An attempt to assess animal pain using brain activity

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Een poging tot het meten van pijn bij dieren aan de hand van hersenactiviteit
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Anthropomorphism is not justified by allusions to evolutionary continuity among vertebrates, because no living vertebrate was ever a descendant of humans, so none could have inherited human traits
Rose JD (May 2007). Anthropomorphism and 'mental welfare' of fishes. Diseases of Aquatic Organisms. 75 139-154

However, since humans are descendants of other vertebrates, they should have inherited traits from these vertebrates. Therefore many human traits are in fact "vertebrate traits", and as such anthropomorphism is justified….
van Oostrom H (August 2007).

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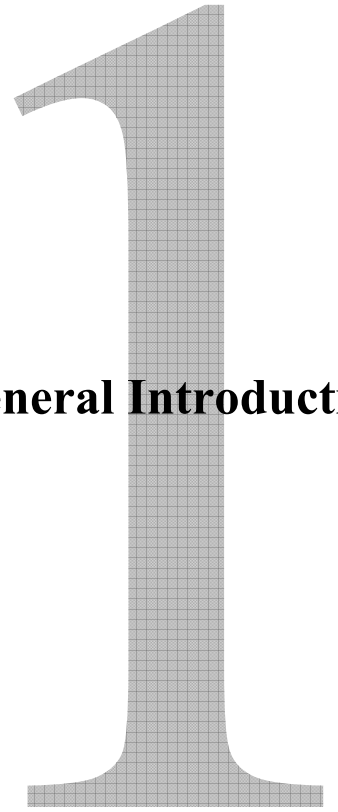

General Introduction

1. Pain and nociception

Pain is *"an emotionally unpleasant sensory experience associated with actual or potential tissue damage, or described in terms of such damage"* (Merskey et al., 1979). The feeling of pain originates from neuronal activity within the nociceptive system. The nociceptive system comprises several different components which will, in brief, be introduced here. Generally, activity within the nociceptive system starts with the activation of peripheral receptors, called nociceptors. These nociceptors are activated by (potentially) painful stimuli. Depending on whether they are actually causing damage or not, these stimuli are called noxious or nociceptive, respectively. The activity from the activated nociceptors is transmitted via peripheral nociceptive nerve fibres to the spinal cord dorsal horn. The peripheral nociceptive nerve fibres are separated into two subclasses, being 1) the A-delta fibres and 2) the C-fibres, which are involved in two different aspects of pain sensation. The relatively fast conducting A-delta fibres (4-30 m/s) mediate the sharp, fast and well localised pain sensations, whereas the slower conducting C-fibres (0.4-1.8 m/s) are responsible for the slow, burning and poorly localised pain sensation, which generally follows the former (Bromm and Lorenz, 1998). From the spinal cord dorsal horn, the activity is relayed, via different spinal tracts, to different structures in the brain such as, among others, the different arousal centres in the brainstem, the thalamus, and finally to the cerebral cortex (Willis and Westlund, 1997; Almeida et al., 2004). It is generally accepted that ultimately, the pain sensation is generated in cerebral structures, with the primary and secondary somatosensory cortex, the insular cortex and the anterior cingulate cortex considered being the most important structures (Shibasaki, 2004). However, up to present, it is not fully understood how the electrical activity arising from the peripheral nociceptor is ultimately conveyed into one of the most unpleasant, if not the most unpleasant, sensations; pain.

Pain is not always initiated by the activation of peripheral nociceptors, as described above. In specific situations such as central pain or neurophatic pain, the pain sensation evolves from "spontaneous" activity in the nervous system somewhere along the pathway from the nociceptors up to the cerebral cortex (Cohen and Abdi, 2002; Zhuo, 2007).

The biological function of pain is threefold, 1) it protects our body against trauma, by withdrawing from potentially hazardous stimuli, 2) it facilitates the regeneration of our body when trauma has occurred, by avoiding the use of traumatized body part as much as

possible and 3) it warns social group members of imminent danger, by the expression of pain by the affected group member (Dennis and Melzack, 1983). The vital importance of the nociceptive system, and the subsequent sensation of pain, becomes convincingly evident when observing the consequences of a disease called congenital analgesia (Danziger and Willer, 2005; Barone et al., 2005; Amano et al., 2006; Butler et al., 2006; Schalka et al., 2006). Patients suffering from this disease can not feel pain, which consequently results in repeated and severe bodily damage (especially during early childhood) such as loss of teeth, tongue, lips and finger tissue, severe ocular damage and skin burns.

Although of vital importance, pain is generally also regarded as a most unpleasant and therefore unwanted sensation, which needs adequate and instant treatment. The presence of pain impairs many individuals in their daily functioning and is thus associated with a high economical impact. The costs involving sick-leave, treatment etc, are estimated to exceed the total costs involved in cardiovascular diseases and cancer together (Thompson, 1997; Loeser, 1999; Sleed et al., 2005). Pain does not always protect our body from damage; it might in fact be harmful itself. In the postoperative period for example, under-treatment of pain has been shown to be associated with an increased susceptibility to infections, delayed wound healing, decreased pulmonary function and cardiac ischemia (Kehlet and Holte, 2001; Bonnet and Marret, 2005, 2007). Due to its unpleasantness and sometimes even clearly damaging effects, man have searched and developed ways for treatment and/or elimination of pain in those situations where it loses its biological function. Eventually, this led to the emergence and further development of a highly relevant and interesting medical field, the field of anaesthesiology.

2. Treatment of pain; analgesic drugs

The treatment of pain, using various techniques and drugs, knows a very long history which will be outlined shortly below. For a more detailed review see Boulton and Wilkinson (1995).

The earliest records about the application of analgesic drugs date from approximately the 20th century B.C. They are found on the Babylonian clay tablets which describe the use of henbane (Hyoscyamus niger) to relieve toothaches. Other of such early descriptions report the use of Cannabis (Cannabis sativa), mandrake (Atropa mandragora), henbane

(Hyoscyamus niger) and poppy (Papaverum somniferum) boiled in water as an analgesic mixture. These plant extracts were used on sponges, which were soaked in the mixture and placed between the lips or into the nostrils of a patient, who subsequently became "anaesthetized".

Up to the Middle Ages, the ancient techniques and ingredients were still used. However, from the Middle Ages to the 18th century the art of anaesthesia was abandoned, due to the opposition against *magic potions* and accusing the ones associated with it of witchcraft. This was very likely due to the fatalities associated with anaesthesia at that time, due to a lack of standardisation of the techniques. However, even then it was still tried to eliminate pain during surgery. Hypnosis, sectioning or compressing the nerves innervating the operation field, crushing of the margins of the proposed incision and icing the operation field were popular techniques to provide analgesia at that time.

The big revolution in anaesthesia came in 1799 when Humphry Davy discovered that inhalation of nitrous oxide (discovered by Joseph Priestly in 1776) made his headaches and toothache disappear and at some occasions made him unconscious for a few minutes.
In 1844 Horace Wells, an American dentist, rediscovered the analgesic effect of nitrous oxide and in collaboration with his associates, extracted many teeth under nitrous oxide anaesthesia. Wells had the opportunity to demonstrate the effects of nitrous oxide at Harvard Medical School but, due to certain problems, he failed and his ideas on nitrous oxide were further ignored.

On October 16th 1846, Thomas Green Morton demonstrated general anaesthesia with ether at the Massachusetts General Hospital and he therefore is often referred to as the founder of ether anaesthesia. However, medical students already knew the anaesthetic effects of ether, before that time, by using it for fun. This "private" use of ether had already led to several successful clinical applications of ether anaesthesia before the "official" demonstration by Morton in 1846.

With the spread of ether anaesthesia, the negative side effects, excitation during induction, retching, excessive salivation and vomiting, were recognized very fast. Due to these negative side effects, other drugs and other routes of administration (e.g. intravenously) were tried. Chloroform, chloral-hydrate, paraldehyde, tri-bromo-ethanol, barbiturates, halogenated gasses (e.g. halothane), opioids etc have all been tried and some of them are still successfully used today.

Nowadays, the field of anaesthesiology to a certain extent still has an empirical character and the choice of the drugs used is still affected by personal preference and experience of the anaesthesiologist (Alwardt et al., 2005). However, drug safety and knowledge of the background mechanisms have been greatly improved by proper scientific studies. For this purpose, studies using animals have been, and still are, of vital importance. In history, the pioneers of anaesthesia already used animals to discover for example the effects of nitrous oxide and ether. Nowadays animal models still play an important role in the improvement of existing, and the development of new, analgesic drugs (Stanley and Paice, 1997; Le Bars et al., 2001; Hogan, 2002). However, since animals lack the possibility to verbally communicate pain, objectively monitoring pain and analgesia in animal models is difficult.

3. Animal models to study (anti)nociception

In experimental pain research involving animals, the terms pain and analgesia are often avoided, and replaced by the terms nociception and anti-nociception. The choice for the latter terminology is based on the fact that animals can not verbally communicate and as such it can not be assessed whether the animals indeed experience unpleasantness from the nociceptive stimuli applied (Stanley and Paice, 1997). The true validity of this line of reasoning is discussed in the general discussion of this thesis, however, in this introduction the terms nociception and anti-nociception will be used, when it comes to animal models.

The ideal (animal) model to study nociception must meet the following criteria (Tjølsen and Hole, 1997)

1) Specificity. Both input and output must be specifically related to nociception, i.e. the stimulus must selectively activate nociceptive fibres and the output parameter must selectively represent a reaction to nociceptive input.

2) Sensitivity. It should be possible to quantify (anti)nociception using the output parameter, i.e. the output parameter must demonstrate a linear change with changes in the nociceptive input, modulated by for example stimulus intensity or administration of analgesic drugs.

3) Validity. It should be possible to differentiate between drug actions on the nociceptive system and actions on other systems, i.e. actions of analgesic drugs on the motor system or the thermoregulation system must not influence the output parameter of nociception.

4) Reliability. The results obtained with a certain model must be consistent, i.e. the same results must be obtained when retesting the animal using an identical test.
5) Reproducibility. It should be possible to reproduce the results obtained with a certain test, i.e. the test should yield the same results when used in different laboratories.

At present, the majority of animal models studying (anti)nociception use behavioural responses of the animal, after applying nociceptive stimuli, as a read-out parameter. Popular examples of such behavioural reactions are the tail-flick, paw withdrawal, and writhing reflex (Stanley and Paice, 1997; Le Bars et al., 2001). However, none of these tests meet all the criteria stated above.

Firstly, many of the behavioural parameters involve spinal reflexes which do not necessarily involve higher structures within the central nervous system, and therefore are not definitively indicating conscious perception of the nociceptive stimuli.

Secondly, the output of the reflexes is generated by the motor system, therefore all drugs that exert co-effects on the motor system of the animal (e.g. increase of muscle tone or muscle relaxation) influence the outcome of the tests, leaving it unclear whether the effects observed reflect drug effects on the motor system or effects on the nociceptive system.

Thirdly, many of the behavioural studies are prone to learning effects. In for example the hotplate and tail-flick tests, animals learn after one stimulus what will happen and therefore start reacting earlier and more vigorously to subsequent stimulations. These learning effects might be erroneously interpreted as an increase in nociception.

Fourthly, since most models work with thermal stimuli, variations of and effects on the basal skin temperature bias the outcome of the tests. For example, a low environmental temperature or an analgesic with vasoconstrictive capacities (e.g. an alpha-2 adrenoceptor agonist) lowers the skin temperature of a rat's tail (a very important structure in the rats thermoregulation) thereby decreasing the tail-flick response to the thermal stimuli applied. Consequently, this can then be misinterpreted as an anti-nociceptive effect.

Fifthly, with these models it is difficult to quantify changes in nociception, since most models convert nociception in an on/off mechanism by either 1) looking at which stimulus intensity a threshold to evoke a nocifensive reflex is reached or by 2) looking at changes in the duration of stimulation needed to evoke a reflex, using a fixed stimulus intensity. Furthermore, the fixed intensity of the stimulus chosen might bias the outcome of the tests.

At a low stimulus intensity the anti-nociceptive effect of a drug might seem rather strong since a weak nociceptive stimulus is readily blocked, whereas at a high stimulus intensity it might seem rather weak, since a strong nociceptive stimulus is more difficult to block.

Sixthly, many of the reflexes can also be evoked by non-nociceptive mechanosensitive input. Therefore, these tests are not always specific for nociception.

Seventhly, not all tests are sensitive to all drug classes. For example the hot-plate and tail-flick test are only sensitive to strictly morphine agonist-like drugs and have even been reported to respond poorly to partial agonistic opioids such as buprenorphine.

Although options to compensate for the shortcomings of these animal models are suggested in the literature, it may be concluded that most of the animal models used are far from perfect. For a highly interesting and in depth review on this subject see Le Bars et al. (2001).

Next to the behavioural animal models of nociception, there is a growing field of research using electrophysiology to study (anti)nociception in animal models. These models vary from single unit recordings in the spinal dorsal horn (You et al., 2005) to recordings of the continuous electroencephalogram (EEG) of the animals (Ichinose et al., 1999; Murrell et al., 2003; Haga and Dolvik 2005; Haga and Ranheim 2005). As stated previously, the final perception of a nociceptive stimulus takes place somewhere in the cerebrum, most likely in the cerebral cortex. Therefore, focussing on monitoring brain activity, i.e. recording and processing data from the EEG, provides the most direct method to study the perception of nociceptive stimuli at the central nervous system level.

Studies using the EEG to study (anti)nociception most often compare the EEG recorded following nociceptive stimulation with the EEG recorded during periods in which nociceptive stimulation is absent (baseline recordings). In such studies the raw EEG is processed using Fast Fourier Transformation (FFT). FFT divides the raw EEG signal in 4 different frequency bands, i.e. the delta, theta, alpha and beta bands (which unfortunately are only well defined for humans and not for animals), and calculates the relative power of each band. Finally, parameters such as the median frequency (MF; the frequency below which 50% of the power of all frequencies are located), the spectral edge frequency (SEF; the frequency below which a predetermined % of the power of all frequencies is located) and the total power of the EEG are extracted. Finally, the effect of nociceptive stimulation on the EEG can be determined by comparing the parameters obtained during nociceptive

stimulation with those obtained during the baseline situation. However, since the raw EEG is composed of all electrical activity in the cerebral cortex, i.e. also the neuronal activity totally unrelated to nociception, it can be questioned to what extent the analysis of the full EEG is discriminative and indicative of the perception of nociceptive stimuli *per se*. Different studies using the full EEG to study (anti)nociception report different, and sometimes even opposite, effects of nociceptive stimulation upon the EEG, indicating the lack of specificity of this technique. For an excellent review see Murrell and Johnson (2006).

A potentially more specific method to study (anti)nociception using the recording of EEG is the recording of evoked potentials. In general, an evoked potential is a small epoch of EEG recorded time-locked to a specific stimulus. Averaging several of such recordings eliminates the unspecific background EEG, leaving only the stimulus-related EEG, i.e. the evoked potential. The evoked potential recorded after nociceptive stimulation (here referred to as the somatosensory evoked potential; SEP) represents the activity of the central nervous system following nociceptive stimuli (Bromm and Lorenz, 1998). In man, it has been shown that the SEP recorded from the centre of the scalp, i.e. the vertex (Vx-SEP), correlates with the subjective pain rating of the stimuli applied (Arendt-Nielsen, 1994; Kakigi et al., 2000). Therefore, the Vx-SEP can be considered to reflect the subjective perception of nociceptive stimuli, eliminating the need of verbal response and making this parameter a very promising tool to study (anti)nociception in animals.

4. Aims and objectives of the present thesis

The aim of this thesis was to investigate whether Vx-SEPs can be used to study (anti)nociception in animal models. First, it was determined whether in animals, similar to the situation in man, the Vx-SEP correlates with the subjective experience of the nociceptive stimuli applied. Second, it was investigated whether SEPs can be recorded in other animal species than the rat, i.e. the dog in this thesis. Finally, the anaesthetic profile of dexmedetomidine in both rats and dogs was studied, using the recording of evoked potentials. Dexmedetomidine was chosen to determine whether this drug can be used in an intensive care setting to provide sedation and analgesia as an alternative to opiates.

Chapter 2 describes a newly developed, integrative paradigm, which combines the recording of SEPs with Pavlov fear-conditioning in rats. By studying the SEPs in relation to

the fear-conditioned behaviour expressed by the animal it is possible to show whether the Vx-SEP is related to the unpleasantness of the nociceptive stimuli.

Chapter 3, 4, and 6 describe studies into the relation between the SEPs and fear-conditioned behaviour under different circumstances to elucidate whether the SEP is truly indicative of the unpleasantness of the nociceptive stimuli, experienced by the animal.

Chapter 5 describes a study investigating the sedative and analgesic effects of dexmedetomidine in the rat, by recording SEPs and auditory evoked potentials (AEP) before, during and after constant rate infusion of dexmedetomidine.

Chapter 7 describes a model to record SEPs in awake dogs.

Chapter 8 describes a study investigating the sedative and analgesic effects of dexmedetomidine in the dog, by recording SEPs and AEPs before, during and after constant rate infusion of dexmedetomidine.

Finally, in **chapter 9 and 10** the results of the chapters 2 till 8 are summarized and reviewed in the light of pain research as a whole, respectively.

Rather than to develop the ultimate animal model to study (anti)nociception, the aim of this thesis was to demonstrate the validity and applicability of the SEP, as an alternative to the behavioural tests, to study (anti)nociception in animals. As discussed before, it has been clear for a long time that the use of behavioural models incorporates numerous experimental flaws. By leaving the concept of behavioural models, the alternative of recording SEPs can introduce a new and innovative approach to study (anti)nociception in animal models, potentially leading to a more ideal model to study (anti)nociception in animals in the future.

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2

Development of a rat model to assess the efficacy of the somatosensory-evoked potential as indicator of analgesia

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Abstract

Drug-induced changes in somatosensory-evoked potentials (SEPs), are considered to reflect an altered nociceptive state. Therefore, the SEP is proposed to be a parameter of analgesic efficacy. However, at present, SEPs have not been studied in relation to animal pain. The present study aims to develop a rat model in which this relationship can be studied based on Pavlovian fear-conditioning. Therefore, rats, implanted with epidural electro-encephalogram recording electrodes, were randomly assigned to either a paired or random-control group and subjected to an aversive-to-appetitive transfer paradigm. During the aversive phase the SEP-stimulation paradigm (5 mA square wave pulses, n=72, of 2 ms duration each, with a stimulus frequency of 0.5 Hz; total duration 144 s) was used as the unconditioned stimulus (US), while a tone (40 s, 1500 Hz , 85 dB sound pressure level) was used as the conditioned stimulus (CS). During the appetitive phase the CS was presented paired to the presentation of a sugar pellet. When compared to the random-control group, the paired group showed significantly more freezing behaviour and significantly less reward-directed behaviour in response to the CS in the appetitive phase. In addition, SEPs were not significantly affected by fear-conditioning. Based on these results, we conclude that the SEP-stimulation paradigm can be successfully employed as a US in fear-conditioning. In future studies, fear-conditioning can be carried out under different levels of an analgesic regimen to allow the changes in SEP parameters to be compared to changes in fear-induced behaviour making this model potentially useful to validate SEP parameters as indicators of analgesia.

1. Introduction

The somatosensory-evoked potential (SEP) is a fragment of electroencephalogram, recorded time-locked to a somatosensory stimulus. Averaging several recordings eliminates unspecific electroencephalographic signals, leaving only the stimulus-related electroencephalogram, the SEP. The SEP evoked after high intensity stimulation of peripheral somatosensory fibres, is believed to represent the neural processing of noxious stimuli.

Although in the rat, SEP components in the 10-30 ms latency range have been largely neglected with respect to nociception and analgesia in the literature, it has been shown that SEP components in this latency range may be of special interest in this respect. A positive peak occurring at approximately 15 ms (P15) recorded from the vertex (Vx-SEP) has been shown to be highly sensitive to increased stimulus frequency and different anaesthetic drugs (Stienen et al., 2003, 2004). These findings have led to our working hypothesis that the Vx-SEP P15 reflects a primary somatosensory mechanism to discriminate between relevant and irrelevant somatosensory stimuli, rather than somatosensation *per se*. Consequently, the Vx-SEP P15 is suggested to be of special interest in relation to nociception and analgesia. However, it remains unknown whether experimental induced changes in the Vx-SEP waveform also involve emotional components, rather than just nociception.

The International Association for the Study of Pain (IASP) defines analgesia as absence of pain in response to stimulation that would normally be painful. Therefore, when the Vx-SEP P15 is to be considered of special interest in relation to analgesia, the stimuli applied to evoke this signal (the SEP-stimulation paradigm) must be painful under normal conditions. Since the IASP definition of pain (an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage) involves an emotional component the important question is whether the SEP-stimulation paradigm is indeed experienced as emotionally unpleasant. Consequently, the next important question is whether the changes in the Vx-SEP P15 are indeed related to changes in the unpleasant emotional experience of the SEP-stimulation paradigm (i.e. changes in perception of pain).

To our knowledge no reports have become available to this date presenting data comparing changes in Vx-SEP components and changes in animal pain. This fundamental

relationship needs to be defined before firm conclusions can be drawn with respect to the Vx-SEP P15 as potential indicator of analgesic efficacy. This study aimed to develop a rat model based on Pavlovian fear-conditioning in which the correlation between Vx-SEP components and animal pain can be investigated.

During Pavlovian fear-conditioning, animals learn that an innocuous stimulus, the conditioned stimulus (CS), predicts an aversive stimulus, the unconditioned stimulus (US). After several CS-US pairings, the CS will evoke fear-conditioned behaviour. The level of fear-conditioned behaviour, expressed after presenting the CS, correlates with the intensity of the shock used as the US (Annau and Kamin, 1961). Therefore, the amount of fear-conditioned behaviour is considered to be a measure for the animal's emotional experience of the aversive stimulus. When the SEP-stimulation paradigm is employed as a US in fear-conditioning sessions, the Vx-SEP can be recorded simultaneously during these sessions. When fear-conditioning different groups of rats under different levels of an analgesic regimen, the relationship between the drug-induced changes in the Vx-SEP components and animal pain can be studied by assessing the amount of fear-conditioned behaviour. This approach is potentially useful to validate the Vx-SEP P15 as parameter of the level of analgesia.

Most Pavlovian fear-conditioning studies use a single aversive stimulus as US, most often a foot-shock. Contrary to this, a SEP-stimulation paradigm consists of repeated stimuli over a longer time span. Therefore, we needed to determine whether the SEP-stimulation paradigm provides an effective US in a fear-conditioning paradigm. In the present study, the effectiveness and applicability of the SEP-stimulation paradigm as a US in a Pavlov fear-conditioning paradigm was investigated. Furthermore, it was determined whether fear-conditioning affected the Vx-SEP components. To this goal rats were subjected to an aversive-to-appetitive transfer paradigm.

An aversive-to-appetitive transfer consists of two phases. During Phase 1, i.e. the aversive phase, the animal is subjected to a Pavlovian fear-conditioning paradigm in which a CS is presented paired with an aversive US. During Phase 2, i.e. the appetitive phase, the same CS is presented paired with an appetitive US, most often a food or water reward. In such a transfer paradigm, the aversive association with the CS made during Phase 1 impedes the appetitive response during Phase 2. Both the aversive behavioural responses and the acquisition rate of the appetitive responses shown after CS presentations during

Phase 2 can be used as parameters for the strength of the association between the CS and the aversive US formed during Phase 1 (Bouton, 1993). In this study, Phase 1 consisted of 10 Pavlovian fear-conditioning trials in which the SEP-stimulation paradigm (5 mA square wave pulses, n = 72, of 2 ms duration each, with a stimulus frequency of 0.5 Hz; total time 144 seconds) was presented either paired (paired group) or randomly (random-control group) to the CS (a 40 seconds 1500 Hz tone, 85 dB sound pressure level). SEPs were recorded for every US presentation. For both groups Phase 2 consisted of 4 subsequent days, on which the CS was presented paired with a sugar pellet for 10 trials a day. During these 4 days, the strength of the association between the CS and the SEP-stimulation paradigm was determined by studying the aversive behavioural response (freezing behaviour) as well as reward-directed behaviour (latencies to, and number of, food magazine-visits).

