and depressive symptoms could result from decreased endogenous opioid system activity in CNS after social losses or separation from others.

2.5 Measuring of Opioid system

2.5.1 Principles of PET imaging

Positron-emission tomography (PET) can be used for quantifying human tissue composition with high biological specificity (Heiss & Herholz 2006). PET provides a non-invasive way for measuring differences in neuroreceptor availability across patient and control subjects to reveal pathophysiological changes in specific neurotransmitter circuits *in vivo* (Gryglewski et al. 2014; Volkow et al. 2009; Whone et al. 2003).

A variety of biological molecules can be labelled with radioactive isotopes, for example 11C, 18F and 15O. When combined with an isotope, the chosen molecule becomes a radioligand, which binding can be measured locally. Positron emitting isotopes are produced in a particle accelerator, usually in a cyclotron. The cyclotron creates an unstable nucleus by forcing an atom under a strong alternating current and accelerating the ionized particle to a high speed in a spiral movement. Finally, one ionized proton is expelled from a cyclotron to bombard stable atoms to become unstable radioactive isotopes (e.g. 11C, 18F). Isotope is then bound with a ligand, chosen as basis of the investigated molecular system. In studies I-III, an unstable 11C isotope is combined with carfentanil, a specific μ-opioid agonist.

In the nucleus of labile isotope, a proton (p) decays to a neutron (η), a positron (e^+) and electron neutrino (υ) (1). After an intravenous radio-tracer injection an isotope exhibits positron (e^+) decay. The unstable ion (e+) travels a short distance (in the tissue), until it meets an electron (e-). When two ions merge, they emit two 511 keV gamma (γ) photons apart in two approximately opposite directions (2). That phenomenon is called an annihilation radiation.

$$p \rightarrow \eta + e^+ + \upsilon \quad (1)$$

$$e^+ + e^- \Rightarrow \gamma + \gamma \quad (2)$$

γ-photons travel through tissue in parallel with an imaginable straight line (the line of response, LOR), and are captured by detectors surrounding the subject (**Figure 1**). This so called coincidence data is reconstructed computationally to form a visual

image by using the local information of the photons (angle, speed) to show where (in tissue) the annihilation originally happened. PET scanner measures the radioactivity of the tracer as a function of time (time-activity curve, TAC) (Lammertsma 2002). Corrections can be made afterwards to reduce the false detections by the PET scanner (scatter, attenuation, random events and dead-time (Turkington 2001).

Afterwards, the PET data is computationally modelled to a more statistically informative form. Radioactivity counts are rather non-specific information that depends on injected dose, tracer-mass, target tissue types and individual differences among study subjects (i.e. BMI). Different kinetic modelling methods have been created to account for tissue-dependent effects on tracer binding (non-displaceable binding) after tracer injection to circulation. Here, we used the simplified reference tissue modelling (SRTM) in processing of the PET data (more in **Chapter 4: Materials and Methods**).

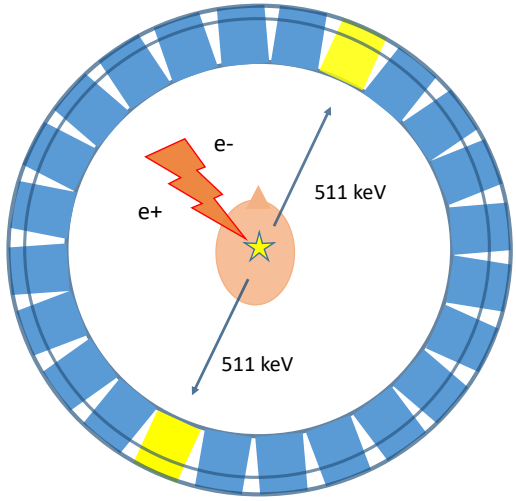

Figure 1. A schematic illustration of PET-instrumentation and annihilation radiation. Blue arrows are depicting the line of response.

2.5.2 Simplified Reference Tissue Model (SRTM)

The clearance of the tracer is dependent on penetrated tissue types and can vary between the subjects. Thus, the aim of modelling is to calculate the distribution of radioactivity concentration of the used tracer in scanned tissue in specific time-window (kBq/cm^3/s). Raw PET data needs to be transformed into a more biologically meaningful format using kinetic modelling, that offers assumptions about rate constants that vary between different compartments in human (or animal) body.

All studies (I-III), utilized the simplified reference tissue model (SRTM) as a kinetic model. SRTM uses tracer's time-activity curves (TACs) from the reference region as an input data (Lammertsma & Hume 1996). SRTM considers tracer concentrations within the time frame of a measurement (**Figure 2**) and has been validated against a full kinetic modelling, that uses arterial blood measurement as an input function. Compared with full kinetic modelling, one big advantage of SRTM in quantitative PET studies is that arterial cannulation is not needed (Lammertsma & Hume 1996). SRTM yields a measure called non-displaceable binding potential, BP_{ND} (Innis et al. 2007). It refers to receptor concentration and affinity to it's target receptors, and is a ratio of specific and non-specific binding in the tissue (Heiss & Herholz 2006). BP_{ND} enables a quantitative, voxel- and ROI (region of interest)- wise approach of receptor availability *in vivo* (Mintun et al. 2004). BP_{ND} can be expressed with the function as follows (3):

$$BP_{ND} = f_{ND} \text{ x affinity x density} \quad (3)$$

,where f_{ND} refers to product of free fraction of non-displaceable compartment, affinity to tracers gravity towards the target receptor and density to the actual concentration of target receptors in the tissue of interest.

SRTM relies on several assumptions: i) only non-specific binding in reference tissue occurs and ii) binding affinity to target receptors is same in reference and in target tissue. In addition, the tissue-level exchange between free ligand and non-specific binding is fast, why they can be considered as one compartment.

In PET-studies, neurotransmitter release in CNS can be indexed by comparing changes in tracer binding potential (BP_{ND}) between two consecutive scans (Colasanti et al. 2012; Nummenmaa et al. 2016). The small changes in radiotracer BP_{ND} between two separate scans (with same study subject) are thought to reflect changes in neurotransmitter release and receptor occupancy in a synaptic space (**Figure 3**). High tracer binding indicates more available receptors, which is thought to reflect low endogenous peptide concentration. In moderate binding of the tracer there is assumed to exist same amount of both endogenous peptides and tracer-ligand, that are competing available target-receptors. Both affinity and density of the target receptors may affect the tracer binding. The commonly used occupancy model assumes that both receptor density and affinity are rather constant over time (Laruelle 2000), so changes in BP_{ND} can be regarded as changes in synaptic neurotransmitter concentration.

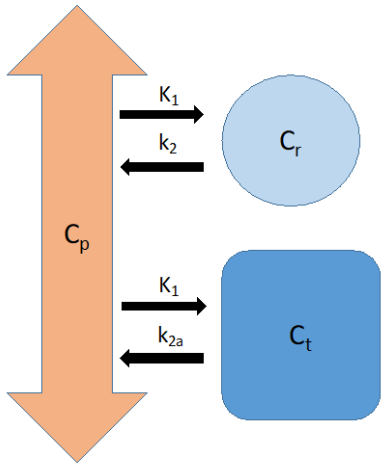

Figure 2. Schematic illustration of SRTM. C_p = tracer concentration in arterial plasma; C_r = tracer concentration in the reference tissue; K1 = rate constant for transfer from arterial plasma to tissue; k2 = rate constant for transfer from tissue to plasma; C_t = tracer concentration in target region; and k_{2a} = apparent rate constant for transfer from tissue to plasma.

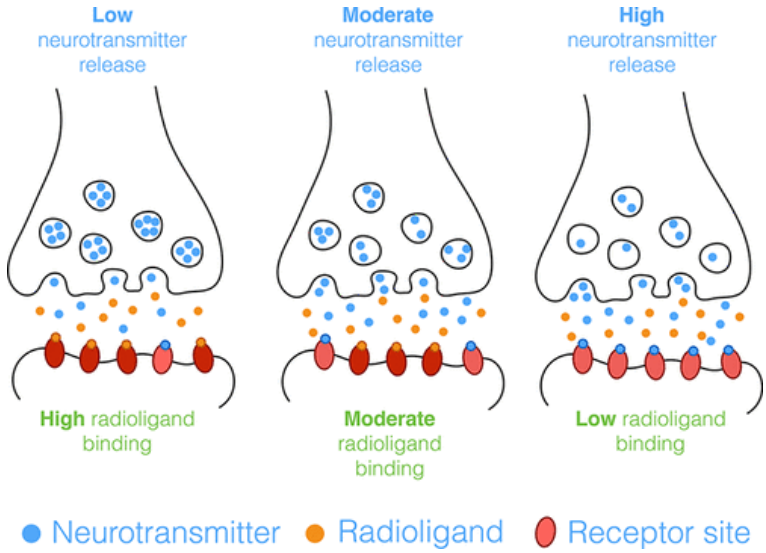

Figure 3. The PET challenge paradigm. Competition between endogenous ligand and radioligand binding in the receptor sites results in different binding potentials depending on the level of endogenous ligand release (from Nummenmaa, Tuominen, et al. 2018).

2.6 Magnetic resonance imaging

Magnetic resonance imaging (MRI) is a noninvasive method for collecting anatomical and functional image data from body structures, that uses resonating magnetic field in image formation. MRI is based on an external radio frequency (RF) energy that is created in transmitter coil and directed to the scanned tissue in a strong external magnetic field. A strong (1.5-3 T) static magnetic field is used in clinical scans. Small particles (protons, neurons and electrons) rotate around their axis in original magnetic field (B_0), where they have their own natural magnetic spin. The hydrogen atom (H+) has only a single proton, which makes it a good resonating target. Fat and water are omnipresent in human tissue, and they both contain abundant number of H+ atoms, therefore MRI is well suited for human tissue imaging.

