Offset Analgesia: Affected by Morphine?

Matias Nilsson

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Matias Nilsson

Supervisor(s): Parisa Gazerani, Associate Professor at the Department of Health Science and Technology, Aalborg University and Christina Brock, Post Doc, DVM and PhD at Center of Mech-Sense, Aalborg Hospital

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* The content of this project is freely available; however disclosure can only be effected as directed by the author

Preface

This Master thesis was written by Matias Nilsson, group no. 1004, Translational Medicine, 10th semester of Medicine with Industrial Specialisation, Aalborg University, Denmark. The thesis was compiled from January 16th, 2012 to May 31st, 2012. The project was supervised by internal supervisor Parisa Gazerani, Associate Professor at the Department of Health Science and Technology, Aalborg University and external supervisor Christina Brock, Post Doc, DVM, PhD at Center of Mech-Sense, Department of Gastroenterology, Aalborg Hospital.

The thesis is an extension of the authors' 9th Semester work, entitled "Offset Analgesia: A Reproducibility Study", which can be found in Appendix A. The present thesis is structured as a scientific article, where the Harvard format is used for referencing. First chapter is an extended introduction giving the reader an overview of topics relevant to the project. The following chapters include materials and methods, results, and discussion, which lead to a general conclusion of the project and future perspectives.

The structure throughout the report as well as in the Table of Contents is based on a four-levelled subdivision of the chapters, where the first three headers are numbered (e.g. 1.; 1.1.; 1.1.1.) and sublevels of these are marked *italic*. Matias Nilsson has constructed all figures.

The knowledge acquired during the course of the project was primarily from scientific articles of high validity found on databases such as PubMed or through the State and University Library (Statsbiblioteket). Keywords used during the literature search included: Offset Analgesia, pain modulation, DNIC, CPM, gate-control, pain pathways, nociception, and endogenous pain inhibition, morphine, opioid receptor, opioidergic system.

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Abbreviations

CNS – Central nervous system CPM – Conditioned pain modulation EEG - Electroencephalography OA – Offset analgesia PAG – Periaqueductal grey RVM – Rostral ventromedial medulla SIA – Stress-induced analgesia VAS – Visual analogue scale

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Offset Analgesia: Affected by Morphine?

Matias Nilsson Dept. Health, Science, and Technology, Aalborg University

Abstract

Pain constitutes the primary reason why patients seek medical attention and chronic pain can be extremely disabling. Moreover, pain poses a substantial socio-economic burden due to health care expenses and absence from the work force. Although huge advances in pain management has been made over the last century, pain continues to be severely complicated to treat due to its wide diversity. The endogenous pain modulating mechanisms are a frequently used target in pain management and with the recent discovery of offset analgesia (OA), this mechanism might open up to novel target sites. Offset analgesia is characterised by a powerful decrease pain perception in response to a discrete decrease in noxious stimulus intensity. However, much has yet to be learned about OA, in order to evaulate its full potential as an experimental or clinical tool. Importantly, studies need to determine whether the physiological and anatomical properties of OA is located peripherally or centrally. The aim of the present study is to evaluate the potential effect of morphine on OA in terms of subjective pain ratings and objective electroencephalographic data. It was found that morphine causes the decrease in pain perception that arises from the OA effect to be significantly greater when the subjects are treated with morphine. The objective EEG data are less clear, but opioidergic influence over OA is established.

1. Introduction

Pain is an important sensory experience that facilitates the survival of a species by warning the organism of actual or potential tissue damage. The ability to respond adequately to threat is crucial for the survival of the individual [Bolles, 1970; Sherrington, 1900]. Because of the unpleasant nature of pain, aversive behaviour such as fear, anxiety, panic, and escape behaviours encourages the individual to avoid similar painful experiences in the future. Accordingly, The International Association for the Study of Pain defines pain as:

"An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage." [IASPTaskforce, 1994].

Accordingly, pain sensation can be divided into three components; the sensory-discriminative component, the affective-motivational component, and the cognitive-evaluative component. The sensory-discriminative component of pain is concerned with determining the location from which pain originates, the intensity of the pain, and which stimulus modality causes the pain (heat/ mechanical/etc.). On the other hand, the affectivemotivational component of pain is responsible for the emotional response to pain including aversion. The affective-motivational component also facilitates a desire to terminate the noxious stimulus. The cognitive-evaluative component is the psychological aspect of pain sensation and is influenced by parameters such as appraisal, cultural values, and distraction [Melzack & Casey, 1968; Treede *et al.*, 1999; Brock, 2009].

Despite being a useful protective sensory experience, pain is the primary reason patients seek medical attention and constitutes a substantial socio-economic burden on the health care sector, as a result of medical expenses, hospitalisation, and absence from work. Moreover, prolonged exposure to pain is socially and functionally disabling for the patients, which reduces quality of life considerably [Langley *et al.*, 2010b; Langley *et al.*, 2010a; Gustavsson *et al.*, 2011; Gore *et al.*, 2011]. Because of the complex nature of pain, treatment is often challenging and the effect of a pharmacological compound varies greatly between patients. Furthermore, the endogenous ability to suppress pain can be incredibly powerful and most available treatment regimes aim at targeting endogenous pain modulating mechanisms [Basbaum *et al.*, 2009; Millan, 1999; DeLeo, 2006]. Considering the number of people suffering from acute or chronic pain, the immediate incentive for research in this area is readily apparent.

1.1. The Somatosensory System

The somatosensory system provides a constant inflow of information to the brain cortices regarding temperature, proprioception, touch, and pain. Specific receptors exist for every sensory modality and occur in higher or lower density throughout the body depending on the kind of receptor and the location in the body. The nerves that conduct information between different sites, are divided into A and C type nerve fibres, and the former is subdivided into A and A δ fibres. Sensory receptors usually conduct their electrical signals through $A\beta$ nerve fibres, characterized by a thick layer of myelin, a relatively large diameter, and the presence of nodes of Ranvier. A β fibres allow very rapid signal conduction reaching conduction velocities of up to 100 m/s. Examples of A β fibres include the median nerve, the radial nerve, and the ulnar nerve, the largest nerves of the hand and forearm, responsible for innervating each their distinct area. However, noxious input from the nociceptors are transmitted through the A δ and C fibres. A δ are thinner and less myelinated than $A\beta$ fibres, and conduct cold pain and well-localized pain (e.g. pin prick), while the C fibres are thin, non-myelinated fibres, which conduct heat-, or mechanically induced pain. The $A\delta$ fibres conduct information rapidly (12 - 30 m/s), while the thinner, non-myelinated C fibres reaches conduction velocities of (0.5 - 2 m/s) [Almeida et al., 2004; Millan, 1999].

1.1.1. Ascending Pain Pathway

Whenever the somatosensory apparatus is presented to a noxious stimulus in the skin, most afferent nerve fibres carrying innocuous as well as noxious information, synapse with second-order neuron in the dorsal horn. The characteristics of the different primary afferent nerve fibres are listed in Table 1. From the dorsal horn, the noxious input primarily ascend to the thalamus, but collateral projections also reach mesencephalic regions including the midbrain periaqueductal grey (PAG), the rostral ventromedial medulla (RVM), and the dorsal reticular nucleus [Willis & Westlund, 1997; Weiss et al., 2005; Millan, 1999]. From the thalamic nuclei, third-order sensory neurons terminate in the "pain-matrix" [Treede et al., 1999; Almeida et al., 2004; Giesler Jr et al., 1994; Loewy, 1990], which includes:

- The primary somatosensory cortex
- The secondary somatosensory cortex
- The anterior cingulate cortex
- Limbic regions including insula and amygdala
- The prefrontal cortices

1.1.2. Endogenous Pain Modulation

In agreement with the affective-motivational component of pain, the experience of pain is heavily modulated by endogenous mechanisms, depending on environmental and psychological circumstances. For example, pain may be inhibited during stress, intense exercise, or during escape from a predator to allow the individual to deal with immediate hazards [Bolles & Fanselow, 1980]. Conversely, the pain-facilitating systems can cause central sensitisation, which urges the organism to be aware of existing or potential tissue injury in order to protect that specific area from further harm [Woolf, 1995;

Fibre Type	Diameter (µm)	Myelin Sheath	Terminates in Lamina(e)	Conduction Velocity (m/s)	Nodes of Ranvier
Αβ	> 10	Thick	III - V	30 - 100	Yes
Αδ	2 - 6	Thin	II and V	12 - 30	No
С	0.4 - 1.2	None	I and II	0.5 - 2	No

 Table 1 Characteristics for the different types of primary afferent nerve fibres.

McMahon *et al.*, 1993]. Taken together, pain perception is the net effect of extremely complex systems involving most parts of the central nervous system (CNS) including intensity coding, affective, behavioural, and cognitive components. Not surprisingly, many of the implicated parameters including endogenous modulation have yet to be fully understood.

The gate-control theory of pain

The gate-control theory of pain was first proposed in 1965 when Ronald Melzack and Patrick D. Wall published a now renowned paper, in which the gate control theory was hypothesized as a potential candidate for the hitherto vaguely described mechanism underlying endogenous pain modulation [Melzack & Wall, 1965]. Briefly, the theory assumed that nociceptive information was carried to the dorsal horn of the spinal cord, by large-diameter fibres and small-diameter fibres. The pain fibres would terminate in substantia gelatinosa as well as central transmission cells, which in turn would relay information to the central nervous system. Moreover, substantia gelatinosa was hypothesized to be capable of decreasing the output from the central transmission cells, thereby giving rise to a decreased pain sensation. The large-diameter fibres terminating in substantia gelatinosa would cause inhibition of the central transmission cells, whilst the small-diameter cells would reduce the inhibition. In effect, this means that during the presence of a noxious stimulus, the painful sensation can be alleviated by innocuous stimuli such as vibration [Melzack & Wall, 1965]. The gate control theory founded the belief in endogenous pain modulation and since 1965 it has been generally accepted that brainstem and supraspinal systems also act to modulate nociception [Basbaum & Fields, 1984; Calvino & Grilo, 2006; Ossipov et al., 2010].

The majority of literature on pain modulation derives from animal experimental data. However, it is clear that descending pain modulation relies heavily on PAG-RVM and locus coeruleus as these structures are able to influence the spinal modulation through inhibitory as well as facilitatory pathways [Gebhart, 2004; Gebhart *et al.*, 1983; Sandkuhler & Gebhart, 1984]. This was first substantiated by Reynolds in 1969, when electrical stimulation of PAG in rats was proven capable of inducing analgesia strong enough to perform laparotomies without any form of chemical anaesthesia [Reynolds, 1969]. Other pain modulating systems include conditioned pain modulation (CPM), stress-induced analgesia (SIA) and offset analgesia (OA).

Conditioned pain modulation

The proverbial "pain inhibits pain" was legitimated and the phenomenon named diffuse noxious inhibitory control following discoveries made by Le Bars and colleagues in 1979, and recently renamed CPM [Le Bars et al., 1979a; Le Bars et al., 1979b]. It was discovered that recordings from the spinal dorsal horn in anaesthetised rats were altered during heterotopic peripheral noxious stimuli to the tail, paws, ears, viscera, and muzzle. In fact, tonic peripheral noxious stimuli of different modalities are able to inhibit the neural responses of convergent dorsal horn units. It has been established, that CPM relies heavily on a spino-bulbo-spinal loop through dorsal reticular nucleus. This is partly attributed to the fact that the dorsal reticular nucleus consist of multiceptive neurons with the whole body as the receptive field [Le Bars, 2002; Pud et al., 2009; Sprenger et al., 2010]. In standardised experimental pain, the effect of CPM can be assessed by applying a test pain before and after a conditioned stimulus. The heterotopic conditioning pain alters the perception of the test pain, allowing before and after-comparison.

Stress-induced analgesia

Stress-induced analgesia was thoroughly investigated in the 1970ies and 1980ies and describes the ability to suppress pain during stressful situations, and from an evolutionary perspective it probably exists to allow unhindered focus on fight or flight [Akil *et al.*, 1976; Amit & Galina, 1986; Bodnar, 1986; Terman *et al.*, 1984]. The models used to induce SIA always consist of two different stimuli; a noxious stimulus and a stressful stimulus. Examples

of noxious/stressful stimuli applied in rodent experiments include radiant heat/continuous cold swim [Bodnar et al., 1978a; Bodnar et al., 1978b] and nitro-glycerine/restraint stress [Costa et al., 2005]. In humans SIA has been elicited using virtual reality video game playing [Hoffman et al., 2001], peripheral electrical stimulation [Abdulhameed et al., 1989] or exposing arachnophobics to spiders [Janssen & Arntz, 1996]. The analgesic effect of SIA is quite powerful and numerous attempts have been made to uncover how this mechanism operates. Several structures have been implicated in the control of SIA, mainly through lesion studies, including the amygdala [Werka, 1994; Werka, 1997], the PAG [Helmstetter & Tershner, 1994], and the hypothalamus [Millan et al., 1980].

Offset analgesia

Offset analgesia, the endogenous pain modulating mechanism around which this master's thesis revolves, was first described in 2002 as:

"A disproportionately large decrease in pain intensity following a relatively small decrease in noxious stimulus intensity" [Grill & Coghill, 2002].

When stimulating the surface of human skin with heat, pain is usually evoked around 45-53 °C [Chery-Croze, 1983; Almeida et al., 2004]. To evoke the OA phenomenon a stimulus train of three different intensities is usually applied. For thermal stimulation, the first temperature (T1) is within the noxious sensory range, for example 46 °C. The second temperature (T2) is T1 + 1 °C, in this example 47 °C. Finally, the last temperature (T3) is the same as T1, (See Figure 1). A slight increase in perceived pain occurs following the transition from T1 to T2 in the stimulus paradigm and in the absence of endogenous pain modulation, a corresponding decrease would be expected from the transition from T2 to T3. However, OA causes the resultant decrease in perceived pain to be far greater. Indeed, Grill & Coghill displayed a decrease in perceived pain following the transition from T2 to T3 of up to 271



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Figure 1 Stimulus paradigm used to evoke offset analgesia. The first phase, T1, consists of a noxious stimulus intensity for five seconds. The sercond phase, T2, is one degree above T1 also for five seconds. The last phase, T3, is the same temperature as T1 but for a 20 second duration. After this, the temperature decreases back to the baseline of 35 °C.

% of increase caused by transition from T1 to T2 [Grill & Coghill, 2002]. Grill & Coghill were the first to describe the concept of OA, although Robinson and colleagues had published a paper about 20 years earlier where they attempted to uncover the human ability to detect incremental increases in noxious stimulus intensity. The experimental setup was constructed in a manner very much like the ones used in most OA studies today, although a completely different end point was investigated. Still, from their results it is readily apparent that OA was in effect [Robinson *et al.*, 1983].

To this day, the underlying mechanism for OA still remains elusive, and the question remains whether OA is a centrally or a peripherally controlled mechanism. However, studies using functional magnetic resonance imaging, has uncovered some of the potentially implicated structures of the central nervous system facilitating OA. These include the PAG, RVM and locus coeruleus [Derbyshire & Osborn, 2009; Yelle *et al.*, 2009]. As mentioned earlier, these supraspinal structures have been identified as key players in the endogenous modulation of pain and their activity during OA indicates that OA is a centrally modulated mechanism.

The recent years have yielded an increasing number of publications on OA and its potential applications. Offset analgesia has been suggested to serve as a temporal contrast enhancement mechanism to amplify awareness of stimulus offset in order to reinforce escape behaviours. Furthermore, disruption of the normal OA mechanism has been hypothesized to play an important role in chronic pain conditions [Grill & Coghill, 2002]. A recent study examined the effect of OA in patients with small fibre neuropathy. Peripheral neuropathy appears to abolish the OA effect, thereby causing the OA stimulus paradigm to inflict pain that reflects the stimulus intensity to a greater extent [Niesters *et al.*, 2011b]. A schematic representation of the OA phenomenon is illustrated in Figure 2.