2. Material and methods

2.1 Animals and Surgery

Animal care and experimentation were performed in accordance with protocols approved by the Science Committee and the local Animal Experimentation Committee (Utrecht University, Utrecht, The Netherlands).

Adult male Wistar rats (HsdCpb:WU, Harlan Netherlands BV, Zeist, body weight 300-350 g, n=20) were anaesthetized with 0.3 mg/kg fentanyl (i.p., Fentanyl Janssen®, Janssen-Cilag BV, Tilburg, The Netherlands, containing 0.05 mg/ml fentanyl citrate) and 0.3 mg/kg medetomidine (i.p., Domitor®, Pfizer Animal Health BV, Capelle a/d IJssel, The Netherlands, containing 1 mg/ml medetomidine hydrochloride) and fixed in a stereotaxic apparatus (Model 963, Ultra Precise Small Animal Stereotaxic, David Kopf Instruments, Tujunga, CA, USA). Epidural electrodes (wired stainless steel screws, tip diameter 0.6 mm, impedance 300-350 Ω, Fabory DIN 84A-A2, Borstlap BV, Tilburg, The Netherlands) were implanted at the vertex (4.5 mm caudal to bregma, 1.0 mm right from midline) and bilateral in the frontal sinus (10.0 mm rostral to bregma, 1.0 mm left and right from midline, respectively) (Stienen et al., 2003). All electrodes were wired to an eight-pin receptacle (Mecap Preci-Dip 917-93-108-41-005, Preci-Dip Durtal SA, Delémont, Switzerland) and fixed to the skull with dental cement (Simplex Rapid, Associated Dental Products, Ltd,

Swindon, UK). At the end of surgery, anesthesia was antagonized with 1 mg/kg atipamezole (s.c., Antisedan®, Pfizer Animal Health BV, Capelle a/d IJssel, The Netherlands, containing 5 mg/ml atipamezole hydrochloride) and 0.15 mg/kg buprenorphine (s.c., Temgesic®, Schering-Plough, Amstelveen, The Netherlands, containing 0.3 mg/ml buprenorphine). Postoperative analgesia was provided by 0.15 mg/kg buprenorphine, administered s.c. at 8 hour intervals for three days after surgery.

After surgery the animals were housed individually in clear plastic cages under climate-controlled conditions on an inversed 12:12 h light/dark cycle (lights on at 18:00 h), with *ad lib* access to food and tap water. Animals were allowed to recover for at least two weeks prior to the start of the experiments.

2.2 Aversive-to-appetitive transfer paradigm

2.2.1 Phase 1: apparatus

Fear conditioning sessions were performed in a darkened experimental room lit by a single 25-watt red light bulb. Ventilators in the room produced a constant background noise of approximately 70 dB sound pressure level. The fear-conditioning box was made of Plexiglas (40×28×30cm) with a grounded stainless-steel bottom, surrounded by a Faraday cage. The box was shielded by polystyrene plates preventing the animals from getting visual cues from outside the fear-conditioning box, only leaving access to a camera for observation of the animal's behaviour. The CS (a 40 seconds 1500 Hz tone, 85 dB sound pressure level) was generated by a sound generator (33120 A, Arbitrary Waveform Generator, Hewlett Packard, Palo Alto, CA, USA) and presented by two speakers mounted in the covering lid of the box. The US (5 mA square wave pulses, $n = 72$, of 2 ms duration each, with a stimulus frequency of 0.5 Hz; total time 144 seconds) was generated by a Grass-stimulator (Model S-88, Grass Medical Instruments, Quincy, Mass, USA) and delivered to a Grass stimulation isolation unit (Model SUI 5, Grass Medical Instruments) and constant current unit (Model CCU 1A, Grass Medical Instruments) to control the intensity.

2.2.2 Phase 1: Procedure

The animals were randomly assigned to either the paired or the random-control group. All fear-conditioning sessions were performed in the "lights off" period. Approximately 30 minutes prior to the start of the fear-conditioning session, the animals were placed in the experimental room to acclimatize. Next, they were fitted in a tight fitting jacket allowing free movement, and an electrical stimulation device was fixed at the left tail base. Subsequently, the animals were placed in the fear-conditioning box. The stimulation device was wired to the Grass-stimulator via a swivel connector. The receptacle at the animal's head was wired via the same swivel-connector to the recording device (described in section 2.3). This approach allowed free movement of the animal during the session as described by Stienen et al. (2003). The fear-conditioning session started after 15 minutes of acclimatization in the box.

The paired group was subjected to a Pavlovian fear-conditioning paradigm in which the CS was presented paired with the US. The US started 10 seconds after the CS, creating a 30 seconds overlap between CS and US. The interval between the CS onsets was 454 seconds. In the random group, the CS was presented randomly with the US, whereby the onset of the CS varied between 72 and 288 seconds after onset of the US. The interval between the US onsets was 454 seconds. The total number of trials per group was 10. SEPs were recorded for every US presentation during the fear-conditioning session.

In order to familiarize the animals with the sugar pellets (Dustless Precision Pellets Sucrose 45 mg, Bio-Serv, Frenchtown, NJ, USA) used in Phase 2 of the experiment, four sugar pellets were fed directly after the fear-conditioning session, at the end of the fear-conditioning day and approximately 2 hours prior to the first session of Phase 2.

2.2.3 Phase 2: apparatus

The Phase 2 sessions were performed in the same experimental room as the Phase 1 sessions, under similar conditions. The sessions were performed, in a Plexiglas box (41×31×20 cm), different from the fear-conditioning box. A plastic tube provided access from outside the box to a food magazine mounted in a corner of the box. Two speakers in the covering lid of the box presented the CS. Similar to the Phase 1 conditions, polystyrene plates shielded the box, only leaving access to a camera, recording the animal's behaviour.

2.2.4 Phase 2: procedure

All Phase 2 sessions were performed during the "lights off" period. In order to make the animals more receptive to the sugar pellets presented during this phase, they were deprived of food two hours before each session. Acclimatization before the actual start of the sessions was the same as in Phase 1 (see section 2.2.2). For each animal, the start of the Phase 2 sessions was counterbalanced over the four days.

During the Phase 2 sessions, the same CS as used in the Phase 1 session was presented paired with the presentation of a sugar pellet. The sugar pellet was delivered to the food-magazine through the plastic tube, 30 seconds after onset of the CS. The interval between the CS-sugar pellet presentations ranged between 180 to 480 seconds. Uneaten pellets were taken away approximately 1.5 minutes before the next CS onset.

2.3 Recordings of the somatosensory evoked potentials

During Phase 1 of the experiment SEPs were recorded in the freely moving animal over one channel, as described by Stienen et al. (2003). In brief, SEPs were recorded from the vertex electrode, the ipsi- and contra-lateral frontal sinus electrodes served as reference and ground, respectively. Signals were amplified 2000 times, band-pass filtered between 15 and 300 Hz and digitized online at 2000 Hz. For each SEP trial, 72 subsequent data segments of 256 data points (25 ms pre-stimulus, 102.5 ms post stimulus) were recorded and averaged. During Phase 2 SEPs were not recorded since only CSs and no USs (i.e. SEP evoking stimuli) were presented.

2.4 Data and statistical analysis

Calculations were performed with the aid of Microsoft Excel 2000. Statistical analysis was performed with SPSS 11.0 for Windows. Differences were considered to be significant when $P<0.05$.

Freezing and reward-directed behaviour were evaluated after the Phase 2 sessions using the camera recordings. Freezing behaviour was defined as the absence of all visible movements with the exception of breathing movements and pendulum motion of the head, while the animal sat in a tensed posture. Pendulum motion has been described to appear under circumstances of emotional excitement, most often fear (Kolpakov et al., 1977). Reward-directed behaviour was studied by determining the latency-time to, and the number

of, magazine-visits in the first 120 seconds after onset of the CS. A magazine-visit was defined as every entrance of the animal's snout into the food-magazine. The latency-time to a magazine-visit was defined as the time between the onset of the CS and the first magazine-visit that followed within 120 seconds. When the first magazine-visit after onset of the CS occurred later than 120 seconds, the latency-time was scored as 120 seconds. The 120 seconds limit was arbitrarily chosen. The behavioural data were scored blind with respect to the fear-conditioning paradigm used. The scorings of the experimenter [HvO] demonstrated a high correlation (Pearson's correlation coefficient r=0.86, n=30, $P \leq 0.01$) with the scorings of a second observer who was unaware of the aims and procedures of this experiment.

SEP data analysis was performed by visual determination of the early peaks of the Vx-SEP waveform. Peak amplitudes and latencies were stored on disk. Parameters extracted for analysis were the amplitude of the P15 with respect to its following negative deflection and its latency. Furthermore, the amplitude of the positive peak occurring at approximately 37 ms (P37) and its latency, were included in the analysis because its consistency within, as well as between, subjects. For both peaks, signal-to-noise ratios were calculated. The signal-to-noise ratio was defined as the amplitude value divided by the root mean square calculated for the pre-stimulus period as described by Stienen et al. (2003). Peak amplitudes with a signal-to-noise ratio <2 were considered to be zero and therefore replaced by the root mean square of the pre-stimulus period. For two animals (one in each group), the SEP waveforms were inconsistent with respect to the other animals due to technical or experimental problems. These two animals were excluded from data analysis, with respect to the SEP data.

All data were statistically analyzed by a two-way repeated measurements analysis of variance (RM-ANOVA), with the repeated factor "trial-block" and fixed factor "group" followed by *post hoc* analysis whenever appropriate.

3. Results

3.1 Behavioural data

3.1.1 Freezing behaviour

Neither the paired group nor the random-control group showed freezing behaviour during the acclimatization period in the box prior to the start of Phase 2. After presentation of the CS, although both groups showed freezing behaviour, there was a significant difference between the groups over the trial-blocks. (Fig.1A; group×trial: $F_{7,126}$=5.636, P<0.001). Both groups showed a significant decrease of freezing behaviour over the trial-blocks (Fig. 1A; *post hoc* one-way RM-ANOVA, factor trial-block: paired group: $F_{7,63}$=14.127, P<0.001 ; random-control group: $F_{7,63}$=8.732, P<0.001). The paired group showed significant more freezing behaviour on trial-blocks 1-5 and 6-10, when compared to the random-control group (Fig. 1A; *post-hoc* independent samples t-test: t_{18}=4.144, P=0.001 and t_{18}=2.819, P=0.018 respectively).

3.1.2 Reward directed behaviour

During Phase 2, both groups shifted the initial aversive response to the CS towards an appetitive response. Both groups showed a significant decrease in latencies to, and an increase in the number of, magazine-visits over time (Fig. 1B and 1C; factor trial-block: latency to magazine- visit: $F_{7,126}$=25.607, P<0.001; number of magazine-visits: $F_{7,126}$=19.507, P<0.001), but there was no difference in the acquisition rate of the appetitive response (group×trial: latency to magazine-visit: $F_{7,126}$=1.609, P=0.139; number of magazine-visits: $F_{7,126}$=1.450, P=0.191). However, the paired group showed significantly longer latencies to, and significantly less numbers of magazine-visits compared to the random-control group (Fig. 1B and 1C; factor group: $F_{1,18}$=6.873, P=0.017 and $F_{1,18}$=28.372, P=0.048 respectively).

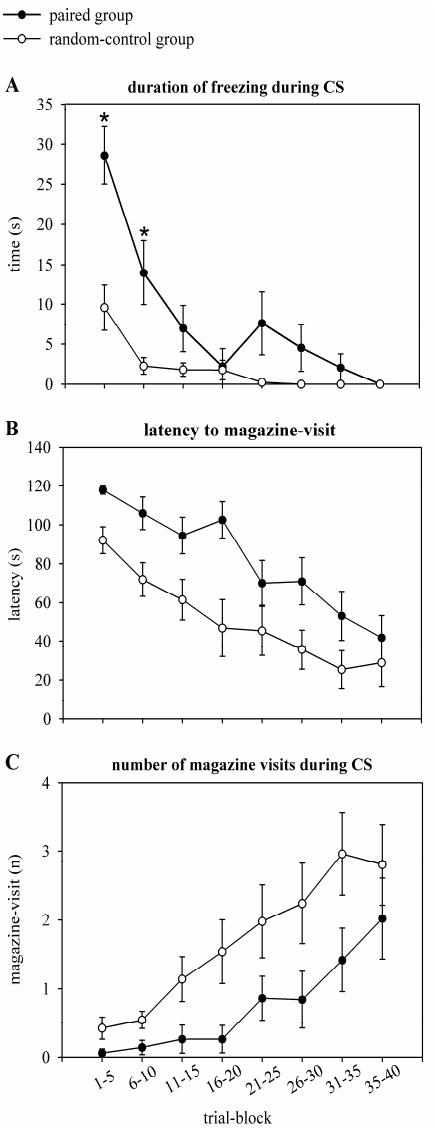

Fig. 1. Aversive and appetitive behavioural responses during Phase 2. (A) Duration of freezing behaviour in the paired and random-control group during the CS presentation. Data are presented as the mean duration of freezing behaviour (s) ± S.E.M (*n*=10) over the subsequent trial-blocks. Trial-blocks with a significant difference between the paired and random-control group are indicated by *; (B) Latency-time to the first magazine-visit in the paired and random-control group. Data are presented as the mean latency the first magazine-visit (s) ± S.E.M (*n*=10) over the trial-blocks; (C) Number of magazine-visits during the CS presentation. Data are presented as the mean magazine-visits ± S.E.M (*n*=10) over the trial-blocks.

3.2 Somatosensory evoked potentials

The grand average waveform of the Vx-SEP with its peak definitions is shown in Figure 2A. The two groups showed no significant differences in the P15 amplitude and latency over the trial-blocks (Fig. 2B and 2C; trial×group, factor trial-block and factor group: amplitude P15: $F_{9,144}$=0.551, P=0.835; $F_{9,144}$=1.290, P=0.247 and $F_{1,16}$=2.032, P=0.173, respectively; latency P15: $F_{9,144}$=0.903, P=0.525; $F_{9,144}$=1.313, P=0.235 and $F_{1,16}$=1.518, P=0.236, respectively).

There were no differences between the two groups in P37 amplitude and latency over the trial-blocks (Fig. 2B and 2C; trial×group and factor group: amplitude P37: $F_{9,144}$=1.723, P=0.089 and $F_{1,16}$ =0.000, P=0.998, respectively; latency P37: $F_{9,144}$=0.739, P=0.673 and $F_{1,16}$=7.376, P=0.586, respectively). However, for both groups, a significant increase in amplitude and a significant decrease in latency were found over trials. (Fig.2B and 2C; factor trial-block: $F_{9,144}$=3.711, P=0.009 and $F_{9,144}$=7.928, P<0.001, respectively).

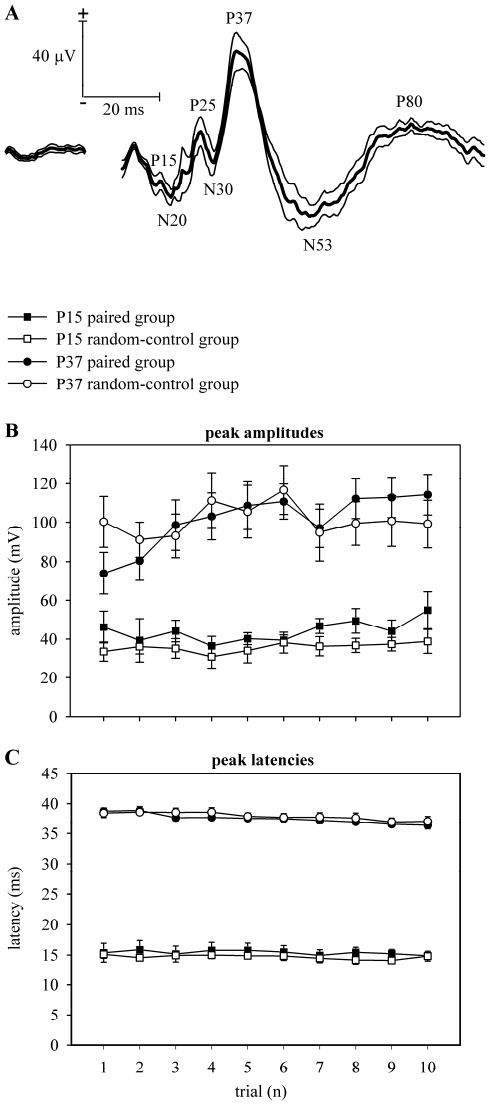

Fig. 2. Effect of fear-conditioning on the Vx-SEP during Phase 1. (A) Grand average waveform of the Vx-SEP-signals obtained from the random-control group during the first fear-conditioning trial. Data are presented as the mean (bold line) ± S.E.M (*n*=9) (plain line). The curve interruption denotes the stimulus onset. Positive deflections (P) and negative deflections (N) are accompanied by their average latency of onset (ms); (B) Effect of fear-conditioning on the amplitudes of the P15 and P37 of the Vx-SEP. Data are presented as mean (µV) ± S.E.M (*n*=9) per trial; (C) Effect of fear-conditioning paradigms on the latencies of the P15 and P37 of the Vx-SEP. Data are presented as the means (ms) ± S.E.M (*n*=9) per trial.

4. Discussion

In the present study, we investigated whether the SEP-stimulation paradigm provides an effective US in Pavlov fear-conditioning and whether the Vx-SEP P15 and P37 were affected by the fear-conditioning paradigm. The principal findings are, first that the paired group showed significantly more freezing behaviour and significantly less reward directed behaviour compared to the random group. Second, we found that fear-conditioning itself did not affect the Vx-SEP P15 and P37. The behavioural findings strongly suggest that the paired group made a stronger association between the CS and the US during Phase 1, when compared to the random-control group and thus is supportive for the fact that the SEP-stimulation paradigm activates peripheral fibres involved in nociceptive processing. The neurophysiologic findings imply that in future studies, using the present model for investigating drug-induced changes in SEPs and fear-conditioned behaviour, the changes in the Vx-SEP components can be interpreted as drug-induced changes and not fear-conditioning induced changes. Based on these findings we conclude that the rat model, as developed in this study, can be used to study changes in the Vx-SEP components in relation to animal pain. In the future, such studies can be performed by fear-conditioning animals under different levels of analgesia, using the present model, which is potentially useful to validate the Vx-SEP components as potential indicator of the quality of analgesia.

Peck and Bouton (1990) found an important effect of fear-conditioning to context, during Phase 1 of an aversive-to-appetitive transfer, upon the behavioural response shown during Phase 2. We suggest that fear-conditioning to context did not affect the behavioural responses found in this study. First, although the context of the experimental room did not differ between the two phases in this experiment, the box used in Phase 1 differed, with respect to context, to the box used in Phase 2. Second, in the acclimatization period prior to the start of Phase 2, freezing behaviour was absent in both groups and only occurred after the CS presentation.

The random-control group showed freezing behaviour after presentation of the CS, during the first trial-blocks, although to a much lesser extent than the paired group. A likely explanation for the freezing behaviour in the random-control group is that in this group some of the CSs overlapped the US presentation due to randomization of the CS presentation. During these trials the animals possibly made an association between the CS and the US. Animals in a random-control group making a CS-US association, although not

as strong as animals in a paired group, has been described (Kremer, 1971; Keller et al., 1977).

The paired group showed less reward-directed behaviour, compared to the random-control group. This finding is consistent with other studies and is explained in terms of excitatory aversive and appetitive motivational brain systems inhibiting one another (Scavio, 1974; Peck and Bouton 1990). According to this explanation, the CS presented during Phase 2 of an aversive-to-appetitive transfer paradigm activates the motivational aversive brain-system, which has an inhibitory effect upon the motivational appetitive system, thus blocking learning an appetitive-conditioning task. Therefore, the appetitive system in the paired group, in the present study, may have been blocked to a greater extent resulting in less reward-directed behaviour. Alternatively, differences in reward-directed behaviour found in this study could also be a result of response competition at the motor level (Scavio, 1974; Peck and Bouton 1990). In this view reward-directed behaviour can be seen as an indirect measure of freezing behaviour, indicating a stronger association between the CS and the SEP-stimulation paradigm in the paired group, when compared to the random-control group.

Since every CS during Phase 2 was reinforced by the presentation of a sugar-pellet, the possibility exists that the animals showed an appetitive response towards presentation of the sugar-pellet itself, rather than to the CS. The graph shown in Figure 1B could possibly be interpreted this way, since in both groups none of the latencies to magazine-visits were shorter than 30 seconds; the time at which the sugar-pellet was dropped. However, two animals in the random-control group did not show an appetitive response in any of the 40 trials of the aversive-to-appetitive transfer paradigm, although the amount of freezing behaviour was comparable to the animals that did show an appetitive response. When excluding the data of the two animals, the mean latency-times (s) ± S.E.M. (n=8) were: trial-block 26-30: 23.95 ± 8.08, trial-block 31-35: 11.35 ± 2.73, trial-block 36-40: 11.95 ± 3.49). This indicates that the animals responded (at least in the later trials) to the CS, rather than to the presentation of the sugar-pellet itself.

The Vx-SEP components, in the latency range studied, are considered to be nociceptive in nature (Stienen et al., 2003, 2004), which is of primary importance when using these components as indicators of analgesic efficacy. Nociceptive processing involves primarily activation of Aδ-fibres (acute pain) and C-fibres (slow pain). It is known that electrical

stimulation as used in the present study activates Aα-fibres involved in motor processing, Aβ-fibres involved in mechanosensation and Aδ- and C-fibres (Schouenborg et al., 1986; Fehlings et al., 1988; Bromm and Lorenz, 1998; Shaw et al., 2001). Based on the conduction velocities (Aα: 60-70 m/s, Aβ: 30-40 m/s, Aδ: 4-30 m/s and C: 0.4-1.8 m/s) (Schouenborg et al., 1986; Fehlings et al., 1988; Bromm and Lorenz, 1998; Shaw et al., 2001) and the distance between the stimulus location and the recording site in this study (approximately 0.15 - 0.20 m), both Aβ-and Aδ-mediated responses can be found within the latency range examined in the present study. It has been shown that the Vx-SEP P15 (Stienen et al., 2003) and P37 (unpublished observations, PJ Stienen) are affected by the μ-opioid receptor agonist fentanyl. Since μ-opioid receptor agonists, selectively modulate nociceptive processing (Aδ- and C-fibres) on both peripheral and supra-spinal level, but not tactile processing (Aβ fibres) (Kalliomäki et al., 1998; Silbert et al., 2003), we consider both the Vx-SEP P15 and P37 to be Aδ-mediated. The fact that the animals in this study clearly demonstrated fear-induced behaviour after presentation of the CS further supports this hypothesis.

In conclusion the findings obtained in the present study strongly suggest that the SEP-stimulation paradigm used, forms an effective US in a Pavlovian fear-conditioning paradigm and that the Vx-SEP P15 and P37 are not affected by fear-conditioning itself. This provides us with a unique tool to study the direct relationship between the characteristics of the Vx-SEP components and animal pain. Therefore, the rat model developed in this study is potentially useful for validation of the Vx-SEP P15, as parameter of the level of analgesia.