RF energy forces H+ to another magnetic energy field (B_1), and the spinning angle of the atom changes. When the RF pulse ends, B_1 returns back to the original direction along B_0 and protons return back to their original spinning state. Electromagnetic waves are produced simultaneously, and this is the signal that can be measured with receiving coils. Coils capture the RF signal created by the change in magnetic energy field and spinning changes. This phenomenon is called relaxation. Relaxation constants, T1 (longitudinal) and T2 (transverse) are measures, that give information about tissue type and it's magnetic features. T1 and T2 relaxation time depend on proton density and molecular structure of the scanned tissue. T1 and T2 weighted scans produce images with opposite contrasts of water and fat tissue. T1 weighted images provide a reliable discrimination of water and fat compartments, so the voxel-wise differentiation between brain gray and white matter sections is possible with T1-MRI method. (Hamberg & Aronen 1992)

2.6.1 Volumetric measures of brain tissue morphology

Enlargement of mammalian cerebral cortex has enabled the brain evolution towards more advanced neurocognitive functions. The human brain is gyrencephalic (formed by gyruses or gyri), which means the surface structure is not flat or smooth, and for example, there is no linear correlation with brain total volume and surface area (Hofman 1985). This needs to be taken into consideration when measuring brain volume. There are different ways for classifying brain tissue morphology: total volume, gray matter volume, cortical thickness and surface area, for instance. Surface area and cortical thickness are individual measures that can effect the size of total gray matter volume (Winkler et. al 2011). Both gray matter volume and cortical thickness are known to decrease during age (Hutton et al. 2009). Gray matter density (GMD) is a probability-measure to indicate those brain regions, where most of the synapses and neuroreceptors and -transporters locate and hence are

neurotransmissionally active areas. Brain gray matter can be reliably separated from white matter from T1 weighted brain MR images using voxel-based morphometric (VBM) approach (Ashburner & Friston 2000). In study III, we used VBM-derived GMD to depict neuropil-rich areas extended from white matter, which is typically constructed by cell bodies and neurofilaments in human brain (Purves 2018).

2.7 Summary of the literature

Accumulating evidence suggests that endogenous opioids are linked with social behavior in mammals and humans (Inagaki 2018; Nummenmaa & Tuominen 2018). The brain opioid theory of social attachment (BOTSA) states that endogenous opioids govern both pleasurable sensations elicited by social bonding as well as painful sensations triggered by social losses. According to BOTSA, endorphins are needed to create motivation, liking and wanting towards other conspecifics and the neurobiological mechanisms of social affiliation may be analogous to drug addictions (Machin & Dunbar 2011). This hypothesis is based on animal studies that highlight the anxiolytic role of opioids in social distress in addition to their well-established link with physiological anti-nociception (Panksepp et al. 1978).

Because opioids are involved in nociception, it is possible that they also govern the social distress-relaxation axis in social encounters. The main goal of this thesis was to assess whether social bonding is mediated through opioidergic system in CNS. We examined, if pro-sociability triggers endogenous opioid release that could act as a safety signal reinforcing the social bonds. Additionally, we examined whether regional variation in MOR availability could explain individual differences in sociability. Finally, this thesis links the receptor and cortical density metrics of brain tissue composition using voxel-wise comparison of gray matter density, to specify the interpretation of the findings in brain PET and MRI studies.

3 Aims

The main objective of this thesis was to investigate neuro-molecular and -structural individual differences in human sociability using multi-modal neuroimaging approach. PET was used for quantifying endogenous opioid system activity and receptor availability, respectively. The tracer binding data were also linked with MRI derived indices of cerebral tissue density.

The specific research questions were:

I Does prosocial behavior trigger endogenous opioid peptide release in brain? (Study I)

II Are individual differences in human sociability associated with brain μ-opioid receptor availability? (Study II)

III Is the receptor availability in brain associated with same regions' gray matter density (defined with VBM)? (Study III)

4 Materials and Methods

4.1 Study design

The study protocols were approved by the ethics board of the Hospital District of Southwest Finland and the studies were conducted in accordance with the Declaration of Helsinki. Subjects were compensated for their time and travel costs and they signed ethics committee- approved informed consent forms. Physical health was examined before the scanning day.

4.1.1 Subjects

Study samples are described in **Table 1**. For study I, 12 healthy men were recruited via e-mail through Finnish universities' mailing lists. Only young males were scanned because age, sex, and women's hormonal circulation are known to influence both MOR availability and the capacity to engage the MOR system (Gabilondo et al. 1995; J.-K. Zubieta et al. 1999; Smith et al. 1998). 50 healthy adults (20 females, ages 19–58, mean age 32 years, SD 6.4 years) volunteered for study II. The exclusion criteria in studies I–II (in addition to standard PET and MRI exclusion criteria) were poor compliance, smoking, excessive alcohol consumption (over 8 weekly doses), substance abuse determined by interview and blood tests, a history of or current neurological or psychiatric disease (confirmed using the structured clinical interview for DSM-IV, medical history and blood tests), and current medication affecting the central nervous system. One subject had to be excluded as MRI indicated a previously unknown neurological disease. In study III, subjects consisted of historical data from multiple different studies at Turku PET Centre. Altogether 325 (151 females, age range 18–55, M_{age} = 35.8, SD_{age} = 13.9 years) brain scans were included in the study III, with a total of 162 [11C]carfentanil, 72 [11C]MADAM and 91 [11C]raclopride scans (**Table 2, p. 35**).

Table 1. Study samples in all studies I-III.

SUBJECTS	N (F)	AGE RANGE	MEAN AGE	SD AGE
STUDY 1	12 (0)	20-32	22.9	3.3
STUDY 2	50 (20)	19-58	32.0	6.4
STUDY 3	325 (151)	18-55	35,8	13.9

4.1.2 Questionnaires

In the prescreening phase, all subjects in studies I-II were interviewed using DSM-IV (Diagnostic and Statistical manual of Mental disorders, 4th edition) (Bell 1994), to rule out recent major mental illnesses. Participants also completed the Beck Depression Inventory II (Beck et al., 1988) and State-Trait-Anxiety Inventory (Spielberger et al., 1983) to rule out minor anxious and depressive symptoms and their potential association with MOR availability.

In study I, subjects were asked to report their current sensations (sleepiness, happiness, irritability, tension, pleasure, amusement, pain and calmness) on a visual analogue scale ranging from 0-100. The answers were recorded by the operator in the beginning (0 min), in the middle (27 min) and in the end (51 min) of each PET scan. A self-report about the current emotional state were also collected in the beginning and in the end of neutral (baseline) and challenge (group laughter) sessions.

In study II participants completed the Experiences in Close Relationships-Revised (ECR-R) questionnaire, that consists of 36 likert-scale questions addressing adult attachment style. ECR-R has been psychometrically validated to capture anxiety and avoidance dimensions of adult attachment style (Fraley et al. 2000). Study III was based on historical scanning data and no specific questionnaire measures were used.

4.1.3 Radiotracers

A valuable radiotracer is simple to produce, has a short half-life ($T_{1/2}$), passes through blood-brain barrier (optimal lipophility), and binds to the target receptor with high specificity. In addition, it should not have brain-penetrant radio-metabolites, but should be measurable from plasma proteins easily and should have fast enough kinetics to allow quantification in next few hours after injection (Fridén et al. 2014; Rosso et al. 2008). All used tracers here were labelled with short-lived carbon-11 (11C, $T_{1/2} \approx 20$ min), that was produced in the cyclotron in Åbo Akademi's

laboratory. The production procedure of [11C]carfentanil has been described in previous studies (Hirvonen et al. 2009; Karlsson et al. 2015) as well as the preparation process of [11C]raclopride and [11C]MADAM (Tuominen et al. 2013; Karlsson et al. 2015). Chemical structures of the used tracers in Studies I-III are presented in **Figure 4.**

The main tracer (used in all studies) was the MOR specific radioligand [11C]carfentanil (Frost et al. 1985). Carfentanil is a potent synthetic opioid agonist with similar molecular structure as fentanyl and heroin, being over 10.000 times more potent as a same unit of morphine. Because of it's potency, the concentration of carfentanil needs to be kept as low as scientifically possible when combined with a radio-active isotope. [11C]carfentanil shows competitive and reversible binding to MORs (with sub-nanomolar affinity, < 0.1 nM), and it has a good test-retest reliability (Hirvonen et al. 2009). Because of a low local specific binding in occipital cortex (OCC), that is typically used as a reference region in quantitative PET studies with [11C]carfentanil (Frost et al. 1985).

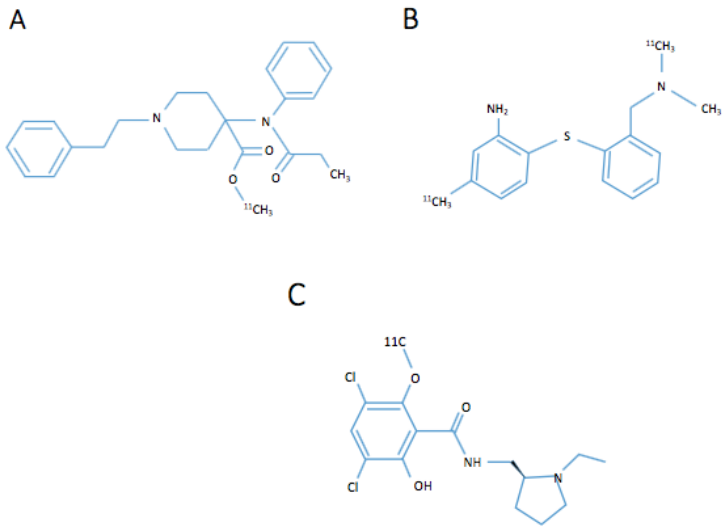

Figure 4. The chemical formulas of A) [11C]carfentanil, B) [11C]MADAM and C) [11C]raclopride.

