Opiate toxicity and the respiratory control centre

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1. Abstract

Opiates have for a long time played an important role in the treatment of severe and chronic pain, but they are also historically associated with drug abuse. Addiction and side effects, the most serious one: respiratory depression, can lead to death which limits the applications, intensity and extent of opiate use. A significant problem in opioid poisoning is that those who suffer do not represent a homogeneous group of individuals, i.e. they are not only opioid addicts. The group is heterogeneous and the concentration of opioid in the blood can vary from remarkably low levels to levels, which could be considered normal. The concentration of metabolites in the bloodstream after a heroin overdose can vary 100 fold between individuals. It is believed that the pattern of tolerance for the different effects of opiates; respiratory depression, analgesia and euphoria, does not have a common progression and/or reversing course. The tolerance mechanism of respiratory depression is not yet fully understood, and in this project a small piece of this large puzzle will be examined. The aim of the present was to examine if pharmacological and/or morphological alterations in the respiratory centre due to opiate poisoning can assist in the forensic investigation of suspected opiate caused deaths.

1.1. Summering

2. Introduction

2.1 Opiate origin, toxicity and tolerance

The Opium Poppy (*Papaver somniferum*) is the original source of opium (Janick, 1997; Samuelsson and Apotekarsocieteten, 2004). Opium remains a widely used natural product in medicine, but it has also been used throughout history in other more spiritual contexts (Brownstein, 1993; Corbett et al., 2006; Janick, 1997; Samuelsson and Apotekarsocieteten, 2004). It is one of the first domesticated plants and the cultivation dates back to hundreds of years BC (Janick, 1997). The reason for its long history in the service for mankind is the alkaloids harvested from the poppies seed pods (Brownstein, 1993; Janick, 1997; Samuelsson and Apotekarsocieteten, 2004). *Papaver somniferum* contains several biologically active alkaloid compounds where two of the major compounds are morphine (with a strong analgesic and sedative effect) and codeine (with a antitussive and a more moderate analgesic effect) (Janick, 1997; Samuelsson and Apotekarsocieteten, 2004).

The exact evolution and the purpose of the alkaloid content are not fully understood, but the general recognized idea is that it has given the plant a survival advantage (Samuelsson and Apotekarsocieteten, 2004). The production of alkaloids has protected the plants from being digested because of the bitter taste and potent effects (Janick, 1997). The opium poppy produces morphine by conversion of tyrosine in a nine-step biosynthetic pathway to S-reticuline (Hirata et al., 2004; Page, 2005) and the final biosynthesis of morphine takes place by an additional seven steps, see Figure 1 (Hirata et al., 2004; Page, 2005).

Opiates, just like the endogenous opioid peptides, such as enkephalins, endorphins, and dynorphins, (Harrison et al., 1998) bind to the same targets. They act as agonists on a group of G protein-coupled receptors (GPCRs), named μ-, κ- and δ-opioid receptors which are widely distributed in the brain, spinal cord and intestines of humans and other mammals (Harrison et al., 1998). Opiates have several biochemical effects and are recognized for their strong analgesic and sedative effects, which are desirable in medical use, but can also produce adverse effects such as respiratory depression, and euphoria (Mohammed et al., 2013; Seldén et al., 2012).
Morphine and other opiates and opioids are widely used as analgesic drugs. However, most of them are also abused because of their sedative and euphoric effects (Contet et al. 2004). Opiates, with heroin and morphine as front figures, constitute the main group of abused drugs for 1.4 millions Europeans according to EMCDDAs annual report 2013 (EMCDDA, 2013). Although the amount of opiates seized by custom and police services have declined in recent years, the opiates still hold a predominant position over other abused drugs and are found in 80% of drug related deaths.

The toxic effect of morphine and heroin is generally considered to be mediated by the binding and activation of \( \mu \)-receptors located on interneurons in the respiratory control center in medulla oblongata. When the receptors are highly stimulated (i.e. after an overdose of opiates) they lower the respiration rate, which might result in respiratory arrest and death (Manzke et al., 2003). The difference between an intoxicating dose and a lethal dose varies widely between individuals and the difference can be minimal. Autopsy findings and analyses

**Figure 1.** The biosynthetic pathway of the production of morphine. The addition of two acetyl groups to morphine results in the synthetic and abused drug heroin.
show that morphine concentrations in the blood can vary 100-fold between individuals (Druid et al., 2007).

Tolerance is a major problem that addicts and patients are afflicted by when using opiates (Harrison et al., 1998; Trujillo, 2002). Tolerance is a phenomenon which describes when an individual over time becomes insensitive and no longer experiences the desired effect of a, compared to when the drug was given the first time. To overcome the tolerance problem and to maintain the desired effect, a continuous increase of the dose is necessary resulting in concentrations that are well above lethal levels for a naïve person (a person that never been exposed to opiates) (Nyberg and Hallberg, 2012). The complete mechanism of the development of opiate tolerance is not fully characterized but the current understanding is that chronic opioid use leads to cellular adaptations through mechanisms like desensitization by phosphorylation, internalization and down-regulation of receptors (Harrison et al., 1998; Koch and Höllt, 2008; Trujillo, 2002). These cellular changes are important in the development of addiction. When opiates are no longer administrated the signaling system becomes more sensitive to stimulation by the endogenous compounds (Darke, 2013), which leads to physiological reactions such as pain, akathisia and tachycardia. Furthermore, psychological symptoms such as paranoia, panic attacks and extreme craving for the drug are common (Darke, 2013). These symptoms and many others are a part of the abstinence and withdrawal syndrome, a highly unpleasant state, although rarely fatal (Chen et al., 2011; Lobmaier et al., 2010). Fortunately, by time the symptoms are relieved by fractional tolerance regression (Darke, 2013; Mohammed et al., 2013).