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3

Vertex-recorded, rather than primary somatosensory cortex-recorded, somatosensory-evoked potentials signal unpleasantness of noxious stimuli in the rat

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Abstract

In the present study, we investigated in the rat whether vertex- or primary somatosensory cortex-recorded somatosensory-evoked potentials (Vx-SEP/SI-SEP, respectively) signal unpleasantness of noxious stimuli. Therefore, initially we characterised fentanyl effects (0, 20, 40 or 50 µg/kg/h) on somatosensory and auditory processing by recording Vx/SI-SEPs and vertex- and primary auditory cortex-recorded auditory-evoked potentials (Vx-/AI-AEPs, respectively). Subsequently, in a separate experiment, the animals were subjected to a Pavlovian fear-conditioning paradigm. The noxious stimuli applied to evoke Vx-/SI-SEPs (unconditioned stimulus (US)) were paired to a tone (conditioned stimulus (CS)) under 'steady state' conditions of 0, 20, 40 or 50 µg/kg/h fentanyl. Vx-/SI-SEPs were recorded simultaneously during these trials. After CS-US presentation, CS-induced fear-conditioned behaviour was analysed in relation to the SEPs recorded during CS-US presentation and the AEPs recorded in the first experiment. While the SI-SEP and AI-AEP were minimally but significantly affected, fentanyl dose-dependently decreased the Vx-SEP and Vx-AEP. The decrease of the Vx-SEP and Vx-AEP was parallelled by the dose-dependent decrease of the amount of CS-induced fear-conditioned behaviour. These results suggest that the dose-dependent decrease of the Vx-SEP amplitude, rather than of the SI-SEP, indicates that the US was experienced as less unpleasant. Next to an altered US processing, altered CS processing contributed to the decrease of the amount of CS-induced fear-conditioned behaviour as indicated by the dose-dependent decrease of the Vx-AEP.

1. Introduction

Electroencephalographic somatosensory-evoked potentials (SEPs) evoked by stimulation of peripheral somatosensory fibres appear as a waveform consisting of positive and negative peaks with different amplitudes and time of onset after stimulation (latency). SEP waveforms evoked by noxious stimulation are considered to represent the processing of the noxious stimulus in the brain (Bromm and Lorenz, 1998; Kakigi et al., 2000). SEP amplitudes and latencies are altered by drugs primarily suppressing the perception of noxious stimuli (analgesic drugs) (Arendt-Nielsen, 1994; Kakigi et al., 2000; Banoub et al., 2003; Stienen et al., 2004). Further, in man, SEP amplitudes correlate well with subjective pain ratings (Bjerring and Arendt-Nielsen 1988; Arendt-Nielsen, 1994; Kakigi et al., 2000; Iannetti et al., 2004; Ohara et al., 2004). Combined, the SEP is considered a potential readout-parameter in pain and anaesthesiologic research.

Recently, differences were established between the vertex-recorded positive-to-negative component at approximately 15 and 20 ms (this component is abbreviated as Vx-SEP throughout this chapter) and the primary somatosensory cortex (SI)-recorded positive-to-negative component at approximately 12 and 20 ms (this component is abbreviated as SI-SEP throughout this chapter) in the rat model. These differences are of special interest to (anti)nociception and to pain and the absence thereof, i.e. analgesia (Stienen et al., 2004; 2005). The Vx-SEP and SI-SEP were both found to be related to nociception (Stienen et al., 2004; 2005). However, the Vx-SEP compared to the SI-SEP, showed an increased sensitivity to filtering out external information and focus attention on newer, more salient stimuli (Stienen et al., 2004; 2005). In addition, the Vx-SEP showed an increased sensitivity to different 'sleep-inducing' (hypnotic) and analgesic drugs, when compared to the SI-SEP (Stienen et al., 2004; 2005). These findings have led to the hypothesis that the Vx-SEP and SI-SEP are related to separate functional pain mechanisms (Stienen et al., 2004; 2005). While noxious stimuli are evaluated on their spatio-temporal localisation and intensity, their 'emotional color' resulting from signalling unpleasantness, is modulated by the individual's state of arousal, attention and memory (Bromm and Lorenz, 1998; Milan, 1999; Price, 2000; Schnitzler and Ploner, 2000). The Vx-SEP is considered to be related to signalling unpleasantness, while the SI-SEP is related to spatio-temporal localisation and intensity quantification of noxious stimuli (Stienen et al., 2004; 2005).

In the present study, we investigated whether the Vx-SEP, rather than the SI-SEP, signals unpleasantness of noxious stimuli. Therefore, fentanyl-induced Vx-SEP and SI-SEP changes were studied using the SEP fear-conditioning model (van Oostrom et al., 2005). In that previously developed model, stimuli applied to the basal part of the tail to evoke Vx-/SI-SEPs (SEP stimulation paradigm) provided an effective unconditioned stimulus (US) in a fear-conditioning paradigm with a tone as conditioned stimulus (CS). It was proposed that the use of a similar approach under different levels of analgesia, would allow to reliably 'translate' analgesic drug-induced SEP changes to its corresponding level of unpleasantness of noxious stimuli (Fig. 1). SEPs represent neural processing of the SEP stimulation paradigm, i.e. the US. Thus analgesic drug-induced SEP changes can be considered to represent changes in US processing during CS-US pairing in our model. The magnitude of the CS-induced fear-response after CS-US pairing, i.e. the strength of the CS-US association represented by the duration of freezing behaviour, is generally accepted as an expression of fear and even considered as an expression of pain experienced from the US (Fanselow and Bolles, 1979; Sewards and Sewards, 2002). Combined, analgesic drug-induced SEP changes during CS-US pairing correlating with a reduced CS-induced fear response, may therefore be assumed to indicate that the US was experienced as less unpleasant by analgesic drug treatment during CS-US pairing (Fig. 1).

Although fentanyl, a μ-opioid, is primarily analgesic and therefore considered to primarily suppress the perception of unpleasantness of the US, fentanyl may also modulate other sensory processes such as hearing. Consequently, using a tone as CS, fentanyl-induced changes in fear-conditioned behaviour could theoretically be ascribed to effects of fentanyl on neural processing of the CS (CS processing). In order to determine potential fentanyl effects on CS processing, next to neural processing of the US, (US processing) and to perform fear-conditioning under 'steady state' conditions of fentanyl, initially we studied the effects of saline and fentanyl (20, 40 and 50 μg/kg/h) on the Vx/-SI-SEPs and auditory-evoked potentials (AEPs) (Experiment 1) to characterise fentanyl effects on somatosensory and auditory processing and to determine whether a steady state effect on the SEP and AEP parameters could be achieved. In Experiment 2, Vx-/SI-SEPs were recorded simultaneously during CS-US pairing, according to the SEP-fear conditioning model (Fig. 1), under steady state conditions of saline, 20, 40 and 50 μg/kg/h fentanyl administration. The CS-induced

fear-response was tested the day after CS-US pairing and studied in relation to the SEPs and AEPs according to the SEP-fear conditioning model (Fig. 1).

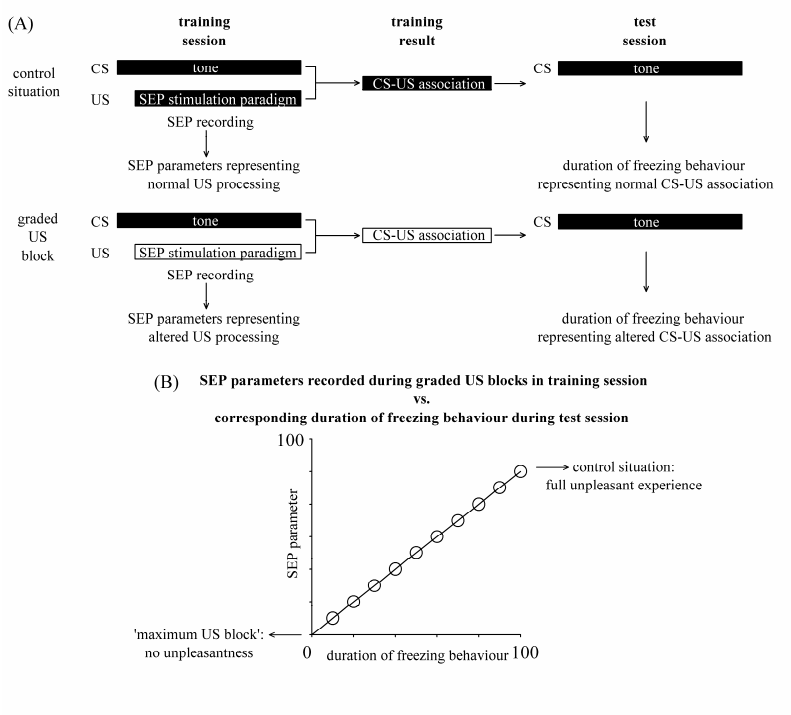

Fig. 1. SEP fear-conditioning model. (A) Black and white boxes indicate 'presence of' and 'graded suppression/absence of', respectively. (B) Data are fictively chosen based on an 'ideal' situation. For explanation, see text.

2. Material and methods

2.1 Animals and surgery

Animal care and experimentation were performed in accordance with protocols approved by the Science Committee and the local Animal Experimentation Committee (Utrecht University, Utrecht, The Netherlands). Adult male Wistar rats (HsdCpb:WU, Harlan Netherlands BV, Zeist, body weight 300-350 g, n=62) were pre-medicated with 0.05 mg/kg atropine sulphate (s.c., Atropine Sulphate®, Eurovet Animal Health B.V., Bladel, The Netherlands) and, subsequently, anaesthetized with 0.3 mg/kg fentanyl (i.p., Fentanyl-B. Braun, B. Braun, Melsungen, Germany) and 0.3 mg/kg medetomidine (i.p., Domitor®, Pfizer Animal Health BV, Capelle a/d IJssel, The Netherlands) and fixed in a stereotaxic apparatus (Model 963, Ultra Precise Small Animal Stereotaxic, David Kopf Instruments, Tujunga, CA, USA). In all animals used in Experiments 1 (n=9) and 2 (n=53), epidural electrodes (wired stainless steel screws, tip diameter 0.6 mm, impedance 300-350 Ω, Fabory DIN 84A-A2, Borstlap BV, Tilburg, The Netherlands) were implanted at the vertex (4.5 mm caudal to bregma, 1.0 mm right from midline (Stienen et al., 2004)), the SI tail representative area (2.5 mm caudal to bregma, 2.5 right from midline (Chapin and Lin, 1984)) and bilateral at the frontal sinus (10.0 mm rostral to bregma, 1.0 mm left and right from midline, respectively (Stienen et al., 2003)). In the animals used in Experiment 1, an epidural electrode was also inserted at the right primary auditory cortex (AI) (4.5 mm caudal to bregma, 4.5 mm ventral with respect to the dorsal surface of the skull, through the lateral skull (Miyazato et al., 1995)). All electrodes were wired to an eight-pin receptacle (Mecap Preci-Dip 917-93-108-41-005, Preci-Dip Durtal SA, Delémont, Switzerland) and fixed to the skull with dental cement (Simplex Rapid, Associated Dental Products, Ltd, Swindon, UK). At the end of surgery, anaesthesia was antagonized with 1 mg/kg atipamezole (s.c., Antisedan®, Pfizer Animal Health BV, Capelle a/d IJssel, The Netherlands) and 0.15 mg/kg buprenorphine (s.c., Temgesic®, Schering-Plough, Amstelveen, The Netherlands). Postoperative analgesia was provided by 0.15 mg/kg buprenorphine, administered s.c. at 8 hour intervals for three days after surgery.

After surgery the animals were housed individually in clear plastic cages under climate-controlled conditions on a reversed 12:12 h light/dark cycle (lights on at 22:00 h),

with ad lib access to food and tap water. Animals were allowed to recover for at least two weeks prior to the start of the experiments.

2.2 Experiment 1: Characterisation of fentanyl effects on somatosensory and auditory processing by SEPs and AEPs, respectively

The experiment was performed in the 'lights off' period in a darkened experimental room. Before the start of the experiment, a 24 gauge cannula (Vasofix®, B. Braun, Melsungen, Germany) was inserted in a lateral tail vein of the rat for intravenous administration of saline (injection fluid containing 0.9% NaCl, Fresenius Kabi B.V., 's Hertogenbosch, The Netherlands) or fentanyl. Next, the rat was fitted in a tight-fitting jacket (developed in house) and individually placed in a purpose-built Plexiglas box with an stainless steel electrically grounded bottom (40×28×30 cm) shielded by a Faraday cage.

For recording of SEPs and AEPs, the rat's head-mounted receptacle was connected, via a swivel connector (SLC-2, Plastics One, Roanoke, VA, USA), to the recording device. SEPs were evoked as previously described in detail (Stienen et al., 2004). In short, an electrical stimulation device was fixed at the left tail base and wired with a separate electrical shielded cable via the open middle of the swivel connector to the stimulation device. This recording/stimulation set-up prevented electrical interference between recording and electrical stimulation and allowed the animal to move freely inside the box. Stimuli for SEP recording were 72 square-wave pulses of 2 ms duration with stimulus frequency 0.5 Hz and stimulus intensity 5 mA (SEP stimulation paradigm). Stimuli for an AEP recording were 72 white noise clicks of 0.2 ms duration with stimulus frequency 0.5 Hz and stimulus intensity 100 dB sound pressure level and were presented by two speakers mounted in the covering lid of the box.

SEPs were recorded from the vertex and SI (Vx-SEP and SI-SEP, respectively). AEPs were recorded from the vertex and AI (Vx-AEP and AI-AEP, respectively). The accompanying ipsilateral frontal sinus electrode served as reference electrode and the accompanying contralateral frontal sinus electrode served as signal ground. Signals were fed via the swivel connector to separate, but identical, amplifiers (Bio-electric amplifier AB 601-G, Nihon Kohden, Tokyo, Japan). The signals were band-pass filtered between 15 and 300 Hz, amplified 2000 times and fed to a personal computer which digitised the signal online at 2.0 kHz. The SEPs and AEPs were at times sensitive to electrical net 50 Hz-

interference. Therefore, a 50 Hz notch-filter was applied to all recording configurations to prevent possible electrical 50 Hz-interference. Power spectral analysis showed that there was a negligible contribution of the 50 Hz-component in regular signals. One SEP/AEP recording consisted of 72 averaged subsequent epochs of 256 data points (pre-stimulus 50 data points, post-stimulus 206 data points) and was stored on disk and analysed offline.

After recording of eight SEPs and AEPs (baseline measurements) over 40 minutes, the cannula was connected, via tubing, to an automatic anaesthesia pump (Graseby 3500, Graseby Medical LTD, Watford Herts, UK) and all animals received a continuous infusion over 108 minutes of either saline (injection fluid containing 0.9% NaCl, Fresenius Kabi B.V., 's Hertogenbosch, The Netherlands) or different doses of fentanyl, i.e. 20, 40 and 50 µg/kg/h. The infusion time and doses of fentanyl were based upon our own laboratory experience (Haberham et al., 1999; Haberham et al., 2000; Sienen et al., 2004) and pilot experiments preceding this study. Fentanyl was diluted in saline, in a way that all animals received an equal volume of injection fluid during each session. Body temperature was maintained at 37-38°C by a water-heating pad placed under the experimental box. The sessions continued until one hour after the end of infusion. Recovery time between the sessions was at least two weeks and the sequence in receiving either saline or the different dosages of fentanyl, was randomly divided over the animals.

During sessions, every 3 minutes, the stimulations (n=72) to elicit a SEP or AEP started alternately.

2.3 Experiment 2: Neurophysiologic parameters in relation fear-conditioned behaviour

Fear-conditioning sessions were performed in the same box and under similar conditions as described in Experiment 1. The box was shielded by polystyrene plates preventing the animals from getting visual cues from outside the fear-conditioning box, only leaving access to a camera for observation of the animal's behaviour. The CS (a 40 seconds, 1500 Hz tone, 85 dB sound pressure level) was generated by a sound generator (Arbitrary Waveform Generator, Model 33120 A, Hewlett Packard, Palo Alto, CA, USA) and was presented by two speakers mounted in the covering lid of the box. The SEP stimulation paradigm (72 square-wave pulses of 2 ms duration with stimulus frequency 0.5 Hz and stimulus intensity 5 mA as in Experiment 1) served as US.

Approximately 30 minutes prior to the start of the experiment, the animals were placed in the experimental room to acclimatize. Next, a 24 gauge cannula was inserted in a lateral tail vein of the rats for intravenous administration of saline or fentanyl. Subsequently, the rats were fitted in a tight-fitting jacket allowing free movement, and the electrical stimulation device was fixed at the left tail base. Then, the animals were placed in the fear-conditioning box and connected to the stimulation and recording device. Next, the cannula was connected to the automatic anaesthesia pump and infusion of either saline or different doses of fentanyl started. The animals (n=53) were randomly assigned to the following groups: 1) CS-US 0 (n=9); 2) CS-US 20 (n=10); 3) CS-US 40 (n=8); 4) CS-US 50 (n=8); 5) US 0 (n=9) and 6) US 50 (n=9). The CS-US 0 and US 0 group received a continuous infusion of saline. The CS-US 20 and CS-US 40 group received a continuous infusion of 20 and 40 μg/kg/h fentanyl, respectively. The CS-US 50 and US 50 group both received a continuous infusion of 50 μg/kg/h fentanyl. Fentanyl was diluted in saline, in a way that all animals in each group received an equal volume of injection fluid. Body temperature was maintained at 37-38°C by a water-heating pad placed under the experimental box. Infusion continued in all groups until the end of the training session.

In all groups, fear-conditioning started after 80 minutes from the beginning of the infusion according to the results of experiment 1 (see section 3.2). The CS-US 0, CS-US 20, CS-US 40 and CS-US 50 groups were each subjected to a Pavlovian fear-conditioning paradigm in which the CS was presented paired with the US. The US started 10 seconds after the CS. The interval between the US onsets was 454 s. In the US 0 and US 50 group, the US was presented without the CS using the same interval between US onsets (454 s). The total number of CS-US pairings per group was 10. In all groups, SEPs were recorded for every US presentation during the fear-conditioning session.

The test sessions were performed in the same experimental room as the fear-conditioning sessions under similar conditions and started approximately 24 hrs after the end of the training sessions. The sessions were performed, in a Plexiglas box (41×31×20 cm) shielded by polystyrene plates, only leaving access to a camera recording the animal's behaviour. The box was different from the fear-conditioning box in dimensions, and cues at the bottom plate and internal walls. Two speakers in the covering lid of the box presented the CS.

All test sessions were performed during the 'lights off' period. Acclimatization in the experimental room before the actual start of the sessions was 30 minutes and in the test apparatus15 minutes. During the test sessions, the same CS as used in the training sessions was presented to all groups. The interval between the CS onsets varied between 180 to 480 seconds. The total number of test trials was 10.

2.4 Data and statistical analysis

Calculations were performed with Microsoft Excel 2000. Statistical analysis was performed with Sigmastat 2.0 and SPSS 11.0. Differences were considered to be significant when $P<0.05$.

2.4.1 Experiment 1

SEP and AEP data analysis were performed by visual determination of the prominent components in the SEP and AEP waves of the individual animals. Parameters extracted for analysis were the amplitude of the positive-to-negative components of the 1) Vx-SEP at approximately 15 and 20 ms; 2) SI-SEP at approximately 12 and 20 ms; 3) Vx-AEP at approximately 13 and 20 ms and 4) AI-AEP at approximately 7 and 10 ms (see Fig. 1A and 2A, upper trace: Vx-SEP P15-N19, SI-SEP P13-N18, Vx-AEP P14-N19 and AI-AEP P7-N10). Further, a signal-to-noise ratio was calculated as previously described (Stienen et al., 2003). Peak amplitudes with a signal-to-noise ratio <2, were assumed to be zero and were, therefore, replaced by the average noise value in the analysis.

For analysis of drug-effects within and between recording sites, the SEP and AEP amplitudes were expressed as the percentage change with respect to the average baseline measurements (8 baseline measurements for both SEP and AEP) for each time point after infusion, each treatment, each recording and each rat separately (relative amplitude). For statistical analysis of drug effect within and between recording sites, the relative amplitude of the SEP and AEP parameters was analysed separately in a three-way repeated analysis of variance (RM-ANOVA) design including repeated factors treatment (saline, 20, 40 and 50 µg/kg/h fentanyl), recording (SEP: vertex and SI; AEP: vertex and AI) and time followed by post hoc analysis whenever appropriate.

2.4.2 Experiment 2

In Experiment 2, determination of the prominent peaks in the SEP waves and parameters extracted for analysis, were similar as described above. In one animal in the US 50 group, neurophysiologic signals could not be recorded most probably due to a short circuit between the epidural electrodes. This animal was excluded from SEP data analysis. The absolute amplitude (μV) of the Vx-SEP P15 and SI-SEP P13, were analysed separately in a two-way RM-ANOVA design including fixed factor group (CS-US 0, CS-US 20, CS-US 40, CS-US 50, US 0 and US 50) and repeated factor trial followed by post hoc analysis whenever appropriate. For the analysis of drug effects between the recording sites, the SEP data were expressed as relative amplitude (%). Therefore, the SEP data of the CS-US 0 (n=9) and US 0 (n=9) and the SEP data of the CS-US 50 (n=8) and US 50 (n=8) were pooled, since there were no significant differences between these groups, respectively (see section 3.3.1). Then, the data were averaged over the ten trials per rat, since there were no significant trial effects (see section 3.3.1). Subsequently, the SEP data were expressed as the percentage change with respect to the average of the measurements of the saline group for each recording site and rat, separately. For statistical analysis of the drug effects between the recording sites, the relative amplitude was analysed in a two-way RM-ANOVA design including fixed factor treatment (saline, 20, 40 and 50 μg/kg/h fentanyl) and repeated factor recording site (vertex and SI) followed by post hoc analysis whenever appropriate.

To study the CS-induced fear-conditioned behaviour in the groups, the amount of freezing behaviour during the presentation of the CS in the test session was evaluated after the experiments using the video-registrations. Freezing behaviour was defined as: the rat adopts a tensed posture with no visible movements except for breathing movements and pendulum motion of the head. Pendulum motion has been described under circumstances of emotional excitement, most often fear (Kolpakov et al., 1977). During behavioural evaluation, the experimenter (PJS) was blind with respect to the groups. The behavioural evaluation of the experimenter correlated highly with the evaluation of a second external observer, who was not aware of the aims and procedures of this experiment (Pearson's correlation, r=0.89, P<0.001, n=35). For statistical analysis, the amount of freezing behaviour was analysed in a two-way RM-ANOVA design including fixed factor group

(CS-US 0, CS-US 20, CS-US 40, CS-US 50, US 0 and US 50) and repeated factor trial followed by post hoc analysis whenever appropriate.

3. Results

3.1 General waveforms of the SEPs and AEPs

Fig. 2A (upper trace) and Fig. 3A (upper trace), show the general average waveforms of the SEPs and AEPs, respectively. Consistent and similar waveforms were recorded across subjects. The traces show a clear Vx-SEP P15 (± 0.19) and N19 (±0.14) (rounded to the nearest mean value ± standard error of the mean (S.E.M)), SI-SEP P13 (± 0.05) and N18 (±0.06), Vx-AEP P14 (±0.10) and N19 (±0.09) and AI-AEP P7 (±0.05) and N10 (±0.09).