[11C]MADAM ([^{11}C]N,N-Dimethyl-2-(2-amino-4-methylphenylthio)benzylamine) is a selective inhibitor of serotonin transporter (5-HTT) in CNS, and can be used for quantifying brain serotonin levels *in vivo* (Chalon et al. 2003). [11C]raclopride is a dopamine receptor antagonist that gravitates to brain D2/D3 (dopamine-2- and dopamine-3)- receptors with medium affinity, and binds mostly to the striatal areas

in brain (Egerton et al. 2013). [11C]MADAM and [11C]raclopride were used in study III, where the aim was to get a comprehensive view about receptor densities and their association to brain gray matter density (GMD) in whole brain area. All used tracers have unique distribution due to their target receptor localization in brain (see **Figure 5**).

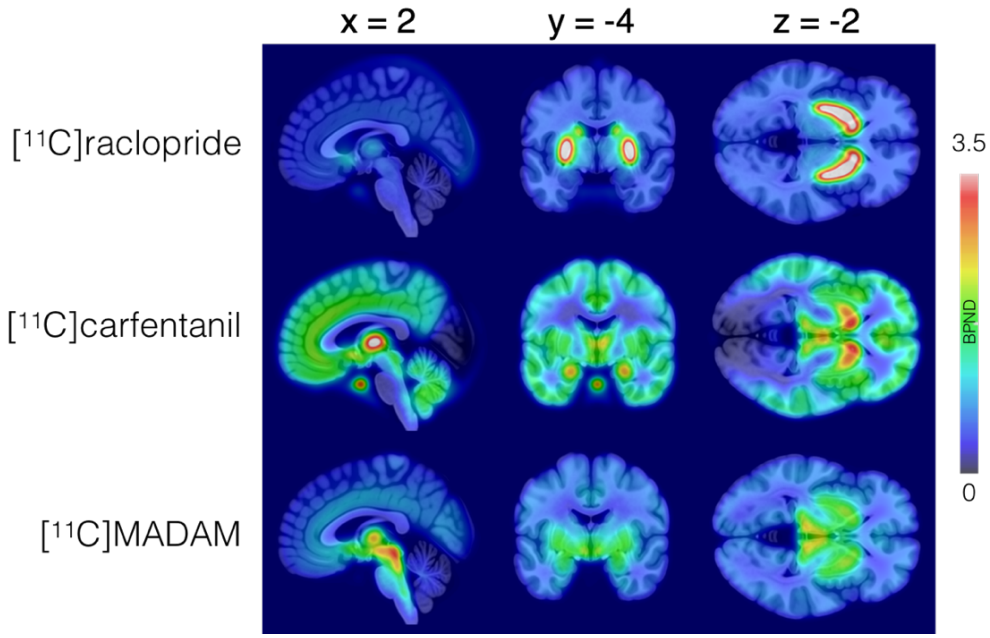

Figure 5. Mean distributions for [11C]carfentanil, [11C]raclopride and [11C]MADAM (expressed in BP_{ND}) shown over T1 weighted structural MR template image (from the original manuscript no III).

4.1.4 PET data acquisition in studies I-III

PET and MRI data were acquired with different scanners at Turku PET Centre: i) Philips Ingenuity PET-MRI scanner was used in study I-III, i) GE Heatlhcare Discovery TM 690 PET/CT in study II and III and iii) multiple PET scanners (ECAT HRRT, Siemens Medical Solutions or Philips Ingenuity 3T PET/MR scanner) in study III (manuscript). During all PET acquisitions, subjects were lying in the PET scanner in supine position covered with light blankets and the lights in the scanner room were dimmed.

Table 2. Detailed scanning information from the used scanners in Study III (from the original manuscript no III).

PET CAMERA	N(MALES)	N(FEMALES)	AGE ± SD	DOSE ± SD
HRRT				
[^{11}C]CARFENTANIL	39	40	42±9	468±59
[^{11}C]MADAM	32	40	43±12	490±30
[^{11}C]RACLOPRIDE	35	0	24±2	299±62
PET-CT				
[^{11}C]CARFENTANIL	10	19	36±14	252±10
[^{11}C]RACLOPRIDE	0	19	43±15	253±17
PET-MRI				
[^{11}C]CARFENTANIL	54	0	25±5	251±14
ECAT				
[^{11}C]RACLOPRIDE	4	11	62±10	198±15
HR+				
[^{11}C]RACLOPRIDE	0	19	23±4	309±112
GE-ADVANCED				
[^{11}C]RACLOPRIDE	0	3	54±8	182±4

4.1.5 Processing of PET data

In all studies (I-III), radiotracers were dosed intravenously through a cannula in right or left cubital vein as a bolus injection in the beginning of each PET scan. After radioligand injection, MOR availability was measured with [11C]carfentanil (in studies I-III), D2R availability with [11C]raclopride and SERT availability with [11C]MADAM (in study III). In PET scans with [11C]carfentanil the duration was 51 min with an in-plane resolution of 3.75 mm, and the targeted injected dose was approximately 250 MBq in studies I-III. PET data were corrected for dead time, decay, and measured photon attenuation and dynamic PET scans were reconstructed using the MRP reconstruction method (Alenius & Ruotsalainen 1997).

Data processing and analysis was performed with statistical parametric mapping-software (SPM8 or 12) in all studies (http://www.fil. ion.ucl.ac.uk/spm/) and complementary analyses were run with in-house code with Matlab R2016b (Math Works, Natick, MA). The processing pipeline involved 1) realigning the PET image frames 2) co-registering the T1-weighted MR images with the summed PET image, and 3) normalization of the PET data to the MNI space using the subject-wise

T1 images. For kinetic modelling in studies I-II, reference regions were drawn manually over the structural T1 images with PMOD 3.4 (PMOD Technologies, Zurich, Switzerland) while FreeSurfer parcellation based regions were used in study III. For SRTM, occipital cortex for [11C]carfentanil, and cerebellum for [11C]raclopride and [11C]MADAM were used as reference regions. TACs were extracted from the reference regions with PMOD 3.4 and with in-house created software. BP_{ND} was calculated for each voxel using the SRTM with the reference tissue TACs as input data (Gunn et al. 1997). The effects of social laughter on MOR availability were then assessed in SPM12 and anxious and avoidant attachment styles in SPM8 (http://www.fil. ion.ucl.ac.uk/spm/) using linear regression model. Statistical threshold was set at $P < 0.05$, FDR corrected at cluster level. In study III subject-wise parametric BP_{ND} images were spatially normalized using the individual flow fields derived from the DARTEL analysis, and finally smoothed with a Gaussian kernel of 6 mm FWHM.

4.1.6 MRI Data Acquisition

T1 weighted MR scans were acquired for normalization of the PET images and for the definition of regions of interests (ROIs) in all studies I-III, and for the VBM-analyses in Study III. MRI was performed using the 3 T Philips Ingenuity PET-MR scanner and the Philips Gyroscan Intera 1.5 T CV Nova Dual scanner in Turku PET Centre. With both scanners, high-resolution (1 mm^3) anatomical MR reference images were acquired using a T1 weighted sequence. Subjects were lying in a supine position in the scanner while the brain MRI acquisition was ongoing. Anatomical MR images were screened afterwards by the recearch group radiologist.

4.1.7 Voxel-based morphometry (VBM)

Voxel-based morphometry (VBM) is an analysis method for T1 weighted MRI brain scans (Ashburner 2009). For each voxel in the brain, VBM defines the likelihood for that voxel belonging to gray or white matter. This allows the division of whole brain tissue to gray and white matter segments from T1 weighted images. VBM allows registering every brain to a template, which removes most of the large differences in brain anatomy among study subjects. Then the brain images are smoothed so that each voxel represents the average of itself and its neighbors. Hence, it is possible to measure subject-wise mesoscopic differences in surface area of the cortex: T1-images are compared voxel-by-voxel to each other to delineate morphological differences in gray and white matter densities (GMD, WMD). After that, a statistical parametric mapping (SPM) can be performed on the segments for voxel-wise comparison over tissue compartments between two different groups.

VBM was used in study III to generate GMD and WMD segments from MRI data. After that, the GMD probability maps were compared with tracer BP_{ND} maps over all study subjects. Structural MR images were analyzed with Matlab R2016b (Math Works, Natick, MA) using SPM12 (www.fil.ion.ucl.ac.uk/spm/) software and the Dartel pipeline which enables automated spatial normalization, tissue classification to WM and GM and radiofrequency bias correction to be combined with the segmentation step using a study-specific template.