The tolerance to opiate-induced analgesia and euphoria seems to follow similar courses, but on the other hand the opiate-induced tolerance to respiratory depression seems to develop slower and to a lesser extent (Mohammed et al., 2013). This means that patients with chronic pain and heavy drug abusers are continuously at risk of experiencing respiratory depression due to the use of high doses of morphine and other opiates.
2.2 Respiration and Pre-Bötzingers complex

The exchange between carbon dioxide (CO\textsubscript{2}) and oxygen (O\textsubscript{2}) is the most important function of the respiratory system (Alheid and McCrimmon, 2008; Del Negro et al., 2010). The respiratory movement is generated by contraction of the diaphragm, which generates a decreased pressure in the thorax, creating an airflow pulling down fresh air in to the lungs and a relaxation of the diaphragm forces air out of the lungs (Del Negro et al., 2010). The respiratory system ensures the release of carbonic acid (H\textsubscript{2}CO\textsubscript{3}) from the bloodstream and by that keeping a constant pH (Alheid and McCrimmon, 2008).

Fluctuations in blood pH are not well tolerated and there are several control areas within the body that are very sensitive to changes in pH as well as changes in oxygen pressure (pO\textsubscript{2}). These stimulate the respiratory system to increase the rate and volume of respiration during a drop of pH (Alheid and McCrimmon, 2008; Del Negro et al., 2010). Carotid bodies in the carotid arteries react to a lowered pO\textsubscript{2} or increased CO\textsubscript{2} pressure, and the activity of the nerve cells in medulla oblongata are subsequently affected (Alheid and McCrimmon, 2008; Del Negro et al., 2010).

The medulla oblongata harbors several important centers like the respiration, vasomotor, cardiac and vomiting systems (Rekling and Feldman, 1998).

The respiratory control centers are located in the lower part of the brain stem (pons) and the upper part of the medulla oblongata (the latter consisting of the ventral and posterior respiratory groups). Subpopulations of respiratory neurons innervate inspiratory pre-motorneurons in the medulla, which in turn project to the motorneurons in the cervical nerves C3-C5 building up the phrenic nerve that stimulates the diaphragm to contract. The most important nucleus implicated in active inspiration is the pre-Bötzinger complex in the rostral part of the ventral respiratory group. Experimental silencing of a somatostatin-expressing subpopulation of pre-Bötzinger neurons will cause severe respiratory depression or even apnea and death. Using enhanced green fluorescent protein (eGFP), the projections of these neurons have recently been determined in the rat. They send projections to several other nuclei within the medulla oblongata and pons including the contralateral pre-Bötzinger complex, the Bötzinger complex, the parafacial respiratory group/retzotrapezoid nucleus and the parabrachial/Kölliker-Fuse nuclei (Tan et al., 2010). These inspiratory interneurons in pre-Bötzinger complex express gamma-aminobutyric acid (GABA), neurokinin-1, adenosine, glutamate and serotonin receptors. Under normal conditions, glutamate is the predominant
stimulatory compound, whereas glycine produces inhibition of the signaling. Having said that, a subpopulation of the pre-Bötzinger neurons as well as neurons within the parafacial respiratory group/retzotrapezoid nucleus are pacemaker neurons, which will produce a regularly bursting, rhythmogenesis, that is essential for involuntary respiration (e.g. during sleep). Exactly how these groups of cells interact is however not yet fully elucidated, and the normal modulation of the activity of these neurons may actually in part also be conferred by pH/CO$_2$-sensitive astrocytes co-located with groups of inspiratory interneurons.

The human counterpart to the pre-Bötzinger complex was recently identified in a discrete region of the ventrolateral medulla oblongata (Schwarzacher et al., 2011). This region contains a similar distribution of different neurons, some of which certain co-express neurokinin-1 and somatostatin, just like the pattern observed in all previously studied mammals. It is believed that most of the connections with other respiratory regulating nuclei also are similar, but this is still not fully explored in the human brain.

2.3 Opiates and respiration

It is well recognized that most of the severe, or even fatal, intoxications by opiates are mediated by respiratory depression (White et al., 1997). Despite significant advantages in opiate treatment, there are a number of potential adverse effects of which respiratory depression remains the most problematic. Cases of life-threatening respiratory depression were reported soon after clinical administration of neuraxial morphine (Sultan et al., 2011). The most opioid sensitive aspect of respiration is rhythm generation, and changes in the respiratory pattern are observed at lower opioid doses than change in tidal volume. Higher opioid doses cause reduction in tidal volume probably due to decreased tonic inputs from opioid sensitive chemoreceptors, which in vivo are partly compensated by increases in carbon dioxide pressure PaCO$_2$ (Pattinson, 2008). Most researchers in the field favor the idea that this respiratory depression is due to an overactivation of µ-opioid receptors on the inspiratory interneurons in the pre-Bötzinger complex (Taylor and Fleming, 2001). Experimentally and in the clinical setting, opiate intoxication will cause apnea or severe depression of respiration, which typically can be counteracted by naloxone, a µ-opioid receptor inverse agonist (antagonist), whereas the circulation can be fairly well maintained (Clarke et al., 2005). The µ-opioid receptor is expressed by most of the neurons in the pre-Bötzinger complex, and most likely respiratory depression by opiates is mediated by activation of these receptors, resulting in a reduced stimulation of the inspiratory pre-motor and motor neurons that causes the diaphragm to contract. It was recently shown that the respiratory depressant effect by opiates
is specifically limited to the activation of \( \mu \)-opioid receptors on the pre-Bötzinger neurons that express the neurokinin-1 receptor (Montadon, 2009). Interestingly, (Manzke et al., 2003) reported that the \( \mu \)-opioid receptor on the pre-Bötzinger neurons are co-localized with serotonin 5-HT(a) receptors, and that a selective 5-HT4(a) agonist, BIMU8 could reverse respiratory depression induced by the \( \mu \)-opioid receptor agonist fentanyl. It could therefore be expected that psychostimulants, such as amphetamines that cause increased serotonin levels in the brain, might counteract opiate toxicity. However, there are no animal data or clinical data to support this notion (White & Irvine, 1999).