3.2 Experiment 1: Characterisation of fentanyl effects on somatosensory and auditory processing by SEPs and AEPs, respectively

3.2.1 Fentanyl effect on SEPs

The relative amplitudes of the Vx-SEP and SI-SEP (Fig. 2B) were not affected by saline infusion. Both the relative amplitudes of the Vx-SEP and SI-SEP were affected by fentanyl treatment over time, but their latencies were negligibly affected (Fig. 2A and B). Visual observation showed both the Vx-SEP and SI-SEP to be stable at 63 minutes after infusion onset of the different treatments. Following the end of the different fentanyl infusions, both parameters gradually recovered over time (Fig. 2B).

Overall statistical analysis over the period from 63 minutes to the end of infusion (t=108 min) showed that the relative amplitude of the Vx-SEP and SI-SEP during all treatments, were not affected over time (treatment×recording×time: $F_{21,168}$=0.748, P=0.778; treatment×time: $F_{21,168}$=0.694, P=0.835; recording×time: $F_{7,56}$=0.648, P=0.714; time: $F_{7,56}$=1.033, P=0.419), but were significantly and differently affected by the specific treatments (treatment×recording: $F_{3,24}$=11.355, P<0.001). Post hoc analysis showed that the relative amplitude of the Vx-SEP was significantly lower during 40 and 50 µg/kg/h fentanyl compared to saline and 20 µg/kg/h fentanyl (Fig. 2C) (one-way RM-ANOVA, factor treatment: $F_{3,24}$=19.558, P<0.001 followed by Student-Newman-Keuls for post hoc comparison (SNK), P<0.05. The relative amplitude of the SI-SEP was significantly lower

during 50 µg/kg/h compared to saline and 20 µg/kg/h fentanyl (Fig. 2C) (one-way RM-ANOVA, factor treatment: $F_{3,24}$=4.952, P=0.008 followed by SNK, P<0.05.

The relative amplitude of the Vx-SEP during 20, 40 and 50 µg/kg/h fentanyl was significantly lower compared to the relative amplitude of the SI-SEP (Fig. 2C) (paired t-test: 20 µg/kg/h fentanyl: t_8=-5.093, P=0.001; 40 µg/kg/h fentanyl: t_8=-4.988, P=0.001 and 50 µg/kg/h: t_8=-9.543, P<0.001).

3.2.2 Fentanyl effect on AEPs

The relative amplitudes of the Vx-AEP and AI-AEP were not affected by saline infusion (Fig. 3B). The relative amplitude of the Vx-AEP was affected by fentanyl treatment, whereas the latency of the Vx-AEP and the AI-AEP and the relative amplitude of the AI-AEP were negligibly affected (Fig. 3A and B). Visual observation showed both parameters to be stable at 69 minutes after infusion onset of the different treatments. After the end of infusion, both parameters gradually recovered over time (Fig. 3B).

Overall statistical analysis over the period from 69 minutes to the end of infusion showed that the relative amplitude of the Vx-AEP and AI-AEP during all treatments, were not affected over time (treatment×recording×time: $F_{18,144}$=1.010, P=0.453; treatment×time: $F_{18,144}$=1.267, P=0.218; recording×time: $F_{6,48}$=0.155, P=0.987; time: $F_{6,48}$=0.917, P=0.491), but were significantly and differently affected by the different treatments (treatment×recording: $F_{3,24}$=12.179, P<0.001). Post hoc analysis showed that the relative amplitude of the Vx-AEP was significantly lower during 40 and 50 µg/kg/h fentanyl compared to saline and 20 µg/kg/h fentanyl (Fig. 3C) (one-way RM-ANOVA, factor treatment: $F_{3,24}$=22.816, P<0.001 followed by SNK, P<0.05). No differences existed between the relative amplitude of the AI-AEP during the different treatments (Fig. 3C) (one-way RM-ANOVA, factor treatment: $F_{3,24}$=2.774, P=0.063).

The relative amplitude of the Vx-AEP was significantly lower during 40 and 50 µg/kg/h fentanyl, when compared to the relative amplitude of the AI-AEP (Fig. 3C) (paired t-test: 40 µg/kg/h fentanyl: t_8=-3.581, P=0.007 and 50 µg/kg/h: t_8=-5.594, P=0.001).

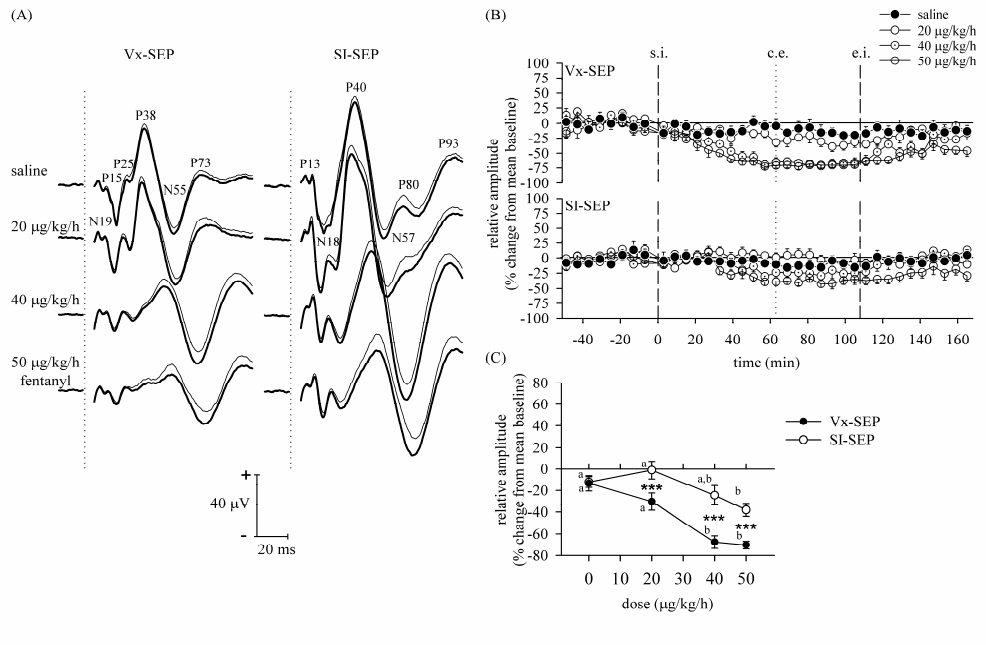

Fig. 2. SEP monitoring during continuous infusion of different doses of fentanyl. (A) Grand mean (bold lines) + S.E.M. (plain lines) waveforms (n=9) of the Vx-SEP and SI-SEP during different doses of fentanyl. Dotted line and curve interruption denote stimulus onset. Peaks are designated by either P (positive peak) and N (negative) peak accompanied by their rounded off mean latency (ms). For each animal, the Vx-SEP and SI-SEP resulted from the average of their corresponding SEP recordings during the period in which continuous infusion of fentanyl resulted in a constant effect on the SEP parameters. (B) Effect of fentanyl on the SEP parameters. Data are represented as the mean relative amplitude (%; +/- S.E.M.; n=9) over time following infusion of either saline or 20, 40, or 50 µg/kg/h. s.i. = start infusion; c.e. = time point that fentanyl infusion resulted in a constant effect on the SEP parameters; e.i.= end infusion. (C) Differences between the effect of fentanyl on the Vx-SEP and SI-SEP in the period after fentanyl infusion resulted in a constant effect on the SEP parameters. Data are represented as the mean relative amplitude (± S.E.M., n=9) against the dose of fentanyl. Data points corresponding to either Vx-SEP or SI-SEP with the same characters are not different from one another [one-way ANOVA, factor dose followed by SNK]. ***P<0.001 [Vx-SEP vs. SI-SEP; post hoc paired t-test].

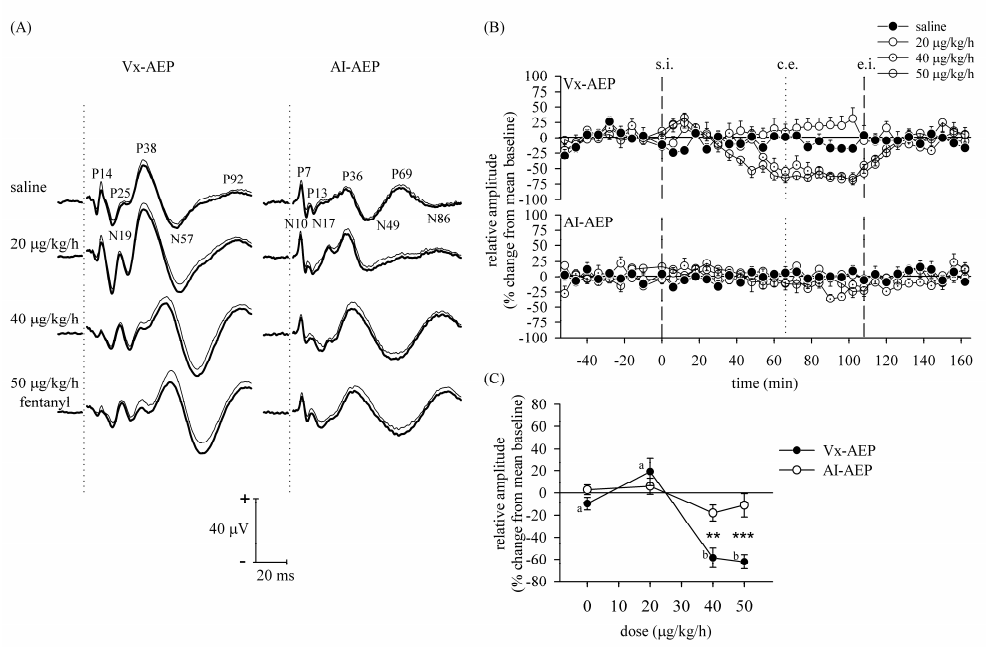

Fig. 3. AEP monitoring during continuous infusion of different doses of fentanyl. (A) Grand mean (bold lines) + S.E.M. (plain lines) waveforms (n=9) of the Vx-AEP and AI-AEP during different doses of fentanyl. Dotted line and curve interruption denote stimulus onset. Peaks are designated by either P (positive peak) and N (negative) peak accompanied by their rounded off mean latency (ms). For each animal, the Vx-AEP and AI-AEP resulted from the average of their corresponding SEP recordings during the period in which continuous infusion of fentanyl resulted in a constant effect on the AEP parameters. (B) Effect of fentanyl on the AEP parameters. Data are represented as the mean relative amplitude (%; ± S.E.M.; n=9) over time following infusion of either saline or 20, 40, or 50 µg/kg/h. s.i. = start infusion; c.e. = time point that fentanyl infusion resulted in a constant effect on the AEP parameters; e.i.= end infusion. (C) Differences between the effect of fentanyl on the Vx-AEP and AI-AEP. Data are represented as the mean relative amplitude (± S.E.M., n=9) against the dose of fentanyl. Data points corresponding to either Vx-AEP or AI-AEP with the same characters are not different from one another [one-way ANOVA, factor dose followed by SNK]. **P<0.01, ***P<0.001 [Vx-SEP vs. SI-SEP; post hoc paired t-test].

3.3 Experiment 2: SEP-fear conditioning model: neurophysiologic parameters in relation fear-conditioned behaviour

3.3.1 SEP parameters

During fear-conditioning, overall statistical analysis showed that for all groups, the absolute amplitude of both the Vx-SEP and SI-SEP were not affected over trials (Fig. 4A, B) (Vx-SEP amplitude: trial×group: $F_{45,414}$=0.887, P=0.681; trial: $F_{9,45}$=1.650, P=0.099; SI-SEP amplitude: trial×group: $F_{45,414}$=1.220, P=0.164; trial: $F_{9,45}$=1.342, P=0.213). The absolute amplitude of the Vx-SEP was different between the groups, whereas the absolute amplitude of the SI-SEP was not different between the groups (Fig. 4A and B) (Vx-SEP amplitude: group: $F_{5,46}$=11.514, P<0.001; SI-SEP amplitude: group: $F_{5,46}$=2.132, P=0.078). Post hoc analysis showed that the amplitude of the Vx-SEP in the CS-US 40, CS-US 50 and US 50 group was significantly lower, when compared to the CS-US 0, CS-US 20 and US 0 group (Fig. 4A) (SNK: P<0.05).

When expressed as the percentage amplitude change with respect to the mean amplitude of the saline group, overall statistical analysis showed that the relative amplitude of the Vx-SEP and of the SI-SEP were affected differently by the different treatments (Fig. 4D) (treatment×recording: $F_{3,48}$=14.773, P<0.001). Post hoc analysis showed that the relative amplitude of the Vx-SEP was significantly lower during 40 and 50 µg/kg/h fentanyl compared to saline and 20 µg/kg/h fentanyl (Fig. 4D) (one-way ANOVA, factor treatment: Vx-SEP: $F_{3,48}$=19.300, P<0.001 followed by SNK, P<0.05). The relative amplitude of the SI-SEP was significantly lower during 50 µg/kg/h fentanyl compared to saline and 20 µg/kg/h fentanyl (one-way ANOVA, factor treatment: SI-SEP: $F_{3,48}$=3.518, P=0.022).

The relative amplitude of the Vx-SEP was significantly lower during 20, 40 and 50 µg/kg/h fentanyl compared to the SI-SEP P13 (Fig. 4D) (paired t-test: 20 µg/kg/h fentanyl: t_9=-3.340, P=0.009; 40 µg/kg/h fentanyl: t_7=-5.926, P<0.001and 50 µg/kg/h fentanyl t_{15}=-9.945, P<0.001).

3.3.2 Freezing behaviour

Although the tone induced freezing behaviour in all groups, the duration of freezing behaviour differed between the groups over trials (Fig. 4C) (trial×group: $F_{45,423}$=3.832,

P<0.001). Post hoc analysis showed that the duration of freezing behaviour in all groups, decreased significantly over the trials (one-way RM ANOVA, factor trial: *P*<0.001 in all groups). In the CS-US 0 group, the duration of freezing behaviour overall was significantly longer over the trials, when compared to the CS-US 20, CS-US 40, CS-US 50, US 0 and US 50 group (Fig. 4C) (one-way ANOVA, factor group followed by SNK: see Fig. 4C for results). Further, although in the first trial, the duration of freezing behaviour in the CS-US 20 group was similar compared to CS-US 40, CS-US 50 and US 0 group, in trial 2-6, the duration of freezing behaviour in the CS-US 20 group overall was significantly longer and the extinction rate of freezing behaviour was significantly less, when compared to the CS-US 40, CS-US 50, US 0 and US 50 group (Fig. 4C) (one-way ANOVA, factor group followed by SNK: see Fig. 4C for results). The duration of freezing behaviour displayed by the different groups, was highly correlated with the level of the Vx-SEP amplitude (Fig. 4E) (Pearson's correlation, *r*=0.71, n=35, *P*<0.001), whereas correlation between the duration of freezing behaviour and the level of the SI-SEP amplitude was absent (Fig. 4F) (Pearson's correlation, *r*=0.29, n=35, *P*=0.09). Finally, the correlation coefficient resulting from the correlation analysis between the duration of freezing behaviour and the Vx-SEP amplitude, was significantly different from that of the correlation analysis between the duration of freezing behaviour and the SI-SEP amplitude (Fisher *r*-to-*z* transformation, $z = 2.35$, $P = 0.019$).

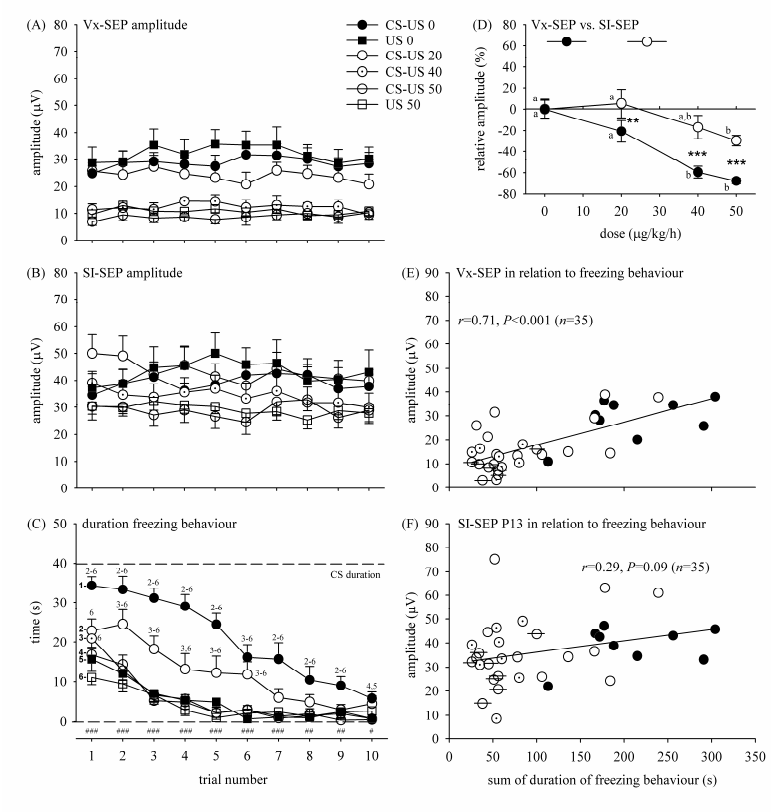

Fig. 4. Effect of fear-conditioning during continuous infusion of different doses of fentanyl on SEP parameters and fear-conditioned behaviour. (A) Effect of fear-conditioning on the amplitude of the Vx-SEP in the different treated groups. Data are represented as the mean amplitude (µV; ± S.E.M.) over the ten training trials. (B) Effect of fear-conditioning on the amplitude of the SI-SEP in the different treated groups. Data are represented as the mean amplitude (µV; ± S.E.M.) over the ten training trials. (C) Duration of freezing behaviour in the different groups during CS presentation. Data are represented as the mean duration of freezing behaviour (s; ± S.E.M.) over the subsequent test trials after fear-conditioning. #P<0.05, ##P<0.01, ###P<0.001 [one-way ANOVA, factor dose; the numbers (1 to 6) refer to significant differences compared to the group(s) that are designated by that number (post hoc SNK)]. (D) Differences between the effect of fentanyl on the Vx-SEP and SI-SEP during fear-conditioning. Data are represented as the mean relative amplitude (%;± S.E.M.) against the dose of fentanyl (dose (µg/kg/h): 0 (saline): n=18; 20: n=10; 40: n=8 and 50: n=16). Data points corresponding to either Vx-AEP or ACx-AEP with the same characters are not different from one another [one-way ANOVA, factor dose followed by SNK]. **P<0.01, ***P<0.001 [Vx-SEP vs. SI-SEP; post hoc paired t-test]. (E) Pearson product-moment correlation of averaged (10 trials) Vx-SEP amplitude (µV) and the sum (10 trials) of duration of freezing behaviour (s). (F) Pearson product-moment correlation of averaged (10 trials) SI-SEP amplitude (µV) and the sum (10 trials) of duration of freezing behaviour (s).

4. Discussion

The major findings of the present study are, firstly, that fentanyl dose-dependently decreased the amplitude of the Vx-SEP, while the SI-SEP was minimally, although significantly, affected. These results support that the Vx-SEP and SI-SEP are related to nociception as previously reported (Stienen et al., 2004; 2005) . Both noxious and tactile responses can be found within the 10-30 ms latency range (Schouenborg et al., 1986; Bromm and Lorenz, 1998; Shaw et al., 1999; Heppelmann et al., 2001). However, with μ-opiate receptor agonists like fentanyl modulating noxious, rather than tactile, processing (Yeomans et al., 1996a; 1996b; Kalliomaki et al., 1998; Casey et al., 2000; Silbert et al., 2003), the Vx-SEP and SI-SEP in the present study can be considered to represent processing of noxious stimuli. Secondly, fentanyl dose-dependently decreased the CS-induced fear-response as measured during the test sessions. This decrease correlated with the dose-dependent decrease of the Vx-SEP, rather than the SI-SEP. Combined, we suggest that the dose-dependent decrease of the Vx-SEP amplitude, rather than of the SI-SEP, indicates that the US was experienced as less unpleasant.

4.1 Fear-conditioned behaviour and the formation of a CS-US association

The tone-induced freezing behaviour in the US 0 and US 50 group in the present study, explicitly represents a 'novelty' response and can not be related to CS-US association, as no CS was presented during training. The duration of freezing behaviour of the CS-US 0 and CS-US 20 group was significantly greater than the duration of freezing behaviour of the US 0 and US 50 group. Therefore, the duration of freezing behaviour of the CS-US 0 and CS-US 20 group can be ascribed to the formation of a CS-US association during conditioning, rather than being a 'novelty' response.

The duration of freezing behaviour of the CS-US 40 and CS-US 50 group was not different with respect to that of the US 0 and US 50 group, but was different with respect to that of the CS-US 0 and CS-US 20 group. Therefore, we suggest that the CS-US 40 and CS-US 50 group formed no CS-US assocation during fear-conditioning.

The fact that the tone-induced freezing behaviour of the US 50 group was similar to that of the US 0 group suggests that fentanyl does not induce a releveant effect on auditory processing and behavioural expression during testing afterwards, i.e. does not increase or decrease freezing behaviour due to a difference in the perception of tones. Therefore, the

duration of freezing behaviour in the CS-US 20, CS-US 40 and CS-US 50 group, can be ascribed to fentanyl-induced effects on the formation of a CS-US association during fear-conditioning, rather than to fentanyl-induced effects on tone processing and behavioural expression during testing afterwards.

4.2 Neurophysiologic parameters in relation to fear-conditioned behaviour

The effects of fentanyl on the Vx-SEP amplitude, rather than on the SI-SEP, correlated with the fentanyl-induced effects on the formation of a CS-US association. Therefore, the fentanyl-induced dose-dependent reduction of the Vx-SEP amplitude, rather than of the SI-SEP, is suggested to be related to a reduced perception of the US indicating that the US was experienced as less unpleasant. Analgesic drug-induced SEP changes can be considered to represent analgesic drug-induced changes in US processing, and the magnitude of the CS-induced fear-response, can be considered a measure of the animal's adversity to the US during fear-conditioning. Combined, analgesic drug-induced SEP changes during fear-conditioning, that correlate with a reduced CS-induced fear-response after conditioning, i.e. the changes of the Vx-SEP rather than of the SI-SEP in the present study, may therefore be assumed to indicate that the US was experienced as less unpleasant.

The AEP results in the present study suggest that, next to an effect on US processing, fentanyl affected CS processing during fear-conditioning. This might have contributed to the less distinct or absence of the formation of a CS-US association in the CS-US 20 and CS-US 40 and CS-US 50 group, respectively, during fear-conditioning.

The Vx-AEP can be considered a specific measure of arousal-dependent perceptual awareness of auditory stimuli. Considerable evidence exists on the involvement of the pedunculopontine nucleus (PPN) and/or its medial thalamic output projections in the activating reticular system (RAS), in the generation of the Vx-AEP component occuring at 11-15 ms (P14-N19 in the present study) (Reese et al., 1995a; 1995b; Miyazato et al., 1999; 2000; Teneud et al., 2000). The AI-AEP occuring at 6-9 ms (P7-N10 in the present study), is well established as primary cortical excitation of the AI in the primary auditory pathway (Barth and Di, 1990). PPN to medial thalamic output projections have been implicated in arousal to auditory stimuli (i.e. 'wake –up, something happened') (Garcia-Rill et al., 2004; Skinner et al., 2004), whereas the AI is involved in auditory spatio-temporal localisation and intensity quantification (i.e. 'what is it') (de Ribaupierre, 1997). Therefore, the

decreases in Vx-AEP amplitude rather than in the AI-AEP in Experiment 1 in the present study, can be considered to be related to a reduced perception of auditory stimuli, and thus, indicate fentanyl effects on CS processing during fear-conditioning in Experiment 2. It should be noted here that studying possible fentanyl effects on auditory processing by AEPs during fear-conditioning following the SEP fear-conditioning model (van Oostrom et al., 2005) was experimentally limited. AEPs are evoked by short, repeated auditory stimuli rather than a continous tone like the CS, which, consequently, is not useful to evoke AEPs during fear-conditioning.