As previously mentioned most pharmacological treatment of pain targets the endogenous pain modulating mechanisms. However, to the best of the author's knowledge, OA is not used in pain management, likely because it is still uncertain how OA works. One of the most widely used treatment options is morphine and investigating whether or not morphine exerts an effect on OA might provide valuable information on the underlying mechanism.



Figure 2 Graph representing the OA phenomenon. Initially, the pain intensity increases and comes to a plateau upon reaching the T1 stimulus temperature. Upon reaching T2, the pain intensity increases slightly, but decreases significantly following the decrease in temperature that occurs in the T3 of the stimulus paradigm. After a while, the pain intensity may start to increase again, as the effects of OA gradually diminishes. Also depicted are three key points in the morphology of the pain ratings that occur due to the OA stimulus paradigm. A) is the plateau, B) is the peak, and C) is the nadir.

1.2. Morphine

The analgesic potential of morphine was discovered as early as 3400 BC in Mesopotamia, where it was discovered that slicing the unripe seedpods of the poppy plant, Papaver somniferum, produced a thick milky substance rich in morphine and codeine, harbouring analgesic effects [Trescot et al., 2008]. This remedy was applied for thousands of years but it was not until 1804 that Friedrich Sertürner was able to isolate the pure substance and he subsequently named it Morphium after Morpheus, the Greek god of sleep. Now, it became a much more reliable drug, as the purification facilitated precise dosing [Klockgether-Radke, 2002]. Although the remainder of the present study will focus primarily on morphine, it is important to note that morphine belongs to a large family of analgesics; namely the opioids. Numerous opioids exist and their pharmacodynamic response depend on the receptor to which each opioid bind, its affinity for that receptor and whether it acts as an agonist or as an antagonist. Up to this day, opioids remain widely used in pain management. An example is the treatment of cancer pain in which the WHO guidelines states that orally administered morphine is the first choice in relieving pain of moderate severity or greater [WHO, 1996]. One of the reasons opioids are so widely used is of course the strong pain-relieving effects. This effect arises from the opioid receptors, which normally respond to the endogenous opioids (endorphine, enkephaline, dynorphine, endomorphine).

1.2.1. Receptors

Four different receptors display affinity towards opioids, namely the μ -receptor, the κ -receptor, the δ -receptor, and the opioid receptor like-1 which displays 65 % sequence homology to the other receptors [Fioravanti & Vanderah, 2008]. The primary analgesic effect of morphine arises from its binding to receptors within the central nervous system but the opioid receptors are widely distributed throughout the body, both centrally and peripherally, as all receptors are synthesised within the dorsal root ganglia and from here, axonal transport brings the

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receptors to nerve terminals in the periphery or in central structures [Epstein & Stein, 1995]. As the opioid receptors are all G-protein coupled receptors, the binding of morphine to the receptor causes part of the G-protein to diffuse to an enzyme or an ion channel and thereby indirectly inhibiting voltage-gated calcium channels, which in turn will decrease the amount of intracellular cyclic adenosine monophosphate. This blocks the release of pain neurotransmitters such as glutamate, substance P, calcitonin gene-related peptide from the A δ and C fibres, thus ultimately causing analgesia (see Figure 3) [Trescot et al., 2008]. Within the CNS, µ-receptors are located primarily in the brainstem and medial thalamus and are therefore responsible for the supraspinal analgesic effects of morphine. The κ-receptors are found in greatest quantities within the limbic system, brainstem and spinal cord and are primarily involved with the spinal analgesia, whereas the δ -receptors are found within the brain but their analgesic effects have yet to be fully understood [Trescot et al., 2008].



Figure 3 Illustration of morphine's mechanism of action. All opioid receptors are G-coupled receptor and upon binding, an intracellular part of the receptor inhibits voltage-gated ion channels or activates specific enzymes which can inhibit the voltage-gated ion channels. This decreases the intracellular cAMP, which inhibits the release of several neurotransmitters including calcitonin gene-related peptide, glutamate, and substance P. cAMP = cyclic adenosine monophosphate.

Peripherally, opioids decrease excitability of A δ and C fibres primarily through u-receptors. At spinal level, within the dorsal horn opioids act to decrease ascending nociceptive information by binding to $\kappa\text{-}receptors$ and $\mu\text{-}receptors$ located around A\delta and C fibre terminals in laminae I and II [Cesselin et al., 1999]. Within the CNS, opioid receptors are widely distributed, thereby accounting for the great multitude of effects morphine exerts within the brain (e.g. supraspinal analgesia, respiratory depression, euphoria, sedation, opioid induced bowel dysfunction and physical dependence). When focusing on supraspinal analgesia, most opioid receptors are located within PAG and RVM (chiefly µ-receptors) [Heinricher & Morgan, 1999]. Although PAG does not directly interact with the dorsal horn, it communicates extensively with RVM, which has a large projection to the dorsal horn [Fields et al., 1995]. Opioids act on the RVM through inhibition of both stimulatory and inhibitory centres. Specifically, morphine is capable of silencing the on-cells and accelerating the firing from the off-cells within the RVM. This facilitates the descending inhibitory control arising from the RVM that ultimately inhibits the nociceptive processing in the spinal cord [Fields, 2004]. Furthermore, studies have shown that selective blockade of the off-cells omits the analgesic effect of morphine, indicating that the activation of off-cells is essential for the pain inhibition of morphine [Heinricher et al., 1999].

1.2.2. Pharmacodynamics

The analgesic effect of morphine relies on the binding to the μ -receptors and the κ -receptors on spinal and supraspinal level. Experimental studies often find that opioids increase the pain threshold only marginally. Conversely, opioids are highly effective in providing pain relief even during intense pain. After opioid administration, patients are often able to acknowledge the pain, but to a lesser extent. This indicates that opioids have a great effect on the affective-motivational component of pain and a slight effect on the sensory-discriminatory component of pain [Brunton *et al.*, 2006].

Because the opioid receptors are present in

many different locations they account for a relatively wide array of different adverse effects in different organs including the gastrointestinal tract (nausea, vomiting, constipation, xerostomia), the skin (itch, sweating), the autonomic nervous system (urinary retention, postural hypotension), and the CNS (drowsiness, cognitive impairment, hallucinations, delirium, respiratory depression) [Cherny *et al.*, 2001].

1.2.3. Pharmacokinetics

Morphine can be administered orally, sublingually, bucally, intravenously, rectally, subcutaneously, epidurally, etc. In general, orally administered opioid analgesics are absorbed completely from the intestinal epithelium, but undergoes extensive first-pass metabolism (up to 40 - 50 %), resulting in a relatively low plasma half-life and a bioavailability of 30 - 40 % [Lugo & Kern, 2002]. Approximately 90 % of a morphine dose is converted into metabolites in the liver, predominantly by way of the hepatic enzyme UGT2B7, yielding 50 % morphine-3-glucoronide (M3G) and 10 % morphine-6-glucoronide (M6G) [Yeh et al., 1977]. Other metabolites are formed from the hepatic enzymes CYP3A4 and the highly polymorphic CYP2D6 [Lötsch et al., 1996; Somogyi et al., 2007; Osborne et al., 1992]. The M3G metabolite is not functionally active and can even cause hyperalgesia in high enough concentrations, whereas the M6G metabolite is believed to exhibit an additional analgesic effect of morphine [Lötsch et al., 1996].

Morphine functions primarily at a central level, and its effect on brain wave activity can be measured using electroencephalography (EEG). Accordingly, it is possible to measure how different stimuli alter brain wave activity (e.g. noxious stimuli). Such measurements may provide important information on how the CNS perceives and processes incoming stimuli in the presence and absence of morphine.

1.3. Electroencephalography

In 1924 the German physiologist and psychiatrist Hans Berger published the first recording of the electrical activity within the human brain [Haas, 2003]. The idea that the brain functioned through electrical signalling and that these signals could be recorded and exploited for information underwent extensive research in the following years. Soon it was discovered that the brain waves that could be measured had different characteristics depending on their wavelengths, and electroencephalographic data was subdivided into delta, theta, alpha, beta, and gamma frequency bands (Table 2). Further analysis of the EEG signals includes several different measurements e.g. power in each frequency band and relative changes in power distribution between bands. These measurements have been used in experimental as well as clinical setups [Tonner & Bein, 2006].

Frequency band	Frequency (Hz)
Delta	0.5 - 4
Theta	4 - 8
Alpha	8 - 12
Beta	12 - 30
Gamma	30 - 80+

Table 2 The five different frequency bands and their respectivefrequency ranges.

Today, EEG is clinically used to diagnose epilepsy [Engel Jr, 1984] monitor depth of anaesthesia [Kortelainen *et al.*, 2009; Anderson & Jakobsson, 2006], determine brain death [Grigg *et al.*, 1987], diagnose sleep disorders [Williams *et al.*, 1974] and much more. In an experimental context, EEG is used in several research areas including neuroscience, cognitive science, cognitive psychology, and psychophysiological research.

2. Aims of the Study and Hypothesis

The aim of the present study is to explore whether morphine exerts an effect on OA in healthy human subjects in terms of subjective psychophysical assessments:

- Relative change in pain intensity between peak and nadir pain rating ($\Delta VAS_{Corrected}$).
- Slope coefficient between peak and nadir.
- Temperature applied to reach VAS 7.

Furthermore, the aim is to investigate the potential effect of morphine on OA assessed by electroencephalographic recordings:

- Degree of reproducibility of EEG data recorded during OA.
- Relative change in five frequency bands (delta, theta, alpha, beta, and gamma) during the OA stimulus paradigm.
- The potential effect of morphine on the different frequency bands.

It is hypothesized that the administration of morphine will alter the effects of OA in healthy human volunteers both as seen in the subjective pain ratings as well as in the objective EEG data.

3. Materials and Methods

3.1. Subjects

Fifteen healthy adult volunteers (7 females and 8 males) were included (see Table 3 for demographic details). Before participating in this study every subject gave written, informed consent acknowled-ging that all methods and procedures used in the experiment were understood and that they were aware of that they were going to experience pain and were free to terminate and withdraw from the experiment at any time. The study protocol was approved by the local Ethics Committee (N-20100046), and the study was GCP-monitored.

Height		Weight		Age	
Males	Female	Male	Female	Male	Female
183.43 ± 1.76	168.20 ± 2.98	83.00 ± 5.12	74.60 ± 6.45	26.72 ± 0.91	25.37 ± 0.40
177.08 ± 2.73		79.50 ± 4.03		26.16 ± 0.57	

Table 3 Demographic details for the healthy volunteers who participated in the study. Data (mean \pm SEM) is displayed for both males and females as well as the mean for both genders in the bottom row.

A medical doctor affiliated with Center of Mech-Sense handled the screening process, in which the subjects discussed their relevant medical history (past medical/surgical treatment, potential drug allergies or other adverse effects related to the use of pharmacological drugs). Any ongoing pharmacological treatment was evaluated and noted in the case report form. This was followed by a physical examination including blood pressure and heart rate. The screening process furthermore entailed the same noxious stimuli as would be applied during the actual OA runs, to ensure the subjects knew what to expect and that they could withstand it.

3.1.1. Inclusion Criteria

Inclusion criteria included giving informed written consent, age between 20 and 65 years, Caucasian race, that the medical doctor responsible for the screening process believed that the test subject fully understood and consented to the protocol and that there was no suspicion of drug abuse. Additionally, the subject should be healthy, without any chronic or recurring pathologies. Included in this definition was having a blood pressure of 140/90 or less, a resting heart rate above 45 beats per minute, although exceptions were made if the medical doctor found it justifiable. All female subjects should be using contraceptives and present a negative pregnancy test before initiation of the study. Furthermore, female subjects were asked to participate in the follicular stage of their menstrual cycle, to avoid fluctuations in gonadal hormonal influence on the nociceptive processing [Riley III *et al.*, 1999]

3.1.2. Exclusion Criteria

Exclusion criteria were pregnancy, known allergy towards morphine, ongoing or prior participation in other clinical drug studies within 30 days of the screening, any prior participation in studies using morphine or other opioids, prior addictive behaviour defined as substance abuse (alcohol, cannabis, opioids or other euphoriant substances) or any first-degree relatives with a history of substance abuse. Further exclusion criteria were prior pain conditions or mental illnesses, expected need of medical treatment, surgery or hospital admission during the course of the study, ongoing use of strong analgesics, and use of any kind of analgesics within 24 hours of the study.

Moreover, none of the subjects displayed any apparent wounds, scars, or tattoos on their forearms. A flowchart of the test subject inclusion/exclusion process can be seen in Figure 4.

3.2. Procedure

Each subject participated in a screening session prior to the start of the actual experiment, which consisted of two different days separated by at least 10 days (mean 20 ± 9 days). The two days of experimentation followed the exact same protocol except for the treatment with either placebo or morphine.

Upon arriving to the research facility, all subjects were fitted with a 64-channel EEG cap (Quick-Cap International, Neuroscan, El Paso, Texas, USA) and



Figure 4 Flowchart of the inclusion/exclusion process. Fifteen subjects entered the screening, 14 entered the actual experiment, 13 completed the study, 12 were suitable for data analysis on subjective pain ratings, and 9 were suitable for data analysis on objective EEG data.

instructed in correct usage of the visual analogue scale (VAS). Subsequently, their left volar forearm was divided into three adjacent, yet distinctive sites (proximal, central and distal site), see Figure 5. As the subjects would be exposed to heat pain in the experiment, their individual pain tolerance levels (VAS 7) towards heat pain were determined on both treatment days. The temperature corresponding to the subject's pain tolerance level was applied as the T2-temperature in the aforementioned stimulus paradigm. The first OA stimulus paradigm (Run 1) was conducted at the proximal site, and subsequently the thermode was moved to the central site and lastly to the distal site. The



Figure 5 The three stimulation sites of the left volar forearm.

OA stimulus paradigm was conducted on two different time points, the first at t = 0 minutes, immediately before administration of placebo/morphine and the second at t = 120 minutes, two hours after administration of placebo/morphine. At each time point three runs of the OA stimulus paradigm were conducted; at the proximal, central, and distal site, respectively.

Each test subject received 30 mg morphine (Batch: 841461, oral liquid mixture 2 mg/mL, Sygehusapoteket RN, Aalborg, Denmark) and placebo (Batch: 802898, oral liquid mixture, Sygehusapoteket RN, Aalborg, Denmark). In order to secure blinding of the taste, both morphine and placebo was mixed into a glass of orange juice and administered orally. A pharmacist, not otherwise included in the study, prepared this mixture. If the subjects were dizzy, nauseous, or otherwise influenced by the morphine after the experiment had concluded, they were observed in the test room until they had recovered. All test subjects were contacted 24 hours after each treatment day and asked to report their general well being and to report the adverse effects they had experienced, if any.

3.3. Thermal Stimulation

Heat stimulation was applied to the volar surface of the left forearm using a 27 mm Standard Thermode (see Figure 6) connected to Medoc's PATHWAY Pain & Sensory Evaluation System (Contact Heat-Evoked Potential Stimulator, CHEPS) with PATHWAY software 4.0.11.0. To minimize the influence of habituation, the volar surface of the forearm was measured from the elbow joint to the wrist, and divided into three adjacent, yet distinctive zones in the middle of the arm, and five centimetres proximal and distal to the central site, see (Figure 5).

3.3.1. Determination of the Stimulus Intensity

Rather than using predefined noxious stimulus intensity, all subjects defined their individual pain tolerance level (VAS 7) prior to the actual experiment. The thermal intensity was gradually increased 1.5 °C/s from a baseline of 35 °C. Upon reaching the pain threshold, the test subject was able to prevent further increase in temperature at the press of a button. Three such measurements were carried out at the distal site of the left forearm and the average was used in the following OA-experiment, as depicted in Figure 7.



Figure 6 The CHEPS thermode. The stimulating area has a diameter of 27 mm, which corresponds to a stimulating area of 572.5 mm². The thermode is fixed with a velcro strap.



Figure 7 Example of the determination of the test subjects' individual VAS 70. Three runs are conducted where the test subject clicks a button upon reaching his/her VAS 7, and the average is used as the T2-temperature in the ensuing stimulus paradigm.