About 100 deaths by heroin toxicity are recorded annually in Sweden and morphine, methadone, buprenorphine and other opioids cause approximately an additional 100 deaths. The diagnosis of fatal opioid overdose is not always straightforward. There are no morphological findings that are specific, even if froth in the airways and massive pulmonary edema in an individual with no history of cardiovascular disease can be a hint. Further, heroin is rapidly broken down to 6-acetylmorphine, and then to morphine, so in some deaths that are delayed, heroin and 6-acetylmorphine may not any longer be detectable in blood. In addition, the morphine levels seen in heroin overdose death vary tremendously, and overlap those seen in living subjects (Jones FSI, 2012). The same is true for other opioids and the explanation is most likely a large difference in tolerance to opiates. Unfortunately, there is no biomarker for opiate tolerance. This means that the interpretation of the autopsy findings and toxicology results in deaths with presence of opioids remains difficult, and as a consequence, the death statistics, including trends, are affected. It should be noted that numbers of acute opiate deaths constitute an often-used means of estimating the drug abuse situation, impacting on health care activities and political decisions.

In the absence of a biomarker of opiate tolerance, different substitutes are used. Hence, to estimate the tolerance, information about previous and recent use of opiates from interviews with relatives, police investigation and/or medical records are often used, when possible. (Merrall et al., 2010) reported that the deaths increased three to eight-fold within the first two weeks after imprisonment compared to longer intervals. Also, hair analysis can provide information about previous opiate exposure. Both (Tagliaro et al., 1998) and Darke et al., 2002 found that the concentrations of morphine in hair samples from heroin overdose deaths were lower than those in hair samples from living street users. However, sometimes drug abusers present with shaved hair, and information about previous opiate exposure from other sources might not be available.
Other means to estimate opiate tolerance are based on our current knowledge of the underlying mechanisms. These are presently not well elucidated and are mostly based on studies on the changes seen in receptors mediating tolerance to pain, which is the predominant reason for opioid treatment. A general appreciation is that acute tolerance is mediated by internalization of the µ-opioid receptor or uncoupling of this receptor complex from the downstream signaling system (Zhang et al., 1998; Zhang et al., 1999). Secondary effects of transcription factors have also been suggested to account for acute tolerance (Williams et al., 2001). Further, pharmacokinetic adaptations may also be involved such as increased metabolism to less potent metabolites and/or different distribution by the action of p-glycoprotein (p-gp), which both may affect the absorption of opioids from the bowel and the transport of opioids to and from the CNS (Oude Elferink and Zadina, 2001). Drug metabolism may also impact on the effects of opiate exposures in naïve and chronic users (Taylor and Fleming, 2001).

Opiate tolerance is a problem in the treatment of pain, but also for opiate abusers, who might misjudge their dosing and administer a dose that the body is not prepared to cope with. Unknown tolerance is further a problem for forensic pathologists in the determination of the opioid concentrations found in a death represent an intoxication or if the detected levels of opiates are an incidental finding. The present study was undertaken to find out if pharmacological and/or morphological changes in the respiratory control centers in the medulla oblongata of fatal opiate intoxications can be used to assist in the diagnosis of opiate overdose death.

Assuming that overstimulation of µ-opioid receptors on inspiratory neurons in the pre-Bötzinger complex mediates respiratory depression, the [35S]GTPγS-assay was applied on fresh frozen medulla oblongata sections from rats dying of opiate overdose, and control rats, and also on sections from human opiate overdose cases and non-opiate users dying of other causes. This autoradiographic assay can show the density of activated G protein-coupled receptors (GPCR) (Sim et al., 1995). By stimulating the particular GPCR of interest with a specific ligand, the activation of these receptors may be estimated. Hence the synthetic opioid peptide [D-Ala², N-MePhe⁴, Gly-ol]-enkephalin (DAMGO) was used to pinpoint the activation of µ-opioid receptors within the respiratory control centers. The method has been used extensively to estimate the activity of several GPCRs and the µ-opioid receptor is among the ones that has been most studied (Sim-Selley and Childers, 2002). The method basically detects increase in guanine nucleotide exchange at G-proteins upon agonist stimulation. With
this technique activation of G protein-coupled receptors upon agonist binding can be detected and, at the same time, the location of activated receptors can be visualized (Sóvágó et al., 2001).

As an alternative, the number of morphine molecules binding to the μ-receptors at the time of death was studied using a proximity ligation assay (PLA). This method is based on the generation of a specific immunohistochemical signal caused by the formation of a DNA-complex formed when two different antibodies, labeled with complementary DNA-strands, bind to epitopes closely co-located (less than 40Å apart) (Figure 2). If the antibodies are not sufficiently specific or if their binding sites are too distant to each other, this analysis does not work.

The third alternative to provide supportive (or non-supportive) data to the forensic pathologist examining deaths tentatively caused by opiate overdose was to examine the number of cells showing a positive reaction to anti-NeuN, which reacts to neuron-specific antigens present in neuronal nuclei in the pre-Bötzinger complex. The rationale for this is the assumption that these cells are critical for the maintenance of inspiratory stimulation and that with fewer cells, a more severe inhibition may be exerted by a given dose of opiates.

For these studies, fresh-frozen postmortem medulla oblongata samples from rats treated with (or without = controls) high doses of morphine or heroin, and from deceased donors dying of opiate intoxication (or not = controls) were used for analysis.
2.4 Scientific question

Given the problems in making the diagnosis *opiate toxicity death*, the present study was conducted to find out if pharmacological and/or morphological changes in the medulla oblongata can assist in the investigation of suspected opiate intoxications.

Our two main hypotheses are (i) that opiate poisoning causes respiratory depression by overstimulation of \( \mu \)-opioid receptors on interneurons in the Pre-Bötzingers complex, (ii) that chronic opiate use reduces the sensitivity of the opioid-receptors to opiate exposure (i.e. tolerance) and opiate toxicity death may occur after a low dose in subjects with a recent discontinuation in opiate use (i.e. loss of tolerance). One reason for such an increased sensitivity after abstinence can be up-regulation of \( \mu \)-opioid receptors on the pre-Bötzinger interneurons. Another possibility is that the number of interneurons in the medulla is decreased in the subjects dying from opiate toxicity. Such a loss of interneurons can either be explained by previous non-fatal overdoses or chronic overstimulation of the \( \mu \)-opioid receptors of these cells.