Since following 20 μg/kg/h fentanyl administration, the Vx-AEP amplitude was not affected, the less distinct CS-US association in the CS-US 20 group, is to be ascribed to effects of fentanyl on US processing, rather than CS processing. This is in accordance with the fact that μ-opioids such as fentanyl are primarily analgesic (20 μg/kg/h fentanyl in the present study), and therefore, can be considered to primarily suppress processing of nociceptive stimuli (US), rather than other sensory processes (CS). The absence of the CS-US association in the CS-US 40 and CS-US 50 group, can be ascribed to an effect of fentanyl on CS processing, next to US processing, since the Vx-AEP amplitude was decreased by 40 and 50 μg/kg/h fentanyl. This is in accordance with the fact that μ-opioids at higher doses (40 and 50 μg/kg/h fentanyl in the present study) do modulate other sensory processes including auditory processing.

From the present data, the possibility that fentanyl affects the ability of associating the CS and US at the level of the amygdala, which is the locus for formation and storage of CS-US associations during fear-conditioning (Maren, 2001; LeDoux, 2003) can not be excluded. Therefore, next to the effects of fentanyl on US and CS processing represented by decreases of the Vx-SEP and Vx-AEP amplitude, respectively, the possibility that fentanyl affects associating the CS and US at the level of the amygdala, might potentially have contributed to the less distinct or absence of the formation of a CS-US association in the CS-US 20 and CS-US 40 and CS-US 50 group, respectively, during fear-conditioning.

An alternative, albeit unlikely, influence on the fentanyl-induced effects on the CS-US association in the CS-US 20, CS-US 40 and CS-US 50 groups, might be found in state-dependent learning. This refers to the situation whereby information learned while the animal is under the influence of a certain drug (‘state’), such information can be recalled only when the animal is in the same ‘state’ (Overton, 1985). However, this phenomenon is

most often very weak or absent (Overton, 1985; Maes and Vossen, 1997) and therefore, is highly unlikely to have a significant role in the present study.

4.3 Conclusions

In summary, the fact that the dose-dependent decrease of the Vx-SEP amplitude, rather than the SI-SEP, was paralleled by a dose-dependent decrease of the duration of freezing behaviour, suggests that the Vx-SEP, rather than the SI-SEP, signals unpleasantness of noxious stimuli, and thus is potentially a useful and reliable readout-parameter in pain and anaesthesiologic research. For a meaningful interpretation of the SEPs in pain assessment, in future studies, the Vx-/SI-SEP is to be investigated in relation to the structure and function of separate functional nociceptive pathways relaying either unpleasantness or spatio-temporal localisation and intensity quantification of noxious stimuli according to the possible mechanisms underlying the differences between the Vx-SEP and SI-SEP as recently described in full detail (Stienen et al., 2004; 2005).

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4

Somatosensory-evoked potentials indicate increased unpleasantness of noxious stimuli in response to increasing stimulus intensities in the rat.

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Abstract

Recently, it has been shown in rats that specific characteristics of somatosensory-evoked potentials (SEPs) recorded from different sites on the scalp correlate differently to the amount of unpleasantness experienced by the animal following noxious stimulation. It was shown that the SEP recorded from vertex (Vx-SEP) did correlate with the unpleasantness, whereas the SEP recorded from the primary somatosensory cortex (SI-SEP) did not. In the present study we further investigated the relationship between the Vx-SEP, the SI-SEP and the unpleasantness of noxious stimuli. Therefore, different groups of rats were subjected to a SEP fear-conditioning paradigm in which the unconditioned stimulus (US), represented by noxious stimuli applied to evoke SEPs, was paired to a conditioned stimulus (CS) represented by a tone. Different stimulus intensities of the US were applied in the different groups. After CS-US presentation, CS-induced fear-conditioned behaviour was analysed in relation to the characteristics of the Vx- and SI-SEP during CS-US presentation. Results showed that increasing stimulus intensities led to increased SEP amplitudes which were paralleled by an increased amount of CS-induced fear-conditioned behaviour. However no differences between Vx-SEP and SI-SEP were found. The increase in the SEPs in parallel with the increased amount of fear-induced behaviour further supports the SEP to be a potentially valuable tool for studying acute pain and analgesia in animals.

1. Introduction

Evoked potentials recorded from the scalp after noxious stimulation (SEPs) represent neuronal processing of the noxious stimulus in the brain (Bromm and Lorenz, 1998; Kakigi et al., 2000; Spiegel et al., 2000). SEPs recorded from vertex (Vx-SEP) in man show interesting characteristics making them useful to study acute pain and analgesia. Vx-SEPs 1) are sensitive to analgesic intervention (Arendt-Nielsen, 1994; Beydoun et al., 1997; Liu et al., 2005), 2) show a positive correlation with the unpleasantness of the noxious stimuli applied to evoke the SEP (Bjerring and Arendt-Nielsen, 1988; Arendt-Nielsen, 1994; Kakigi et al., 2000; Kanda et al., 2002; Ianetti et al., 2004) and 3) are absent or altered in patients suffering from deficits in pain sensation (Bromm et al., 1991; Lorenz et al., 1996a; 1996b; Kakigi et al., 2000; Spiegel et al., 2003; Lefaucheur and Creange, 2004).

In animals, SEPs are also of interest to study acute pain and analgesia. SEPs recorded in animals 1) are sensitive to analgesic intervention (Beydoun et al., 1997; Kalliomaki et al., 1998; Logginidou et al., 2003; Shi et al., 2004; Stienen et al., 2004;2005;2006) and 2) a recent study in rats showed a correlation between the Vx-SEP and the unpleasantness of the noxious stimuli applied to evoke the SEP (Stienen et al., 2006) using a model based on Pavlovian fear-conditioning (van Oostrom et al., 2005). This Pavlovian fear-conditioning model consists of two sessions executed on consecutive days. On day 1 (training session) rats are subjected to a paradigm in which an innocuous tone, the conditioned stimulus (CS), is presented paired to the unconditioned stimulus (US), which is represented by noxious stimuli applied to evoke a SEP (SEP stimulation paradigm). On day 2 (testing session) the strength of the rat's CS-US association is investigated by determining the duration of freezing behaviour after presenting the CS without the US. The literature provides ample evidence that the duration of freezing behaviour is to be considered to reflect the unpleasantness of the noxious stimuli experienced by the animal (Fanselow and Bolles, 1979; Anagnostaras et al., 2000; van Oostrom et al., 2005). Thus, correlating the SEPs recorded on day 1 with the duration of freezing behaviour recorded on day 2 shows whether the SEP indicates the unpleasantness of the noxious stimuli. In a previous study, using this model in combination with the μ-opioid analgesic fentanyl applied to modulate the unpleasantness of the US, it was shown that SEPs recorded from vertex (Vx-SEP) did signal the unpleasantness of noxious stimuli whereas SEPs recorded from the primary somatosensory cortex (SI) (SI-SEP) did not (Stienen et al., 2006). However, analgesic

drugs can influence the outcome of Pavlovian fear-conditioning tasks by other mechanisms than analgesic actions alone (Davies et al., 2004; Zarrindast and Rezayof, 2004; Pietersen et al., 2006). Therefore the relation between the Vx-SEP and the unpleasantness of the noxious stimuli, found in the previous study (Stienen et al., 2006), might be confounded by the use of fentanyl. Although the possible co-influences of fentanyl were argued to be of little importance in the previous study (Stienen et al., 2006), the present study further investigated the relation between both the Vx-SEP and the SI-SEP and the unpleasantness of the noxious stimuli. To this aim, we used the SEP fear-conditioning model while modulating the US unpleasantness by using different stimulus intensities instead of a pharmacological intervention.

2. Materials and Methods

2.1 Animals and surgery

Animal care and experimentation were performed in accordance with protocols approved by the Science Committee and the local Animal Experimentation Committee (Utrecht University, Utrecht, The Netherlands).

Adult male Wistar rats (HsdCpb:WU, Harlan Netherlands BV, Horst, The Netherlands, body weight 300-350 g, *n*=48) were permanently instrumented with epidurally placed EEG recording electrodes. After induction of anaesthesia with 0.3 mg/kg fentanyl (i.p., Fentanyl Janssen®, Janssen-Cilag BV, Tilburg, The Netherlands) and 0.3 mg/kg medetomidine (i.p., Domitor®, Pfizer Animal Health BV, Capelle a/d IJssel, The Netherlands), the animals were fixed in a stereotaxic apparatus (Model 963, Ultra Precise Small Animal Stereotaxic, David Kopf Instruments, Tujunga, CA, USA). Epidural electrodes (wired stainless steel screws, tip diameter 0.6 mm, impedance 300-350Ω, Fabory DIN 84A-A2, Fabory, Nieuwegein, The Netherlands) were implanted at the vertex (4.5 mm caudal to bregma, 1.0 mm right from midline), the primary somatosensory cortex (2.5 mm caudal to bregma, 2.5 mm right from midline), and bilateral in the frontal sinus (10.0 mm rostral to bregma, 1.0 mm left and right from midline, respectively) (Stienen et al., 2004). All electrodes were wired to an eight-pin receptacle (Mecap Preci-Dip 917-93-108-41-005, Preci-Dip Durtal SA, Delémont, Switzerland) and fixed to the skull with dental cement (Simplex Rapid, Associated Dental

Products Ltd, Swindon, UK). At the end of surgery, anaesthesia was antagonized with 1 mg/kg atipamezole (s.c., Antisedan®, Pfizer Animal Health BV, Capelle a/d IJssel, The Netherlands) and 0.15 mg/kg buprenorphine (s.c., Temgesic®, Schering-Plough, Amstelveen, The Netherlands). Postoperative analgesia was provided by 0.15 mg/kg buprenorphine administered s.c. at 8 hour intervals for three days after surgery. One animal did not survive surgery.

2.2 Fear-conditioning (training session)

The fear-conditioning sessions on day 1 (training session) were performed under the same conditions and using the same procedures as described previously (van Oostrom et al., 2005; Stienen et al., 2006). In brief, after 30 minutes of acclimatization in the experimental room the animals were fitted in a tight fitting jacket and an electrical stimulation device (two brass electrodes of 3 mm in diameter tapered towards the end, fixed in a silicon tube, which enclosed the tail base (Stienen et al., 2004)) was fixed at the left tail base and tightened by Velcro tape for maximal fixation. Subsequently, the animals were placed in the fear-conditioning box, and the receptacle at the animal's head was wired, via a swivel-connector, to the SEP recording device. Through the open middle of this swivel-connector, the stimulation device was wired to a Grass-stimulator via a separate swivel-connector. This approach allowed free movement of the animal during the session. The sessions consisted of 10 CS-US pairings and started after 15 minutes of acclimatization in the box. The US (square wave pulses, n = 72 of 2 ms duration each, with a stimulus frequency of 0.5 Hz; total time 144 seconds) was presented 10 seconds after onset of the CS (a 40 seconds 1500 Hz tone, 85 dB sound pressure level) creating a 30 seconds overlap. Time between subsequent CS onsets varied between 358 and 502 seconds. The US intensity was 0.0, 0.5, 1.0, 2.0, 3.0 or 5.0 mA for group 0.0, 0.5, 1.0, 2.0, 3.0 and 5.0 respectively (n=8 for every group except for group 0.0, n=7). During the fear-conditioning sessions SEPs were recorded in the freely moving animal for every US presentation, as described previously (Stienen et al., 2003). In brief, SEPs were recorded from the vertex and the primary somatosensory cortex electrodes with the ipsi- and contra-lateral frontal sinus electrodes serving as reference and ground, respectively. Signals were amplified 2000 times, band-pass filtered between 15 and 300 Hz and digitized online at 2000 Hz. For each SEP trial, 72

subsequent data segments of 256 data points (25 ms pre-stimulus, 102.5 ms post stimulus) were recorded and averaged online.

2.3 Assessment of CS-US association (testing session)

The sessions to assess the CS-US association on day 2 (testing session) were performed under the same conditions and using the same procedures as described previously (van Oostrom et al., 2005; Stienen et al., 2006). In brief, after 30 minutes of acclimatization in the experimental room the animals were placed in the testing box. After 15 minutes of acclimatization in the box the testing session, consisting of 10 CS presentations, started. Time between subsequent CS onsets varied between 196 and 370 seconds. During these sessions the behaviour of the animals was videotaped continuously.

2.4 Data and statistical analysis

Calculations were performed with the aid of Microsoft Excel 2000. Statistical analysis was performed with SPSS 11.0 for Windows. Differences were considered to be significant when $P<0.05$.

The SEP signals were evaluated using the Rate Dispersion Factor (RDF. The RDF, an expression of the overall shape of the SEP waveform in the latency range studied, is obtained by averaging the absolute differences between all pairs of subsequent sampled data points y_k in a specified latency range from x to m (see equation 1) (Mantzaridis and Kenny, 1997; Haberham et al., 2000; Stienen et al., 2004). Both decreases in amplitude and increases in latency of the SEP components, decrease the value of the RDF, whereas both increases in amplitude and decreases in latency of the SEP components increase the value of the RDF. When choosing the RDF latency range (x to m) in the group with the strongest signals (5.0 mA group), individual signals at all stimulus intensities can be analyzed using this fixed latency range. Therefore the RDF is a highly objective method to evaluate SEP signals without the need of choosing peak amplitudes in individual signals which is prone to confounding errors, especially when signals are weak.

For the Vx-SEP the RDFs were calculated for the latency range (x to m) of the positive-to-negative complex previously described as the P15-N20 complex (Stienen et al., 2006), recorded in the 5.0 group. For the SI-SEP the RDFs were calculated for the latency range (x to m) of the positive-to-negative complex previously described as the P12-N20

complex (Stienen et al., 2006), recorded in the 5.0 group. For both the Vx-SEP and the SI-SEP, the RDF was calculated as the percentage change over groups, using the mean RDF value of both Vx-SEP and SI-SEP of the 5.0 group as 100%, since in this group the signals had the highest amplitudes and the shortest latencies, resulting in the highest RDFs.

$$\text{Equation 1}: \text{RDF} = \frac{1}{m - x} \sum_{k=x+1}^{m} \left| y_k - y_{k-1} \right|$$

The duration of freezing behaviour during the testing sessions was analyzed using the video recordings. Freezing behaviour was defined as the absence of all visible movements with the exception of breathing movements and pendulum motion of the head, while the animal sits in a tensed posture (Kolpakov et al., 1977). Freezing was scored only during presentation of the CS. The behavioural data were scored blindly with respect to the stimulus intensity used and the scorings of the principal investigator [HvO] demonstrated a high correlation with the scorings of a second observer who was unaware of the aims and procedures of this experiment (Pearson's correlation coefficient r=0.959, P<0.001, n=36).

For each animal the SEP data of the ten trials were averaged for both recording sites and subsequently analysed using a repeated measurement analysis of variance with fixed factor "group" and repeated factor "recording site". The freezing data of the ten trials were averaged and subsequently analysed using a one-way analysis of variance with factor "group".

3. Results

The grand average waveforms of the Vx-SEP and SI-SEP are shown in Fig. 1A and 1B respectively. At the intensity of 5.0 mA the Vx-SEP P15 and SI-SEP P12 occurred at a latency of 14.37 ± 0.09 and 13.95 ± 0.06 ms, respectively (mean ± S.E.M.). The Vx-SEP N20 and SI-SEP N20 occurred at a latency of 18.51 ± 0.37 and 18.21 ± 0.1 ms, respectively (mean ± S.E.M.)

As shown in Fig. 1C, increasing stimulus intensities resulted in increasing RDF values for both Vx-SEP and SI-SEP. Relative RDFs calculated over the specific latency range (x

to *m*) did not show differences for recording site (recording site×group: $F_{5,41}$=0.578, P=0.717; recording site: $F_{1,41}$=3.028, P=0.089) but significantly increased with higher stimulus intensities (group: $F_{5,41}$=7.064, P<0.001).

None of the experimental groups showed freezing behaviour during the 15 minutes of acclimatization in the testing box. Onset of the CS, however, induced freezing behaviour. The duration of freezing behaviour increased significantly with increasing stimulus intensities (group: $F_{5,41}$=6.207, P<0.001). As shown in figure 1C, the duration of freezing behaviour paralleled the increase in RDFs of the SEPs.

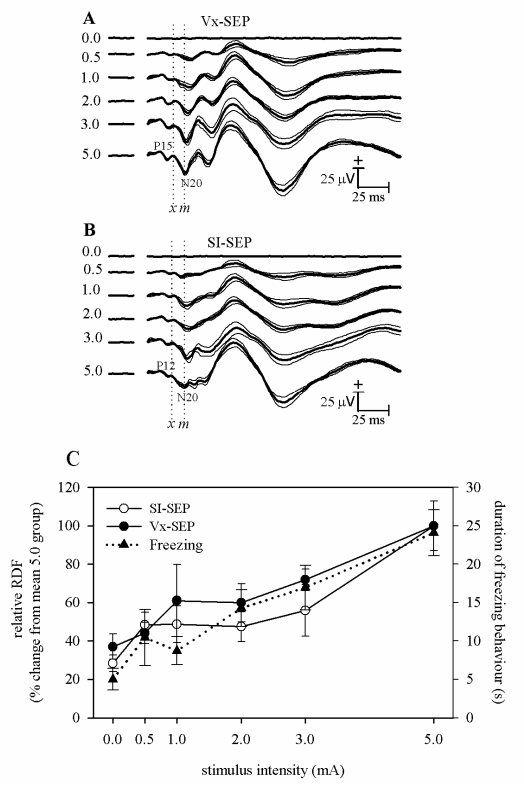

Fig. 1. SEPs in relation to stimulus intensity. (A) and (B) Grand mean (bold lines) ± S.E.M. (plain lines) waveforms of the Vx-SEP and SI-SEP, respectively, at the different stimulus intensities (n=8 for every intensity except for intensity 0.0, n=7). Dashed lines *x* and *m* indicate the latency range for which the RDF was calculated (see text). (C) Percentage change in the RDFs calculated over the latency range *x* to *m* for both the Vx-SEP and the SI-SEP in relation to the duration of freezing behaviour. Data are presented as mean ± S.E.M. (n=8 for every intensity except for intensity 0.0, n=7). Note the two different y-axes; left for the RDF and right for the duration of freezing behaviour.

4. Discussion

Data from the present study show that increasing stimulus intensities result in an increase of the RDF of both the Vx-SEP and the SI-SEP, which is paralleled by an increase in the duration of freezing behaviour. From these findings it is concluded that the SEPs indicate the unpleasantness of the noxious stimuli experienced by the animals. Although previous studies demonstrated that increasing stimulus intensities of noxious stimuli 1) lead to an increase of SEP amplitudes (Stienen et al., 2003; 2004; 2005) and 2) lead to an increase in the duration of freezing behaviour (Fanselow and Bolles, 1979; Anagnostaras et al., 2000), these data were obtained in separate and unrelated studies. In the present study, however, the data were generated in an integrative approach, providing the possibility to demonstrate a direct relation between the SEPs and the unpleasantness of the noxious stimuli.

The SEP fear-conditioning model in this study was used without pharmacological intervention since (analgesic) drugs might influence the outcome of Pavlovian fear-conditioning tasks by other mechanisms than the principle (analgesic) actions on the US alone. First, administering drugs can impair the formation of a CS-US association at the level of the amygdala, while both CS and US were perceived normally by the animal (Kandel et al., 1996; Davies et al., 2004; Pietersen et al., 2006). Second, training animals in a drugged state might introduce state-dependent learning. State-dependent learning is described as a situation in which a subject can only recall an association formed under a certain state (e.g. a drugged state) when it is in that state again, (Overton, 1985; Maes and Vossen, 1997; Zarrindast and Rezayof, 2004). Third, administration of drugs from several classes have been shown to reduce auditory evoked potential amplitudes, as such indicating a reduction in the perception of auditory input, i.e. the CS (Pypendop et al., 1999). Taken together, 1) impairment of a CS-US association at the level of the amygdala, 2) state-dependent learning and 3) a reduced or absent perception of the CS, might result in a reduced or absent CS-US association, leading to short durations of freezing behaviour, while the animal possibly did experience the US as unpleasant. Therefore, one might mistakenly conclude that the reduced SEP amplitude, paralleled by a reduced duration of freezing behaviour, indicates a reduction in the perception of noxious stimuli. Although a

previous study that used the SEP fear-conditioning model (Stienen et al., 2006) already argued these drug-induced confounding co-influences to be of limited importance, the present study explicitly demonstrates that SEPs indicate the unpleasantness of noxious stimuli in the rat, without possible confounding drug effects.

In this study, no differences could be demonstrated between the Vx-SEP and SI-SEP in relation to the unpleasantness of noxious stimuli. Although seemingly contradictory to previous work (Stienen et al., 2006) , the present findings are in line with 1) the hypothesis previously presented regarding the background of the Vx-SEP and the SI-SEP (Stienen et al., 2004; 2005; 2006) and 2) the differences regarding the experimental set-up between the two studies.

It was hypothesized that the Vx-SEP and SI-SEP are related to separate functional pain mechanisms, being a mechanism signalling emotional affective aspects (unpleasantness) of noxious stimuli and one signalling sensory discriminative aspects (e.g. place, duration, intensity) of noxious stimuli, respectively (Stienen et al., 2004; 2005; 2006). In the present study noxious stimuli of different stimulus intensities were used to modulate the impact of the US. When using different stimulus intensities, the unpleasantness and the sensory discriminative aspects of the noxious stimuli are modulated in parallel (Willis and Westlund, 1997; Price, 2000; Sewards and Sewards, 2002). This explains the absence of dissociation between the Vx-SEP and the SI-SEP with respect to their correlation with the unpleasantness of the noxious stimuli. In contrast, using the SEP fear-conditioning model in combination with a μ-opioid agonist to modulate the impact of the US has been shown to result in dissociation between the Vx-SEP and the SI-SEP with respect to their correlation with the unpleasantness of the noxious stimuli (Stienen et al., 2006). This is likely caused by the fact that the neuroanatomical pain pathway involved in unpleasantness contains more μ-opiate receptors than the pathway encoding for sensory discriminative aspects (Atweh and Kuhar, 1983; Jones et al., 1991; Casey et al., 2000; Silbert et al., 2003). Combined, the present and previous findings (Stienen et al., 2006) show that both the Vx-SEP and the SI-SEP indicate the perception of noxious stimuli but that only the Vx-SEP indicates the unpleasantness of the noxious stimuli.

The SEPs were generated using electrical stimulation, which in principal, can co-activate Aα-fibers involved in motor processing, Aβ-fibers primarily involved in tactile sensation next to Aδ- and C-fibers primarily involved in nociception (Bromm and Lorenz

1998). However, the SEP components in the latency range studied have been reported to be primarily related to nociception (Shi et al., 2004; Stienen et al., 2003; 2004), which is of primary importance when using these components as indicators of pain and analgesia.

First, conduction velocities are considered to be specific for different classes of nerve fibers, e.g. motor (Aα: 60-70 m/s), tactile (Aβ: 30-60 m/s) or nociceptive (Aδ: 4-30 m/s and C: 0.4-1.8 m/s) fibers (Bromm and Lorenz, 1998). Based on these conduction velocities and the distance between the stimulus location and the recording site in this study (approximately 0.15 - 0.20 m), primarily Aδ-fiber mediated responses will be found within the latency range examined in the present study. According to the international literature Aδ-, compared to Aβ-fibers, primarily mediate nociceptive responses. However, up to 60% of the nociceptive responses in rats (and other species) are reported to be mediated by Aβ-fibers, which is generally neglected (Djouhri and Lawson, 2004). Therefore, conduction velocities alone can not definitively distinguish between responses representing nociceptive or tactile sensations.