4.1.8 Laughter elicitation protocol for Study I

Study I used a PET challenge paradigm to measure endogenous opioid release following social laughter. Every subjects went through two (2) PET scans per examination day: a laughter scan and a baseline scan. Before the laughter scan, subjects spent 30 min with their two close friends watching videos from the previously selected YouTube-list (https://www.youtube.com/playlist?list=PLwD5Oy7K3-z5zljneoLPqHrnG70h0a9a2; note that some of the videos have been already removed from the list by the service provider). They could also choose any videos they wanted to watch from the list themselves. Group laughter was recorded with a digital audio recorder (Olympus Digital Recorder VN-711PC) and the frequency of laughter bursts was annotated after the experiment. After watching the videos for 30 min, the subjects were asked to complete a self-report about their current mood (sleepiness, happiness, irritability, tension, pleasure, amusement, pain and calmness verbally). The same questions were asked also in the beginning (0 min), in the middle (27 min) and in the end (51 min) of the both PET-scans. Before the baseline scan, the subjects spent 30 min alone in neutral condition in the research center and were not able to connect with any friends via mobile phone or laptop. Both scans (challenge and baseline) were done in the same day for each same subject and the order of the scans were counterbalanced over the subjects, to minimize the possible time-dependent results in [11C]carfentanil binding (morning vs. midday). Scans were separated by 2 h break to allow tracer decay.

5 Results

5.1 Study I: Social Laughter Triggers Endogenous Opioid Release in Human

Laughter is an universal prosocial signaling that is used for displaying affiliation and amusement (Dunbar 2012). Also, laughing in a social situation is highly contagious (Provine 1992) and triggers pleasurable sensations via primitive bonding mechanisms and by alleviating stress levels (Hatfield et al. 1992; Keltner & Bonanno 1997). Because social laughter also elevates pain threshold (a proxy for opioidergic activation) in humans (Dunbar et al. 2012), it may also mediate social bonding via EOS. Here, we examined if group laughter triggers endorphin release in brain, indexed with changes in [11C]carfentanil $BP_{ND.}$

12 healthy adult males underwent two separate PET scans in same examination day. Before the *laughter scan* subjects watched laughter-inducing comedy clips with their 2 close male friends that they had chosen themselves. The participants watched humorous videos for 30 minutes. Laughter-bursts were recorded with a digital voice recorder and quantified manually after the experiment: laughter bursts more than 3 seconds apart were considered as separate. Before the *baseline scan*, participants spent 30 minutes alone in a neutral condition. After both conditions, MOR availability was measured with PET using [11C]carfentanil. The order of the baseline and laughter conditions were counterbalanced among subjects.

Laughter manipulation evoked social laughter reliably in the participants with mean rate of 1.04 (SD 0.60) laughter bursts per minute. At the subjective level, social laughter increased pleasurable sensations and calmness (**Figure 6**). In whole-brain analysis of the PET data, endogenous opioid release – indexed with decreased [11C]carfentanil BP_{ND} – was detected in thalamus, caudate nucleus, and insular, cingulate and frontal cortices ($p < 0.05$, FDR corrected). Decreased opioid release was observed in middle cingulate cortices (**Figure 7**). Results from anatomical ROI analyses did not differ significantly from full-volume analysis. Baseline MOR availability was positively associated with social laughter induced by the comedy clips (r^2s > 0.38, *p*s 0.05) in the cingulate and orbitofrontal cortices and ventral striatum.

The results supports our hypothesis that social laughter releases endorphins in human brain.

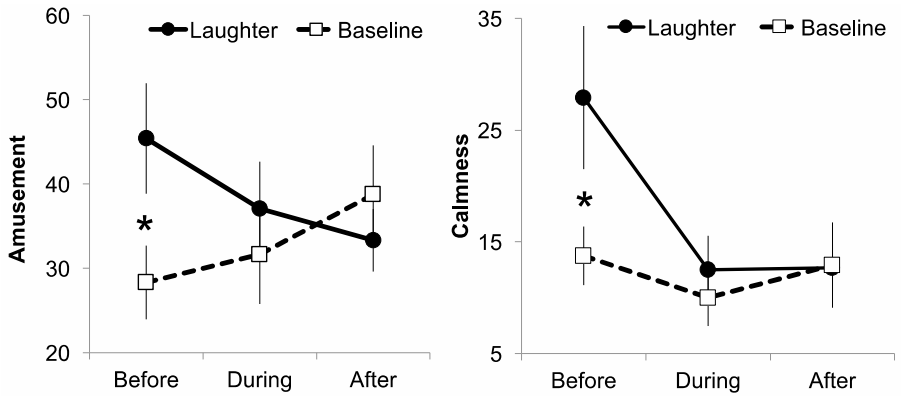

Figure 6. Self-reported amusement and calmness in laughter and baseline conditions. Data were collected before, during and in the end of each PET scan (51 min). It is notable, that the Before-timepoint was recorded immediately after the video-task, just before entering the PET camera (from the original publication no I).

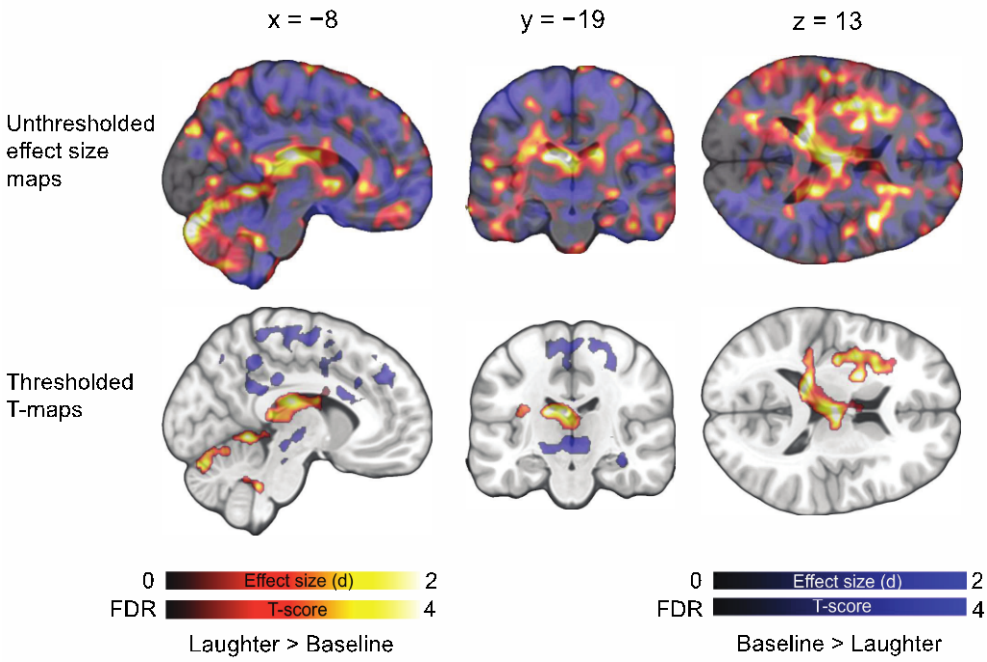

Figure 7. Brain regions showing endogenous opioid release in baseline and social laughter conditions. Top row shows unthresholded effect size maps, bottom row T-contrast maps thresholded at $p < 0.05$, FDR corrected at the cluster level. Color-bars denote the d / T statistic rangers (from the original publication no I).

5.2 Study II: Human Attachment Style is Associated with Brain Baseline MOR-levels

MOR availability is expected to be linked with adult attachment style, according to vast range of previous knowledge about endogenous opioids mediating human and animal social behavior (Nummenmaa & Tuominen 2018), as well as with asocial personality traits in humans (Ross et al. 2005). Here, we expanded these findings to the domain of individual differences in romantic attachment. Insecure attachment style is linked with mental illnesses and may predispose an individual to drug addictions and personality disorders later in life (Brennan & Shaver 1998). Revealing the neurobiological mechanisms of insecure attachment style is crucial for better understanding both normal and abnormal human social behavior.

Fifty (50) healthy adults (20 females, ages 19–58, mean age 32, SD 6.4 years volunteered for a brain PET study with [11C]carfentanil. Attachment traits avoidance and anxiety were measured with the ECR-R scale before the scanning, and the questionnaire results were statistically compared with [11C]carfentanil BP_{ND} levels in baseline PET scan using SPM8 (http://www.fil. ion.ucl.ac.uk/spm/).

Avoidant attachment style was associated negatively ($P < 0.05$, FDR corrected) with cerebral MOR availability in amygdala, thalamus, insula, anterior, middle and posterior cingulate cortices, medial and lateral prefrontal cortices, orbitofrontal cortex and ventral striatum (**Figure 8**). No significant associations were observed between MOR availability and the anxiety dimension of attachment in any region. MOR availability or avoidance and anxiety scores did not differ across males and females (ts (47) < 1.67, $Ps > 0.11$) and were not correlated with each other (r = 0.08). Finally, neither depression nor anxiety scores were associated with MOR availability.

In the ROI-based analysis the results were consistent with the whole-brain analysis. Attachment avoidance (but not anxiety) was negatively associated with BP_{ND} in orbitofrontal cortex, amygdala, dorsal striatum and thalamus (Ps < 0.05 in one-tailed test). However, associations were not significant in insula, anterior cingulate cortex, ventral, and dorsal striatum (Ps > 0.05).

The results pinpoint the endogenous opioids underlying individual differences in tendency to seek and maintain social affairs.