The aim was to investigate the number of interneurons and \( \mu \)-opioid receptors within the pre-Bötzinger complex of individuals who died from opiate poisoning compared to individuals who have never encountered opiates.
3. Materials and Methods

The primary objective of this project was to identify an indicator of opioid cases where there is an explicit tolerance and allowing these cases to be distinguished from those who have never used opiates. The methods that were supposed to be used was (i) the autoradiographic method $[^{35}\text{S}]\text{GTP} \gamma \text{S}$, the experiment was already conducted but the analyses of the collected data were carried out, (ii) the proximity ligation assay (PLA) was evaluated with the intention to see if this assay be an easier and more refined method that can be used in place of the somewhat more complex and extensive $[^{35}\text{S}]\text{GTP} \gamma \text{S}$. Immunohistochemistry (IHC) and Western Blot were used partly as the primary platform for experimenting with the antibodies for the PLA. IHC also served as a complementary method in order to stain for NeuN with the view of counting the number of interneurons in the pre-Bötzinger complex (pBC) to see if there is a difference between the groups.

3.1 Biological materials and ethical aspects

Prior to this project, samples from rat experiments and deceased opiate toxicity deaths had already been collected, partly processed and stored at -80 °C and were available for further use.

In total 89 rats (male, Sprague-Dawley, Scanbur BK AB, Sollentuna, Sweden) were available for use, weighing between 216 and 376 g. The rats were separated into six groups containing six to fourteen individuals kept in separate cages and were acclimatized for one week prior to the start of the experiments. The rats had free access to fresh water and food (ad libitum) and were kept in 12:12 hour light-dark cycles. The six groups received different opiate or non-opiate treatments (Table 1).

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Intravenous injection with morphine</td>
</tr>
<tr>
<td>2</td>
<td>Intravenous injection with saline</td>
</tr>
<tr>
<td>3</td>
<td>Intraperitoneal injection with saline + abstinent + Intravenous injection with saline</td>
</tr>
<tr>
<td>4</td>
<td>Intravenous injection with heroin</td>
</tr>
<tr>
<td>5</td>
<td>Intraperitoneal injection with morphine + Intravenous injection with heroine</td>
</tr>
<tr>
<td>6</td>
<td>Intraperitoneal injection with morphine + abstinent + Intravenous injection with heroin</td>
</tr>
</tbody>
</table>

*Table 1. The different opiate or non-opiate treatments given to the rats.*

The human material was collected from deceased donors where the individual, family or close relatives had given informed consent to the use of tissues for research purposes. Ten
individuals were chosen for this project, five of them died due to opiate intoxication and the remaining five died of other causes (Table 2).

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Cause of death</th>
<th>Hair analysis</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND003</td>
<td>23</td>
<td>Buprenorphine intoxication</td>
<td>No opiates</td>
<td>No history of opiate abuse</td>
</tr>
<tr>
<td>ND004</td>
<td>31</td>
<td>Heroin intoxication</td>
<td>Heroin*</td>
<td>Abuse of heroin and cocaine for years</td>
</tr>
<tr>
<td>ND015</td>
<td>25</td>
<td>Heroin intoxication</td>
<td>Heroin*</td>
<td>Known addict of heroin and cocaine</td>
</tr>
<tr>
<td>ND042</td>
<td>38</td>
<td>Morphine intoxication</td>
<td>No hair obtained</td>
<td>Chronic addict, opiates unknown</td>
</tr>
<tr>
<td>ND049</td>
<td>35</td>
<td>Methadone intoxication</td>
<td>No hair obtained</td>
<td>Chronic addict, opiates unknown</td>
</tr>
<tr>
<td>ND014</td>
<td>36</td>
<td>Hanging</td>
<td>Not analyzed</td>
<td>Control</td>
</tr>
<tr>
<td>ND002</td>
<td>29</td>
<td>Hanging</td>
<td>Not analyzed</td>
<td>Control</td>
</tr>
<tr>
<td>ND020</td>
<td>33</td>
<td>Hanging</td>
<td>Not analyzed</td>
<td>Control</td>
</tr>
<tr>
<td>ND071</td>
<td>23</td>
<td>Multiple injuries</td>
<td>Not analyzed</td>
<td>Control</td>
</tr>
<tr>
<td>ND075</td>
<td>39</td>
<td>Hanging</td>
<td>Not analyzed</td>
<td>Control</td>
</tr>
</tbody>
</table>

Table 2. Characteristics of the human subjects. All subjects were male and between 23 and 39 years old. *Both were negative for opiates in the two most recent hair segments, but positive for heroin metabolites in segment 3 (exposure more than 3 weeks before death).

The animal experiments were carried out in line with approved animal ethics applications (Reg. no 264/07, 265/07, 110/05 and 178/09, Animal ethics committee at Karolinska Institutet, Stockholm). The collection, handling and use of the human material were approved by the Regional Ethics Board in Stockholm (Reg. no 2002-371, 2006/1211-31 and 2010/313-31/3).
3.2 Autoradiography and [$^{35}$S]GTPγS data analysis

The raw data from the already performed [$^{35}$S]GTPγS-assay was obtained from the Research Laboratory of Forensic Medicine in Stockholm. The experimental work had been conducted prior to the start of the current project, but not statistically analyzed.