Second, μ-opiate receptor agonists specifically modulate nociceptive processing at both peripheral and (supra-)spinal levels but not tactile processing (Kalliomaki et al., 1998; Silbert et al., 2003). Since the μ-opioid receptor agonist fentanyl affects both the Vx-SEP and SI-SEP (Stienen et al., 2005; 2006), these responses are considered to be primarily related to nociception and not tactile sensations.

Finally, the correlation between the duration of freezing behaviour and Vx-SEP and SI-SEP, studied after presentation of the CS, further supports these signals to be primarily related to nociception rather than to tactile sensations.

In conclusion, the SEP components in the latency range studied in the present study are considered to be related to nociception which is in line with other studies using SEPs evoked by electrical stimulation to study nociception (Bromm and Meier, 1984; Inui et al., 2002; Shi et al., 2004; Stienen et al., 2003; 2004; 2005; 2006; van Oostrom et al., 2005).

Although many animal models exist to study acute pain and analgesia, many of these models are based on nocifensive reflexes (e.g. tail flick, nocifensive withdrawal reflex) which have been shown to be present also in the decerebrate or spinalized animal (Le Bars et al., 2001). Therefore, these models do not necessarily involve higher cerebral structures and functions and consequently, do not provide unequivocal insight in the animal's emotional unpleasant experience. Furthermore, these models have limited suitability for

studying the different classes of analgesic drugs and may be easily confounded by learning effects and many other environmental factors (Le Bars et al., 2001).

In contrast to the approaches discussed above, we argue that recording of SEPs after noxious stimulation is a powerful tool to study pain and analgesia in animals, rather than nociception and anti-nociception. First, SEPs represent the neural processing of noxious stimuli at the cerebral level (Bromm and Lorenz, 1998; Kakigi et al., 2000; Spiegel et al., 2000). Second, the Vx-SEP, is sensitive to different classes of analgesic drugs (Stienen et al., 2004). Third, SEPs can be recorded and analyzed in a highly objective and standardized way, reducing animal and environmental influences to a minimum. Fourth, combining the results of the present and previous study (Stienen et al., 2006) demonstrates that the Vx-SEP potentially is a reliable indicator for unpleasantness whereas the SI-SEP potentially is a reliable indicator for sensory discriminative aspects of noxious stimuli in the rat. Since pain primarily involves the emotional unpleasant component of nociception, the Vx-SEP is considered to be the preferred signal when it comes to studying acute pain and analgesia in animals.

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5

Evaluation of analgesic and sedative effects of dexmedetomidine continuous infusion by measuring somatosensory and auditory evoked potentials in the rat

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Abstract

In this study, analgesic and sedative effects of different constant rate infusion (CRI) of dexmedetomidine in the rat were characterised by measuring specific electro-encephalographic parameters. Parameters recorded were somatosensory evoked potentials (SEPs) and auditory evoked potentials (AEPs), which have been shown to be related to analgesia and sedation respectively. Nine male Wistar rats (HsdCpb:Wu, Harlan Netherlands BV, body weight 300-350 g). SEPs were recorded from the primary somatosensory cortex and the vertex location (SI/Vx-SEPs). AEPs were recorded from the primary auditory cortex and vertex location (AI/Vx-AEPs). SI/Vx-SEPs and AI/Vx-AEPs were recorded alternately, during CRI of dexmedetomidine (4.0, 10.0, 20.0 µg/kg/h) and studied in comparison with control recording (saline). While the SI-SEP and AI-AEP were not affected or minimally (yet significantly) affected respectively, dexmedetomidine dose-dependently decreased the Vx-SEP and Vx-AEP. A maximum effect on the Vx-AEP was reached at lower doses than a maximum effect on the Vx-SEP. Based on the present findings, it is suggested that CRI of dexmedetomidine provides profound sedation at low doses, whereas higher doses are needed to provide concurrent analgesia. CRI of dexmedetomidine can be a valuable adjunctive in strategies aiming to provide sedation and/or analgesia. However, it seems that analgesia cannot be present without sedation and that sedation is not necessarily accompanied by equal amounts of analgesia.

1. Introduction

Dexmedetomidine, a potent and highly selective alpha-2 adrenoceptor agonist, is the active D-isomer of the racemic mixture medetomidine (Doze et al., 1989; Savola and Virtanen, 1991). Dexmedetomidine 1) has analgesic, sedative, anxiolytic and sympatholytic properties, 2) induces limited respiratory depression and 3) is easily antagonized by the specific alpha-2 adrenoceptor antagonist atipamezole (Doze et al., 1989; Savola and Virtanen, 1991; Bol et al., 1999; Bhana et al., 2000; Scholz and Tonner, 2005; Granholm et al., 2006). The sedative and analgesic profile of dexmedetomidine has been studied in different clinical and laboratory settings on the basis of reflex testing, ventilatory, cardiovascular and continuous electroencephalographic (EEG) effect measures. However, the behavioural, haemodynamic as well as applied EEG effect measures were considered to have limited value to predict deeper levels of analgesia and hypnosis of dexmedetomidine (Bol et al., 1999).

In contrast, several studies using specific EEG parameters, i.e. somatosensory- and auditory evoked potentials (SEPs and AEPs, respectively), have shown these parameters to be indicative for analgesia and sedation, respectively (Haberham et al., 2000; Stienen et al., 2006; van Oostrom et al., 2007). SEPs and AEPs are fragments of the EEG recorded time-locked to a somatosensory or auditory stimulus, respectively. Averaging a sufficient number of recordings eliminates unspecific EEG activity, leaving only the stimulus-related EEG, i.e. SEP or AEP. SEPs and AEPs appear as a waveform with successive positive and negative peaks characterized by their latency (time of onset after stimulation) and amplitude. Anaesthetic drugs increase the latencies and decrease the amplitudes of the SEPs and AEPs, changes which are considered to be related to a decreased perception of nociceptive and auditory stimuli, respectively. Therefore, SEPs and AEPs are considered to be useful to monitor the analgesic and sedative actions of different anaesthetic drugs (Jensen et al., 1998; Haberham et al., 2000; Stienen et al., 2003; 2004; 2005; 2006; Murray et al., 2006).

Over the recent years, differences were found between SEPs and AEPs recorded from different locations on the scalp in the rat. SEPs and AEPs recorded from their corresponding primary cortical area (primary somatosensory cortex (SI) (SI-SEPs) and primary auditory cortex (AI) (AI-AEPs), respectively) are considered to be related to noxious and auditory localisation and intensity coding (i.e. ‘what happens?’; sensory-

discriminative component) (Miyazato et al., 1995; 1999; Homma et al., 2003; Stienen et al., 2004; 2005; 2006). In contrast, SEPs and AEPs recorded from the vertex (Vx-SEPs and Vx-AEPs, respectively) are considered to be related to signalling emotional aspects of noxious and auditory stimuli, respectively (i.e. 'what do I feel (unpleasantness) or hear?'; emotional-affective component) (Miyazato et al., 1995; 1999; Homma et al., 2003; Stienen et al. 2004, 2005, 2006; van Oostrom et al., 2007). Therefore, the differential recording of SI/Vx-SEPs and AI/Vx-AEPs, rather than conventional anaesthetic effect measures as mentioned above, is considered to be a useful tool to determine the actions of dexmedetomidine on the different perceptual systems and consequently, its analgesic and sedative actions.

In the present study, we characterized the analgesic and sedative effects of different infusion rates of dexmedetomidine by alternately recording SI-/Vx-SEPs and AI-/Vx-AEPs, under different infusion rates of dexmedetomidine (4.0, 10.0, 20.0 μg/kg/h) and in comparison with control recording (saline).

2. Materials and methods

2.1 Animals and surgery

Animal care and experimentation were performed in full accordance with the protocols approved by the Science Committee and the institutional Animal Experimentation Committee (Utrecht University, Utrecht, The Netherlands).

Adult male Wistar rats (HsdCpb:Wu, Harlan Netherlands BV, body weight 300-350 g, n=9) were anaesthetized with 0.3 mg/kg fentanyl (i.p., Fentanyl Bipharma, Hameln Pharmaceuticals GmbH, Hameln, Germany, containing 0.05 mg/ml fentanyl citrate) and 0.3 mg/kg medetomidine (i.p., Domitor®, Pfizer Animal Health BV, Capelle a/d IJssel, The Netherlands, containing 1 mg/ml medetomidine hydrochloride) and fixed in a stereotaxic apparatus (Model 963, Ultra Precise Small Animal Stereotaxic, David Kopf Instruments, Tujunga, CA, USA). Epidural electrodes (wired stainless steel screws, tip diameter 0.6 mm, impedance 300-350 Ω, Fabory DIN 84A-A2, Borstlap BV, Tilburg, The Netherlands) were implanted at the vertex (4.5 mm caudal to bregma, 1.0 mm right from midline), at the SI (2.5 mm caudal to bregma, 2.5 mm right from midline), at the AI (4.5 mm caudal to bregma, 4.5 mm ventral with respect to the dorsal aspect of the skull) and bilateral in the

frontal sinus (10.0 mm rostral to bregma, 1.0 mm left and right from midline, respectively) (Stienen et al., 2006). All electrodes were wired to an eight-pin receptacle (Mecap Preci-Dip 917-93-108-41-005, Preci-Dip Durtal SA, Delémont, Switzerland) and fixed to the skull with dental cement (Simplex Rapid, Associated Dental Products, Ltd, Swindon, UK). At the end of surgery, anaesthesia was antagonized with 1 mg/kg atipamezole (s.c., Antisedan®, Pfizer Animal Health BV, Capelle a/d IJssel, The Netherlands, containing 5 mg/ml atipamezole hydrochloride) and 0.15 mg/kg buprenorphine (s.c., Temgesic®, Schering-Plough, Amstelveen, The Netherlands, containing 0.3 mg/ml buprenorphine). Postoperative analgesia was provided by 0.15 mg/kg buprenorphine administered s.c. at 8 hour intervals for three days after surgery.

After surgery the animals were housed in a living apart together system (two animals in one clear plastic cage, separated by a wire-mesh fence), under climate-controlled conditions on an inversed 12:12 h light/dark cycle (lights on at 21:00 h), with ad lib access to food and tap water. Animals were allowed to recover from the surgery for at least two weeks prior to the start of the experiments.

2.2 Recordings of the SEPs and AEPs

SEPs and AEPs were recorded in the freely moving animal, as described in detail by Stienen et al. (2004) and Haberham et al. (2000), respectively. In brief, SEPs were recorded from the SI and vertex (SI-/Vx-SEP). AEPs were recorded from the AI and vertex (AI-/Vx-AEP). The accompanying ipsi- and contra-lateral frontal sinus electrodes served as reference and ground, respectively. The stimuli for SEPs consisted of 32 square-wave electrical pulses of 5 mA, 2 ms in duration with a stimulus repetition rate of 0.5 Hz, generated by a Grass stimulator (Model S-88, Grass Medical instruments, Quincy, Mass., USA), which was triggered by dedicated software build in house (AD) in a Labview environment (Labview 7.2, National Instruments Netherlands B.V., Woerden, The Netherlands). The stimuli were delivered to a Grass stimulation isolation unit (Model SUI 5, Grass Medical Instruments) and a constant current unit (Model CCU 1A, Grass Medical Instruments) controlling the stimulus intensity. The stimuli for AEPs consisted of 72 white noise clicks of 80 dB sound pressure level, 0.2 ms in duration with a stimulus repetition rate of 0.5 Hz generated by a Grass stimulator, which was triggered by the data acquisition

software. The clicks were presented by two speakers mounted in the covering lid of the experimental box.

For each SEP and AEP trial, respectively 32 and 72 subsequent data segments of 150 ms were recorded and averaged online, using a delay of 25 ms. All signals were amplified 5000 times, band-pass filtered between 15 and 300 Hz, using an additional 50 Hz notch-filter, and digitized online at 10000 Hz by data acquisition hardware (National Instruments PCI-6251, Instruments Netherlands B.V., Woerden, The Netherlands) and data acquisition software build (AD) in the same Labview environment responsible for stimulus triggering as mentioned above.

2.3 Effect assessment of different infusion rates of dexmedetomidine on SI-/Vx-SEPs and AI-/Vx-AEPs

The animals were placed in the experimental room and were allowed to acclimatize. After 30 minutes of acclimatization a 24-gauge catheter was inserted in a lateral tail vein, the animals were fitted in a tight fitting jacket and an electrical stimulation device was fixed at the left tail base (Stienen et al., 2004). Subsequently, the animals were placed in the experimental box. Via an infusion tube, the catheter in the tail vein was connected to a syringe, operated by a Graseby infusion pump (Graseby Anaesthesia Pump 3500, Graseby Medical LTD, Watford Herts, United Kingdom). The receptacle at the animal's head was wired via a swivel-connector to the SEP and AEP recording device. Through the open middle of this swivel-connector, the stimulation device was wired to the Grass-stimulator via a separate swivel-connector. This set-up allowed the animals to move freely during the session.

Next, a bolus infusion with either saline or dexmedetomidine (Dexdomitor, Orion Corporation, Espoo, Finland) was given. After administering the bolus, the infusion with either saline or dexmedetomidine was continued as a constant rate infusion (CRI). All rats (n=9) received the following four treatments over different recording sessions: 1) 10 ml/kg/2 min saline bolus followed by 10 ml/kg/h saline CRI (session 0.0), 2) 10 ml/kg/2 min dexmedetomidine (0.4 µg/ml) bolus followed by 10 ml/kg/h dexmedetomidine (0.4 µg/ml) CRI (session 4.0), 3) 10.0 ml/kg/2 min dexmedetomidine (1.0 µg/ml) bolus followed by 10 ml/kg/h dexmedetomidine (1.0 µg/ml) CRI (session 10.0) and 4) 10 ml/kg/2 min dexmedetomidine (2.0 µg/ml) bolus followed by 10 ml/kg/h dexmedetomidine (2.0

µg/ml) CRI (session 20.0). During CRI, SEPs and AEPs were recorded alternately. Time between subsequent SEP/AEP measurements was approximately 1 minute. The total recording time during infusion was 1 hour and 21 minutes. After the infusion was terminated, the recordings proceeded for 1 hour. Signals were recorded for each treatment in each rat. The time between two subsequent recording sessions in one rat was at least two weeks. Treatment sequence was randomly determined for each rat.

2.4 Data and statistical analysis

Calculations were performed with the aid of Microsoft Excel 2003. Statistical analysis was performed with SPSS 12.0.1 for Windows. Differences were considered to be significant when $P<0.05$.

The AEP and SEP signals were evaluated using the Rate Dispersion Factor. The RDF, an expression of the overall shape of the evoked potential waveform in the latency range studied, was obtained by averaging the absolute differences between all pairs of subsequent sampled data points y_k in a specified latency range from x to m (equation 1).

$$\text{Equation 1}: \text{RDF} = \frac{1}{m - x} \sum_{k=x+1}^{m} \left| y_k - y_{k-1} \right|$$

Therefore, both decreases in amplitude and increases in latency of the evoked potential components, decrease the value of the RDF, whereas both increases in amplitude and decreases in latency of the evoked potential components increase the value of the RDF (Mantzaridis, 1997; Haberham et al., 2000; Stienen et al., 2004).

The RDF was calculated for the latency range (x to m) of the 1) SI-SEP positive-to-negative complex at approximately 14 to 19 ms, 2) Vx-SEP positive-to-negative complex at approximately 15 to 18 ms, 3) AI-AEP positive-to-negative complex at approximately 9 to 12 ms and 4) Vx-AEP positive-to-negative complex at approximately 14 to 21 ms. The corresponding latency ranges were determined in the grand average waveforms of the saline recordings (control) for each recording site (Fig. 1 and 2). When choosing the RDF latency range (x to m) in the group with the strongest signals (0.0 group, saline), individual signals at all time points during each treatment can be analyzed using this fixed latency

range. Therefore the RDF is a highly objective method to evaluate evoked potential signals without the need of choosing peak amplitudes in individual signals which is prone to confounding errors, especially when signals are weak (van Oostrom et al., 2007).

The RDFs of the SEPs and AEPs were averaged over 5 trials, for each rat, session and recording site separately. This resulted in 5 time-blocks, 3 during infusion and 2 after infusion, of 5 recording trials each. Finally, all averaged RDF values were calculated as a percentage of the mean RDF during the control session (relative RDF). For statistical analysis, changes of the relative RDFs were analyzed using a repeated measurement analysis of variance (RM-ANOVA) with repeated factors "treatment" (session 0.0, 4.0, 10.0 and 20.0), "recording site" (primary cortex vs. vertex) and "time" (time-block 1 to 5), followed by a post-hoc analysis when appropriate.

3. Results

3.1 SEPs

Fig. 1A and 1B show the grand average waveforms and the peak definitions of the Vx-SEP and SI-SEP respectively, in the four different sessions. Across the subjects, similar and consistent waveforms were recorded.

Statistical analysis showed that the relative RDFs of the SI-SEP and Vx-SEP were significantly and differently affected over time by the specific treatments (time×treatment×recording site: $F_{12,96}$=3.248, P=0.001). Post hoc analysis showed that the relative RDF of the SI-SEP was not affected by the treatments during infusion, but after infusion, treatment-dependent changes were found to be significant on time-block 4 and 5 (Fig. 3B; time×treatment:$F_{12,96}$=1.859, P=0.049; time-block 4: treatment: $F_{3,24}$=7.513, P=0.001 followed by Sidak multiple comparisons: session 0.0 vs. 20.0: P=0.002 and session 4.0 vs. 20.0: P=0.04; time-block 5: treatment: $F_{3,24}$=4.220, P=0.016 followed by Sidak multiple comparisons: session 0.0 vs. 20.0: P=0.004).

In contrast, the relative RDFs of the Vx-SEP in group 10.0 and 20.0 were significantly lower in comparison with the control session (Fig. 3A; treatment: $F_{3,24}$=25.056, P<0.001 followed by Sidak multiple comparisons: session 0.0 vs. 10.0: P<0.001; session 0.0 vs. 20.0: P<0.001). The relative RDF of the Vx-SEP was not affected over time for all treatments (Fig. 3A)

Compared to the SI-SEP, the relative RDF of the Vx-SEP, was significantly lower in session 4.0, 10.0 and 20.0 (Fig. 3A vs. 3B; treatment×recording site, $P<0.05$ for all time-blocks; time×recording site followed by paired t-test, $P<0.05$ in session 4.0, 10.0 and 20.0)

3.2 AEPs

Figure 2A and 2B show the grand average waveforms and the peak definitions of the Vx-AEP and the AI-AEP respectively, in the four different sessions. Across the subjects, similar and consistent waveforms were recorded.

Statistical analysis showed that the relative RDFs of the AI-AEP and Vx-AEP were significantly and differently affected over time by the specific treatments (time×treatment×recording site: $F_{12,96}=4.385$, $P<0.001$). Post hoc analysis showed that during infusion, the relative RDF of the AI-AEP in session 10.0 and 20.0 was significantly lower in comparison with the relative RDF of the control session (Fig 4B; treatment×time: $F_{12,96}=3.852$, $P<0.001$; one-way RM-ANOVA factor treatment $P<0.05$ in all time-blocks, followed by Sidak multiple comparisons $P< 0.05$: session 0.0 vs. 10.0 for time-block 1 and 2; session 0.0 vs. 20.0 for time-block 1,3,4; session 4.0 vs. 10.0 for time-block 3; session 4.0 vs. 20.0 for time-block 3 and 5; session 10.0 vs. 20.0 for time-block 5). After infusion, the relative RDF of the AI-AEP in session 10.0 and 20.0 gradually returned to control level (Fig. 4B; time: $P<0.05$ for treatment 10.0 and 20.0, followed by Sidak multiple comparisons, $P<0.05$: treatment 10.0: time-block 1 vs. 5, 2 vs. 4 and 5, 3 vs. 5, 4 vs. 5; treatment 20.0: time-block 3 vs. 5).

During infusion, the relative RDF of the Vx-AEP in session 4.0, 10.0 and 20.0 was significantly lower in comparison with the control session (Fig. 4A; treatment: $F_{3,24}= 35.238$; $P<0.001$ followed by Sidak multiple comparisons: session 0.0 vs. 4.0 $P< 0.001$; session 0.0 vs. 10.0 $P=0.002$; session 0.0 vs. 20.0 $P=0.001$). After infusion, the relative RDF of the Vx-AEP in session 4.0, 10.0 and 20.0 gradually returned to control level. (Fig. 4A; time: $F_{4,32}=4.563$, $P=0.005$ followed by Sidak multiple comparisons: time-block 3 vs. 5 $P=0.043$).

Compared to the AI-AEP, the relative RDF of the Vx-AEP was significantly lower in session 4.0 and 10.0 (Fig. 4A vs 4B; treatment×recording site followed by paired t-test, $P<0.05$ for time-blocks 2-5; time-block 1: recording site: $F_{1,8}=32.173$, $P<0.001$; time×recording site followed by paired t-test, $P<0.05$ in session 10.0).

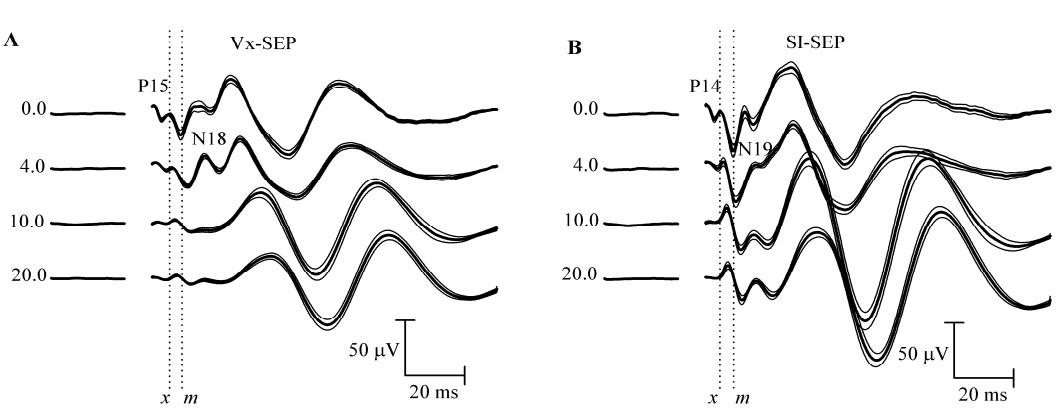

Figure 1. Changes in SEP waveforms during CRI of dexmedetomidine. Grand mean (bold lines) and S.E.M. (plain lines) waveforms of the Vx-SEP (A) and SI-SEP (B) during different infusion rates of dexmedetomidine. Curve interruption and dotted vertical lines denote stimulus onset and the latency range (x to m) for which the RDF is calculated, respectively. Peaks are designated by P (positive peak) and N (negative peak) accompanied by their rounded off mean latency (ms).