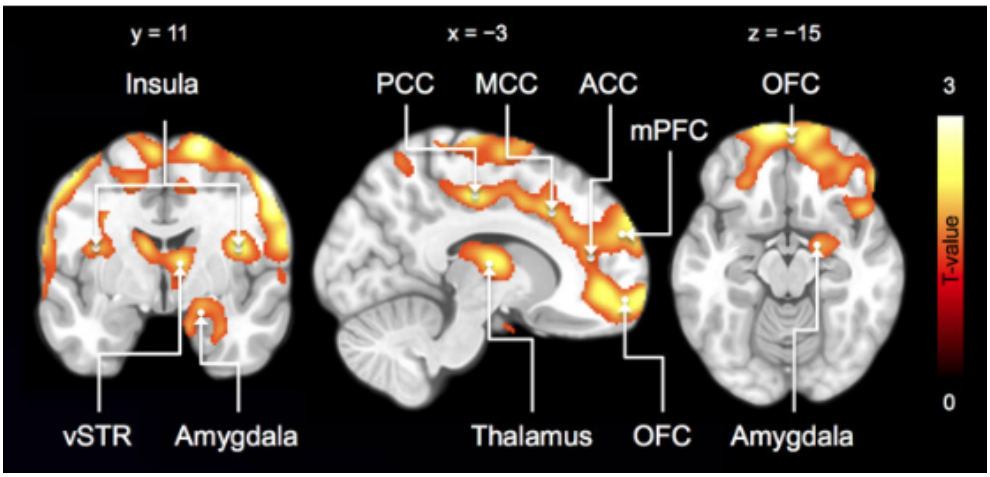

Figure 8. Statistically significant association between MOR availability and avoidant attachment style was found in several brain regions. ACC= anterior cingulate cortex, MCC= middle cingulate cortex, mPFC= medial prefrontal cortex, OFC= orbitofrontal cortex, PCC= Posterior cingulate cortex, vSTR= ventral striatum. Data are thresholded at P < 0.05, FDR corrected. Colourbar indicates the t statistic range (from the original publication no II).

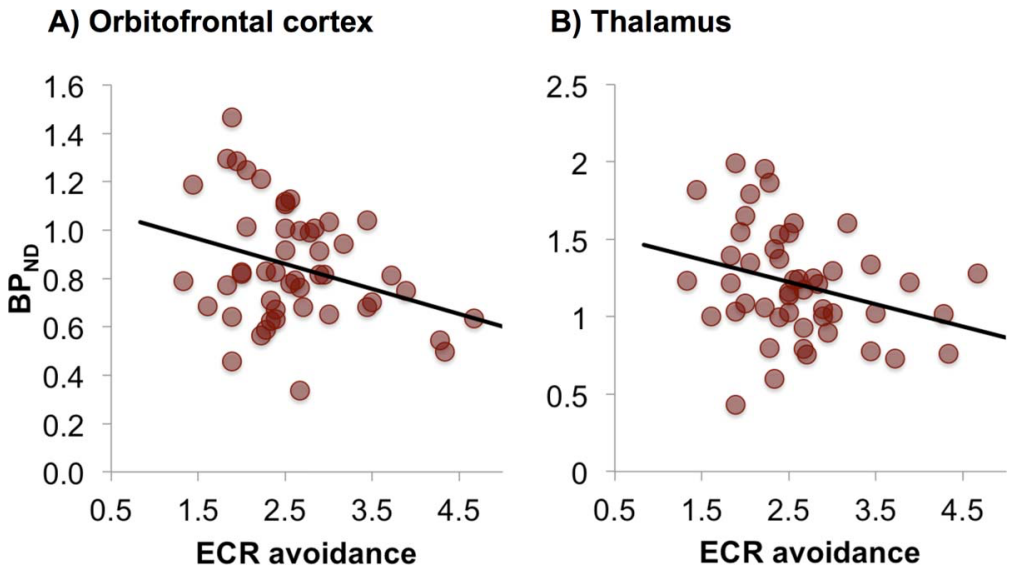

Figure 9. Scatterplots showing the negative correlation between MOR availability and avoidant attachment style in A) OFC and B) Thalamus (from original publication no II).

5.3 Study III: Mesoscopic variation in brain structure is associated with neuroreceptor availability: A combined PET and MRI study

It is not yet well understood how mesoscopic brain variations (in GM and WM densities) are linked with the receptor availability quantified with PET. Even that VBM allows quantification of gross atrophy on the basis of the T1 weighted MR images, it cannot specifically pinpoint the neuroreceptor systems involved in the detected tissue atrophy. Here, we tested whether regional brain GMD is linked with tracer binding using three differently distributed radioligands: [11C]carfentanil, [11C]MADAM and [11C]raclopride. The association between brain GMD and tracer binding was addressed retrospectively from 325 healthy adults, using the ratio of specific to non-displaceable binding (BP_{ND}) to reflect the tracer uptake in brain tissue and VBM-based indices of GMD. Tracer-wise BP_{ND} and brain GMD were correlated voxel-by-voxel method to reveal the associations, while controlling for age, sex and the scanners.

The results revealed a significant correlation with GMD and BP_{ND} in specific brain areas with all three tracers. GMD correlated positively with [11C]raclopride in caudate nucleus, putamen and ventral striatum. With [11C]MADAM and [11C]carfentanil, the significant positive correlations were seen in amygdala, caudate nucleus, thalamus and ventral striatum. With [11C]MADAM and [11C]carfentanil a weak negative correlation was shown in putamen and unsignificant negative correlation in OFC. Because ageing promotes cortical atrophy and neuroreceptor availability in normally aging brain, we ruled out the effect of age and sex by using partial correlations. The results remained essentially unchanged, showing that the association between cortical density and BP_{ND} does not simply reflect age and gender dependent atrophy or decline in receptor binding.

The present results show how neuroreceptor availabilities measured with PET are associated with MRI derived mesoscopic structural changes. These effects were also independent of age-related normal brain atrophy.

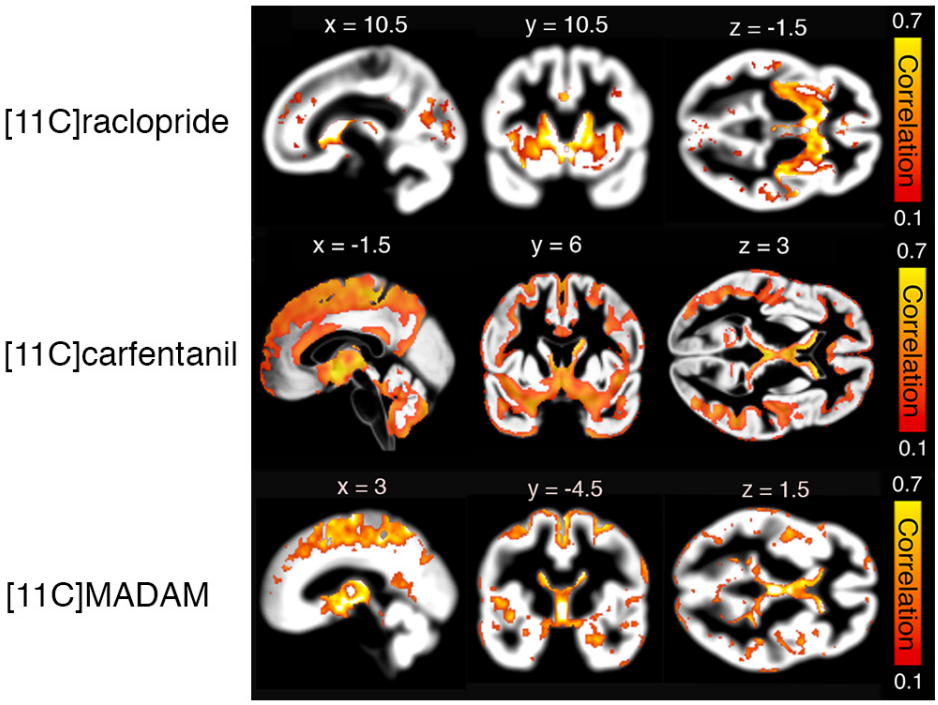

Figure 10. Voxel-vise BP_{ND}-GMD correlation maps, showing different spatial pattern of associations across all tested tracers. The data is thresholded at $p < 0.05$, FDR corrected. Colourbar indicates the correlation coefficient. From original manuscript no III.

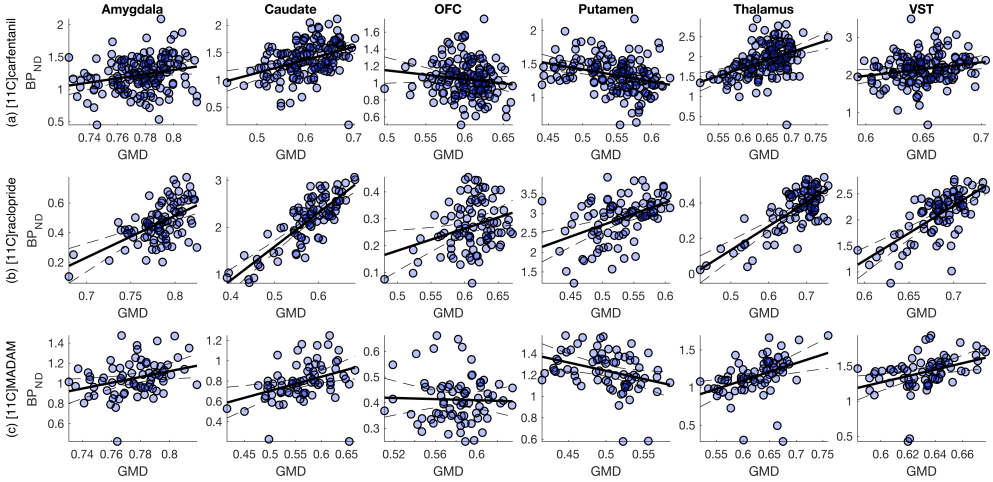

Figure 11. Scatterplots with regression line and its 95% CI for GMD and BP_{ND} in chosen ROIs with [11C]carfentanil (top), [11C]raclopride (middle) and [11C]MADAM (bottom). From original manuscript no III.