The material available comprised autoradiographic sections from medulla oblongata from 23 rats divided into six groups, which received different treatment (Table 1), and from ten human cases divided into two groups (Table 2). Eight sections from the mid-portion of the pre-Bötzinger complex were selected from each individual based on anatomical landmarks. The right angle shape of linear nucleus along with the shape of the XIIth cranial nerve and the arrangement of the windlings of the pyramidal tract were used to determine this level in the rat medullas. For the human samples, the shape of the accessory olivary nucleus, the location of nucleus ambiguus and the size of the ventrolateral groove were used as landmarks to determine the level. The results of the autoradiographic densities were compiled by calculating the averages for each pre-Bötzinger complex, when sufficiently intact, the average for each subject, and the average for each group.
3.3 Proximity ligation assay (PLA)

All included reagents, solutions and chemicals in this assay were ready-made solutions and preparations from the DuoLink-II kit (Olink Bioscience, Uppsala, Sweden). The assay was performed according to the manufacturer's protocol.

In short, the areas of interest of the fresh frozen tissue from rat and human (opiate deaths and controls) were encircled with a para-pen. The sections were defrosted and dried at room temperature for one hour. Samples were washed in Duolink wash buffer A (NaCl, Tris base, Tween, dH2O and pH adjusted to 7.4 with HCl) for ten minutes. One drop per sample of Duolink Hydrogen Peroxidase solution was added to each tissue sample followed by incubation for five minutes. This peroxidase quenching is important to reduce the non-specific background by irreversibly inactivating the endogenous peroxidase, which otherwise is a risk when using horseradish peroxidase (HRP)-conjugated antibodies. The samples were washed in Duolink wash buffer A twice five minutes under agitation. Duolink blocking buffer was added one drop per sample and the slides were incubated in a pre-heated humidity-chamber for one hour at 37 °C. After incubation the blocking buffer was removed and the primary antibodies anti-morphine (Aviva systems Biology Corp, San Diego, USA) and anti-μ-opioid-receptor (Enzo Life Sciences Inc, New York, USA), diluted in Duolink antibody diluent (Table 3), were immediately added to the samples followed by an overnight incubation at °C in a humidity-controlled chamber.

The following day, the slides were washed two times five minutes under agitation in Duolink wash buffer A. Duolink PLA probes (Rat MINUS, Rat PLUS, Mouse MINUS and Mouse PLUS) were mixed and diluted (1:5) in Duolink antibody diluent and the PLA probe solutions were added to the slides and incubated for one hour at 37 °C in a pre-heated humidity-chamber. Then the slides were washed two times five minutes under agitation. The Duolink ligation stock was diluted 1:5 in high purity water and the ligase enzyme (diluted to 1:40) was added immediately prior to addition to the slides. The slides were incubated for 30 minutes in a pre-heated humidity-chamber at 37 °C and subsequently washed in Duolink wash buffer A for two times two minutes under agitation. The subsequent steps of the assay contained photosensitive reagents and were therefore performed in darkness. The Duolink amplification solution diluted to 1:5 by addition of high purity water and Duolink polymerase (dilution 1:80) were added to the samples and incubated for 120 minutes in a pre-heated humidity-chamber at 37 °C. Followed by washing two times two minutes in Duolink washing buffer A under gentle agitation. The Duolink detection brightfield solutions (HRP-labelled probes)
were added to the samples and incubated at room temperature (RT) for 60 minutes in a humidity-controlled chamber. The components for the Duolink substrate solutions were diluted in high purity water and mixed (solutions A 1:70, B 1:100, C 1:100 and D 1:50). Thereafter the substrate solution was added to the slides and incubated for five to ten minutes at RT, followed by washing in high purity water for two times two minutes. One drop per sample of the Duolink nuclear stain was added and incubated for two minutes at RT, followed by washing under running tap water for ten minutes. Dehydration was performed by dipping the slides in increasing concentrations of ethanol (96% 2 x 2 min, 99.7% 2 x 2 min) and finally in xylene for ten minutes. The slides were mounted in a 20% glycerol solution and stored at -20°C in darkness until analysis in an epifluorescence microscope.

### 3.4 Immunohistochemistry – fluorescence microscopy

A series of immunohistochemistry stainings were performed on fresh frozen tissue sections (14 μm thick) from both rat and human samples (opiate cases and controls). The surroundings of the tissue samples were marked with a para-pen and were thawed and dried for one hour at RT. Rehydration comprised two times ten minutes in phosphate buffered saline (PBS). Fixation of the samples was carried out by 30 minutes incubation in 4% formaldehyde solution. Formaldehyde acts by cross-linking the proteins with methylene bridges. This protocol can sometimes conceal epitopes for certain antibodies, which is why fresh frozen tissue along with a short fixation time were used. The samples were rinsed and washed two times for ten minutes. Cold blocking buffer (BB), which contained bovine serum albumin (BSA), NaN₃, Triton-X, PBS and mixed with normal donkey serum (NDS), was added to the slides and incubated in a humidity-controlled chamber for one hour at 4 °C. Incubation with BB was done to prevent or at least minimize possible cross-reactivity between the antibodies and nonspecific antigens. BB reacts with these nonspecific reactive areas and reduces the background interference. After the incubation BB was removed from the slides and the primary antibodies diluted in BB were added (Table 3) followed by incubation in a humidity controlled chamber at 4 °C overnight.
The incubated samples were rinsed and washed three times for ten minutes each (3X10 min) with PBS and subsequently blocking buffer was added and then incubated in a humidity-controlled chamber for one hour at RT. The two secondary antibodies goat-anti rabbit IgG conjugated to Alexa Fluor 555 (Invitrogen, Oregon, USA) and goat-anti-mouse IgG conjugated to Alexa Fluor 488 (Invitrogen, Oregon, USA) were diluted (Table 3) with BB together with 0.2 μL of Hoechst (Invitrogen, Oregon, USA). Hoechst, which is a nuclear stain, and the secondary antibodies, are light sensitive so the subsequent steps were conducted in a dark room. Samples were incubated in a humidity-controlled chamber for one hour at RT. This was followed by rinsing and washes three times during ten minutes each. The samples were mounted with 20% glycerol in PBS enclosed by a cover slip and stored at 4 °C away from light exposure until analysis with an epifluorescence microscope.