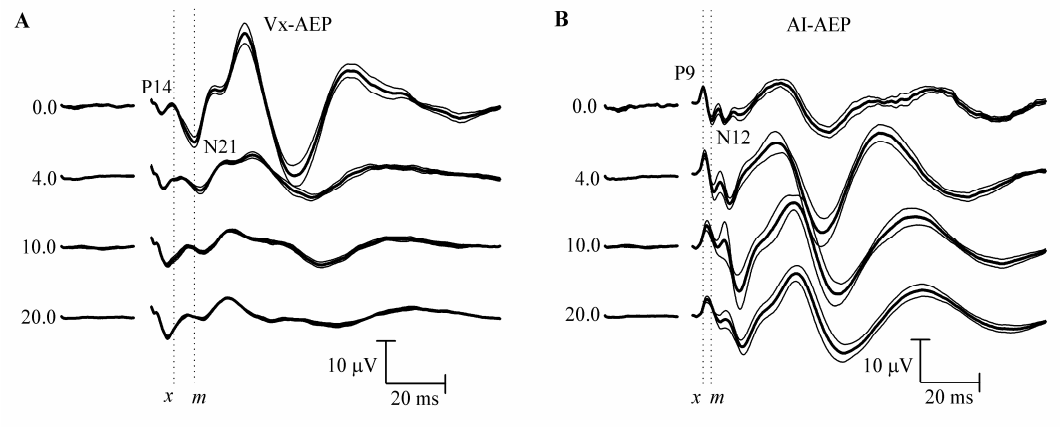

Figure 2. Changes in AEP waveforms during CRI of dexmedetomidine. Grand mean (bold lines) and S.E.M. (plain lines) waveforms of the Vx-AEP (A) and AI-AEP (B) during different infusion rates of dexmedetomidine. Curve interruption and dotted vertical lines denote stimulus onset and the latency range (x to m) for which the RDF is calculated, respectively. Peaks are designated by P (positive peak) and N (negative peak) accompanied by their rounded off mean latency (ms).

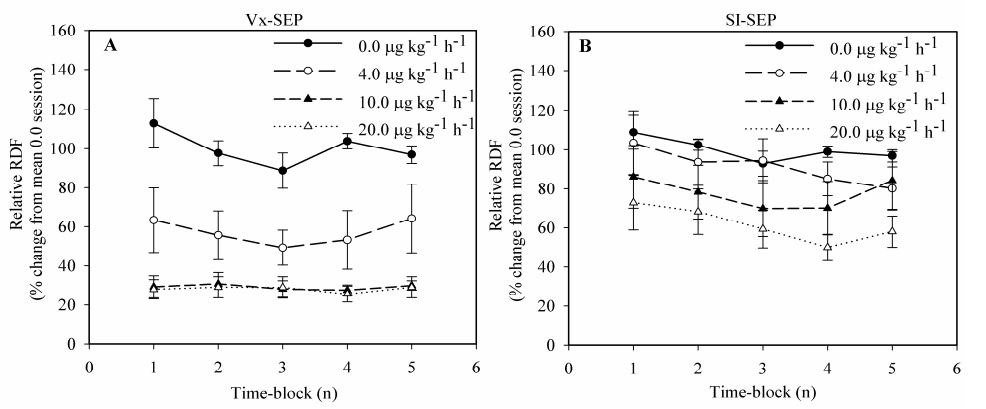

Figure 3. RDF changes by different doses of dexmedetomidine for the Vx-SEP and SI-SEP in the latency range x to m (see fig 1). Data are presented as the mean relative RDF (+/- S.E.M., n=9) over 5 subsequent time blocks for the four different treatments. Time-block 1, 2 and 3 represent the data during infusion of dexmedetomidine, whereas time-block 4 and 5 represent the data after terminating the CRI.

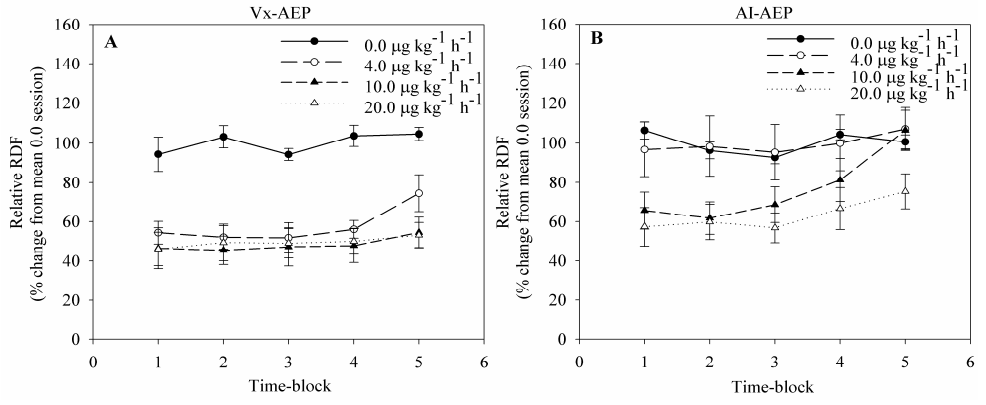

Fig. 4. RDF changes by different doses of dexmedetomidine for the Vx-AEP and AI-AEP in the latency range x to m (see fig 2). Data are presented as the mean relative RDF (+/- S.E.M., n=9) over 5 subsequent time blocks for the four different treatments. Time-block 1, 2 and 3 represent the data during infusion of dexmedetomidine, whereas time-block 4 and 5 represent the data after terminating the CRI.

4. Discussion

In the present study, we characterized the analgesic and sedative effects of different infusion rates of dexmedetomidine by recording SI-/Vx-SEPs and AI-/Vx-AEPs, respectively. The present study showed that dexmedetomidine dose-dependently decreased the RDF of the Vx-SEP and Vx-AEP, while the SI-SEP and AI-AEP were not affected or only minimally (yet significantly) affected, respectively. In addition, a maximum effect on the Vx-AEP was obtained at a lower dose than the dose needed for a maximum effect on the Vx-SEP. These results suggest that relatively low doses of dexmedetomidine provide sedation, while higher doses are needed to provide analgesic effects.

The Vx-SEP and Vx-AEP can be considered as specific measures of unpleasantness of noxious stimuli and perceptual awareness of auditory stimuli, respectively. Data from previous studies suggest the involvement of separate but parallel pain and auditory processing systems underlying SI-/Vx-SEPs and AI-/Vx-AEPs, respectively (Simpson and Knight, 1993; Stienen et al., 2004; 2005). On one hand, nociceptive and auditory stimuli are evaluated on their spatio-temporal localisation and intensity (sensory-discriminative processing). On the other hand, their 'emotional colour' is modulated by the individual's state of arousal, attention and memory (emotional-affective processing) (Miyazato et al., 1995; 1999; Homma et al., 2003; Stienen et al., 2004; 2005; 2006). SI-SEPs and AI-AEPs, originating from the respective primary cortical areas, are considered to be related to the first process, while Vx-SEPs and Vx-AEPs are considered to be related to the second process (Miyazato et al. 1995; 1999; Homma et al., 2003; Stienen et al., 2004; 2005; 2006). Moreover, the dexmedetomidine-induced decrease of the amplitude of the Vx-SEP and Vx-AEP, rather than the SI-SEP and AI-AEP, indicates that dexmedetomidine actually exerts its primary effects on emotional-affective, rather than sensory-discriminative, processing. This is consistent with the alpha-2A adrenoceptor subtypes distribution in pathways underlying emotional-affective and sensory-discriminative processing, respectively (Scheinin et al., 1993). The predominant subtypes of the alpha-2 adrenoceptors (alpha-2A) involved in the mediation of the antinociceptive and sedative actions of dexmedetomidine (Hunter et al., 1997) are highly dominant in the locus coeruleus, the structure that plays a central role in emotional-affective processing of nociceptive as well as auditory stimuli (Willis and Westlund 1997; Miyazato et al., 2000; Rauschecker and Tian, 2007). Combined, the dexmedetomidine-induced decreases of the Vx-SEP and Vx-AEP, rather

than that of SI-SEPs and AI-AEPs, are considered to be indicative of the analgesic and sedative properties of dexmedetomidine in the present study.

The finding that dexmedetomidine provides sedation at a relatively low dose and both sedation and analgesia in higher doses, is consistent with findings in previous studies with CRI of dexmedetomidine in rats and man. In rats, as well as in man, increasing concentrations of dexmedetomidine resulted in progressive increases of primarily sedation followed by analgesia (Bol et al., 1999; Ebert et al., 2000; Hall et al., 2000).

The SI-/Vx-SEP and AI-/Vx-AEP were not affected over time during CRI, although the Vx-SEP and Vx-AEP were affected by the specific treatments. These findings suggest that dexmedetomidine exerts its effects almost immediately after bolus infusion. This is consistent with findings in man showing significant sedation directly after bolus infusion of dexmedetomidine (Hall et al., 2000).

In conclusion, based on the neural processes thought to underlie the differentiation between SI-/Vx-SEPs and AI-/Vx-AEPs, it is suggested that CRI of dexmedetomidine at a relatively low dose provides profound sedation, whereas higher doses are needed to provide concurrent analgesia. CRI of dexmedetomidine is therefore considered to be a valuable adjunctive in strategies aiming to provide sedation and/or analgesia.

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6

Dexmedetomidine suppresses fear-conditioning only at doses attenuating processing of sensory input

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Submitted for publication

Abstract

In the present study, it was investigated whether continuous rate infusion (CRI) of the alpha-2 adrenoceptor agonist dexmedetomidine can suppress fear-conditioning by other mechanisms than reducing the processing of sensory input. To this aim, different groups of rats infused with either saline or dexmedetomidine (2.0, 4.0 or 10.0 µg/kg bolus, followed by 2.0, 4.0 or 10.0 µg/kg/h CRI respectively) were subjected to a somatosensory-evoked potential (SEP) fear-conditioning paradigm. This SEP fear-conditioning paradigm combines the pairing of an innocuous stimulus (CS) and a noxious stimulus (US), of which the latter is used to generate the SEPs (training phase). Subsequently, this fear-conditioning paradigm is used to evaluate the perception of the US during the training phase. The day following the training phase, the CS-US association is assessed by presenting the CS only and subsequently scoring the resulting duration of freezing behaviour (testing phase). Data showed that freezing behaviour during the testing phase was reduced only in those groups which demonstrated a reduced SEP during the training phase. Based on these findings, it is concluded that dexmedetomidine suppresses fear-conditioning only at doses attenuating processing of sensory input.

1. Introduction

Alpha-2 adrenoceptor agonistic drugs are applied in both veterinary and human clinical anesthesiology for their sedative, analgesic and anxiolytic actions (Sinclair, 2003; Paris and Tonner, 2005; Tobias, 2007). Dexmedetomidine, the dextro-optical isomer of medetomidine, is a relatively novel and highly specific alpha-2 adrenoceptor agonist. In veterinary medicine, it is used as a sedative, analgesic and pre-anesthetic drug, whereas in human medicine it is used as a sedative agent in ICU settings. Alpha-2 adrenoceptor agonistic drugs exert their anesthetic effects by binding to pre-synaptic alpha-2 adrenoceptors. By binding to these receptors, the noradrenaline release from primarily the Locus Coeruleus (LC) neurons is inhibited, resulting in subsequent sedation and analgesia (Svensson et al., 1975).

Besides the two most common actions of dexmedetomidine, i.e. sedation and analgesia, amnesic actions have been reported as well (Ebert et al., 2000; Hall et al., 2000). These drug-induced amnesic actions may be of clinical importance, since it may prevent subjects developing fearful memories of procedures or interventions experienced. These fearful memories may subsequently lead to aversive responses when revisiting the clinic, potentially resulting into problems such as increased risk of postoperative pain (Vaughn et al., 2007) and agitation/delirium at emergence from general anesthesia (Aono et al., 1999; Kain et al., 2004; Aouad and Nasr, 2005). Therefore, inducing amnesia might be of extra benefit to the patient, especially when this can be achieved with a drug already used for its sedative and analgesic actions.

The induction of amnesic effects of the alpha-2 adrenoceptor agonists in animal models remains controversial throughout the literature. Several animal studies report amnesia induced by alpha-2 adrenoceptor agonists (Zarrindast et al., 2003; Davies et al., 2004; Galeotti et al., 2004a; 2004b), while others, on the contrary, report memory enhancement (Riekkinen and Riekkinen, 1999; Tanila et al., 1999). These contradictory findings can be ascribed to the dose used, the type of memory involved (e.g. working memory *versus* associative memory) and the timing of administration (e.g. before or after the training phase) (Sirviö et al., 1992). Studies addressing the amnesic effects of dexmedetomidine in man also differ in their conclusions. Some studies report amnesic effects of dexmedetomidine infusion at sedative doses (Ebert et al., 2000; Hall et al., 2000), whereas others do not (Venn et al., 1999; Ustün et al., 2006). Combined, the controversy on the

potentially amnesic effects of alpha-2 adrenoceptor agonists such as dexmedetomidine, in both animal models and man, clearly requires further research.

It has not been adequately investigated whether the doses of dexmedetomidine, capable of attenuating a learning task in an animal, attenuate sensory processing in that same animal during the learning phase of the learning task. Therefore it remains unclear whether dexmedetomidine attenuates learning tasks by reducing the perception of sensory input or other mechanisms. The present study will investigate this question, using a special type of Pavlov fear-conditioning paradigm, called the SEP fear-conditioning paradigm (van Oostrom et al., 2005). During Pavlov fear-conditioning an innocuous, conditioned stimulus (CS), e.g. a tone, is presented paired with a noxious, unconditioned stimulus (US), e.g. an electric shock. After several CS-US pairings (training phase) the animal associates the CS with the noxious US and, consequently, the CS given alone (testing phase) evokes a fear-related behavioural response, i.e. freezing behaviour in rats. The duration of the freezing behaviour is indicative of the strength of the association between the CS and the aversive US (Fanselow and Bolles, 1979; Anagnostaras et al., 2000), and moreover is considered a measure of memory formation. With a special fear-conditioning paradigm (van Oostrom et al., 2005), the so-called SEP fear-conditioning paradigm, it is possible to study 1) the perception of the US during the training phase by the recording of somatosensory evoked potentials (SEP), as well as, 2) the CS-US association by scoring the duration of freezing behaviour during the testing phase, in the same animal. SEPs are small epochs of electroencephalogram (EEG) recorded time-locked to a somatosensory stimulus. Averaging several recordings eliminates the unspecific background EEG, leaving only the stimulus related EEG, i.e. the SEP. The SEP represents the activation of the central nervous system by the somatosensory stimuli (Bromm and Lorenz, 1998). In both man and animals, it has been shown that the amplitude of the vertex-derived SEP (Vx-SEP) correlates with the perception of somatosensory stimuli (Arendt-Nielsen, 1994; Kakigi et al., 2000; Stienen et al., 2006; van Oostrom et al., 2007). Combined, the SEP fear-conditioning paradigm provides a unique approach to study the formation of a CS-US association simultaneously with the perception of the US and therefore can be used to study whether dexmedetomidine can suppress fear-conditioning by other mechanisms than reducing the processing of sensory input.

To study the potential amnesic effects of dexmedetomidine in parallel with its effects on sensory perception, different groups of rats were subjected to the SEP fear-conditioning paradigm and trained under either saline or different doses of dexmedetomidine CRI (2.0, 4.0 and 10.0 μg/kg/h). The day following the training, the duration of freezing behaviour after presentation of the CS only was investigated and studied in relation to the SEP amplitudes obtained during training.

2. Materials and Methods

2.1 Animals and surgery

Animal care and experimentation were performed in accordance with protocols approved by the Science Committee and the local Animal Experimentation Committee (Utrecht University, Utrecht, The Netherlands).

Adult male Wistar rats (HsdCpb:WU, Harlan Netherlands BV, Horst, The Netherlands, body weight 300-350 g, *n*=33) were permanently instrumented with epidurally placed EEG recording electrodes. After induction of anesthesia with 0.3 mg/kg fentanyl (i.p., Fentanyl Bipharma, Hameln Pharmaceuticals GmbH, Hameln, Germany, containing 0.05 mg/ml fentanyl citrate) and 0.3 mg/kg medetomidine (i.p., Domitor®, Pfizer Animal Health BV, Capelle a/d IJssel, The Netherlands), the animals were fixed in a stereotaxic apparatus (Model 963, Ultra Precise Small Animal Stereotaxic, David Kopf Instruments, Tujunga, CA, USA). Epidural electrodes (wired stainless steel screws, tip diameter 0.6 mm, impedance 300-350Ω, Fabory DIN 84A-A2, Fabory, Nieuwegein, The Netherlands) were implanted at the vertex (4.5 mm caudal to bregma (0.0 with skull surface flat), 1.0 mm right from midline), the primary somatosensory cortex (2.5 mm caudal to bregma, 2.5 mm right from midline), and bilateral in the frontal sinus (10.0 mm rostral to bregma, 1.0 mm left and right from midline, respectively) (Stienen et al., 2004). All electrodes were wired to an eight-pin receptacle (Mecap Preci-Dip 917-83-208-41-005101, Preci-Dip Durtal SA, Delémont, Switzerland) and fixed to the skull with dental cement (Simplex Rapid, Associated Dental Products Ltd, Swindon, UK). At the end of surgery, anesthesia was antagonized with 1 mg/kg atipamezole (s.c., Antisedan®, Pfizer Animal Health BV, Capelle a/d IJssel, The Netherlands) and 0.10 mg/kg buprenorphine (s.c., Temgesic®, Schering-

Plough, Amstelveen, The Netherlands). Postoperative analgesia was provided by 0.10 mg/kg buprenorphine administered s.c. at 8 hour intervals for three days after surgery.

After surgery the animals were housed in a living apart together system (two animals in one clear plastic cage, separated by a wire-mesh fence), under climate-controlled conditions on an inversed 12:12 h light/dark cycle (lights on at 21:00 h), with *ad lib* access to food and tap water. Animals were allowed to recover from surgery for at least two weeks prior to the start of the experiments.

2.2 Fear-conditioning (training phase)

The animals were placed in the experimental room 30 minutes prior to the start of the experiment, to acclimatize. After acclimatization, a 24 gauge catheter was placed in a lateral tail vein, using a tube restrainer. Subsequently the animals were fitted in a tight fitting jacket developed in-house which stayed on throughout the recording sessions. An electrical stimulation device (two brass electrodes of 3 mm in diameter tapered towards the end, fixed in a silicon tube enclosing the tail (Stienen et al., 2004)) was fixed at the left tail base and tightened by Velcro tape for maximal fixation. Subsequently, the animals were placed in a Plexiglas box (40×28×30cm), with a grounded stainless steel floor and two speakers in the covering for presentation of the CS. The complete box was shielded by a Faraday cage. After placing the animal in the box, the receptacle at the animal's head and the stimulation device at the tail base were wired via separate swivel-connectors, to the SEP recording device and a stimulator, respectively (for details see further). Finally, the catheter in the tail vein was connected to a syringe containing either saline or dexmedetomidine (Dexdomitor, Orion Corporation, Espoo, Finland), driven by a Graseby infusion pump (Graseby Anaesthesia Pump 3500, Graseby Medical LTD, Watford Herts, United Kingdom). After checking for correct functioning of all equipment, infusion was started by giving a bolus of either saline (saline control group, n=8) or dexmedetomidine 2.0 (group 2.0, n=9), 4.0 (group 4.0, n=9) or 10.0 (group 10.0, n=8) µg/kg given over a one minute period, followed by CRI of either saline or dexmedetomidine 2.0, 4.0 or 10.0 µg/kg/h. Concentrations of dexmedetomidine solutions were prepared in a way that infused volumes for the different groups were equal, i.e. 10.0 ml/kg bolus followed by 10.0 ml/kg/h CRI.

The actual fear-conditioning training phase started 15 minutes after the start of the bolus infusion and consisted of 10 CS-US pairings. The US (square-wave pulses, n = 32 of

5.0 mA, 2 ms duration each, with a stimulus frequency of 0.5 Hz; total time 64 seconds) was presented 10 seconds after onset of the CS (a 40 seconds 1500 Hz tone, 85 dB sound pressure level) creating a 30 seconds overlap. Time between subsequent CS onsets varied between 300 and 460 seconds. During the fear-conditioning sessions SEPs were recorded in the freely moving animal for every US presentation, as described previously (Stienen et al., 2003). In brief, SEPs were recorded from the vertex and primary somatosensory cortex (Vx-/SI-SEP, respectively). The accompanying ipsi- and contra-lateral frontal sinus electrodes served as reference and ground, respectively. The stimuli to evoke the SEPs (US) were generated by a Grass stimulator (Model S-88, Grass Medical instruments, Quincy, Mass., USA), which was triggered by dedicated software build in house (AD) in a Labview environment (Labview 7.2, National Instruments Netherlands B.V., Woerden, The Netherlands). For each SEP recording, 32 subsequent data segments of 150 ms were recorded and averaged online, using a delay of 25 ms. All signals were amplified 5000 times, band-pass filtered between 15 and 300 Hz, using an additional 50 Hz notch-filter, and digitized online at 10 kHz.

2.3 Assessment of CS-US association (testing phase)

The testing phase was performed 24 hours after the training phase. Thirty minutes prior to the testing phase the animals were placed in the same experimental room as used during the training phase. Subsequently, the animals were placed in a Plexiglas box (41×31×20 cm) different from the one used for the training phase. After 15 minutes of acclimatization in the box, the testing phase, consisting of 10 CS presentations, started. Time between subsequent CS onsets varied between 200 and 420 seconds. During these sessions the behaviour of the animals was videotaped continuously.

2.4 Data and statistical analysis

Calculations were performed with the aid of Microsoft Excel 2003. Statistical analysis was performed with SPSS 12.0.1 for Windows. Differences were considered to be significant when $P \leq 0.05$, unless indicated otherwise.

The SEP signals were evaluated using the Rate Dispersion Factor (RDF). The RDF, an expression of the overall shape of the SEP waveform in the latency range studied, is obtained by averaging the absolute differences between all pairs of subsequent sampled

data points y_k in a specified latency range from x to m (see equation 1) (Mantzaridis and Kenny, 1997; Haberham et al., 2000; Stienen et al., 2004; van Oostrom et al., 2007). When choosing the RDF latency range (x to m) in the group with the strongest signals (saline control group), individual signals recorded during all treatments can be analyzed using this fixed latency range. Therefore the RDF is a highly objective method to evaluate SEP signals without the need of choosing peak amplitudes in individual signals which is prone to confounding errors, especially when signals are weak.

For the Vx-SEP, the RDFs were calculated for the latency range (x to m) of the positive-to-negative complex previously described as the P15-N20 complex (Stienen et al., 2006), recorded in the saline control group. For the SI-SEP the RDFs were calculated for the latency range (x to m) of the positive-to-negative complex previously described as the P12-N20 complex (Stienen et al., 2006), recorded in the saline control group. For both the Vx-SEP and the SI-SEP, the RDF was calculated as the percentage change over groups, for both the Vx-SEP and SI-SEP using the mean RDF value of the saline control group as 100%, since in this group the signals had the highest amplitudes and the shortest latencies, resulting in the highest RDFs.

$$\text{Equation 1}: \text{RDF} = \frac{1}{m - x} \sum_{k=x+1}^{m} \left| y_k - y_{k-1} \right|$$

The duration of freezing behaviour during the testing sessions was analyzed using the video recordings. Freezing behaviour was defined as the absence of all visible movements with the exception of breathing movements and pendulum motion of the head, while the animal sits in a tensed posture (Kolpakov et al., 1977). Freezing was scored only during presentation of the CS. The behavioural data were scored blindly with respect to the dose of dexmedetomidine used. The scorings of the principal investigator [HvO] demonstrated a high correlation with the scorings of a second observer who was unaware of the aims and procedures of this experiment (Pearson's correlation coefficient r=0.945, P<0.001, n=36).

For each animal, the SEP data of the ten trials were averaged for both recording sites and, subsequently, analyzed using a two-way repeated measurement analysis of variance (two-way RM-ANOVA) with fixed factor "group" and repeated factor "recording site",

followed by *post-hoc* analysis when appropriate. The freezing data of the ten trials were averaged and subsequently analyzed using a one-way ANOVA with factor "group", followed by *post-hoc* analysis when appropriate.