6 Discussion

We were for the first time able to quantify the neurobiological consequences of social laughter in human brain *in vivo*. According to the results, laughing with close friends does not only feel pleasant, but also triggers endorphin release in brain. This is in line with the prior evidence that social laughter increases pain threshold – a well-known proxy for endogenous opioid release (Dunbar et al. 2012). Study I suggests individual differences in MOR availability being associated with pro-sociality, indexed as a tendency to laugh in social group. Thus, laughter-induced endorphin release may function as *a social glue* that tightens the initial bond and keeps us wanting the social togetherness, due to rewarding molecular effect of pleasant social situation.

Study II showed that avoidantly attached individuals have lower amount MORs in brain areas crucial for a multitude of socioemotional functions (Vrtička & Vuilleumier 2012). People vary in tendency to seek social contact, and according to Study II, such variability can be partially explained in terms of MOR availability: the more opioid receptors individuals have, the more prone to pro-sociality they are. This is further supported by Study I, where a positive correlation was found between baseline MOR-availability and self-reported amount of laughter (during watching video-clips). This supports the earlier evidence about endogenous opioids being a crucial neurotransmitter in human socio-emotional functions (Nummenmaa & Tuominen 2018; Hsu et al. 2013) and other mammalian prosocial behavior (Panksepp et al. 1980; Panksepp et al. 1997).

The main findings from Studies I-II were that 1) social laughter enhanced endogenous opioidergic tonus in brain and 2) baseline MOR availability is a considerable neurobiological marker for sociability in humans. These results provide a neurobiological explanation for why people differ in social behavior: how they seek, create and maintain proximate relationships.

Finally, the multimodal analysis in Study III with VBM and PET revealed that cortical density (measured from MR images) is associated with neuroreceptor and transmitter availability (indexed with tracer BP_{ND}) in many of the regions involved also in social processing. Individual differences in GMD - not restricted to only age-related atrophy – was linked with tracer binding in subjects' brain with differently

distributed tracers. In comparison with previous evidence of GM density and radiotracer binding, we observed effects with higher statistical significance due to improved statistical power (Kraus et al. 2012; Woodward et al. 2010; Winkler et. al 2011). Altogether these results suggest that availability of these neuroreceptors and transmitters, as measured with PET, is reflected in the density of cerebral tissue as measured with MRI.

6.1 Laughter as a social glue

Social laughter triggered endorphin release in brain was paralleled with increases in subjective amusement ratings among study subjects, which is in line with the knowledge about comic-induced laughter activating also reward sites in human brain (Iwase, 2002). Laughter-induced endorphin release in CNS could act as a safety signal promoting maintenance of affiliative social bonds, as the importance of EOS in sociability is already well-proved. There was also an opposite effects of the group laughter in cortical midline regions (BP_{ND} was increased) (**Figure 7**). The interpretation of these results is not clear, even similar effects have been shown in previous works with [11C]carfetanil (Hsu et al. 2013). One rough supposition could be that the primary rewarding neural effects of endorphins are elicited in deeper parts of brain (hedonic 'hot spots' in mesolimbic area (Berridge & Kringelbach 2015). The exact cellular conformational changes behind this phenomenon cannot be determined within the used design.

People are more prone to co-operate and act altruistic towards those who share similar taste of humor (Curry & Dunbar 2013). Recent fMRI study also showed, that similar neural responses during cinema viewing predicts friendship (Parkinson et al. 2018). In Study I, we employed triads of friends in the laughter manipulation, because of the contagious nature of laughter (Provine 1992) and because it occurs significantly more likely in groups rather than alone (Andrus 1946; Levy 1979). Finally, as MOR availability was associated to the tendency to laugh during video exposure, that could offer an explanation for individual differences in sense or at least a neural *sensitivity* to humor (even if that was not directly tested).

Laughter-induced endorphin levels could complement group cohesion maintenance with touching or social grooming (Dunbar 2012). Primate studies suggest that laughter maintains social cohesiveness by signaling safety, relaxation and playfulness, and the absence of external threats (Van Hooff 1972; Preuschoft 2010). Laughing helps to improve conversational flow, synchronize emotional states, enhance social bonding (Vugt & Hardy 2010) and increase psychological closeness to others (Gray et al. 2015). In addition, laughing could strengthen the existing social bonds by i) improving the mood among laughers (Neuhoff & Schaefer 2002); ii) strengthening the group cohesiveness by alleviating stress (Vinton 1989); iii)

activating the rewarding sites in the brain when hearing others laugh (Bachorowski & Owren 2001) and iv) promoting the psychological well-being that in turn enables better co-operation and group function (Vugt & Hardy 2010).

Laughter has also other social functions. It can be used for displaying aggression or status over weaker conspecifics and negatively tuned "malicious" laughter may give an individual a feeling of social abandonment and exclusion (Gruner 1997). In addition to positive effects, laughing can also have less-known *negative* effects on somatic health; such as increased blood pressure, cardiac arrhythmias, aspiration of foreign obstacle and precipitate headaches, for instance (Ferner & Aronson 2013). Even though the relative frequency of these effects is not well known, it seems likely that the positive effects of laughter outweigh the negative ones.

Nevertheless, we suggest that laughing with proximate others is an important, non-linguistic communication mechanism that human share with other social primate species, and it is regulated through brain's endogenous opioid systems by eliciting endorphin release in brain reward and socio-emotional circuits.

6.2 Adult attachment style and opioid system

Study II revealed that insecure attachment trait, avoidance (but not anxious), is directly linked with specific neurotransmitter system alterations measured *in vivo*. The results indicate that persons who prefer being alone (lowered reliance to others) have decreased MOR levels and conversely, those seeking social contact have on average higher MOR levels. That is in line with the previous knowledge about EOS function in both positive and negative social experiences (Inagaki 2018) and reward circuits of brain during social interaction in humans (Vrtička et al. 2008).

Social losses and rejections are psychologically painful situations and might modulate opioidergic tone in CNS by decreasing MOR availability, as it is known that the rewarding effects of sociability are downshifted during periods of loneliness and separation from others (Panksepp et al. 1997; Niesink & Van Ree 1989). Endogenous opioids might be one neurobiological link to depression, as repeated and prolonged social losses and continuous social isolation may lead to profound alterations in MOR activation (Hsu et al. 2015). Insecurely attached individuals are more prone to psychological adjustment problems, substance abuse and psychopathologies (Brennan & Shaver 1995; Mikulincer & Shaver 2007). They also have more pain management difficulties compared to securely attached ones (Andersen 2012). Clinically unexplained pain syndromes are a major problem in public healthcare, and those patients are most typically frustrated with societal health services (Dow et al. 2012). There is also increased risk for self-treatment with exogenous opioids, alcohol and/or other drugs that manage the same neurobiological system as somatic pain. The present results may explain why

individuals react differently to social stressors: EOs might act as a buffer against negative emotions, thus individual differences in MOR availability may contribute to coping abilities.

It is not yet clear if individual differences in childhood or adult attachment styles are genetically determined or if they can change in adulthood or during the brain development, even though attachment style is fairly stable throughout lifespan (Fraley 2002). A twin-study with human adolescents showed stronger similarity among monozygotic twins' attachment styles compared to dizygotic ones (Fearon et al. 2014) advocating the hereditary ground of attachment styles. However, other behavioral genetic studies show conflicting effects of genetic vs environmental influence. Some of them even propose that *the shared attachment environment* would be more determinative for infant attachment formation than other environmental or genetic factors (Bokhorst et al. 2003).

Animal studies have showed variations in MOR coding gene, OPRM1, effecting social affiliative behavior in maternal bonding in mice and primates. OPRM1-gene knock-out mice showed permanent deficit in infant-maternal attachment (Moles et al. 2004). In Rhesus maqacues (*Macaca Mulatta*) variation in the OPRM1 allele C77G is associated with increased vocalization in separation distress and reward during reunion (Barr et al. 2008) in addition to unresponsive maternal caring (Higham et al. 2011). In humans, variation in the MOR- coding gene A11G predicts improved parent-child interactions (Copeland et al. 2011), suggesting a role of OPRM1 genes in the architecture of early attachment. A11G-polymorfism is also related to increased risk to develop alcohol dependence (Bart et al. 2005), which fits with the behavioral profile of insecure attachment. Yet, it is not clear how stable or unstable these effects are at the receptor level. Numerous environmental factors likely affect attachment formation along with genetic determination (Fraley 2002).

Altogether, Study I-II results show that i) social bonding is modulated by central opioidergic activation (Study I) and ii) baseline receptor level differences are associated with tendency to seek and maintain social affairs in human adults (Study II).

6.3 Brain mesoscopic variation is associated with availability of neuroreceptors and -transporters

Both adult and childhood attachment styles are associated with brain structure in socio-emotional brain regions in MRI studies (Acosta et al. 2018; Benetti et al. 2010). We wanted to test the link between MR indices of brain tissue composition and PET indices of neuroreceptor and transmitter availability. In Study III, brain GMD was associated with receptor BP_{ND} with all used tracers: [11C]carfentanil, [11C]raclopride and [11C]MADAM. The associations paralleled the well-known

binding sites of each tracers. For example, with [11C]raclopride effects were not seen in extra-striatal regions according to the plausible dopamine receptor-distribution in brain (Egerton et al. 2013). In general, associations were observed in regions with receptor and transporter binding for radioligands. Yet, the effect was not restricted to only high-binding areas with employed tracers (for example brainstem for [11C]MADAM). Results were also independent from subjects' age and sex.