3.4. Experimental Runs

Each experimental run consisted of three contiguous phases; an initial noxious stimulus temperature [T1, VAS 7 - 1 °C, 5-second duration], a 1 °C increase to a second temperature [T2, VAS 7, 5-second duration], and a decrease back to the temperature used in T1 [T3, VAS 7 - 1 °C, 20-second duration] as depicted in Figure 1. Following T3, the temperature was decreased back to baseline (35 °C) at a velocity of 1.5 °C/s. The individual temperature corresponding to VAS 7 was found on both experimental days, as this could differ between days for the same test subject. After determination of the VAS 7 temperature three OA runs were conducted first on the proximal site, then the central and finally the distal site. The first run at the proximal site was considered a test run, in which the subjects could familiarize themselves with the pain experience without focusing on scoring the evoked sensation on the CoVAS instrument. The experimental setup can be seen in Figure 8.



Figure 8 Schematic representation of the study protocol. A = Screening, B = Placebo day, and C = Morphine day. Note that both placebo day and morphine day are identical except for the treatment. All OA runs marked in gray were discarded.

3.5. Stimulation Perception and Intensity Rating

During the last two OA runs, the subjects were asked to evaluate the pain intensity continuously using Medoc's CoVAS (Computerized Visual Analogue Scale), see Figure 9. The subjects were instructed to evaluate both innocuous sensation and noxious sensation, as both innocuous as well as noxious ranges were included in the utilized scale (see Figure 10).



Figure 9 Experimental setup. The OA stimulus paradigm was applied to the left forearm, leaving the right hand free to operate the CoVAS instrument. Simultaneously, EEG data was recorded.

	10	Unbearable/worst imaginable pain
Innocuous Noxious	9	Great pain
	8	Moderately great pain
	7	Moderate pain
	6	Mild pain
	5	Discomfort/Pain threshold
	4	Moderate sensation, clearly felt
	3	Moderate sensation, barely felt
	2	Mild sensation, clearly felt
	1	Mild sensation, barely felt
	0	No sensation

Figure 10 VAS applied in the present study. Please note, that the scale contains the innocuous sensory range as well as the noxious sensory range in a continuous succession.

3.6. Adverse effects

All subjects were asked to score their perception of the most common adverse effects associated with a single dose of morphine (regardless of having received placebo or morphine); itching, sweating, dizziness, and nausea. They were asked at t = 15, 30, 45, 120, and 150 minutes after administration.

3.7. Data

3.7.1. Subjective pain ratings

Subjective pain ratings obtained from the last two runs at each time point (t = 0 and t = 120) were averaged over 1-second periods, and the peak and nadir were determined manually for all subjects. The decrease in pain ratings that occurred between peak and nadir was determined as ΔVAS (see Figure X). In other studies this value is known as the magnitude of OA [Martucci et al., 2011; Martucci et al., 2012; Quevedo et al., 2009] or Δ_{a} VAS [Niesters et al., 2011a; Niesters et al., 2011b] The ΔVAS scores was corrected for the peak value to circumvent a potential artefact arising from variability in peak VAS scores between subjects by dividing Δ VAS with the peak VAS value ((peak-nadir)/peak). The corrected data for Δ VAS was termed Δ VAS_{Corrected}. Furthermore, the slope coefficient between peak and nadir was determined and examined across runs and days to determine whether a significant difference in the velocity of ΔVAS pain decrease was present. Both $\Delta \text{VAS}_{\text{corrected}}$ and the slope coefficient had previously proved highly reproducible (Appendix A). When performing statistical analyses between treatment days the data from both treatment days was baseline corrected by subtracting the t = 0 data points from the t = 120 data points, which allowed for optimal comparison.

3.7.2. Objective EEG data

Electroencephalographic recordings were carried out in the complete duration of the OA experiment (30 seconds) in order to obtain the spontaneous brain activity that occurred in response to the OA stimulus paradigm. The signals were recorded using a SynAmp 2 system (Neuroscan, El Paso, Texas, USA) and a standard 64 channel cap with Ag/AgCl surface electrodes mounted according to the extended 10/20 system [Knott, 2000]. An additional four electrodes detected eye movement allowing this to be extracted. The impedance was kept below 5 k Ω by applying Electro-gel (Electrocap international, Inc., Eaton, Ohio, USA). Recordings were performed in AC-mode with sampling frequency of 1000 Hz and online band pass filter from 0.5 to 200 Hz. All recordings took place in a quiet room with dimmed light and all unnecessary equipment was turned off to minimize background noise and artefacts. The volunteers were instructed to keep as still as possible while the OA runs were being conducted.

The EEG recordings were subjected to offline post-processing using Neuroscan 4.3.1., (Compumedics, El Paso, Texas, USA). The post-processing included notch filtering and visual inspection of the data quality for all 64 channels. All channels displaying abnormal signals in terms of level and/ or shape were discarded and replaced by interpolating the adjacent channels. For three subjects the majority of channels were abnormal, leaving little room for interpolation, and all recordings from these subjects were discarded. At this point the data was still 30-second recordings of raw EEG data. In order to analyse the data, it was crucial to separate the different frequencies (delta, theta, alpha, beta, and gamma from one another). This was done using a wavelet transform which gives a time-frequency representation of the EEG recording. This allowed for inspection of the frequency distribution as a function of time (Figure 11). Subsequently, all 30-second raw EEG recordings were divided into six epochs, each lasting for five seconds. Within each five-second epoch, the distribution of frequency bands (delta, theta, alpha, beta, and gamma) was determined and normalised to yield a 100 percent in total. The percentage-wise composition of wavelengths within the six epochs enabled comparison between epochs to examine whether the frequency distribution changed over time.

Reproducibility was examined both within each treatment day and between the two treatment days for the five frequency bands within each of the six epochs. When performing statistical analyses between the treatment days, the data was baselinecorrected by dividing the t = 120 data with the t = 0 data. From the results in Appendix B it was feasible to conduct statistical analyses on the data from the present study to investigate if an alteration in frequency distribution can be detected due to morphine treatment. Based on EEG recordings from the OA reproducibility study (9th semester) the method proved reproducible. All reproducibility results are presented in Appendix B.



Figure x The wavelet transform. A = Raw EEG data from one electrode. Here we can see the total current as a function of time. B = the wavelet transform. This separates the total current into the relative distribution of the five frequency bands. The ranges of the respective frequency bands is depicted in the right side.

3.8. Statistical Analysis

3.8.1. Subjective pain ratings

Within both the placebo day and morphine day, a Two Way Repeated Measures (RM) ANOVA (with factors Time point (t = 0 and t = 120) and Run (Run 1 and Run 2) was used on parameters $\Delta VAS_{corrected}$ and slope coefficient. When appropriate, a Tukey's Post Hoc test was performed. *P*-values < 0.05 were considered significant.

3.8.2. Objective EEG data

A Two Way RM ANOVA with factors Epoch (1, 2, 3, 4, 5, and 6) and Time point (t = 0 and t = 120)) was used to investigate whether changes occurred between time points. Similarly, a Two Way RM ANOVA with factors Treatment (placebo and morphine) and Frequency band (delta, theta, alpha, beta, and gamma) was used within all six epochs to investigate whether changes occurred between treatments. A Tukey's Post Hoc test was performed when appropriate. *P*-values < 0.05 were considered significant.

4. Results

Fifteen healthy volunteers that were included in the present study, and thirteen completed. Two subjects were excluded; one was excluded due to adverse effects and one due to personal reasons. Of the thirteen that finished, one subject was excluded from the subjective pain rating data analysis due to poor data quality and an additional three subjects were excluded from the EEG data analysis due to poor data quality. A few of the remaining 12 subjects experienced slight adverse effects, but all reported no adverse effects at the 24-hour follow-up.

4.1. Subjective pain ratings

Graphs depicting the mean pain ratings within the placebo day (Figure 12) and within the morphine day (Figure 13).



Figure 12 Graphs depicting the pain ratings on the placebo day. The light green curve represents the mean pain ratings at t=0, whilst the dark green curve represents the mean pain ratings at t=120. The shaded areas represent the respective standard error. The thin red line represents the stimulus temperature.



Figure 13 Graphs depicting the pain ratings on the morphine day. The orange curve represents the mean pain ratings at t=0, whilst the red curve represents the mean pain ratings at t=120. The shaded areas represent the respective standard error. The thin red line represents the stimulus temperature.

4.1.1. Reliability of data

Placebo

No differences were detected between Run 1 and Run 2, within t = 0 and t = 120 for either of the parameters $\Delta VAS_{Corrected}$ (F = 0.02, P = 0.90), and slope coefficient (F = 0.95, P = 0.35). (See Figure 14 and Figure 15).



Figure 14 Bar chart displaying $\Delta VAS_{corrected}$ on the placebo day. The light green bars represent $\Delta VAS_{corrected}$ from the two runs at t=0 and the mean between the two runs. The dark green bars represent $\Delta VAS_{corrected}$ from the two runs at t=120 and the mean between the two runs.



Figure 15 Bar chart displaying the slope coefficient on the placebo day. The orange bars represent $\Delta VAS_{Corrected}$ from the two runs at t=0 and the mean between the two runs. The red bars represent $\Delta VAS_{Corrected}$ from the two runs at t=120 and the mean between the two runs.

Morphine

No differences were detected between Run 1 and Run 2, within t = 0 and t = 120 for either of the parameters $\Delta VAS_{Corrected}$ (F = 1.61, P = 0.23), and slope coefficient (F = 0.45, P = 0.52). (See Figure 16 and Figure 17).



Figure 16 Bar chart displaying $\Delta VAS_{corrected}$ on the morphine day. The orange bars represent $\Delta VAS_{corrected}$ from the two runs at t=0 and the mean between the two runs. The red bars represent $\Delta VAS_{corrected}$ from the two runs at t=120 and the mean between the two runs.



Figure 17 Bar chart displaying the slope coefficient on the morphine day. The orange bars represent the slope coefficient from the two runs at t=0 and the mean between the two runs. The red bars represent the slope coefficient from the two runs at t=120 and the mean between the two runs.

The fact that the degree of reliability within each time point was high, as no statistically significant differences could be detected, allowed for further investigation of the data. Therefore, Run 1 and Run 2 were pooled within each time point in order to examine whether a statistically significant difference in $\Delta VAS_{Corrected}$ or slope coefficient appeared between the two time points for each treatment day.

4.1.2. Within treatment days

Placebo day

No alteration in $\Delta VAS_{Corrected}$ was seen within the placebo day (F = 0.13, P = 0.72). However, the slope coefficient decreased at t = 120 (-7.65 ± 0.79 vs. -5.74 ± 0.44; P = 0.01). (See Figure 14 and Figure 15).

Morphine day

Within the morphine day, $\Delta VAS_{Corrected}$ increased significantly at t = 120 (0.76 ± 0.07 vs. 0.85 ± 0.05; P < 0.01), whereas the slope coefficient was not altered (F = 0.73, P = 0.41). (See Figure 16 and Figure 17).

4.1.3. Between treatment days

 $\Delta VAS_{Corrected}$ was significantly higher on the morphine day (0.01 ± 0.03 vs. 0.12 ± 0.03; *P* < 0.01) compared to the placebo day. The slope coefficient was unaltered (*P* = 0.29). (See Figure 18 and Figure 19). Finally, no statistically significant difference was detected in the temperature applied to reach a pain rating of VAS 7 between days (*P* = 0.72). (See Figure 20).



Figure 18 Bar chart displaying $\Delta VAS_{corrected}$ between the placebo day and the morphine day. The light green bar represent $\Delta VA-S_{corrected}$ on the placebo day and the orange bar represent $\Delta VA-S_{corrected}$ on the morphine day. Note that both bars have been baseline corrected so the y-axis is displayed in arbitrary units.



Figure 19 Bar chart displaying the slope coefficient between the placebo day and the morphine day. The light green bar represent the slope coefficient on the placebo day and the orange bar represent the slope coefficient on the morphine day. Note that both bars have been baseline corrected so the y-axis is displayed in arbitrary units.



Figure 20 Bar chart displaying the mean temperature required to reach a pain rating af VAS 7 between the placebo day and the morphine day. The light green bar represent the VAS 7 temperature on the placebo day and the orange bar represent the VAS 7 temperature on the morphine day.

4.2. Objective EEG data

4.2.1. Reproducibility of EEG data

The degree of reproducibility for the EEG data was very high both within the same treatment day as well as between different treatment days (Appendix B) and the present data was suitable for further statistical analysis.

4.2.2. Within treatment days

Within the placebo day, a decrease in delta band content during epoch 2 (27.1 \pm 1.59 % vs. 23.45 \pm 1.81 %; *P* = 0.02) and an increase in beta band content during epoch 2 (29.58 \pm 1.18 % vs. 32.21 \pm 1.3 %; *P* = 0.03) was seen at t = 120.

During morphine administration no alterations in frequency distribution were seen (all *P*-values > 0.14)

4.2.3. Between treatment days

No alterations in baseline-corrected frequency distribution between treatments were seen (all Pvalues > 0.11)

5. Discussion

The present study finds that morphine does exert an effect on OA, in terms of $\Delta VAS_{Corrected}$ being increased in healthy human volunteers treated with morphine. Conversely, the slope coefficient decreases when healthy human volunteers are administered placebo, but not so when they are administered morphine. Moreover, a change in the EEG data is only found between time points on the placebo day. Taken together, this suggests a general opioidergic influence on the control of OA. Because opioid receptors are found both peripherally and centrally, the fact that the OA effect is enhanced on the morphine day can not stand alone in providing a definitive answer as to whether OA is a peripheral or centrally located mechanism.

5.1. Methodological considerations

5.1.1. Modified pain scale

The pain scale that was utilized in the study was an extension of the classic VAS scale which is a 10 cm continuous line with two extremes as end points only evaluating painful sensation (e.g. "no pain" and "the most intense pain imaginable") [Price et al., 1983]. The modified version utilized in the present study included both the innocuous and noxious sensory range. Additionally, eight anchor points in between the two extremes had been added to guide the subjects in their subjective score as it was expected that it was more difficult to score both innocuous and noxious sensation accurately at the same time. Additionally, it should be kept in mind that different receptors and different afferent nerve fibres respond to and convey information from innocuous and noxious stimuli. Hence, the modified scale could be expected to be less reliable than other previously used pain scales.

Nevertheless, the scale has been validated in a previous study [Drewes *et al.*, 2003], and successfully applied in numerous pain studies [Brock *et al.*, 2008; Brock, 2009; Andresen *et al.*, 2010; Staahl *et al.*, 2006b].

5.1.2. Dosing

In the clinic, the typical dose range for orally administered morphine in adults is 10-30 mg every four hours. The present study administered a single dose of 30 mg orally, which is in concordance with other similar morphine studies [Olesen *et al.*, 2010; Staahl *et al.*, 2006a; Säwe *et al.*, 1981; Säwe *et al.*, 1983; Kaiko *et al.*, 1992]. The OA experiment was conducted 120 minutes after administration, and all subjects were opioid naïve. As the pharmacokinetic properties of morphine cause the peak plasma concentration to occur between one and three hours after oral administration, we believe that the present dosing was sufficient to attain a pharmacological effect in healthy volunteers.

5.1.3. Offset Analgesia Stimulus Paradigm

In the present study, all subjects defined their individual pain tolerance level (VAS 7), on each of the investigation days. As described in Section 3.3.1. this temperature is applied as stimulus temperature during the T2 phase, and the T1 and T3 phases are 1 °C below that. This method ensures that all subjects experience the same subjective pain level, as the perception of pain has been shown to be highly variable both inter-individually and intra-individually [Melzack & Casey, 1968; Treede et al., 1999; Brock, 2009]. This method has its limitations, however. When determining each subject's VAS 7 level each day, we did three stimulations at the same location on the arm and averaged them to obtain the VAS 7 level. In order to avoid temporal summation or habituation at the distal site during these three runs, the temperature was increased quite rapidly (1.5 °C/s), which might have made it difficult for the subjects to hit the button at the exact time that VAS 7 was reached. Although three runs were done to alleviate this methodological downside, the subjects were exposed to 30 seconds close to the predetermined VAS 7 level during each OA run. This is a significantly prolonged period of time compared to rapidly reaching VAS 7 and then returning to baseline (35 °C). Therefore, it might have been more painful than intended during the OA experiments.