**Table 3.** List of primary and secondary antibodies and the different dilutions used for immunohistochemistry (IHC) and proximity ligation assay (PLA).
3.5 Tissue preparation and protein determination for Western Blot

Fresh frozen brain tissue (300 mg each) from two rats were collected, one that had received a fatal dose of heroin and one control rat that only had received saline solution. The tissues were homogenized in a glass tube with a piston (Dounce’s homogenizer) together with ice-cold buffer A (0.32 mM sucrose, 1mM EGTA and proteinase inhibitor (PI cat#P8340, Sigma)). The homogenate solutions were dispersed by sonication (pulse amplitude 60) for three seconds.

Membranes containing the μ-opioid-receptor were isolated by centrifugation of the homogenate at 800 g for twelve minutes at 4 °C in order to remove the nuclei. The supernatant were further high speed centrifuged at 22 000 g for 20 minutes at 4°C and the pellets were re-suspended in buffer A. The Bradford method (Bio-Rad Protein Assay kit) was used to define the protein content in the homogenate. Serial dilution (0 mg/ml, 0.25 mg/ml, 0.5 mg/ml, 1.0 mg/ml and 1.4 mg/ml) of BSA was used as standard solution to generate a standard curve. Bradford reagent (cat# B6916, Sigma-Aldrich) was brought to RT and 1.5 mL of the reagent was mixed with 50 μL of diluted sample solution or standard solution (BSA) (Table 4) in a cuvette, the absorbance was measured at 595 nm in a spectrophotometer.

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<tr>
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<tr>
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<tr>
<td>5</td>
<td>50 µl</td>
<td>1.4 mg/ml</td>
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<tr>
<td>6</td>
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<td>2.0 mg/ml</td>
<td>1.5 ml</td>
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</table>

Table 4. The dilution series and sample volumes loaded into the cuvettes.
3.6 Western Blot

A series of Western blot assays were performed to analyze and verify that the polyclonal anti-μ-opioid-receptor (anti-MOR) (Enzo Life Sciences Inc., New York, USA) was specific enough to be used in the proximity ligation assay (PLA).

950 μL of Lammeli sample buffer (Bio-Rad Laboratories, Inc.) and 50 μL of beta mercaptoethanol were mixed and added to the sample solutions (1:1). 25 μL of the different sample solutions were loaded onto a GTX gradient gel (BioRad cat#456-1083) together with 5 μL of gel loading buffer (Sigma-Aldrich). The mixture was heated at 95 °C for three minutes and then the gel was loaded in a Mini-Protean Terta Cell (Bio-Rad laboratories Inc.) and was run in Running Buffer (Tris-base 25 mM, Glycine 192 mM, 0,1% SDS) at 150 volt (V) for one hour. A polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories Inc.) was activated by incubation with methanol for ten seconds. The gel and the membrane were loaded in a Trans-Top Turbo System (Bio-Rad Laboratories Inc.) according to the manufacturers description (Figure 3). It was run in transfer buffer (Tris-base 25 mM, Glycine 192 mM, Methanol 20% (v/v)) at 150 V for 100 minutes. The membrane was incubated at RT for one hour under gentle agitation in blocking buffer, 5% non-fat milk dissolved in PBS. The blocking buffer was removed and replaced with anti-MOR (Enzo Life Sciences Inc., New York, USA) diluted 1:750 in blocking buffer followed by incubation overnight at 4 °C.

The membrane was washed rapidly three times with PBS, followed by three washes, each ten minutes, in wash buffer (0,1% tween in PBS). The secondary goat anti-rabbit antibody was diluted 1:2000 in wash buffer and added to the membrane and incubated under gentle agitation for two hours at RT. After the incubation the membrane was washed rapidly three times in wash buffer followed by three longer washes each 20 minutes. The membrane was placed in a plastic wrapping and just before the picture development, a mixture 1:1 of clarity western peroxidase and clarity western luminol/enhancer (Bio-Rad Laboratories Inc.) were added to the membrane. The developing was performed in a FUJIFILM Luminescent Image Analyzer LAS.

Figure 3. The assembly of the Top Turbo system, cropped and modified from (Bio-Rad Laboratories Inc).
3.7 **Immunohistochemistry – light microscopy**

Single-staining with the HRP-conjugated antibodies was performed as follows. Four fresh frozen human medulla oblongata sections (14 μm) from five opiate toxicity deaths and four controls were marked with para-pen, and thawed and dried for one hour at RT. The sections were rehydrated in PBS for three ten minutes periods. Fixation was performed by incubation with 4% formalin solution for ten minutes, followed by three ten minutes washes in PBS. Endogenous peroxidase was blocked by incubation with 0.3% H₂O₂ for 30 minutes in darkroom. The sections were rinsed and washed in PBS four times each five minutes. Blocking buffer was added to the slides and incubated for ten minutes in a humidity-controlled chamber at RT. The primary antibody mouse anti-NeuN1 (Merck Millipore) was diluted 1:500 in dilution buffer (PBS, 0.1% TritonX-100 and NaN₃) and added to the slides, which were incubated overnight at 4°C in a humidity-controlled chamber.

The slides were washed in PBS four times for five minutes. The secondary HRP-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch Labs) was diluted 1:100 in dilution buffer and added to the sections and incubated for one hour in a humidity-controlled chamber at RT. The incubation was followed by a series of four washes, each for five minutes in PBS. For detection, 5 mL 3,3'-diaminobenzidine (DAB) were added to the sections, which then were incubated for ten minutes at RT. The slides were washed with distilled water four times for five minutes. The slides were washed under running tap water for nine minutes and then counterstained with hematoxylin for ten seconds. Dehydration of the sections was done by a series of increasing concentrations of ethanol, 70%, 95% and absolute ethanol, and finally with Xylene. The slides were mounted with a mixture of distyrene, a plasticizer, and xylene (DPX) enclosed with a covering slip. The slides were analyzed under a light microscope.
4. Results

4.1 Density of activated μ-opioid receptors in the pre-Bötzinger complex ([35S]GTPγS).

The autoradiographic densities of μ-opioid receptors were measured in the pre-Bötzinger complex of medulla oblongata from rats and human deceased donors, and compared with the densities of control areas. The rats were administered high doses of heroin (or saline). The distribution of the metabolite morphine 15 min after intravenous injection was found to be uniform with a good correlation between the concentration in the blood and medulla (Figure 5).