Brain gray matter contains most of the neuropils and synapses, where most of the neurotransmission happens (Purves 2018). Post-mortem studies show that neuronal integrity does not correlate with dopamine transporter availability in Parkinson patients (Saari et al. 2017). This suggests that not necessarily the raw number of neurons, but merely the amount of active neuroreceptors or -transporters in synaptic space could predict the MRI derived density estimates in each area. Because of the low specificity at the molecular level of T1 weighted MRI, it is hard to tell in general if the GM signal is from neuron- or receptor-rich area. Study III shows that by using VBM, significant amount of the MR signal can be explained by the neuroreceptor availability in numerous brain areas. Individual differences in mesoscopic brain architecture thus can predict the density of locally available neuroreceptors.

In many neurogenerative disorders (as Alzheimer's and Parkinson's disease), disruptions in specific neuro-molecular systems affect brain function in tandem with mild or progressive brain atrophy (Heiss & Herholz 2006; Terry et al. 1991; Tuite et al. 2013). Also in normal aging brain tissue shrinks and may cause behavioral changes and amnesia (Buschke et al. 2017; Gunning-Dixon et al. 2010), and modulates receptor binding in different neurotransmitter systems (Rinne et al. 1993; van Dyck et al. 2000; J. K. Zubieta et al. 1999). Study III results suggest that brain tissue degeneration in normal aging cannot however explain alterations in neuroreceptor or -transporter availability in healthy adults. Instead, other individual differences in brain mesoscopic structure may be coupled with the receptor/transporter availability in certain areas.

From a methodological viewpoint this is an important finding, as comparative tracer-binding studies are common in neuroscientific studies addressing neurotransmitter systems behind the psychopathologies (for example, serotonin system in major depression: Gryglewski et al. 2014). Thus, variations in individual mesoscopic brain structure should be determined first to allow reliable estimation of tracer binding and comparison between two separate groups.

6.4 Limitations of the studies

In study I, we assumed, that the changes in [11C]carfentanil BP_{ND} between two separate scans with same subject would reflect the amount of endogenous opioid release in brain. That is a common presumption in neurotransmission system studies, as the more precise knowledge of the molecular functioning is yet unclear. Increased or decreased radioligand occupancy may however reflect either the i) raw number of available receptors ii) receptor affinity or iii) receptor internalisation in the neuron cell surface (Henriksen & Willoch 2008). It is impossible to rule out these detailed alternatives in a single study. There was seen also decreased opioidergic activity (BP_{ND} was increased) in cortical midline regions after laughter exposure. The interpretation of this is not clear, although similar effect has been shown in earlier studies with [11]carfentanil (Hsu et al. 2013). Anyway, it is hard to state, if the increased BP_{ND} in those areas results from the alterations in endogenous opioid tonus or above mentioned cellular factors.

Here, we used video-derived humor to evoke laughter in subjects. Humor is very strongly bound with the cultural background (Apte 1985) and may differ across countries, due to workplaces and in different social alliances. We did not specify the *type* of humor of the study subjects before they participated in the study. Hence, there might have been a lack of suitable humor for some of the group members, even that participants were able to choose videos themselves from previously collected video list. To control for this, we recorded the frequency of laughter bursts in the experiment and confirmed that there were numerous laughter bursts in every group. The objective laughter recordings corresponded with subjects' self-reports of laughter and amusement. We measured the endorphin release *in vivo* soon after laughter-episodes. Therefore, this study tells only about the acute neural effects of social laughter, and does not reveal the long-term consequences of (frequent) social laughter. Finally, our baseline condition consisted of subjects spending time alone in a laboratory room without a phone or active contact to other people. Thus, it is possible that the presently observed results may be partially driven by mere presence of other people in the laughter condition, as this factor also differed across the scans.

Another limitation in Study I is that formal power analyses were not performed because comparable studies do not simply exist. Accordingly, sample size was decided based on prior challenge studies using [11C]carfentanil (e.g. Karlsson et al. 2016; Nummenmaa et al. 2016; J.-K. Zubieta et al. 1999). We scanned healthy young men, so the results can't be extended directly to other age groups or sexes, even though the additional task in Study I showed no difference in pain tolerance change between males and females.

Study II was a cross-sectional examination with healthy adults. It is well known, that childhood attachment style stays stable from infancy to early adulthood (Fraley 2002). Accordingly, our data might reflect the attachment style formed in the

childhood, but this cannot be resolved from the present data. Our data cannot tell if the results reflects genetically determined effects of the MOR expression in attachment formation - or whether the individual differences in MOR availability are a consequence of differential attachment behavior patterns (learned and transacted during life). Longitudinal studies are needed to assess the causal relationship between MOR availability and attachment formation.

In Study III we analyzed retrospective data. Thus, PET scanners varied between the subject groups, potentially affecting the binding measures. Calibrated PET scanners with using BP_{ND} as a binding measure should together yield consistent data in that case (Johansson et al. 2013; Gunn et al. 1997). Furthermore, using PET scanner as a covariate in the statistical analysis did not alter the overall pattern of results. Also, different subjects were scanned with different tracers, which precludes direct comparative analysis between the tracers. Our main outcome measure was the GM density as indexed by VBM, as this allows voxel-wise analysis. We did not use the Freesurfer meshes in the analyses, as the PET data were preprocessed and analyzed as volumes (rather than surfaces). Finally, this kind of cross-sectional study cannot reveal whether the association between neurotransmitter activity and GMD is altered during the life course. Longitudinal multi-ligand studies would be needed to clarify this, yet radiation exposure from multiple injections essentially prohibits them in humans, thus primate or rodent imaging could be used for resolving the issue.

Because our main focus was on full-volume analysis, we did not control for partial volume effects. This might influence the results, but it must be stressed that the ROI-level and full-volume analyses yielded comparable results, even some differences were also found. These most likely result from the statistical thresholding of the ROI-level versus voxel-level data, as well as noise levels in single-voxel versus ROI-level estimates of binding potential. Nevertheless, the partial volume effect might also contribute to the effects.

6.5 Implications in psychiatry

How could the discovery of laughter-induced opioid release in brain help in psychiatric field in general? In addition to diagnostic systems in psychiatry (DSM-IV or V), for example brain MRI can determine certain outcomes in severe mental illnesses in group level (i.e.: Sankar et al. 2016). Lack of pleasure, increased anhedonia, anxiety and neuroticism are typical symptoms of major depressive disorder (MDD) (AllenLiao et al. 2018). From a biological point of view, affective disorders are linked with disrupted function of the neurotransmitter systems. For example, weakened endogenous opioid activity has been found in MDD (Hsu et al. 2015). Lowered tendency to laugh to humorous events is recognized to be

characteristic for some severe conditions in psychiatric patients (Martin & Lefcourt 1983): laughter structure analysis has even suggested to have a diagnostic potency in the onset and evolution of depression (Navarro et al. 2014).

Laughing emerges very early in infancy, and it is a crucial communicative element for childhood social, cognitive and affective development of intimate, dyadic relationship with early caregivers (Reddy 2001; Mireault et al. 2015). Laughter has also been studied as a coping tool in psychotherapy: laughing helps patients to cope with painful life events and is thought to deepen the relationship between patient and a therapist, which may add to the efficacy of the therapeutic work (Dziegielewski et al. 2003). One possible pathway for this effect is thought to be the laughter-evoked endorphin release and its anxiety-alleviating properties (Dunbar et al. 2012). An individual's capacity to recognize and express their feelings to conspecific and other species is an important skill in nature (Darwin 1956). Those skills are impaired or flattened in major psychiatric disorders such as schizophrenia or in MDD (Kohler et al. 2008; Rottenberg et al. 2002). Flattened emotional expressions gives important information about the overall condition of the patient. Laughing during an interview - adequate or inadequate, emotional versus 'cold' – can also provide clues to the patient's present mental status. Furthermore, the type and amount of laughing during the therapeutic sessions can index the severity of patient's disorder and their attachment style (Gupta et al. 2018). Increased amount of shared humor during the session can tell the physician/therapist about the psychic recovery of the patient (Holmes 2017).

Laughing and continuous humor creation can also be psychologically defensive behavior (Freud 1960), potentially acting as a protective element for a person going through bereavement or depression (Keltner & Bonanno 1997). Laughing and tendency to humorous thinking provides psychological distance from difficult issues and hence, helps the individual see their situation more objectively. Laughing and sense of humor also objectively release stress (Martin & Lefcourt 1983; Martin 2004). For their own study (1983) Martin and colleagues validated two instruments for measuring subjects' sense of humor and their reactivity to humorous cues : The Coping Humor Scale (CHS) and a Situational Humor Response Questionnaire (SHRQ). These allow objective structural assessment of individual differences in laughter and humor creation. CHS and SHRQ measure subject's tendency to use humor in stressful or surprising life-situations. Those who scored high on either of these questionnaires experienced less mood disturbances after negative life events (Martin & Lefcourt 1983). A questionnaire about subject's sense of humor (Sense of Humor Questionnaire (SHQ) can also be used as a measurement tool in research purposes (Svebak 1974). Here, laughing with significant others altered the brain neurobiological mechanisms (MOR system) that are in charge of hedonic processes.