5.1.4. Subjective Pain Parameters

Based on findings from the previous OA reproducibility study, the investigated parameters ΔVAS_{cor-} and the slope coefficient were used, as they were the most reliable parameters both within the same days and between different days (Appendix A). In the present study, these findings were supported as assessment of these parameters once again was very robust and the author recommends their use in future studies investigating OA.

5.1.5. EEG Epoch Length

The 30 seconds of EEG recording from each OA experiment was subdivided into six five-seconds epochs which corresponded to the changes in temperature in the stimulus paradigm and allowed for investigation of the dynamics in EEG data over time. Considering that there is some degree of latency between the temperature changes on the arm until the new level of pain is fully comprehended in the brain, these epochs might not adequately reflect the way pain is perceived. Because each epoch is a mean of all the activity within each band for the full five-seconds duration, this mean might be obscured if this latency is heavily pronounced. For example, we would get an "untrue" mean value if we have a five-second epoch and the first two seconds have strong activation of the delta band, and the three last seconds have no activation of the delta band. In this case we could end up with a mean for that epoch where this dramatic change in delta activity went unnoticed. The EEG was recorded with a sampling frequency of 1000 Hz, so we have 30,000 data points, which have been reduced to the present six data points. This might be a too rough reduction, so that important information is lost, with as low resolution as used here. However, we started out with one-seconds epochs, i.e. 30 data points, from which it was apparent that the changes that occurred were not outspokenly rapid, nor abrupt, so for the sake of convenience a resolution of six epochs were chosen. Furthermore, the reproducibility tests conducted on the data set from Appendix A, proved that EEG data organised this way is highly reproducible, as seen from Appendix B.

5.1.5. EEG Method

The question can be raised exactly what is seen in the EEG data. Is it alteration of the neural processing during OA, is it the morphine effect or is it altered afferent input to the brain due to peripheral adaptation? First of all, it should be recognised that when the input to the CNS is altered at the peripherally, as occurs when we change the temperature in the stimulus paradigm, it will reflect the EEG activity. Moreover, when doing continuous EEG recordings it is important the subject sits completely still to avoid muscle artefacts. The fact that we both applied pain to the volar forearm for 30 seconds and that we had the subjects using their arm to control the CoVAS instrument might have caused a potential motor artefact in the EEG data. However, the motor contribution to the EEG recordings was present in all recordings and on both treatment days.

5.2. Present findings

In the present study, a significant difference in the relative change in pain perception ($\Delta VAS_{corrected}$) between t = 0 and t = 120 was found only within the morphine day. It is found that morphine causes the effect of OA increased. This finding supports the notion that the opioidergic system plays a part in the control of OA, in that the pathway that usually facilitates the effect of OA already is activated to a greater extent than during baseline due to the administration of morphine. Morphine naturally activates opioidergic receptors and hence the opioidergic system, which includes central structures such as PAG/RVM, thalamus, dorsolateral prefrontal cortex, midcingulate cortex, and the insular cortex.

Additionally, it was found that the slope coefficient was significantly different between t = 0 and t =120 only on the placebo day. It is important to note that the slope coefficient was steeper at t = 0 i.e. analgesic effect occurred faster at t = 0. In order to avoid a potential confounding effect, the stimulus paradigm was blinded to the subjects. However, as the stimulus paradigm was identical between runs it could be speculated that the subjects realized that the OA stimulus paradigm was identical every time, and consequently, become more proficient in rating the pain accordingly. Furthermore, the effect of such a learning curve would be expected to be least pronounced within the morphine treatment day where the subjects expectedly are slightly sedated, or merely paying attention to the overall effects of being treated with morphine, therefore not paying as much attention to the stimulus paradigm. Although the study was double-blinded several subjects expressed their subjective experience of whether or not they had been administered morphine. Taken all together, it is likely that such a learning curve would be most pronounced within the placebo day.

Concerning the EEG data, several tendencies have been observed in human subjects exposed to tonic pain. These tendencies include an increase in delta power [Huber *et al.*, 2006], decrease in alpha power [Backonja *et al.*, 1991], and increase in beta power [Chang *et al.*, 2004]. Often, the cold-pressor test is utilized to induce tonic pain during EEG recordings [Backonja *et al.*, 1991; Chen & Rappelsberger, 1994], but other examples exist, including injections of capsaicin into skin and muscle [Chang *et al.*, 2001b; Chang *et al.*, 2001a], and injections of hypertonic saline into muscle [Chang *et al.*, 2002].

Similarly, Huber and colleagues exposed their subjects to tonic heat pain and saw a generalized increased delta activity, diminished fronto-temporal theta activity, a fronto-temporal decrease in alpha activity and a left-sided temporal increase in beta activity. It is interesting however, that when the same subjects were exposed to innocuous heat stimulation the same changes in EEG activity was seen. Therefore, the observed changes in EEG activity may be general for somatic sensation and not specific for pain [Huber *et al.*, 2006].

5.3. Comparison with other studies

Controversy exists in the literature, concerning the potential effect of opioids on OA. In a recently published study, the effect of the μ -opioid antagonist naloxone and the μ -opioid agonist remiferitanil on several psychophysical end points of the OA phen-

omenon was investigated [Martucci *et al.*, 2012]. This study did not detect any changes in OA despite the administration of naloxone and remifentanil, and therefore the authors concluded that the opioidergic system plays an insignificantly small part (if any) in the control of OA. Our findings challenge this theory, as a direct effect of morphine was observed, and the question has to be asked why two studies with very similar setups can be in fundamental disagreement?

In order to answer this question, we need to examine the methodology between the two study groups. It has already been established that OA is able to inhibit perceived pain quite vigorously on its own [Grill & Coghill, 2002; Yelle et al., 2008; Derbyshire & Osborn, 2008; Derbyshire & Osborn, 2009; Martucci et al., 2011], to the point of complete pain relief [Niesters et al., 2011a; Niesters et al., 2011b]. However, instead of only focusing on the noxious sensory range, the present study has included the innocuous sensory range in a combined scale as displayed in Figure 10. This allows the investigator to study the phenomenon in both the noxious sensory range, but also in the innocuous sensory range. The findings from the present study reveals that the effect of OA appears deeply into the sensory range, and it became apparent that OA is capable of diminishing the perception of innocuous heat (Appendix A). As it is seen from Figure 12 and Figure 13, it is also within the innocuous range that the OA effect appears to be amplified in healthy human subjects under the influence of morphine. If all morphine-related modulation of the OA effect occurs within the innocuous sensory range, this will account for the discrepancy between the conclusions in the present study and that of Martucci and colleagues. Moreover, the finding of the effect of naloxone on OA, does not completely rule out the role of the opioidergic system as it has been shown that naloxone only to a certain extent negates the opioidergic system and appears to be dose-dependent. Studies using naloxone doses at 0.14 mg/kg [Sprenger et al., 2010] and 0.15 mg/kg [Leonard et al., 2010] succesfully revert the effect of opioids, whilst 0.02 mg/kg [Leonard et al.,

2010] and 0.01 mg/kg (as used in the discussed OA experiment) does not have an effect on opioidergic activation [Martucci *et al.*, 2012]. Therefore, it would be very exciting to see whether an increased dose of naloxone would affect OA.

Another important methodological difference in the stimulation paradigm exists in the temperatures applied in the OA stimulus paradigm. Where the present study applies individually determined VAS 7-temperatures, Martucci and colleagues applies a predetermined stimulus paradigm for all subjects. Additionally, the average temperature needed to attain a pain rating of 7 on the VAS scale was on either of the experiment days was well below the predefined stimulus temperature in the study by Martucci and colleagues where the stimulus paradigm always was [T1 = 48 °C (5 seconds). T2 = 49 °C (5 seconds). T3 = 48 °C (20 seconds)]. Naturally, their method ensures constant stimulus intensity across subjects, but one should be aware that large inter-individual differences in perceived pain might occur, which might obscure the results obtained especially for the magnitude of OA (ΔVAS) , because this parameter varies greatly depending on the peak pain intensity. In that regard, it would be highly interesting to see if Martucci and colleagues would display an effect of naloxone/remifentanil if the magnitude of OA was corrected for the peak value.

Given that we pursue the idea that OA is controlled at least partially by the opioidergic system then what can we learn from this information? First of all, this information can be used in the determination of OA as a peripherally or centrally controlled mechanism.

5.4. Offset Analgesia: A Peripheral or Central Phenomenon?

It is known that opioid receptors exist in the periphery on primary afferent neurons where they are capable of exerting both anti-inflammatory and analgesic properties [Epstein & Stein, 1995; Stein *et al.*, 2003; Joris *et al.*, 1987]. As described in Section 1.2.1. both μ , δ , and κ -opioid receptors are synthe-

sised in the dorsal root ganglia and transported to the periphery intra-axonally. In order to assess the effects of opioids on the periphery, the central effects have to be negated as much as possible. This has been achieved in both preclinical and clinical studies by administering opioids with high hydrophilicity, which therefore do not readily cross the blood brain barrier. It was discovered that topically applied loperamide effectively exerts anti-hyperalgesic properties towards heat pain in animal studies [Nozaki-Taguchi & Yaksh, 1999]. Furthermore, peripherally restricted selective κ-opioid receptor agonists have been found capable of inducing analgesia without any centrally mediated adverse effects [Kumar et al., 2000; Kumar et al., 2005]. Although morphine is able to cross the blood brain barrier, its peripheral effects have been assessed in studies where morphine was administered intra-articularly in the knee joint post-operatively. Here, it provided strong analgesia comparable with patients receiving intravenously administered morphine, thus suggesting potent peripheral effects of the opioid receptors [Stein et al., 1991; Kalso et al., 2002].

In relation to OA, the fact that morphine is able to modulate nociception both at peripheral and central level, one should be careful to draw conclusions as to OA as a peripheral or central mechanism.

Additionally, Huber and colleagues were able to determine that somatic stimuli (both noxious and innocuous) generally increase power in the delta frequency band of EEG recordings. In the present study, these changes were not detected, but it is important to keep in mind that the present stimulus paradigm was dynamic rather than tonic. Moreover, fundamental methodological differences exist between the studies, where the abovementioned studies investigate changes in frequency distribution over time in response to a tonic stimulus, the present study investigates changes in frequency distribution between different time points and different treatment days in response to a dynamic stimulus. It is not surprising that disagreement between findings appear.

However, if we suppose that the intensity of

somatic sensation can be directly seen in the relative distribution of delta waves in the EEG data, it is interesting that the present study finds that the delta power is decreased within epoch 2 at t = 120 on the placebo day, and that nothing happens on the morphine day. It is within the second epoch where the highest stimulus temperature occurs and we would expect to see strong delta power in this epoch. The fact that it is significantly lower at t = 120 on the placebo day indicates that the OA stimulus paradigm is less painful after two hours of placebo treatment. As the stimulus paradigm was completely identical between runs this is highly unlikely. Rather, the decrease could be a result of the aforementioned learning curve, which is likely to be more pronounced during the placebo day. If the subjects had learned that the stimulus paradigm was completely identical between runs, they might expecting the temperature to increase around 5-10 seconds (epoch 2) and be less surprised by the temperature increase as it occurred. This would decrease the negative emotional response to pain, which has been thoroughly investigated in the past, and there is clear consensus that negative emotional responses to an existing pain (aversion, frustration, anger, sadness) correlates positively to the perceived pain intensity [Wade et al., 1990; Wade et al., 1996; Okifuji et al., 1999; Fernandez & Milburn, 1994; Fernandez & Turk, 1995; Keefe et al., 2001]. Furthermore, if OA was a peripheral phenomenon, the delta power would be expected to decrease dramatically due to the OA effect (probably in epochs three to five where the OA occurs), if the sensory input reaching the CNS was limited peripherally. The fact that the delta power is unaltered within these epochs even on the morphine day, where we saw an amplified effect of the subjective pain ratings at t = 120, might be an indication that the input from the periphery is unaltered, thus speaking in favour of OA being a centrally located mechanism.

Studies investigating OA coupled with functional magnetic resonance imaging have displayed evidence that several brain regions mediating pain relief (PAG/RVM) were more active during OA runs opposed to constant noxious temperature runs

[Derbyshire & Osborn, 2009]. Accordingly, the same study displayed a decreased activation of several brain regions associated with pain perception (thalamus, midcingulate cortex, S1 and S2) during the OA runs opposed to the constant temperature runs. A similar study also found increased activation of PAG, cerebellar regions, thalamus, dorso-lateral prefrontal cortex, midcingulate cortex, and insular cortex during OA [Yelle *et al.*, 2009]. The fact that these areas are more active during the OA stimulus paradigm than during constant pain speaks in favour of OA at least in part, being a centrally controlled mechanism.

On the other hand, a recent study investigated the potential effect of remifentanil and naloxone on OA [Martucci *et al.*, 2012]. Here, it was discovered that administration of the opioid agonist remifentanil and the opioid antagonist naloxone did not affect the OA phenomenon. This contradicts the notion that the abovementioned brain structures are involved in the elicitation of OA because areas such as PAG is strongly associated with opioid analgesia [Mansour *et al.*, 1988]. Therefore, naloxone would effectively bind to the μ -receptors within the PAG, thereby at least suppressing its effect. The fact that OA persists despite the administration of naloxone defies that the opioidergic system mediates OA.

Another line of evidence against OA being a centrally controlled mechanism comes from the discovery that ketamine infusion did not affect OA [Niesters et al., 2011a]. Ketamine binds to the Nmethyl-D-aspartate receptors (NMDAR) within the spinal cord where it acts as an antagonist, thereby negating the pain facilitating function of the NM-DARs which in turn causes pain relief [Petrenko et al., 2003]. The selective binding of the NMDAR prevents central sensitization within the spinal cord and as an effect this decreases the ascending input to higher order structures and consequently decreases the perception of pain [Quibell et al., 2011]. The fact that ketamine alters the normal pain processing of the glutamatergic system, and that OA remains unchanged indicates that the glutamatergic system plays an insignificant part in the control of OA.

Because a large part of the central pain processing relies on the opioidergic and the glutamatergic system and these appear to be without great influence on OA, it speaks against OA being centrally controlled.

In order to further substantiate whether OA is a peripheral or a central mechanism, future studies could utilize microneurography, a method that allows measuring amplitude and firing rate of individual nerves [Wessberg et al., 2003; Schmelz & Schmidt, 2010; Weidner et al., 2002; Schmidt et al., 2002]. Microneurography conducted in the lateral/ medial antebrachial cutaneous nerve would give a definitive answer as to whether the afferent signal is modulated during OA. If the afferent signal decreases in amplitude and firing rate corresponding to the decrease in pain perception, then OA is likely a peripheral phenomenon. However, if the afferent signal remains unchanged, the pain modulation would have to occur at spinal or supraspinal level. Microneurographic recordings have previously been conducted from these nerves in humans [Vallbo et al., 1993; Vallbo et al., 1995; Macefield, 2005; Olausson et al., 2000] and monkey [Slugg et al., 2000].

A clear definition of OA is still needed if the true clinical potential is to be discovered. For the moment we can only speculate that OA can be used as a prognostic tool, in diseases such as diabetic neuropathy. Furthermore, as most pain management aims at either suppressing pain-facilitating mechanisms or enhancing pain-diminishing mechanisms OA might be a potential therapeutic target in future pain management. Although the analgesic effect we are able to elicit experimentally, only lasts some 20 seconds, it can be speculated that detailed elucidation of the OA pathway would allow for more long-lasting activation. Considering how powerful the analgesic effect appears to be, "OA treatment" could possibly benefit a very large population of pain patients.

Other potential uses include experimental usage. Due to the high reproducibility of OA, and the very short period of time it takes to conduct an OA experiment, it is easily implemented into pain research and future analgesic drug trials.