![Morphine (blood vs medulla), ng/g](image)

**Figure 4.** Concentrations of morphine in venous blood and upper cervical medulla 15 min after i.v. injection of heroin (0.1-30 mg/kg B.W.).

However, the autoradiographic measurements of activated μ-opioid receptors did not reveal any significant differences between rats exposed to opiates versus controls (Table 5; ANOVA p>0.05 for all comparisons, SPSS). Measurements of the densities in the human medulla provided confusing results. Figure 5 shows the results for controls and opiate toxicity deaths. Even if the densities on average were higher in opioid toxicity death, several of the measurements provided negative values (after subtraction of non-stimulated from DAMGO-stimulated slides), indicating a methodological problem. Hence, in our hands, the
[^35]S-GTPγS-assay, can apparently not be used to assist in the diagnosis of opiate toxicity death.

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Table 5. Densities of activated μ-opioid receptors in pre-Bötzinger complex of rat medulla as measured by[^35]S-GTPγS assay.

Figure 5. Autoradiographic densities of activated μ-opioid receptors within pre-Bötzinger complex of the human medulla oblongata. The five bars to the left are controls, and the five to the right are opioid toxicity deaths.
4.2 Proximity ligation assay

A series of proximity ligation assays (PLA) were performed with Duolink-II kit (Olink) on human and rat medullar sections, using anti-morphine antibodies (Aviva Systems Biology), 1:100 and 1:50, and anti-MOR (Enzo life science) 1:500 and 1:50. The combination of anti-morphine (1:100) and anti-MOR (1:100) did not give any significant signals in red spectra (Figure 6). An increase of antibody concentrations to 1:50 for both did not help (Figure 7). Hence, even if both antibodies apparently showed a specific staining in isolation, the epitopes were probably not as closely co-localized to provide a reaction with this method.

Figure 6. Human medulla treated with anti-morphine (1:100) and anti-MOR (1:100). (A) Shows a signal (arrow) in red fluorescence (positive) in 20X magnification, which has no corresponding signal (C) in green fluorescence (negative). Higher magnification (60X) (same area) in red fluorescence (B) reveals several signals (arrows), (D) which also are easily identified in the same magnification in green fluorescence (arrows). If positive one should only see signals in red fluorescence (A) and (B) but not in green (C) and (D).
Figure 7. Human medulla treated with anti-morphine (1:50) and anti-MOR (1:50). (A) Shows a cluster of signals (ring) in red fluorescence (positive) in 20X magnification, which has corresponding signals (C) in green fluorescence (negative). Higher magnification (60X) in red fluorescence (B) reveals a group of signals (ring) that are also easily identified in the same magnification in green fluorescence (D). If positive one should only see signals in red fluorescence (A) and (B) but not in green (C) and (D).
4.3 Immunohistochemistry – fluorescence microscopy

A series of immunohistochemistry assays were performed on both human and rat medulla sections. The aim was to evaluate the anti-MOR and anti-morphine antibodies specificity and define the optimal dilutions for PLA. The most promising dilution was 1:100 for anti-morphine (Figure 8A) and 1:500 for anti-MOR (Figure 8B). The antibodies showed convincing specificity for their respective antigen compared with the negative and positive controls (Figure 8 E and F).

![Figure 8](image_url)

**Figure 8.** Rat medulla sections at x20 magnification; Morphine 3A6-antibody 1:100 (A), 1:500 (B), Anti-MOR 1:100 (C) and 1:500 (D). Positive control KI-67-antibody 1:250 (E) and negative control Alexa Fluor 488 1:500 with no primary antibody added (F).
4.4 Western blot
The western blot assay was performed on brain tissue from two rats, one that had been treated with heroin and a control that had received saline solution. The samples were purified from cell membranes by centrifugation and the protein content was determined. The anti-MOR antibody showed a conspicuous positive staining of a band corresponding to 50 kD. The results of these experiments supported using the anti-MOR antibody in the proximity ligation assay.

4.5 Immunohistochemistry – light microscopy
36 sections from nine humans were used. The staining with the HRP-conjugated antibodies against NeuN was satisfactory with a very good signal to noise appearance between background and neurons (Figures 9 and 10). Unfortunately six sections were damaged to such an extent during the preparation that it was impossible to determine the exact location of the pre-Bötzinger complex.

![Figure 9. Human medulla, cluster of neurons positive for NeuN (Arrow), 1:500, x20.](image1)

![Figure 10. Human medulla, neuron positive for NeuN (Arrow), 1:500, x60.](image2)

The results from the cell counting shows a decreased number of cells in opiate users compared with controls (Figure 11 and table 6). However, six of the slides were damaged to such extent that it was impossible to make measurements.
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Table 6, ND014, ND002, ND020 and ND071 died of non-opiate cause, ND004, ND015, ND049, ND042 and ND003 died of opiate poisoning. Four different sections each divided into two parts; Left and Right. (-) means that it was not possible to count cells due to damage. The mean value and standard deviation were calculated based on the number of cells counted to get a representative view over the combined areas (left and right).

**Figure 11.** The mean values of the cell counting, ND014, ND002, ND020 and ND071 represents controls and ND004, ND015, ND049, ND042 and ND003 are opiate cases.
5. Discussion

The basic aims of this study was to find out if the investigation of suspected opioid toxicity deaths could be more firmly concluded after additional, non-routine analyses. This study showed that [35S]GTPγS assay is a cumbersome methodology that requires very accurate conditions, and thus not practical to use in the postmortem casework when estimating the status of the opioid system. Further, the results obtained in these studies were based on rat and human material previously collected. Parts of the tissues were previously used for toxicology and other analyses and the plan was that the [35S]GTPγS analyses as well as immunohistochemistry should have been performed at an earlier point in time, but instead were stored for several years at -20 °C. As a result, the documentation and the condition of the material did not specifically serve the purpose of these studies. Consequently, the project was modified and adapted to existing circumstances.