The theory of brain opioids and social attachment suggests that disruption of the endogenous opioid system could explain why insecurely attached people are more prone to succumb to mental health problems, alcoholism or drug abuse (Brennan & Shaver 1995; Mikulincer & Shaver 2007). In behavioral addictions such as pathological gambling, a dysregulation of endogenous opioid system has been suggested (Mick et al. 2016; Majuri et al. 2016) as well as in alcoholism (Gianoulakis 1996). For example in Gianoulakis' study, an increased release of β-endorphin followed the exposure to alcohol in ethanol-preferring animals, but it also correlated with higher density of MOR availability in NA and VTA (key regions in reward). Conversely, Study II showed lowered MOR availability in subjects with avoidant attachment style. That is not directly parallel with aforementioned alcohol-study, but may provide a biological explanation why differences in attachment styles are manifested in patterns of drug dependency (Schindler et al. 2005). Also, it could underlie the appearance of insecurely attached adults experiencing opiates as particularly pleasant over other drugs (e.g. stimulants or cannabis) (Insel 2003; Schindler et al. 2009).

Early insecure attachment between mother and child is related to mental health problems later in life, including somatization and depressive and anxious symptoms (Egle et al. 2002). An effective psychotherapeutic treatment can evoke functional and receptor-level changes in patients' brains towards more secure attachment profile (Cervenka et al. 2012; Linden 2006). A reciprocal, warm humor between a client and a therapist is a key index of progress in psychotherapy (Malina 2006), while avoidant attachment style is linked with self-disruptive (antisocial) laughter during therapeutic sessions (Gupta et al. 2018). Because i) opioidergic tone is altered during laughter bursts and ii) MOR-availability predicts avoidant attachment style it is possible that psychotherapy could modulate both attachment style and MOR levels. A similar study has already been done for serotonin system: psychotherapeutic treatment increased tracer binding to 5-HT1a-receptors more compared to medication treatment (fluoxetin) in frontal, temporal and parietal cortices (Karlsson et al. 2009). Thus, it would be interesting to examine similar contributions of psychotherapeutic treatment to brain opioidergic system.

Study III stood for the methodological part of this thesis, and the results pinpointed a subject-wise correlation between brain gray matter density (GMD) and neuroreceptor availability (BP_{ND}) with three radioligands that are widely used for measuring opioid-, dopamine- and serotonin systems. There is only limited previous data existing about the relation of brain macrostructure and neurotransmitter uptake (Kraus et al. 2012; Woodward et al. 2010), and our findings had a strong statistical power with over 300 subjects. As a subjects' age is known to affect both brain tissue atrophy and receptor level changes (Resnick et al. 2003; J.-K. Zubieta et al. 1999), it is notable, that not age or sex, but other individual differences in brain

macrostructure correlated with receptor density in brain gray matter area. As these two measures (BP_{ND}, GMD) were observed to give complementary information about brain integrity, that should be taken into consideration in future PET and MRI analysis in future studies in neuroscience and psychiatry. In patient studies, this could provide more reliable information about neurotransmitter system alterations when addressing the baseline receptor/transporter availability in patients with mental illnesses, such as major depression or anxiety, for instance.

7 Conclusions

The main conclusions of this thesis are:

I) Endorphin release during social laughter is a candidate neurobiological mechanism promoting affection and affiliative behavior towards others.
II) The association between MOR availability and avoidant adult attachment style proposes that individual differences in sociability is based on endogenous opioid system activity.
III) Due to the observed linkage between brain gray matter density (GMD) and neuroreceptor/-transporter binding (BP_{ND}), brain morphology should be taken into account in comparative, multimodal imaging studies focusing on tracer binding.

As humans, we should not be arrogant towards the fact that we share similar neurobiological mechanisms with other social mammals when it comes to vital acts such as love and grief. Defining the neurobiological underpinnings behind affective behavior might lead us towards more inclusive understanding of social behavior, psychopathologies and emotions. The presented thesis suggests that the endogenous opioid system plays a key role in maintenance and establishment of human social and affective bonds.

Acknowledgements

The studies presented in this thesis were conducted in the Department of Psychiatry and Turku PET Centre at the University of Turku during the years 2014–2019. I would like to thank the Director of the Turku PET Centre, professor Juhani Knuuti, for enabling the use of a state-of-the-art PET scanner and other facilities in Turku PET Centre for these studies. Also, I want to thank professor Juha Rinne, the Manager of Cerebral scans in Turku PET Centre, who enabled the scanning-procedures in practice. A large number of people in Turku PET Centre have helped me with this thesis and without their significant contribution the work would not have been possible to carry out.

These studies were financially supported by Alfred Kordelin Foundation, The Finnish Medical Society Duodecim, the Turku University Foundation (Kosti Hämmärö Fund), the Hospital District of Southwest Finland (VTR) and the Academy of Finland. I want to thank all of these financiers, who trusted on my research and enabled the full-time working alongside with the clinical working periods.

I want to express my appreciation and gratitude to my supervisors, professor Lauri Nummenmaa and professor Jarmo Hietala: I have been very lucky to have supervisors like you. I want to thank Lauri for believing on me from the day one until the end of this process. I may have not been the easiest student with my tendency to panic, when everything is not going smoothly. Under your wise and original guidance, I have had an opportunity to learn about the basics of neuroimaging, data analysis, statistics and scientific writing – and about neuroscientific field in general. I admire your capability to conduct your research group with such enthusiasm (not to mention your marvelous sense of humor)! I also want to thank Jarmo, for your patience and wise comments on my thesis and articles during the writing phase. You have a vast knowledge of psychiatry and especially of adult attachment, which helped me a lot during this process. Also, I would like to thank professor Hasse Karlsson for being a member of the monitoring committee and encouraging me to push forward.

I would like to acknowledge the contributions of all the co-authors: especially M.D. PhD Lauri Tuominen, who introduced me to the basics of PET analysis methods when I started 4–5 years ago, and after that contributed in all published

articles and manuscripts in this thesis with his reasonable view. Also PhD Tomi Karjalainen needs to be mentioned, due to his wisdom in data analysis and his patient way to discuss about the possible meaning of the given results. Docent Jussi Hirvonen served as a research radiologist in a vast amount of PET scans and also gave his scientific knowledge into the articles. I want to thank all the other co-writers of the articles: Juha Rinne, Riitta Hari, Mikko Sams, Robin I. Dunbar, Valtteri Kaasinen, Juho Joutsa, Kari K. Kalliokoski, Iiro P. Jääskeläinen, Eveliina Arponen, Pirjo Nuutila – your contributions were essential for this thesis.

The people at the Turku PET Centre have been extremely helpful during the whole project, and this work would not have been possible without the help of scientifically minded radiographers and laboratory technicians such as Minna Aatsinki, Anne-Mari Jokinen, Tarja Keskitalo and Eija Salo, and many many others. I am extremely grateful for everyone who helped me with the studies in practice. Rami Mikkola and Marko Tättäläinen are acknowledged for their help in the IT-issues. During these years, all of the bureaucracy was easier to accomplish with the helpful secretaries Lenita Saloranta and Mirja Jyrkinen at Turku PET Center, and the Chief Academic Officer Outi Irjala in the Faculty of Medicine.

I have had a great and relaxing time on social events and during lunch breaks with all 'PET boys and girls', who have also helped me during the process. Special thanks for Kerttu Seppälä, for your encouraging spirit and our endless conversations about life and science; Tiina Saanijoki, for sharing the workstation in 'Mordor' (PET analyysitila) during the last 1-2 years and for helping me to cope with occasional stress (by laughing); Janne Isojärvi, for collecting information from the abyss of different databases whenever I dare to ask (quite often); Jouni Tuisku, for reading and commenting the 'PET methods'- chapter. Also, I am thankful for the unique team-spirit and all other members of the *Emotion-lab* - Mordor rules! (Extra-thanks goes to the lab-idol, Yngwie - because as we all know, *More is more).*

I am very grateful to my fellow brain researchers and colleagues for mind-expanding talks about psychiatry, science and life in general. Thank you M.D. Reetta-liina Armio, for the unique and important friendship that started in Medical school and continued ever since; M.D. PhD Anna Lautamäki and M.D. Elina Sormunen, for sharing the PhD-process and having good times in Medical school and also in other areas of life. M.D. PhD Jetro Tuulari and M.D. PhD Henry Karlsson - your company has always been delightful during these years!

I want to thank the official reviewers of this thesis, Simon Cervenka and Leonhard Schilbach, for their constructive criticism and helpful comments on the thesis manuscript.

Also I am thankful for all of my friends and beloved ones, for the support, relaxing moments, jokes and love. I am lucky to have people like you in my life!

Above all, I want to express my deepest gratitude to my family. I am lucky to have parents like Pasi and Hanna Manninen (and grandparents like Anssi and Ulla-Maija Kuusela and Sinikka Manninen); your endless love and encouragement on my endeavors in all area of life has guided me to reach beyond my limits. I am thankful to have a sister like you, Adalmiina (Ammi), and brother like you, Carolus. With both of you I will always share the connection that no one else can understand. Thank you also Mikko, for being a real support wherever I go or whatever I decide to accomplish. I am happy and proud that you all stand next to me today.

Finally, Dear Med, thank you for your understanding and unconditional support and love, even it was not easy all the time. You make my heart laugh every day, I love you.

Turku, September 2019
Sandra Manninen

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ISBN 978-951-29-7772-7 (PRINT)
ISBN 978-951-29-7773-4 (PDF)
ISSN 0355-9483 (PRINT) ISSN 2343-3213 (ONLINE)

Painosalama Oy, Turku , Finland 2019