6. Conclusion

Morphine exerts an effect on OA, which reflects in the subjective pain ratings. Specifically, morphine amplifies the effect of OA, causing an alteration in the sensory profile, most prominent in the innocuous sensory range. The complete clinical potential of OA has yet to be discovered but it exhibits very promising potential due to its powerful effect and high degree of reproducibility. Future studies investigating OA using microneurography is exceptionally relevant, as this will provide a pivotal piece of information regarding the anatomical and physiological location of OA.

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Appendix A

Offset Analgesia: A Reproducibility Study

9th Semester Project Group 942 Medicine with Industrial Specialisation

Matias Nilsson



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Matias Nilsson

Supervisor(s): Parisa Gazerani, Associate Professor at the Department of Health Science and Technology, Aalborg University and Christina Brock, Research Assistant, DVM and PhD at Center of Mech-Sense, Aalborg Hospital

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Preface

This report was written by Matias Nilsson, group no. 942, Translational Medicine, 3rd semester of the Master's Degree in Medicine with Industrial Specialisation, Aalborg University, Denmark. The project was compiled from September 15th, 2011 to January 4th, 2012. The project was supervised by internal supervisor Parisa Gazerani, Associate Professor at the Department of Health Science and Technology, Aalborg University and external supervisor Christina Brock, Research Assistant, DVM and PhD at Center of Mech-Sense, Department of Gastroenterology, Aalborg Hospital.

The report is structured as a scientific article, where the Harvard format is used for referencing. First chapter is an introduction giving the reader an overview of topics relevant to the project. The following chapters include materials and methods, results, and discussion, which lead to a general conclusion of the project and future perspectives.

The structure throughout the report is based on a four-leveled subdivision of the chapters, where the first three headers are numbered (e.g. 1.; 1.1.; 1.1.1.) and sublevels of these are marked *italic*. Matias Nilsson has constructed all figures.

The knowledge acquired during the course of the project was primarily from scientific articles of high validity found on databases such as PubMed or through the State and University Library (Statsbiblioteket). Keywords used during the literature search included: Offset Analgesia, pain modulation, DNIC, CPM, gate-control, pain pathways, nociception, and endogenous pain inhibition.

Abbreviations

- ACC Anterior cingulate cortex AUC – Area under the curve CNS - Central nervous system CPM - Conditioned pain modulation CV – Coefficient of variation DNIC - Diffuse noxious inhibitory control DRN - Dorsal reticular nucleus EEG - Electroencephalography fMRI – Functional magnetic resonance imaging IASP - International Association for the Study of Pain ICC - Intraclass correlation coefficient OA – Offset Analgesia PAG – Periaqueductal grey RSE – Relative standard error RVM – Rostral ventromedial medulla SEM – Standard error of the mean SFN - Small fibre neuropathy SG – Substantia gelatinosa SI – Primary somatosensory cortex SII – Secondary somatosensory cortex VAS – Visual analogue scale WDR – Wide Dynamic Range
- STAI State-trait anxiety inventory

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Offset Analgesia: A Reproducibility Study

Matias Nilsson Dept. Health, Science, and Technology, Aalborg University

Abstract

Pain affects a tremendously large amount of people, and because pain is a complex interplay between a large variety of factors it can often prove difficult to understand the full extent of the mechanisms underlying pain, and ultimately treat pain patients effectively. Furthermore, pain is a very individual experience and painful stimuli of equal intensity may be perceived very differently between different people. Part of the explanation lies in descending pain modulation, which covers a large number of factors such as emotional state, cultural values, and distraction, and which is able to influence the perception of pain. A new piece to this puzzle was recently discovered and termed offset analgesia (OA). This pain modulating mechanism is defined as a disproportionately large decrease in pain perception in response to a decrease in noxious stimulus intensity. Offset analgesia appears to be distinct from previously described pain modulating mechanisms and is rather powerful in its pain inhibitory capabilities. The aim of the present study is to evaluate whether the OA phenomenon is reproducible within the same day and between different days in 18 healthy human volunteers by examining continuous pain intensity ratings. It was found that a high degree of reproducibility was present both within the same day as well as between different days.

1. Introduction

1.1. Definiton of Pain

The ability to sense and act upon a stimulus that potentially will cause tissue injury is one of the most basic impulses of higher-order organisms, as the survival of individuals of all species is deeply founded in the ability to react adequately to threat. Aversive behaviour such as fear, anxiety, panic and escape behaviours is an important part of an organism's defense system and it is facilitated by exposure to painful stimuli (nociception) or the prediction of imminent aversive events [Bolles, 1970; Sherrington, 1900]. Similarly, The International Association for the Study of Pain (IASP) defines pain as:

"An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage." [IASPTaskforce, 1994].

Concordantly, pain sensation can be divided into three central components: the sensory-discriminative component, the affective-motivational component, and the cognitive-evaluative component. The sensory-discriminative component of pain is concerned with determining the location from which pain originates, the intensity of the pain, and which stimulus modality causes the pain (heat/ mechanical/etc.). On the other hand, the affectivemotivational component of pain is responsible for the emotional response to pain including aversion. The affective-motivational component also facilitates a desire to terminate the painful stimulus. The cognitive-evaluative component is the psychological aspect of pain sensation and is influenced by parameters such as appraisal, cultural values, and distraction [Melzack & Casey, 1968; Treede et al., 1999; Brock, 2009].

In agreement with the affective-motivational component of pain, the experience of pain is heavily modulated by endogenous mechanisms, depending on environmental and psychological circumstances. For example, pain may be inhibited during stress, intense exercise, or during an escape from a predator to allow the individual to deal with immediate hazards [Bolles & Fanselow, 1980]. Conversely, the pain-facilitating systems can cause central sensitisation, which urges the organism to be aware of existing or potential tissue injury in order to protect that specific area from further harm [Woolf, 1995; McMahon *et al.*, 1993]. Taken together, pain perception and processing are the net effect of extremely complex systems involving most parts of the central nervous system (CNS) including intensity coding, affective, behavioural, and cognitive components. Many of the implicated parameters including endogenous modulation have yet to be fully understood.

Meanwhile, pain is the primary reason patients seek medical attention and constitutes a substantial socio-economic burden on the health care sector, as a result of medical expenses, hospitalisation, and absence from work. Moreover, prolonged exposure to pain is socially and functionally disabling for the patients, which reduces the individual quality of life considerably. However, because of the complex nature of pain, treatment is often challenging and the effect of a pharmacological compound varies greatly between patients. Furthermore, the endogenous ability to suppress pain can be incredibly powerful and most available treatment regimes aim at targeting endogenous pain modulating mechanisms [Basbaum et al., 2009; Millan, 1999; DeLeo, 2006]. Considering the number of people suffering from acute or chronic pain, the immediate incentive for research in this area is readily apparent.

1.2. The Somatosensory System

The somatosensory system provides a constant inflow of information to the brain cortices regarding temperature, proprioception, touch and pain. Specific receptors exist for every sensory modality and occur in higher or lower density/cm² throughout the body depending on the kind of receptor and the location in the body. For example, as the skin is constantly presented to touch and temperature, a large amount of corresponding receptors are present here, to allow the individual to sense and act upon any changes that might occur. Similarly, nociceptors, detecting pain, are present in practically all somatic parts of the body, with highest density at the fingertips, lips, and genitalia and lowest density at the back. The receptors themselves are free nerve endings, having their cell bodies located outside the spinal column in the dorsal root ganglia. Noxious stimuli can be induced by chemical, thermal, or mechanical stimuli [DeLeo, 2006; Millan, 1999]. The different receptors of the somatosensory apparatus can be seen in Figure 1.

The somatosensory system is distinct from the viscerosensory system, which deals with sensation in the internal organs. Several parameters separate visceral pain from somatic pain, including:

- Not all viscera is responsive to pain (e.g. liver, kidneys, and lung parenchyma).
- Visceral pain does not always occur upon tissue damage (cutting the intestine is not painful but produces tissue damage, whilst stretching of the bladder wall produces pain but not tissue damage).
- Visceral pain is diffuse and poorly localized (e.g. stomach ache).
- Visceral pain may be referred to other locations (e.g. pain associated with myocardial infarction may radiate into the left arm and jaw).
- Visceral pain is often associated with motor and autonomic reflexes such as nausea, vomiting, lower-back muscle tension, etc. [Cervero & Laird, 1999; Cervero & Laird, 2004].

Moreover, the peritoneum and the parietal serous membranes of the lungs, heart, and liver are outfitted with their own parietal nerve supply (pleura parietale and peritoneum parietale), which embryologically derives from the mesoderm and hence exhibits a nervous supply comparable to the skin. As a result, pain originating from these structures are distinct and highly localised, during e.g. pleuritis, endocarditis, and liver metastasis involving the inner capsula [Bonica, 1990]. However, the viscerosensory system is beyond the scope of this project, and the remainder of the present project will focus only on the somatosensory system.



Figure 1 Schematic overview of the different kinds of receptors and their location in human skin. Merkel's Discs detect light touch and pressure, Pacinian corpuscles are responsible for the detection of deep pressure, vibration and proprioception. The hair follicle receptors detects light touch, Meissner's corpuscles are involved in touch (e.g. two-point discrimination), and Ruffini's end organs are involved in continous touch or pressure. The free nerve endings are highly involved in the detection of noxious stimuli as well as innocuous temperature, joint-movement and proprioception.

1.2.1. Primary Afferent Nerves of the Somatosensory System

The afferent axons, throughout the body, are divided into A and C type nerve fibres, and the former is subdivided into A β and A δ fibres. Sensory receptors usually conduct their electrical signals through A fibres, characterized by a thick layer of myelin, a relatively large diameter, and the presence of nodes of Ranvier.

A β fibres allow very rapid signal conduction reaching conduction velocities of up to 100 m/s. Examples of A β fibres include the median nerve, the radial nerve, and the ulnar nerve, the largest nerves of the hand and forearm, responsible for innervating each their distinct area. However, input from the nociceptors are transmitted through the A δ and C fibres. A δ are thinner and less myelinated than A β fibres, and conduct cold pain and well-localized pain (e.g. pin prick), while the C fibres are thin, non-myelinated fibres, which conduct heat-, or mechanically induced pain. Bessou & Perl described a subpopulation of C fibres as "polymodal" in 1969. These fibres are able to sense both heat- and mechanically induced pain [Bessou & Perl, 1969]. The A δ fibres conduct information rapidly (12 - 30 m/s), while the thinner, non-myelinated C fibres reach conduction velocities of (0.5 - 2 m/s) [Almeida *et al.*, 2004; Millan, 1999].

1.3. Ascending Pain Pathway

The afferent nerve fibres carrying innocuous as well as noxious information, synapse with the secondorder neuron in the dorsal horn. The grey matter of the spinal cord can be divided into 10 distinct laminae, and six of these comprise the dorsal horn of the spinal cord; lamina I (the marginal layer), lamina II (substantia gelatinosa), laminae III and IV (nucleus propius) and laminae V and VI (deep layers) [Besson & Chaouch, 1987; Dubner & Bennett, 1983; Rexed, 1952; Willis & Coggeshall, 2004]. The A β fibres terminate in nucleus propius and the deep dorsal horn (laminae III-V) [Brown, 1981; Schneider, 2005], A δ fibres terminate in both the superficial and the deep dorsal horn (laminae II and V), and C fibres generally terminate in laminae I and II [Lu & Perl, 2005; Light & Perl, 1979]. Generally, the spinal cord neurons from laminae I and II relay nociceptive specific information from A δ and C



Figure 2 Illustration of the dorsal horn laminae. Also depicted are the three different types of primary afferent neurons, coming in through the dorsal root ganglion. The A δ fibres terminate in laminae I and V, the C fibres terminate in laminae I and II, and the A β fibres terminate in laminae III, IV, and V. Here, all fibres synapse with the second order neurons that reach higher regions of the central nervous system (not shown). Note that both A δ and A β fibres terminate in lamina V, giving rise to wide dynamic range second order neurons.

fibres, whilst spinal cord neurons from laminae III and IV carry innocuous information from A β fibres. Finally, lamina V receives converging neurons carrying both innocuous and noxious input through wide dynamic range (WDR) neurons (see Figure 2). These neurons are capable of weak activation from innocuous mechanical stimulation, as occurs with A β fibres as well as more intense activation from noxious mechanical or thermal stimuli. This gives rise to the "wide dynamic range" of the WDR neurons, indicating the ability to carry sensory information within a broader spectrum than regular neurons [Mendell, 1966; Mayer *et al.*, 1975; Price & Mayer, 1975]. The characteristics of the different primary afferent nerve fibres are listed in Table 1.

Both Aδ and C fibres synapse with second order neurons which travel in the contralateral spinothalamic tract [DeLeo, 2006; Almeida *et al.*, 2004]. The spinothalamic tract splits into the lateral neospinothalamic tract and the medial paleospinothalamic tract which carries Aδ and C fibres, respectively. Noxious input chiefly targets the thalamus, but collateral projections also reach mesencephalic regions including the midbrain periaqueductal grey (PAG), the rostral ventromedial medulla (RVM), and the dorsal reticular nucleus (DRN), see Figure 3 [Willis & Westlund, 1997; Weiss *et al.*, 2005; Millan, 1999]. From the thalamic nuclei, third-order sensory neurons terminate in the "pain-matrix", which includes:

- The primary somatosensory cortex (SI)
- The secondary somatosensory cortex (SII)
- The anterior cingulate cortex (ACC)
- Limbic regions including insula and amygdala
- The prefrontal cortices [Treede *et al.*, 1999; Almeida *et al.*, 2004; Giesler Jr *et al.*, 1994; Loewy, 1990].

Fibre Type	Diameter (µm)	Myelin Sheath	Terminates in Lamina(e)	Conduction Velocity (m/s)	Nodes of Ranvier
Αβ	> 10	Thick	III-V	30 - 100	Yes
Αδ	2 - 6	Thin	II and V	12 - 30	No
С	0.4 - 1.2	None	I and II	0.5 - 2	No

Table 1 Characteristics for the different types of primary afferent nerve fibres.



Figure 3 Simplified representation of the somatic pain pathway. (A) A noxious stimulus is presented to the skin surface of the arm and the nociceptors (free nerve endings) in the epidermis transmit the information through the arm (B) and to the dorsal horn (C) where the first order neuron synapses with the second order neuron and crosses over to the contralateral side and contionues into the brainstem and thalamus (D). From thalamus, third order neurons project into cortical and central structures.

1.4. Descending Pain Modulation

1.4.1. The Gate-control Theory

In 1965 Ronald Melzack and Patrick D. Wall published a now renowned paper, in which the gate control theory was hypothesised as a potential candidate for the hitherto vaguely described mechanism underlying endogenous pain modulation. The gate control theory was based on the observation of central control over afferent input combined with the recently discovered evidence supporting spinal pain modulating mechanisms. Briefly, the theory assumed that nociceptive information was carried to the dorsal horn of the spinal cord, by large-diameter fibres and small-diameter fibres. The pain fibres would terminate in substantia gelatinosa (SG) as well as in central transmission cells, which in turn would project information to the central nervous system. Moreover, SG was hypothesized to be capable of decreasing the output from the central transmission cells through inhibitory interneurons, thereby giving rise to a less pronounced pain sensation. The large-diameter fibres terminating in SG would facilitate the inhibition of the central transmission, whilst the small-diameter cells would decrease the inhibition. In effect, this means that during the presence of a noxious stimulus, the painful sensation can be alleviated by innocuous stimuli such as vibration. Tactile sensation activates $A\beta$ nerve fibres which enters the dorsal horn where it activates an inhibitory interneuron, which will stabilize the nociceptors and prolong the period for depolarisation of the nociception afferent [Melzack & Wall, 1965]. A schematic representation of the gate-control theory can be seen in Figure 4.