The aim with this project was to establish if deceased subjects with no history of opiate use compared to known opiate addicts, differ regarding cellular changes in the pre-Bötzinger complex and compare that data with a previously performed [35S]GTPγS-assays. This was conducted to see if it was possible to methodologically distinguish between opiate addicts and non-opiate users. The main method thought to be used was the proximity ligation assay (PLA), which is an excellent and specific method for detection of interaction between a receptor protein and an agonist/antagonist. The PLA assay would not only show the position of interactions but also give their numbers that otherwise requires both IHC to see numbers of cells and GTPγS to measure activated receptors.

The theoretical principle behind the use of the PLA assay for this purpose was to use two primary antibodies, one directed against the receptor and the other against the agonist (in our case opiates). When they have bound to their respective target two secondary antibodies that are conjugated with a small oligonucleotide sequence are added, and if the primary antibodies are close enough the oligonucleotides can polymerize creating a single nucleotide sequence. To avoid non-specific signals a third oligonucleotide are required, together with the already polymerized nucleotide sequence it forms a DNA-circle. The circle can be amplified by rolling-circle amplification resulting in a massive ball of DNA, which can be stained and detected by microscopy. This method requires highly selective and specific primary antibodies, properly prepared tissue sections, careful handling and fresh reagents.
Unfortunately no reliable results were obtained from the PLA-assay, despite the effort of minimizing sources of errors. New antibodies were purchased and thoroughly tested and evaluated and the tissue sections were carefully selected and handled. The anti-µ-opioid-receptor antibody was tested during a series of western blot analysis. Brain tissues from two rats that had been treated with either high doses of opiates or saline solutions were used. Two different types of homogenate were created for testing, one that was purified by high speed centrifugation to only contain cell membranes and the other contained the complete cellular content. The results received were positive for the µ-opioid-receptor (band corresponding to 50 kD) for both homogenates. Further testing consisted of immunohistochemistry (IHC) assays on both rat and human tissue sections, which was performed in order to evaluate which antibody concentration that was most promising for use in the PLA and also, equally important, to verify that the antibody worked on human tissue. The result from the IHC indicated that the dilution 1:100 was the optimal concentration for both rat and human samples.

The anti-morphine antibody was tested through IHC assays on human and rat sections and the signal was neither equally strong nor as trustworthy as for the anti-µ-opioid-receptor antibody (see Figure 6A-B). This can be explained by the fact that the morphine molecule is distributed to the brain by the bloodstream and is therefore more evenly dispersed in the brain, but also the molecule is a much smaller target (285.34 g/mol) than a receptor (50 KD) and by far more easily concealed than a membrane bound receptor.

The lack of results regarding the PLA was probably due to the fact that the DuoLink II reagents had been previously opened, used, and unfortunately past its expiration date. In addition, the tissues were old and therefore possibly not optimal for these kind of assays. Yet some signals were obtained as seen in Figure 4 and 5, which are most likely the result of contaminations during the preparation.

As an alternative method, a single staining assay with HRP-conjugated antibodies directed against NeuN was performed to visualize and count the number of interneurons in Pre-Bötzingers complex. When looking at the results it seems that our experimental data suggest that the number of neurons declines during a long-term opiate abuse (see Figure 9). This data supports our hypothesis that a chronic and long-term opiate use results in receptor and cellular adaptations.
A potential reason behind this cellular decline can be that it is the result of a long-term adaptation by the brain to overcome the stimulatory effect mediated by the constantly activated neurons. This adaption probably arises only after desensitization, internalization or down-regulation of the receptors, which no longer manage to counteract the constant increasing dose and stimulatory effect of opiates. This can partially explain the deaths due to respiratory failure caused by opiates. Due to the loss of interneurons in the Pre-Bötzingers complex, the addict has lowered the threshold for respiratory depression to such a level that it lies in the borderline between a functional level and complete respiratory failure. During a period of abstinence the remaining interneurons regain full receptor capacity and can maintain the regulatory functions, but if relapses occur, the stimulatory effect of the receptors, combined with the low numbers of remaining neurons results in a depressive effect that becomes so massive that it leads to a total stop of respiration.

Elucidation of the mechanism of tolerance development against breathing depression and the great variation of opiate concentrations found in post mortal blood samples are the main questions to be answered and explained. The work and results presented in this thesis is just a small part in the important task of providing a better understanding and description of the mechanism(s) behind opiate toxicity.

In completion, this project aimed primarily to determine if PLA could be used to see cellular adaptations in the Pre-Bötzingers complex. PLA is a great method if properly designed and executed. A complementing and very interesting approach lies in the investigation of epigenetic changes that occur during a long-term opiate use, in terms of receptor gene expression and regulation. This will need a new set of carefully designed animal studies intended for this particular aim. A tempting thought with the epigenetic approach is that one can compare the different areas that are effected by opiates; respiratory, pain and euphoric centers with each other, to see how the progression and regression of adaptations change over time. I think this will provide a broader and more extensive picture of how the different parts of the brain react in a state of tolerance, abstinence and relapse.

It may be so that our genetic heritage has equipped us with different abilities to handle various opiates, we are all unique and it is very likely that it also applies to our reactions against opiates but that is something that future investigation will bring into light.
6. Acknowledgements

I would like to express my sincere gratitude to Professor Henrik Druid for the privilege of working in your laboratory, for your extraordinary encouragement and diligent supervision. Without you, the completion of this thesis would not have been possible. Furthermore, I would like to acknowledge with sincere appreciation and gratitude Dr. Ahmed Mousa for your guidance, good advices and company during this project. Last but not least my deepest thanks to Dr. Kanar Alkass, Dr. Tara Wardi and Charlotte Ovensen for your assistance and support.
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