In summary, the gate control theory founded the belief in endogenous pain modulation and since 1965; it has been generally accepted that brainstem and supraspinal systems also act to modulate nociception [Basbaum & Fields, 1984; Calvino & Grilo, 2006; Ossipov *et al.*, 2010]. This was substantiated by Reynolds in 1969, when electrical stimulation of the periaqueductal grey (PAG) in rats was proven capable of inducing anaesthesia strong



Figure 4 Illustration of the Gate-control theory. Green synapses signify facilitation, whilst red synapses signify inhibition.

enough to allow laparotomies without any form of chemical anaesthesia [Reynolds, 1969]. Further research has implicated other important supraspinal structures involved in descending pain modulation including the RVM and locus coeruleus [Gebhart, 2004; Gebhart *et al.*, 1983; Sandkuhler & Gebhart, 1984]. Supraspinal pain modulating systems include conditioned pain modulation (CPM) and offset analgesia (OA).

1.4.2. Conditioned Pain Modulation

The proverbial "pain inhibits pain" was legitimated and the phenomenon named diffuse noxious inhibitory control (DNIC) following discoveries made by Le Bars and colleagues in 1979. It was discovered that recordings of the spinal dorsal horn in anaesthetized rats were altered during heterotopic peripheral noxious stimuli to the tail, paws, ears, viscerae, and muzzle. In fact, tonic peripheral noxious stimuli of different modalities are able to inhibit the neuronal responses of convergent dorsal horn units. The fact that such noxious stimuli can be applied all over the body, gives rise to the diffuse element of DNIC [Le Bars et al., 1979a; Le Bars et al., 1979b]. More recently, the DNIC phenomenon was renamed conditioned pain modulation (CPM), to describe the psychophysical paradigm used to elicit DNIC. Furthermore, it has been established, that a spino-bulbo-spinal loop through DRN holds a pivotal role in CPM. This is partly attributed to the fact that DRN consist of multiceptive neurons with the whole body as the receptive field [Le Bars, 2002; Pud et al., 2009; Sprenger et al., 2010]. Furthermore, both temporal and spatial summation plays a part in the inhibitory intensity of CPM; the noxious stimulus needs to be applied on a relatively large area and for an extended period of time [Le Bars et al., 1979a; Le Bars et al., 1979b]. In standardised experimental pain, the effect of CPM can be assessed by applying a test pain before and after a conditioned stimulus. The heterotopic conditioning pain alters the perception of the test pain, allowing before and after-comparison.

1.4.3. Offset analgesia

Offset analgesia in man was first described as "A disproportionately large decrease in pain intensity following a relatively small decrease in noxious stimulus intensity" [Grill & Coghill, 2002]. When stimulating the surface of human skin with heat, pain is usually evoked around 45-53 °C [Chery-Croze, 1983; Almeida et al., 2004]. To evoke the OA phenomenon a stimulus train of three different intensities is usually applied. For thermal stimulation, the first temperature (T1) is within the painful range, for example 46 °C. The second temperature (T2) is T1 + 1 °C, in this example 47 °C. Finally, the last temperature (T3) is the same as T1, (See Figure 5). A slight increase in perceived pain occurs following the transition from T1 to T2 in the stimulus paradigm and in the absence of endogenous pain modulation, a corresponding decrease could be expected from the transition from T2 to T3. However, OA causes the resultant decrease in perceived pain



Figure 5 Stimulus paradigm used to evoke offset analgesia. The first phase, T1, consists of a noxious stimulus intensity for five seconds. The sercond phase, T2, is one degree above T1 also for five seconds. The last phase, T3, is the same temperature as T1 but for a 20 second duration. After this, the temperature decreases back to the baseline of 35 °C.

to be far greater. Indeed, Grill & Coghill displayed a decrease in perceived pain following the transition from T2 to T3 of up to 271 % of the increase caused by transition from T1 to T2 [Grill & Coghill, 2002].

Grill & Coghill were the first to describe the concept of OA, although Robinson and colleagues had published a paper about 20 years earlier where they attempted to uncover the human ability to detect incremental increases in noxious stimulus intensity. The experimental setup was constructed in a manner very much like the ones used in most OA studies today, however, they only investigated a different outcome. Still, it is apparent from their results that the OA effect was present [Robinson *et al.*, 1983].

To this day, the underlying mechanism for OA still remains elusive, but studies using functional magnetic resonance imaging (fMRI), has uncovered some of the potentially implicated structures of the central nervous system facilitating OA. These include the PAG, RVM and locus coeruleus [Derbyshire & Osborn, 2009; Yelle *et al.*, 2009]. As mentioned above, these supraspinal structures have been identified as key players in the endogenous modulation of pain.

In effect, evidence points towards the OA phenomenon being a centrally controlled phenomenon, and it has been suggested to serve as a temporal contrast enhancement mechanism to amplify awareness of stimulus offset in order to reinforce escape behaviours. Furthermore, disruption of the normal OA-mechanism has been hypothesised to play an important role in chronic pain conditions [Grill & Coghill, 2002]. A recent study examined the effect of OA in patients with small fibre neuropathy (SFN). Peripheral neuropathy appears to render the OA effect ineffective, thereby causing the OA stimulus paradigm to inflict pain that reflects the stimulus intensity to a greater extent [Niesters *et al.*, 2011b]. A schematic representation of the OA phenomenon is illustrated in Figure 6.



Figure 6 Graph representing the OA phenomenon. Initially, the pain intensity increases and comes to a plateau upon reaching the T1 stimulus temperature. Upon reaching T2, the pain intensity increases slightly, but decreases significantly following the decrease in temperature that occurs in the T3 of the stimulus paradigm. After a while, the pain intensity may start to increase again, as the effects of OA gradually diminishes. Also depicted are three key points in the morphology of the pain ratings that occur due to the OA stimulus paradigm. A) is the plateau, B) is the peak, and C) is the nadir.

2. Aim of the Study and Hypothesis

The aim of the present study is to explore withinday and between days reproducibility of the offset analgesia phenomenon in healthy human subjects in terms of psychophysical assessments:

- Morphology of continuous pain ratings (Plateau, Peak, and Nadir)
- Time Points of Plateau, Peak, and Nadir
- Relative change from Plateau to Peak and from Peak to Nadir (Δ VAS A and Δ VAS B)
- Slope Coefficients between Peak and Nadir
- Area under the curve of VAS ratings
- Anxiety levels
- Stimulus temperature

It is hypothesized that data can be reproduced both between different days as well as within the same day, in the sense that healthy subjects perceive thermal noxious stimuli similarly upon repeated exposures.

Aalborg University, 2012

3. Materials and Methods

3.1. Subjects

All 18 subjects (6 females + 12 males) were healthy, normal subjects between the ages of 21 to 56 years (34.00 ± 3.15 years, mean ± SEM). None of the test subjects displayed any apparent wounds, scars, or tattoos on their forearms, nor did they have a history of diabetes or any chronic pain condition. Subjects were not treated with any analgesics within 48 hours of the study. All female subjects asked to participate in the follicular stage of their menstrual cycle, to avoid fluctuations in gonadal hormonal influence on the nociceptive processing that would be assessed in the reproducibility study [Riley III et al., 1999]. Before participating in this study every subject gave written, informed consent acknowledging that all methods and procedures used in the experiment were understood and that they were aware of that they were going to experience pain and were free to terminate and withdraw from the experiment at any time. The study protocol was approved by the local Ethics Committee (N-20090008). For study protocol see Figure 7.

3.1.1. Procedure

Upon arriving to the research facility, all subjects were asked to fill out a Spielberger State-Trait Anxiety Inventory (STAI), described in detail below. The test subjects were instructed in correct usage of the visual analogue scale (VAS) and their individual pain tolerance levels were found on both days. The temperature corresponding to the subject's pain tolerance level was applied as the T2-temperature in the following experimental trial. The test subjects were asked to score the VAS rating, for the full duration of the experiment, using a handheld VASmeter described below. A total of three recordings were carried out at three adjacent, yet distinct sites in the volar surface of the non-dominant forearm.

3.2. Thermal Stimulation

Heat stimulation was applied to the volar surface of the non-dominant forearm (6 left-handed, 12 right-handed) using a 27 mm Standard Thermode (see Figure 8) connected to Medoc's PATHWAY Pain & Sensory Evaluation System (Contact Heat-Evoked Potential Stimulator, CHEPS) with PATHWAY software 4.0.11.0. To minimize the influence of habituation, the volar surface of the forearm was measured from the elbow joint to the wrist, and divided into three adjacent, yet distinctive zones in the middle of the arm, and 5 centimetres proximal and distal to the central site, see (Figure 9).



Figure 8 The CHEPS thermode. The stimulating area has a diameter of 27 mm, which corresponds to a stimulating area of 572.5 mm². The thermode is fixed with a velcro strap (not shown).



Figure 7 Study protocol. Preparation included obtaining informed consent and filling out the STAI questionnaire.



Figure 9 Stimulation sites on the volar side of the non-dominant forearm. The central site is located in the middle of the test subject's forearm and the distal and proximal site 5 centimetres adjacent to the central site.

Determination of the Stimulus Intensity

Rather than using predefined painful stimulus intensity, all test subjects defined their individual pain tolerance level (VAS 70) prior to the actual experiment. The thermal intensity was gradually increased 1.5 °C/s from a baseline of 35 °C. Upon reaching the pain threshold, the test subject was able to prevent further increase in temperature at the press of a button held in the dominant hand. Three such measurements were carried out at the distal site of the non-dominant forearm and the average was used in the following OA-experiment, as depicted in Figure 10.

3.3. Experimental Trials

Each experimental trial consisted of three contiguous phases; an initial painful stimulus (T1, VAS 70 – 1 °C, 5-second duration), a 1 °C increase to a second temperature (T2, VAS 70, 5-second duration), and a decrease back to T1 (T3), equal to T1 but for a duration of 20 seconds (see Figure 5). Following T3, the temperature was decreased back to baseline (35 °C) at a velocity of 1.5 °C/s. The test subjects participated in the study on two separate days, with a minimum of four days between test days. Both days, the individual VAS 70 was found, as this could differ between days within the same



Figure 10 Example of the determination of the test subjects' individual VAS 70. Three runs are conducted where the test subject clicks a button upon reaching his/her VAS 70, and the average is used as the T2-temperature in the ensuing stimulus paradigm.

test subject. Afterwards, three OA experiments were conducted first on the proximal site, then the central and finally the distal site. The first run at the proximal site was considered a training session, in which the test subjects could familiarize themselves with usage of the CoVAS instrument and how to score continuously while receiving noxious stimuli.

3.4. Stimulation Perception and Intensity Rating

During the full duration of the OA runs, the test subjects were asked to score the pain intensity continuously using Medoc's CoVAS (Computerized Visual Analogue Scale). The test subjects were instructed to score both sensation and pain ratings, as both innocuous as well as noxious ranges were included in the utilized scale (see Figure 11).



Figure 11 VAS applied in the present study. Please note, that the scale contains the sensory (innocuous) range as well as the painful (noxious) range in a continuous succession.

3.5. Individual Anxiety Assessment

The Spielberger State-Trait Anxiety Inventory (STAI) was used to assess the level of anxiety with each individual subject [Hodges & Spielberger, 1969]. This questionnaire stands out as the most effective way of measuring an individual's anxiety level as at clearly differentiates between the temporary "state anxiety" (STAIS) and more general long-term quality of "trait anxiety" (STAIT). The STAIS section of this questionnaire allows for quantification of anxiety levels of a given person at a particular time point. Furthermore, the STAI questionnaire enables detection of differences in anxiety levels between different days. Both STAIT and STAIS consist of completing a 20-point weighted questionnaire.

3.6. Data and Statistical Analysis

The OA data collected during the training runs were discarded and the VAS ratings from the last two runs at the central and distal site were averaged over 1-second periods. The Plateau, Peak, and Nadir were determined along with the corresponding time points they occurred. The changes in VAS that resulted from the shifts in temperature going from T1 to T2 and going from T2 to T3 was determined as Δ VAS A and Δ VAS B (see Figure 12). The Δ VAS B-scores was corrected for the peak value to circumvent a potential artefact arising from variability in peak VAS scores between subjects by dividing the difference in VAS with the peak VAS value ((Peak-Nadir)/Peak). The corrected data for Δ VAS B was



Time

Figure 12 Schematic representation of a VAS curve. The Plateau, Peak, and Nadir can be read along with their corresponding time points, and the Δ VAS A and Δ VAS B can be calculated from these.

termed Δ VAS B_{Corrected}. Furthermore, the slope coefficient between Peak and Nadir was determined and examined across runs and days to determine whether a significant difference in the velocity of OA temperature decrease was present. Finally, the AUC was calculated, as this measurement represents the cumulative sensation-level for the full duration of each experiment.

To assess within-day and between-days reproducibility, data from all subjects were compared between both runs and days using T-tests when data was distributed normally and Mann-Whitney Rank sum test when data was not distributed normally. P-values < 0.05 were considered significant.

When performing a reproducibility study, the statistical analysis must not be based solely on P-values. Obtaining P-values > 0.05 must not be interpreted as data being statistically similar; rather, the fact that there is no statistically significant difference provides incentive to examine data more closely. Several reproducibility/reliability tests were used, including intraclass correlation coefficients (ICC), coefficient of variation (CV), and relative standard error (RSE). ICC values \geq 0.6, CV values < 1, and RSE values < 30 were regarded as indicating reproducibility.

4. Results

4.1. Within-day Reproducibility

The results for all statistical tests in within-day reproducibility are presented below in Table 2. The graphs depicting the mean VAS curves for run 1 and run 2 for both day 1 and day 2 are illustrated in Figure 13 and Figure 14 on the next page, and a table containing data for all parameters examined in the within-day reproducibility part of the present study is presented in Table 3. Statistical analyses have not been carried out on STAI scores and heater values, as data for these parameters were obtained once per day, thus excluding analyses between runs (i.e. within-day). Deviations from the predefined limits for each statistical analysis are summarised on the following pages.

ICC		CV				RSE			P-value				
		Run 1 vs. Run 2		Day 1		Day 2		Day 1		Day 2		Run 1 vs. Run 2	
		Day 1	Day 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	Day 1	Day 2
	Plateau	0.71	0.94	0.46	0.46	0.41	0.47	11.40	11.40	10.23	11.82	0.91	0.65
VAS	Peak	0.98	1.00	0.13	0.14	0.28	0.30	3.22	3.57	6.93	7.47	1.00	0.96
	Nadir	0.99	0.97	1.15	1.20	0.81	0.73	28.77	29.96	20.35	18.20	0.97	0.51
Time Points	Plateau	0.89	0.85	0.35	0.30	0.31	0.19	8.95	7.63	8.00	4.86	0.51	0.62
	Peak	0.62	0.63	0.18	0.16	0.25	0.26	4.70	4.22	6.50	6.74	0.17	0.97
	Nadir	0.82	0.97	0.09	0.12	0.16	0.16	2.29	3.04	4.04	4.05	0.85	0.93
	ΔVAS A	0.80	0.98	0.45	0.45	0.81	0.67	11.36	11.36	20.29	16.85	0.90	0.86
ΔVAS	ΔVAS B	0.99	0.95	0.30	0.37	0.42	0.53	7.56	9.19	10.40	13.32	0.89	0.44
	$\Delta VAS B_{Corrected}$	0.99	0.97	0.32	0.37	0.45	0.52	8.07	9.23	11.15	13.10	1.00	0.47
Slope	Coefficients 0.60 0.95 0.41 0.59 0.66 0.80 10.34 1		14.68	16.44	19.94	0.97	0.46						
	AUC	0.99	0.99	0.34	0.35	0.30	0.31	8.60	8.87	7.41	7.68	0.95	0.92

Table 2 Results for the within-day reproducibility.



Figure 13 Graph of the averages of Run 1 and Run 2 on day 1.



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Day 2: Run 1 vs Run 2
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Figure 14 Graph of the averages of Run 1 and Run 2 on day 2.

ffset Analgesia: A R	eproducibility Study			A	alborg University, 2
Parameter		Time of m	easurement	Mean	Std. Error
		Day 1	Run 1	32.57	3.71
	Distance	Day 1	Run 2	31.99	3.65
	Plateau –	D 2	Run 1	40.58	4.15
		Day 2	Run 2	37.79	4.46
		5 4	Run 1	72.48	2.33
1/40		Day 1	Run 2	72.74	2.59
VAS	Реак –	Day 2	Run 1	76.19	5.28
			Run 2	75.11	5.60
			Run 1	16.91	4.86
		Day 1	Run 2	18.11	5.42
	Nadir –		Run 1	25.72	5.23
		Day 2	Run 2	30.84	5.61
		Day 1	Run 1	7.8	0.69
			Run 2	8.2	0.62
	Plateau –	Day 2	Run 1	7,6	0.60
			Run 2	7.5	0.36
		Day 1 -	Run 1	18.7	0.88
			Run 2	17.1	0.72
Time Points	Peak -	Day 2 -	Run 1	17.7	1.15
			Run 2	17.8	1.20
		Day 1 -	Run 1	27.5	0.63
			Run 2	27.3	0.83
	Nadir	Day 2 –	Run 1	27.6	1.11
			Run 2	27.7	1.12
			Run 1	39.91	4.53
		Day 1 -	Run 2	40.75	4 62
	ΔVAS A		Run 1	35.61	7.22
		Day 2	Run 2	37 31	6.28
			Run 1	55.57	4 20
		Day 1	Run 2	54.63	5.02
∆VAS	ΔVAS B		Run 1	50.46	5.02
		Day 2		44.26	5.89
			Run 1	0.78	0.06
		Day 1	Run 2	0.76	0.07
	$\Delta VAS B_{Corrected}$		Run 1	0.63	0.07
		Day 2	Run 2	0.56	0.07
			Run 1	-6.96	0.07
Slope Coefficients		Day 1			1 03
			Run 1	-5.22	0.85
		Day 2 -	Run 2	-3.22	0.85
			Run 1	-4.04	152.62
		Day 1 —	Kuii 1	1750.00	152.03
AUC				1/59.02	150.09
		Day 2	Kun 1	2086.70	154.59
			Run 2	2065.37	158.61

Table 3 The data used to perform within-day statistical analyses. VAS data is presented as measured on the scale (0-100), Time Points data is measured in seconds, Δ VAS and AUC units are arbitrary, STAI data is measured on a scale of 20-80, and the heater data is measured in °C.

4.1.1. VAS

Plateau

All statistical analyses were within the predefined limits.

Peak

All statistical analyses were within the predefined limits.

Nadir

The CV values for the Nadir data, on day 1 (CV = 1.15 and 1.20) were above the predefined limit.

4.1.2. Time points

Plateau

All statistical analyses were within the predefined limits.

Peak

All statistical analyses were within the predefined limits.

Nadir

All statistical analyses were within the predefined limits.

4.1.3. ∆VAS

$\Delta VAS A$

All statistical analyses were within the predefined limits.

$\Delta VAS B$

All statistical analyses were within the predefined limits.

$\Delta VAS B_{Corrected}$

All statistical analyses were within the predefined limits.

4.1.4. Slope Coefficients

All statistical analyses were within the predefined limits.

4.1.5. AUC

All statistical analyses were within the predefined limits.

4.2. Between days Reproducibility

A table containing results for all statistical tests performed on the between days reproducibility-part of the present study is presented below in Table 4. The graph depicting the mean VAS curves for day 1 and day 2 is illustrated in Figure 15, and all data used to perform statistical analyses regarding between day reproducibility is presented in Table 5 on the following page. Deviations from the predefined limits for each statistical analysis are summarised below.

		ICC	CV		R	RSE		P-value		
		Day 1 vs.	Run 1 + Run 2 Avg		Run 1 + Run 2 Avg		Day 1 vs. Day 2			
		Day 2	Day 1	Day 2	Day 1	Day 2	Run 1	Run 2	Avg	
	Plateau	0.76	0.38	0.41	9.46	10.35	0.16	0.32	0.18	
VAS	Peak	0.52	0.13	0.28	3.30	7.11	0.08	0.18	0.13	
	Nadir	0.76	1.15	0.74	28.84	18.47	0.16	0.07	0.17	
Time Points	Plateau	0.91	0.30	0.23	7.84	5.99	0.98	0.76	0.69	
	Peak	0.71	0.14	0.24	3.82	6.07	0.18	0.64	0.90	
	Nadir	0.78	0.09	0.15	2.29	3.84	0.96	0.76	0.87	
	ΔVAS A	0.53	0.40	0.72	10.10	17.97	0.62	0.66	0.62	
ΔVAS	ΔVAS B	0.80	0.33	0.45	8.14	11.16	0.45	0.19	0.27	
	$\Delta VAS B_{Corrected}$	0.77	0.34	0.46	8.48	11.57	0.14	0.07	0.81	
Slope (Coefficients	0.75	0.39	0.69	9.76	17.16	0.13 0.07		0.07	
STA	l-scores	0.65	0.15	0.10	0.84	0.52	N/A N/A		0.24	
н	leater	0.69	0.04	0.05	0.98	1.11	0.30 0.30		0.30	
	AUC	0.58	0.34	0.30	8.55	7.39	0.16	0.18	0.16	

Table 4 Results for the between day reproducibility.



Figure 15 Graph of the averages of Day 1 and Day 2.

Parameter		Time of measurement	Mean	Std. Error
	Distance	Day 1	32.28	3.05
	Plateau	Day 2	39.18	4.05
MAG	Deel	Day 1	72.61	2.39
VAS	Реак	Day 2	75.65	5.38
	Nie dtu	Day 1	15.51	5.05
	Nadir	Day 2	28.28	5.22
	Distant	Day 1	8.00	0.62
	Plateau	Day 2	7.53	0.45
Time Deinte	Deel	Day 1	17.93	0.68
Time Points	Реак	Day 2	17.76	1.07
	Nadir	Day 1	27.43	0.62
		Day 2	27.66	1.06
		Day 1	40.33	4.07
	Δνας α	Day 2	36.46	6.55
A)/AC	ΔVAS Β	Day 1	55.10	4.48
ΔVAS		Day 2	47.36	5.28
		Day 1	0.77	0.06
	AVAS B _{Corrected}	Day 2	0.60	0.06
<u> </u>	a officients	Day 1	-6.98	0.68
Slope C	oemcients	Day 2	-4.93	0.84
		Day 1	1766.61	151.05
AUC		Day 2	2076.03	153.48
STAI		Day 1	23.70	0.83
		Day 2	21.52	0.52
		Day 1	46.77	0.45
Heater		Day 2	46.25	0.51

Table 5 The data used to perform between day statistical analyses. All data points are averages of run 1 and run 2 for each day. VAS data is presented as measured on the scale (0-100), Time Points data is measured in seconds, Δ VAS and AUC units are arbitrary, STAI data is measured on a scale of 20-80, and the heater data is measured in °C.

4.2.1. VAS

Plateau

All statistical analyses were within the predefined limits.

Peak

The ICC value for Peak (ICC = 0.52) was below the predefined limit.

Nadir

The CV value for the Nadir, for day 1 (CV = 1.15) was above the predefined limit.

4.2.2. Time Points

Plateau All statistical analyses were within the predefined limits.

Peak

All statistical analyses were within the predefined limits.

Nadir

All statistical analyses were within the predefined limits.

4.2.3. ∆VAS

$\Delta VAS A$

The ICC value for VAS A (ICC = 0.53) was below the predefined limit.

$\Delta VAS B$

All statistical analyses were within the predefined limits.

$\Delta VAS B_{Corrected}$

All statistical analyses were within the predefined limits.

4.2.4. Slope Coefficients

All statistical analyses were within the predefined limits.

4.2.5. STAI Scores

All statistical analyses were within the predefined limits.

4.2.6. Heater

All statistical analyses were within the predefined limits.

4.2.7. AUC

The ICC value for AUC (ICC = 0.58) was below the predefined limit.

5. Discussion

The present study set out to determine whether or not it was possible to reproduce the effects of OA in healthy human volunteers within the same day and between different days.

Numerous parameters were investigated in the present study, and inspection of the results generally points toward a high degree of reproducibility, both within the same day as well as between different days. Specifically, reproducibility was clearly evident within the same day for parameters VAS (Plateau + Peak), Time Points (Plateau + Peak + Nadir), Δ VAS (Δ VAS A + Δ VAS B + Δ VAS B_{corrected}), Slope Coefficients and AUC. Moreover, reproducibility was also established between different days for parameters VAS (Plateau), Time Points (Plateau + Peak + Nadir), Δ VAS (Δ VAS B + Δ VAS B_{corrected}), Slope Coefficients, STAI, and Heater.

There appears to be a certain logical pattern in which parameters were reproducible within the same day and which were reproducible between different days. For example, VAS results were highly reproducible within the same day, which can be explained by the fact that the settings were highly alike between runs on the same day. The stimulus intensity was completely the same within the same day, and although the STAI questionnaire was only filled out upon arrival, it is reasonable to assume that no major fluctuations in anxiety level occurred within the same day, based on dialogue with, and observations of, the test subjects. Therefore, it is likely that the test subjects experience the experimental pain very similar on the different runs within the same day. The stimulus paradigm was not disclosed to the test subjects in detail, to obtain as unbiased results as possible. Instead, they were told that they would be presented to heat of varying intensity for 30 seconds in total, and the stimulus intensity would not exceed the VAS 70 they had determined themselves. The fact that the test subjects were not completely aware of what to expect in terms of pain sensation may have facilitated some degree of cautiousness in determining their VAS 70. Upon their second arrival, one could speculate that they had a better understanding of the stimulus paradigm, and were less cautious when determining VAS 70. This could explain that the VAS ratings (both Plateau, Peak, and Nadir value) and subsidiary, the AUC, generally were higher on day 2 and resultantly are reproducible within the same day to a greater extent than between different days.

All of the remaining parameters investigated deviated from a single reproducibility test but fulfilled the criteria for the remaining tests. In these cases, one should not expect reproducibility, nor dismiss it. Large inter-individual differences in the VAS ratings occurred, which to some extent explain the discrepancies in the psychophysical parameters: VAS, Time Points, Δ VAS, Slope Coefficients, and AUC. Importantly, it should be kept in mind that when analysing many different parameters with several different statistical tests, chances are high that a few discrepancies, such as those observed in the present study, will occur. This should not discourage further investigation; rather, it is highly feasible that including a larger sample size may alleviate such problems. Still, it is the author's belief that future studies should focus on the $\Delta VAS B_{corr}$ rected parameter. The change that occurs from Peak to Nadir represents the actual effect of OA. However, the higher the test subject scores the peak value, the greater the decrease in pain sensation due to OA. Therefore, it is reasonable to correct the ΔVAS B results by the peak value to even out peakrelated discrepancies and obtain a more uniform expression of the OA effect.

Importantly, the Δ VAS B_{Corrected} parameter, being a direct expression of the OA effect, proved reproducible both within the same day and between different days.

5.1. Methodological Considerations

5.1.1. Thermode

The use of contact heat applied to the skin surface to elicit pain responses has been commonly used since the 50ies. The methods to apply heat were introduced by Goldscheider in 1884 and either through immersion of a limb in hot water, or by touching the skin with heated materials [Hardy et al., 1951; Hardy et al., 1952; Hall, 1953]. From the 70ies and up until today, contact stimulators such as the CHEPS used in the present study has rendered the former technology obsolete. Such contact stimulators, or thermodes, are able to change temperature very rapidly and when not stimulating it can return to a predefined baseline (often 32-35 °C), thereby directly cooling the skin after heat has been applied. Specific for the CHEPS thermode, is that its effective stimulation area of 572.5 mm² facilitates stimulation of a relatively large skin area for a clear pain response and the accompanying software ensures rapidly responding stimulus paradigms.

The CHEPS thermode was first applied in human experimental trials and provided promising results [Granovsky et al., 2005]. The reliability of this particular thermode has been investigated by Chen and colleagues, who used noxious thermal stimulation to investigate the effect on brain waves. Gender, height, and different sites of stimulation were correlated to the results to determine which factors might alter the results. No statistically significant factors were found, but reliability appears to be highest when stimulating the volar forearm [Chen et al., 2006]. The aforementioned reproducibilitystudy investigated a different oucome than that of the present study, meaning that it might be difficult to compare directly, but their results indicated a high degree of reproducibility for the thermode. However, some of the more recent studies investigating the OA phenomenon have also used the CHEPS thermode, and although no particular comments appear regarding the reproducibility of the thermode, there are no indications against it [Derbyshire & Osborn, 2008; Derbyshire & Osborn,

2009]. Furthermore, this system has been successfully applied in a variety of different studies and has proven reliable in inducing thermal stimuli to different sites of the body including the chest wall [Hobson *et al.*, 2010], the skin near cervical vertebra C7 [Xu *et al.*, 2009], peroneal area [Chen *et al.*, 2006] and on the dorsum of the foot [Wong & Chung, 2011].

The results from the previous literature taken together with those of the present study, strongly indicate that the thermode itself is reliable when used within the same day and when used between different days.

5.1.2. Handedness

Chen and colleagues has investigated whether there is a difference in the detection of heat pain between left and right volar forearm, and found no statistically significant differences [Chen *et al.*, 2006]. Similarly, many studies use the Medoc PATH-WAY system to stimulate the volar forearms of their test subjects, irrespective of handedness [Niesters *et al.*, 2011a; Roberts *et al.*, 2007; Derbyshire & Osborn, 2008; Derbyshire & Osborn, 2009]

The present study stimulated the non-dominant arm, to allow the dominant hand to operate the CoVAS instrument. It can be argued whether it is harder to control the CoVAS instrument with the non-dominant hand, but having all subjects use the dominant hand excludes the potential confounder from occurring, if any change does exist.

5.1.3. Gender

The gender representation in the study population was unevenly distributed with half as many females as males (6 females and 12 males), as it was believed that gender does not influence the effect of OA and test subjects were recruited at random. Previous literature has suggested a gender difference in the perception of heat pain towards males generally having a higher pain threshold than do females [Paulson *et al.*, 1998; Goolkasian, 1985]. Furthermore, the female menstrual cycle has been proven to affect pain perception significantly [Riley III *et al.*, 1999; Sherman & LeResche, 2006]. Including the six females at the same relative time in their menstruation cycle circumvented this, and created a comparable point of reference. Moreover, investigation of gender differences in heat induced evoked potentials, by stimulating the volar forearm yielded no significant results [Chen *et al.*, 2006].

Finally, the subjective nature of the VAS meant that all test subjects should experience the same level of pain (VAS 70) regardless of individual stimulus intensity.

5.1.4. Adaptation/Habituation and Primary Afferent Fatigue

Adaptation is usually defined as a progressive decrease in sensation in response to a stimulus of constant intensity [Theunissen *et al.*, 2000; Price *et al.*, 1977]. Habituation is typically defined by a gradual decrease in responsiveness to, or sensation of, a given stimulus upon repeated exposure [Rankin *et al.*, 2009; Thompson & Spencer, 1966]. The OA effect that occur following the shift from T2 to T3 in the stimulus paradigm has previously been differentiated from simple adaptation [Grill & Coghill, 2002; Derbyshire & Osborn, 2008; Yelle *et al.*, 2008]

Spatial discrimination of heat induced pain varies little across different skin areas (17.1 mm on the foot [Jørum et al., 1989], 9.5-16.0 mm on the dorsum of the hand [Koltzenburg et al., 1993; Moore & Schady, 1995], 10.5 mm on the palm of the hand, and 7.5 mm on the fingers [Ochoa & Torebjørk, 1989]) and by moving the thermode 5 centimetres between runs, it can be considered feasible that the new site is completely distinct from the previous site. Concordantly, a recent study sought to investigate whether spatial differentiation of perceived heat pain intensity occurred in healthy volunteers when presented systematically to a noxious stimulus of 49 °C for five seconds along the arm and leg. It was found that spatial summation of pain intensity was not an issue when moving the thermode 5, 10, 20, and 30 centimetres between runs [Quevedo & Coghill, 2007]. Additionally, at least one minute was allowed to pass between runs to further ensure reliable results, as has been described in previous literature [Yelle et al., 2008]. In concordance with the existing literature, we did not see any influence of habituation, substantiated by the results from within-day reproducibility.

5.1.5. Determination of VAS 70

The determination of VAS 70 may be the source of a potential confounder. During the three repeated stimuli used to average the test subjects' individual VAS 70, the temperature rose quite rapidly and the test subject terminated the stimulation by clicking a button upon reaching VAS 70 in each of the three stimuli, thereby ending further increase in temperature and rapidly returning the heater temperature to 35 °C. It is reasonable to believe that the latency between cognitive recognition of VAS 70 and clicking the button is rather small. This means that the test subjects experienced the stimulus intensity corresponding to their VAS 70 for a very brief period of time. However, in the actual experiment, they were presented to one degree below that intensity for five seconds in T1, the equal intensity for five seconds in T2, followed by 20 seconds of stimulation one degree below. This prolonged exposure to the stimulus may facilitate temporal summation, which would cause the perceived pain intensity to be greater during the actual experiment than it was during the VAS 70 determination, thus explaining why not all test subjects reached a peak of VAS 70, but generally scored the peak pain intensity slightly above 70.

5.2. Psychophysical Assessments

The visual analogue scale utilized in the present study is distinct from those of earlier OA studies in the way that this scale both measures innocuous sensation (VAS 0-49) as well as painful sensation (VAS 50-100). Previous studies have solely measured perception within the painful range (VAS 50-100 on the modified scale). This fact explains, at least partly, the discrepancy in morphology of the VAS curve, which is seen when the grand mean VAS curve of the present study is compared to other groups, who have used pain assessment scales exclusively focusing on sensation within the painful range [Price *et al.*, 1994; Price *et al.*, 1989; Price *et al.*, 1983; Gracely & Kwilosz, 1988; Gracely, 1992]. However, the fact that the present VAS also contains the innocuous sensory spectrum provides new insight into the vigorous effect of OA: Not only does OA inhibit pain, its effect is so powerful that it also inhibits innocuous sensation. The present study successfully demonstrates an inhibitory effect of OA that is powerful enough to rapidly decrease the sensory perception to round 30 VAS (moderate sensation, barely felt), despite the presence of a constant noxious stimulus.

5.3. Anxiety Score

As described in section 1.1., the affective-motivational component of pain processing influences the perception of pain [Melzack & Casey, 1968]. The relationship between pain and emotion has been thoroughly investigated in the past, and there is clear consensus that negative emotional responses to an existing pain (fear, anxiety, frustration, anger, sadness) correlates positively to the perceived pain intensity [Wade et al., 1990; Wade et al., 1996; Okifuji et al., 1999; Fernandez & Milburn, 1994; Fernandez & Turk, 1995; Keefe et al., 2001]. Typically, studies have shown that negative emotional states increase pain ratings and decrease tolerance [Phillips et al., 2003; Palit et al., 2011; Zelman et al., 1991; Weisenberg et al., 1998; Tang et al., 2008] whereas a positive emotional state increases pain tolerance and decreases pain ratings [Zelman et al., 1991; Keogh et al., 2000]. Furthermore, anticipatory anxiety also exacerbates ensuing pain in clinical studies [Sullivan & D'Eon, 1990; Sullivan et al., 2001]. Finally, studies have shown that high trait anxiety can result in greater expectation and altered experience of pain [Tang & Gibson, 2005; Jones et al., 2003]. Positive correlations have also been demonstrated between the personality trait of neuroticisim (higher neuroticisim is characterized by a tendency to experience negative emotions such as anxiety) and the intensity of pain reported [Ramirez-Maestre et al., 2004]. Therefore, in order to optimize the homogeneity of the study population and reduce the confounding effects of anxiety on pain perception and reporting it was of the utmost importance to evaluate individual trait and state anxiety score before including each volunteer into the study. The mean trait and state anxiety levels reported were low and the range of scores was small reflecting the fact that all volunteers scored within the normal range of anxiety. This is not surprising as we studied healthy volunteers, nevertheless the data, which also shows no change in anxiety levels between days, is important and suggests any inter-individual and/or intra-individual (i.e. between days) differences in pain levels and pain response that occurred was not influenced by anxiety level in the current study.

Due to the very low range of scores in the STAI questionnaire (23.70 \pm 0.83 on day 1 and 21.52 \pm 0.52 on day 2), no correlations were performed against VAS ratings.

5.4. Offset Analgesia as Part of the Inhibiting System

Offset analgesia seems to hold a place somewhere in the pain matrix, but the question is, whether it is a completely distinct mechanism that operates irrespective of the other systems or it is an integrated part, or subsystem, of other systems, such as CPM. The complex nature of the pain matrix and the interactions that occur between different centres strongly suggest that OA is integrated somewhere, as practically all endogenous pain modulation is interrelated.

Offset analgesia and CPM are distinct mechanisms in several ways. Firstly, CPM is evoked through heterotopic tonic stimulation, where OA is evoked by dynamic stimulation of the same location. Furthermore, Niesters and colleagues describe at least one other parameter, where CPM and OA are distinct, namely how the two mechanisms are influenced by ketamine. The effect of CPM is altered towards a pain-facilitating function rather than the pain-inhibiting function CPM usually accounts for. However, OA is unaffected thereby indicating a difference between the two [Niesters *et al.*, 2011a; Niesters *et al.*, 2011b].

Important additional information in the attempt to pinpoint the localisation of OA comes from re-
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cent OA studies, implementing fMRI. Here, it is found that there is significant activation of the PAG/ RVM axis during OA, as well as a decrease in activation of the insula [Derbyshire & Osborn, 2009; Yelle et al., 2009]. Activation of the PAG/RVM axis has frequently been associated with pain relief, whilst activation of the insula often appears during pain. Comparison to the CPM system provides additional distinction in this regard, as lesions of PAG/RVM in animal studies does not appear to decrease the effects of the CPM system. However, lesions of the DRN severely impair the effects of CPM. Conversely, OA does appear to function through PAG/RVM activation, where CPM are more exclusive towards DRN activation [Villanueva & Le Bars, 1995; Le Bars, 2002].

In summary, OA is distinct from CPM in several aspects, although both mechanisms appear to function on brainstem-level. The precise location of OA has yet to be discovered, and future studies should aim at obtaining a deeper basic understanding of the OA phenomenon before attempts to divulge its potential in clinical application can be made.

5.5. Offset Analgesia in Future Clinical Application

5.5.1. OA as Therapeutic Target

Recent studies have sought to investigate whether the endogenous pain-inhibiting systems may be involved in the pathology underlying certain chronic pain states. If a pathological defect in pain modulation causes the pain-facilitating systems to enhance the perception of pain, thorough knowledge of these systems might facilitate their surgical or pharmacological inhibition [Porreca et al., 2002; Dubner & Ren, 2004; Vanegas & Schaible, 2004]. As described in the introduction, most therapies available today aim at activating or inhibiting specific parts of the endogenous pain-modulating matrix. As an effect, the discovery of a new pathway such as OA is highly important in the sense that it broadens the spectrum of possible target sites, at least if we can unravel the specific location and pathway of the OA effect. However, the underlying cause of chronic pain is often due to disruption of the normal pain modulation. Such observations have been made for CPM in numerous different chronic pain states including fibromyalgia, osteoarthritis of the knee, temperomandibular disorder, irritable bowel syndrome, and complex regional syndrome [Julien et al., 2005; Ossipov et al., 2010; Lautenbacher & Rollman, 1997; King et al., 2009; Seifert et al., 2009]. The disturbance of CPM can therefore contribute to the development of chronic pain, and CPM can in these cases not be used as therapeutic target. However, the differences between OA and CPM might indicate that OA could be functional in cases where CPM is not, making OA available as a therapeutic target. Needless to say, further research into the OA phenomenon is required, but the present reproducibility of the phenomenon suggests that such research might prove valuable.

5.5.2. Testing Novel Pharmacological Compounds

The effect of novel analgesics can possibly be tested using OA. Niesters and colleagues showed no effect of ketamine, morphine or placebo on the OA effect, despite sharp reductions in pain scores, although the effect of CPM was altered [Niesters *et al.*, 2011a; Niesters *et al.*, 2011b]. It can be speculated whether novel pharmacological compounds can be evaluated to some degree based on their potential influence on OA. Naturally, this is only possible when a more thorough understanding of OA has been established, but if a novel pharmacological compound enhances or inhibits OA this information can be used to understand its mechanism of action.

Offset analgesia might be hard to describe in animal studies, due to the subjective nature of the VAS ratings. It is possible to determine whether or not pain is present in an animal based on aversive behaviour and autonomic responses such as elevated heart rate but changes in pain intensity over a relatively short time span as occur with OA can be difficult. However, if an objective neurophysiologic measure could be implemented into the OA protocol this problem might be alleviated, and the testing of novel pharmacological compounds could be evaluated in animal studies as well using OA. Such a measure could be electroencephalography (EEG) data, as this has been performed in animals for several decades [Lindsley *et al.*, 1950; Buzsáki *et al.*, 1983].

5.5.3. EEG and OA

Combining the subjective VAS ratings during OA with an objective neurophysiologic measure could provide deeper insights into the underlying mechanism facilitating OA. The PAG/RVM axis has been implicated in the OA effect, but it would be interesting to investigate the temporal development in different frequencies of brainwaves during OA. Furthermore, EEG data can provide complementary data to those of the fMRI, as fMRI requires activation of one or more distinct locations over several seconds because it is a measure of the blood flow to different brain regions. EEG however, can be used to describe developments occurring down to the range of milliseconds. The information gathered using EEG could build on top of the fMRI data, and determine especially the potential cortical involvement. Luckily, collection of EEG data is relatively easily incorporated into the OA study protocol.

6. Conclusion

Offset analgesia is highly reproducible both within the same day and between different days in healthy human volunteers. None of the investigated parameters showed statistically significant differences, and most of them displayed reproducibility in all applied reproducibility tests. This strongly encourages further research to unravel both the anatomical and physiological identity of OA. A deeper understanding of this phenomenon may prove very valuable, and because OA is both easily and quickly performed, it can be used as a convenient bedside diagnostic tool in clinical practice and/or clinical research, in the sense that disturbances in the OA mechanism are relatively easy to monitor as an OA experiment can be conducted in 10 minutes.

One can speculate that the same concept can be applied to determine the effective progression of diabetic neuropathy and other such pathologies.

7. References

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Appendix B

		ICC		CV				RSE			
		Run 1 vs Run 2		Day 1		Day 2		Day 1		Day 2	
		Day 1	Day 2	Run 1	Run 2						
Delta	Plateau 1	0.86	0.73	0.34	0.31	0.33	0.27	8.90	7.95	8.55	7.02
	Plateau 2	0.98	0.77	0.29	0.36	0.30	0.30	7.40	9.38	7.79	7.85
	Plateau 3	0.82	0.63	0.26	0.34	0.30	0.28	6.71	8.81	7.79	7.26
	Plateau 4	0.97	0.88	0.3	0.23	0.27	0.26	7.79	6.03	7.05	6.73
	Plateau 5	0.94	0.88	0.25	0.20	0.24	0.28	6.49	5.11	6.26	7.13
	Plateau 6	0.85	0.91	0.32	0.25	0.30	0.23	8.36	6.35	7.63	5.93
Theta	Plateau 1	0.97	0.95	0.3	0.27	0.30	0.32	7.81	6.90	7.86	8.29
	Plateau 2	0.93	0.98	0.24	0.26	0.31	0.34	6.32	6.81	7.91	8.82
	Plateau 3	0.92	0.98	0.25	0.24	0.30	0.29	6.56	6.29	7.72	7.46
	Plateau 4	0.98	0.99	0.25	0.22	0.27	0.24	6.44	5.74	6.99	6.20
	Plateau 5	0.98	0.99	0.24	0.25	0.27	0.25	6.29	6.55	6.94	6.46
	Plateau 6	0.99	0.96	0.24	0.24	0.27	0.25	6.08	6.14	7.00	6.50
Alpha	Plateau 1	0.81	0.92	0.26	0.21	0.28	0.28	6.80	5.30	7.13	7.17
	Plateau 2	0.81	0.93	0.25	0.25	0.26	0.33	6.58	6.48	6.65	8.46
	Plateau 3	0.73	0.98	0.27	0.29	0.28	0.25	6.86	7.39	7.28	6.35
	Plateau 4	0.98	0.92	0.25	0.22	0.30	0.22	6.35	5.80	7.78	5.72
	Plateau 5	0.97	0.94	0.24	0.28	0.28	0.22	6.23	7.28	7.28	5.73
	Plateau 6	0.98	0.97	0.25	0.22	0.30	0.21	6.33	5.74	7.73	5.41
Beta	Plateau 1	0.96	0.95	0.14	0.19	0.19	0.17	3.53	4.87	4.91	4.50
	Plateau 2	0.97	0.98	0.16	0.19	0.18	0.17	4.11	5.01	4.61	4.49
	Plateau 3	0.97	0.91	0.18	0.19	0.17	0.16	4.69	4.96	4.42	4.00
	Plateau 4	0.99	0.96	0.18	0.16	0.18	0.15	4.69	4.08	4.59	3.95
	Plateau 5	0.99	0.98	0.16	0.16	0.17	0.14	4.02	4.09	4.48	3.68
	Plateau 6	0.98	0.97	0.19	0.16	0.17	0.13	4.96	4.02	4.27	3.38
Gamma	Plateau 1	0.94	0.82	0.36	0.24	0.33	0.34	9.34	6.32	8.46	8.90
	Plateau 2	0.88	0.86	0.25	0.28	0.34	0.38	6.34	7.26	8.76	9.83
	Plateau 3	0.81	0.97	0.25	0.24	0.39	0.40	6.34	6.32	9.98	10.21
	Plateau 4	0.90	0.99	0.23	0.18	0.37	0.30	5.83	4.58	9.67	7.86
	Plateau 5	0.94	0.99	0.24	0.21	0.37	0.33	6.07	5.48	9.65	8.62
	Plateau 6	0.86	0.98	0.22	0.21	0.41	0.34	5.81	5.52	10.66	8.89

 Table 1. Within-Day reproducibility tests on EEG data from 9th semester.

		ICC		
		Day 1 v	s Day 2	
		Run 1	Run 2	
	Plateau 1	0.47*	0.59*	
	Plateau 2	0.57*	0.60*	
Dalta	Plateau 3	0.66	0.63	
Deita	Plateau 4	0.77	0.81	
	Plateau 5	0.89	0.8	
	Plateau 6	Day 1 Run 1 0.47* 0.57* 0.66 0.77 0.89 0.65 0.92 0.90 0.82 0.83 0.79 0.82 0.79 0.72 0.82 0.91 0.72 0.82 0.93 0.74 0.82 0.93 0.74 0.82 0.79 0.79 0.79 0.71 0.82 0.79 0.79 0.79 0.77 0.82 0.93 0.91 0.93 0.91 0.93 0.91 0.87 0.68 0.60* 0.66 0.69	0.68	
	Plateau 1	0.92	0.95	
	Plateau 2	0.92	0.94	
Thata	Plateau 3	0.90	0.90	
Ineta	Plateau 4	0.82	0.79	
	Plateau 5	0.89	0.93	
	Plateau 6	0.83	0.90	
	Plateau 1	0.86	0.82	
	Plateau 2	0.79	0.78	
Alaba	Plateau 3	0.77	0.90	
Аірпа	Plateau 4	0.89 0.83 0.86 0.79 0.77 0.82 0.74 0.82	0.90	
	Plateau 5		0.95	
	Plateau 6	0.82	0.81	
	Plateau 1	0.82	0.89	
	Plateau 2	0.93	0.82	
Deta	Plateau 3	0.91	0.82	
Bela	Plateau 4	0.91	0.82	
	Plateau 5	0.98	0.94	
	Plateau 6	0.91	0.95	
	Plateau 1	0.87	0.90	
	Plateau 2	0.68	0.83	
Comme	Plateau 3	0.60*	0.74	
Gamma	Plateau 4	0.57*	0.78	
	Plateau 5	0.66	0.69	
	Plateau 6	0.69	0.70	

Table 2. Between day reproducibility tests on EEG data from $9^{\mbox{th}}$ semester.