3. Results

3.1 Vx-SEPs and SI-SEPs

One animal in the saline control group had exceptionally high peak amplitudes for the SI-SEP. When calculating a Grubbs test for outliers over these data, there was statistical ground to exclude this animal from the SEP data analysis. Another animal in this group did not have a SI-SEP signal due to technical problems; consequently this animal was also excluded from the SEP data analysis.

The grand average waveforms of the Vx-SEP and SI-SEP are shown in Fig. 1A and 1B respectively. In the saline control group, the Vx-SEP P15-N20 complex and the SI-SEP P12-N20 complex both occurred in the latency range of 14.5-19.5 ms.

As shown in Fig. 1C, dexmedetomidine dose-dependently decreased the RDF values for the Vx-SEP, but not for the SI-SEP. The two-way RM ANOVA showed a significant interaction between the factors "group" and "recording site" (group×recording site: $F_{3,27}$=7.167, P=0.001). Subsequent *post-hoc* analysis showed a significant group effect for the Vx-SEP but not for the SI-SEP (one-way ANOVA Vx-SEP: group: $F_{3,27}$=6.833, P=0.001; one-way ANOVA SI-SEP: group: $F_{3,27}$=1.931, P=0.148). Bonferroni multiple comparisons for the Vx-SEP showed that the saline control group had significantly higher RDFs than groups 4.0 and 10.0 (P=0.013 and P=0.003) and that group 2.0 had significantly higher RDFs than group 10.0 (P=0.05). A paired t-test followed by Bonferroni correction (level of significance at $P\leq$0.0166) showed that the RDF of the SI-SEP was significantly higher than the RDF of the Vx-SEP at doses 4.0 (t_8=5.884, P<0.001) and 10.0 (t_8=4.916, P=0.003).

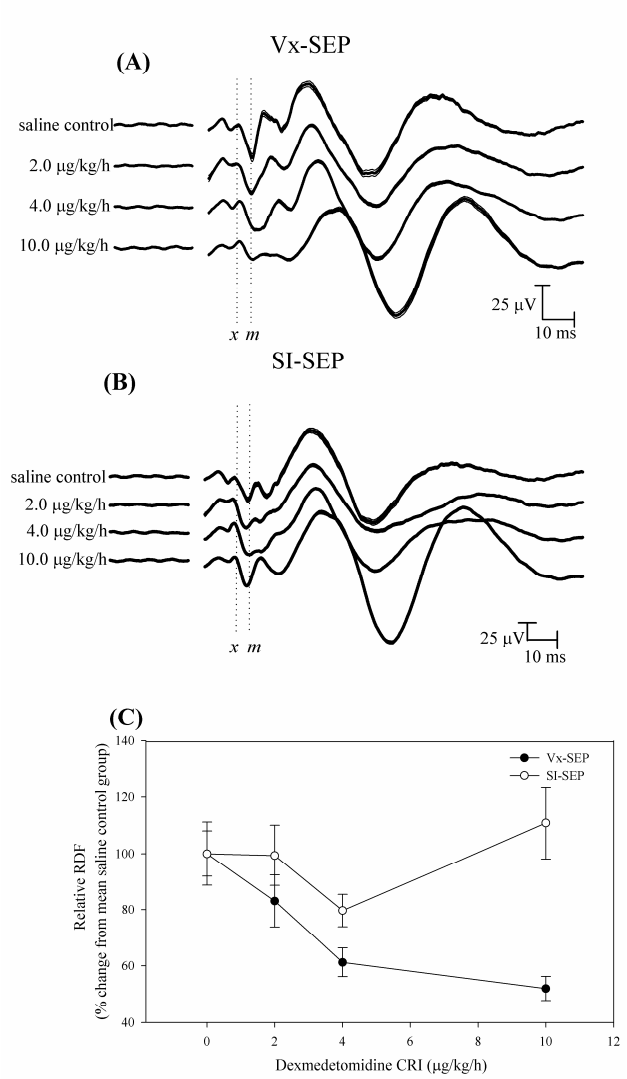

Figure 1. SEPs in relation to the dose of dexmedetomidine. (A) and (B) Grand mean (bold lines) ± S.E.M. (plain lines) waveforms of the Vx-SEP and SI-SEP, respectively, at the different stimulus intensities (*n*=6 for saline control, *n*=9 for group 2.0 and group 4.0 and *n*=8 for group 10.0) Dashed lines *x* and *m* indicate the latency range for which the RDF was calculated (see text). Interruption in the waveforms indicate stimulus onset. (C) Percentage change in the RDFs calculated over the latency range *x* to *m* for both the Vx-SEP and the SI-SEP. Data are presented as mean ± S.E.M. (*n*=6 for saline control group, *n*=9 for group 2.0 and group 4.0 and *n*=8 for group 10.0).

3.2 CS-US association

From one animal in the 10.0 group the behavioural data were lost due to technical problems.

In none of the experimental groups, animals showed freezing behaviour during the 15 minutes of acclimatization in the testing box. Onset of the CS, however, induced freezing behaviour as shown in Fig 2. CRI of dexmedetomidine during the training phase dose-dependently decreased the duration of freezing behaviour in the testing phase (one-way ANOVA group: $F_{3,28}$=7.097, P=0.001). Bonferroni multiple comparisons showed significant differences between the saline control group and group 10.0 (P=0.009 as well as between group 2.0 and group 10.0 (P=0.001).

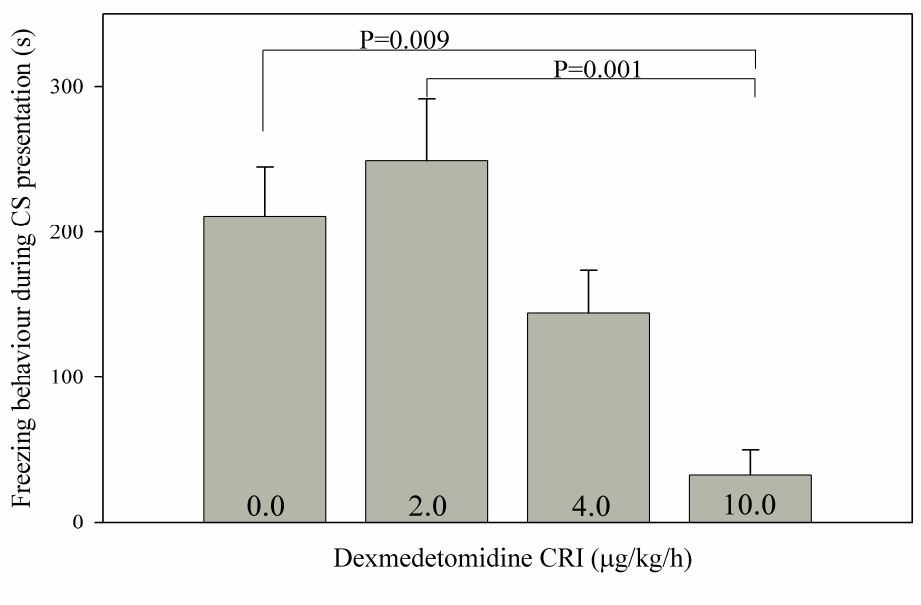

Figure 2. Duration of freezing behaviour in relation to the dose of dexmedetomidine. Mean ± S.E.M. duration of freezing behaviour summed for the 10 CS presentations during the testing phase of the fear-conditioning paradigm (n=8 for saline control, n=9 for group 2.0 and group 4.0 and n=7 for group 10.0).

4. Discussion

The present study shows that CRI of dexmedetomidine during the training phase of a Pavlov fear-conditioning task in rats, dose-dependently reduced the amplitude of the Vx-SEP generated by the US and, in a parallel fashion, reduced the duration of freezing behaviour following exposure to the CS only. These data indicate that dexmedetomidine, given as CRI, suppresses fear-conditioning only at doses that attenuate the processing of sensory input in the recipient.

Dexmedetomidine dose-dependently decreased the Vx-SEP but not the SI-SEP. This finding can be explained by the proposed differences in origin of the two signals (Stienen et al., 2005). Previous work argues that the Vx-SEP originates from a pathway signaling arousing somatosensory input, whereas the SI-SEP originates from a pathway involved in somatosensory discrimination (Stienen et al., 2004; 2005; 2006). An important brain area involved in signaling arousing sensory input, is the Locus Coeruleus (LC) (van Bockstaele, 1998; Miyazato et al., 2000; Jones, 2003; Aston-Jones, 2005). The activity of the LC is reduced by alpha-2 adrenoceptor agonists such as dexmedetomidine, through binding to the presynaptic alpha-2 autoreceptors (Svensson et al., 1975). While dexmedetomidine leaves the SI-SEP, reflecting the primary somatosensory pathway activity, relatively unaffected, the dexmedetomidine-induced attenuation of the Vx-SEP may be ascribed to a dexmedetomidine-reduced activity of the LC. Combined, the dexmedetomidine-induced decrease of the Vx-SEP, rather than that of SI-SEP, is considered to indicate a reduced processing of arousing somatosensory stimuli, i.e. the US in the present study.

The dexmedetomidine-induced suppression of the Pavlov fear-conditioning documented in this study can be ascribed to two different mechanisms. First, by disruption of CS and/or US processing during the training phase and second, by a reduction in noradrenergic input to the amygdala. The alpha-2 adrenoceptor agonist dexmedetomidine has been shown to attenuate the processing of both auditory and somatosensory stimuli (Franken et al., 2007) As such, dexmedetomidine can disrupt Pavlov fear-conditioning by inhibiting the CS and/or the US processing. Second, noradrenalin is required to form and consolidate memories in different brain areas (Gibbs and Summers, 2002), including the amygdala which is the key structure for memory formation in a Pavlov fear-conditioning task (Maren, 2001). Alpha-2 adrenoceptor agonists inhibit the noradrenalin release from the noradrenergic tracts by binding to alpha-2 adrenergic autoreceptors (Svensson et al., 1975;

Bucheler et al., 2002). As such, dexmedetomidine can disrupt the CS-US association in a Pavlov fear-conditioning task, by reducing the noradrenergic input to the amygdala. The present data show that the duration of freezing behaviour was only reduced at doses of dexmedetomidine that reduced US processing. In addition, the doses of dexmedetomidine that reduced freezing behaviour in the present study (≥ 4.0 μg/kg/h) have previously been shown to attenuate auditory perception, i.e. CS processing (Franken et al., 2007). Combined, these data suggest that the primary mechanism responsible for absence of a CS-US association is reducing the processing of sensory input. Although, it cannot be excluded whether dexmedetomidine, next to the attenuation of sensory processing, also affected the formation and consolidation of memory in the amygdala, the present study clearly demonstrates that non-sedative and non-analgesic doses do not suppress fear-conditioning. This is in contrast with a previous study showing that dexmedetomidine suppressed fear-conditioning at non-sedating and non-analgesic dose levels (Davies et al., 2004). In addition, other alpha-2 adrenoceptor agonists have been shown to disrupt passive avoidance learning in non-sedating doses (Galeotti et al., 2004a; 2004b). These findings do suggest that alpha-2 adrenoceptor agonists can suppress learning in an aversive learning task by reducing noradrenergic input to brain areas involved in the formation and consolidation of memory, since the non-sedating and non-analgesic doses used suggest that sensory processing was intact.

A possible explanation for the discrepancy between the present and the previous studies (Davies et al., 2004; Galeotti et al., 2004a; 2004b) is the difference in the aversive character of the learning paradigms used. Galeotti et al., (2004a; 2004b) used the passive avoidance task (Jarvik and Kopp, 1967), in which the animals were shocked once for 1 second at 0.5 mA during the training phase (Galeotti et al., 2004a; 2004b). Therefore, the animals were reinforced only once during the training phase. Davies et al., (2004) used a classic Pavlov fear-conditioning paradigm. This paradigm consisted of three trials during which a tone of 33 seconds (CS) was coupled for the last 3 seconds to a scrambled foot shock (US) of 0.75 mA. Consequently, the animals were reinforced three times during the training phase. In contrast, in the present study the animals were subjected to 10 trials during which a 40 seconds tone (CS) was given paired to a pulse train of 32 shocks of 5.0 mA (US). This specific and more intense US, was required for generating the SEPs. In the present study the animals were therefore reinforced more often and consistently stronger

during the training phase than the animals in the other studies. Consequently, the present paradigm might have activated the LC, which is the primary site of noradrenergic input to the brain, to a greater extend than the paradigms in the previous studies, since the LC is activated by arousing sensory stimuli (van Bockstaele, 1998; Miyazato et al., 2000; Jones, 2003; Aston-Jones, 2005). The activation of the LC by the present paradigm might have lead to a concomitant release of noradrenalin, adequate for memory formation (Gibbs & Summers, 2002), which could not be suppressed by the lowest dose of dexmedetomidine investigated in the present study.

A second explanation for the discrepancy might be found in the different test systems used to assess whether the doses of alpha-2 adrenoceptor agonists used were sedative or not. The behavioural tests used by Davies et al (2004) and Galeotti et al (2004a; 2004b) might not have been sensitive enough to detect subtle changes in processing of sensory input by the central nervous system. These subtle changes can, however, be detected by the recording of SEPs, as was described earlier by Shaw et al. (2001). In the present study the recording of SEPs was used to detect changes in US processing during the training phase. This might explain why the present study found suppression of fear-conditioning only at doses that did change sensory perception, whereas the other studies found a suppression of fear-conditioning and a reduced performance in the passive avoidance task at doses which were argued to be non-sedating and non-analgesic.

Finally, the classification of non-sedating and non-analgesic doses of alpha-2 adrenoceptor agonists in the previous studies (Davies et al., 2004; Galeotti et al., 2004a; 2004b) can be debated. The LC, providing the noradrenergic input needed for memory formation and consolidation, is involved in signaling arousing sensory input (van Bockstaele, 1998; Miyazato et al., 2000; Jones, 2003; Aston-Jones, 2005). When the performance in a learning task is attenuated by reducing the noradrenergic input to the relevant brain areas, through administration of an alpha-2 adrenoceptor agonist, this inherently indicates that the activity within the LC and thus the level of arousal is reduced. A reduction of arousal means that, at least at the central nervous system level, there is sedation. It has indeed been shown that a reduction in the activity of the LC leads to sleep (or sleep like states) and a decrease in levels of attention towards external stimuli (van Bockstaele, 1998; Miyazato et al., 2000; Jones, 2003; Aston-Jones, 2005). Therefore, the doses of alpha-2 adrenoceptor agonists impairing performance in a learning task, by

definition can not be regarded as non-sedating. The present study supports this view, since a suppression of fear-conditioning was only seen at doses of dexmedetomidine that suppressed sensory processing, as was assessed by direct recording of the activity of the central nervous system in response to somatosensory input, using the SEP.

It may also be questioned whether aversive learning paradigms are suitable to study effects of anesthetic drugs, such as alpha-2 adrenoceptor agonists, on memory function. In aversive learning paradigms, drug effects on memory function are assessed by changes in fear-related behaviour such as 1) the duration of freezing behaviour, or suppression of ongoing behaviour in classic Pavlov fear-conditioning tasks (Selden et al., 1990; 1991; Davies et al., 2004) or 2) the retention/step-down latencies in passive avoidance/step down tasks (Zarrindast et al., 2003; Galeotti et al., 2004a; 2004b). These measures indicate the extent of the animal's fear of the CS in classic Pavlov fear-conditioning or for the environment in passive avoidance and step down tasks, since these stimuli were related to an electrical shock. However, the well known analgesic effects of alpha-2 adrenoceptor agonists (Sinclair, 2003; Paris and Tonner, 2005) can be considered to reduce the unpleasantness of the electrical shock during the training phase. Under these conditions, the animal might have formed a normal memory of the events during the training phase, but shows less fear-induced behaviour since the experience during the training phase was less/not unpleasant. The reduction in fear-related behaviour can then be mistakenly interpreted as drug effects on memory function, i.e. intrinsic amnesic effects of the drug. In the present study, a reduction of freezing behaviour was only seen at doses of dexmedetomidine that reduced the Vx-SEP, i.e. the perception of the unpleasant US. Therefore, it can be questioned whether dexmedetomidine impaired the function of memory in the present experiment or whether it reduced freezing behaviour by inducing analgesia during the training phase. Based on the reduction of the Vx-SEP the latter is more plausible.

In conclusion, the present data show that continuous rate infusion of dexmedetomidine does not suppress fear-conditioning in the rat at non-sedating/non-analgesic doses. For the clinical application, this means that amnesia should not be expected at doses of dexmedetomidine at which the patients are still responsive to sensory input.

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Nociception-related somatosensory evoked potentials in awake dogs recorded after intra epidermal electrical stimulation

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Abstract

At present, the specific neurophysiologic methodology of recording pain-related evoked potentials is considered a most promising approach to objectively quantify pain in man. This study was designed to characterise and evaluate the use of somatosensory evoked potentials to study nociception in a canine model. To this aim, somatosensory evoked potentials were evoked by intra-epidermal electrical stimulation and recorded from the scalp in 8 beagle dogs. Characteristics determined were 1) the conduction velocities of the peripheral nerve fibres involved, 2) the stimulus intensity response characteristics and 3) the evaluation of possible disturbance of the signals by muscular activity from the hind paw withdrawal reflex (EMG artefact). The results showed 1) the conduction velocities to be in the A-delta fibre range (i.e. fibres involved in nociception), 2) an increase in amplitude and a decrease in latency of the evoked potential following increasing stimulus intensities and 3) the absence of EMG artefact in the signals. These data indicate that the evoked potentials recorded, are related to nociception and thus are suited to quantitatively characterise the perception of noxious stimuli making this model useful for pain- and analgesia-related research.

1. Introduction

A promising neurophysiologic approach to study (anti)nociception in animals is the recording of somatosensory evoked potentials (SEPs) (Murrell and Johnson, 2006). SEPs to study nociception are fragments of electro-encephalogram (EEG) recorded time-locked to a noxious somatosensory stimulus. Averaging several of such recordings, excludes the non-specific background EEG, leaving only the stimulus related EEG; the SEP. Different ways of noxious stimulation to record SEPs have been used, e.g. electrical stimulation (Bromm and Meier, 1984), fast heating contact thermode stimulation (Granovsky et al., 2005) and high intensity laser stimulation (Carmon et al., 1976). Laser stimulation is proposed as the most appropriate method, since it allows for short stimulus durations and as been shown to primarily activate nociceptive A-delta and C-fibers (Kakigi et al., 2000).

Laser stimulation has been used to study SEPs in both anaesthetized and awake primate and rodent models (Beydoun et al., 1997; Shaw et al., 1999; Baumgartner et al., 2006). However, from both an ethical and technical point of view, its use in animal models can be questioned. First, skin burns might result from too high stimulus intensities, since animals can not communicate the critical determinant of the exact stimulus intensity needed to evoke a pain sensation. Second, retinal damage might occur since awake animals are not easily equipped with protective goggles to protect the retina from the laser light. Finally, the animal needs to remain immobile by either bodily fixation or anaesthesia to allow the exact positioning of the laser beam at the small size stimulus site. This immobilization is to be considered stressful. Combined, alternative ways of selective nociceptive fibre stimulation in animal models should be evaluated for their methodological potential.

Intra-epidermal electrical stimulation has been shown to be a suitable alternative to laser stimulation in man (Inui et al., 2002b). With this type of stimulation, a push pin type needle electrode is used. The needle penetrates the superficial part of the epidermis, locating the tip of the electrode in the direct vicinity of the nociceptive nerve endings. With the application of small currents, this type of electrode has been shown to preferentially activate A-delta nociceptive fibres and to yield SEPs equivalent to those recorded after laser stimulation (Inui et al., 2002b). Intra-epidermal electrical stimulation has been successfully used in a multitude of human experimental studies (Inui et al., 2002a; 2002b; 2003a; 2003b; 2006; Wang et al., 2004; Ogino et al., 2005; Katsarava et al., 2006). However, up to present, it has not been described in animal SEP models of nociception.

In the present study, the applicability of intra-epidermal electrical stimulation to record SEPs in awake dogs was investigated. The major research questions were 1) are the SEPs evoked following this type of stimulation related to nociception, 2) do the characteristics of these SEPs allow for quantification of nociception, 3) are these SEPs are devoid of interference of muscular activity (EMG artefact) and 4) can similarities between these SEPs and SEPs recorded in other species be established.

2. Materials and Methods

2.1 Animals

Animal care and experimentation were performed in full accordance with the protocols approved by the Science Committee and the institutional Animal Experimentation Committee (Utrecht University, Utrecht, The Netherlands).

Beagle dogs (6 female, 2 male) found to be healthy upon clinical examination, in age ranging between 6-9 years, and weighing 12.9 ± 1.2 kg (mean ± SD) were studied. The dogs were housed individually and allowed daily outdoor exercise in groups. Prior to the experiments, the dogs were accustomed to the experimental surroundings and trained to remain quiet and still during the recording sessions.

2.2 Recording of evoked potentials

During recording sessions, the dogs were placed in a hammock through which the hind legs protruded towards the ground. To record the evoked potentials, needle EEG recording electrodes (stainless steel needle electrodes 15.0×0.7 mm, 13L60, Dantec Medical, Skovlunde, Denmark) were used. The electrodes were cleaned mechanically and prepared electrolytically before each session according to the manufacturer's instructions. After proper preparation and anesthetizing of the insertion sites (0.3 ml lidocaine s.c., lidocaine HCl, B. Braun 2%, B. Braun, Melsungen, Germany), the active electrode was placed at the vertex (halfway between nasion and inion at the midline of the scalp) with the reference electrode placed at the left ear and the ground electrode placed at the nape of the neck. The electrodes were fixated to the animal's skin using adhesive tape and connected to a bio-electric amplifier (Bio-electric amplifier AB 601-G, Nihon Kohden, Tokyo, Japan).

For the intra-epidermal electrical stimulation, the stainless steel concentric bipolar stimulation electrode (Inui et al., 2006) was used. For recording of a SEP, 32 square-wave electrical stimuli of 2 ms duration were delivered with a stimulus frequency of 0.2 Hz. All signals were band-pass filtered between 1.59-30 Hz and amplified 10,000 times. Each evoked potential recording consisted of 32 sweeps of 512 ms epochs with a sampling frequency of 1,000 Hz, using a delay of 25 ms.

2.3 Intensity response characteristics

In all dogs, stimulus intensity response characteristics of the SEPs were studied. SEPs were recorded during stimulation of the lateral aspect of the right metatarsus at four different stimulus intensities (0.0, 0.2, 0.5, and 1.0 mA). Three evoked potentials were recorded per animal per stimulus intensity (i.e. 12 recordings per animal). The sequence of the stimulus intensities used was randomised.

2.4 Determination of conduction velocity

In order to investigate whether the SEPs were specifically related to activation of nociceptive fibres, the conduction velocity of the fibre type underlying the consistent peaks of the SEPs was calculated. This calculation used the latency shift of the complex following both distal and proximal stimulation of the hind paw. The proximal stimulation site was located at the level of the right stifle joint and the distal stimulation site was located at the lateral aspect the right metatarsus. For both locations, 5 responses were recorded, i.e. a total of 10, at a stimulus intensity of 0.5 mA. The sequence of the stimulus locations (proximal and distal) was randomized over the 10 recordings. After the recording sessions, the locations of the stimulation electrodes were marked and the distance between the two sites was determined.

2.5 Detection of possible EMG artefact from the hind paw withdrawal reflex.

In order to investigate whether the SEPs recorded from the scalp were free from EMG artefact from the hind paw withdrawal reflex, in 4 dogs evoked potentials from the scalp were recorded simultaneously with the recording of the EMG from the biceps femoris muscle of the stimulated hind paw. For recording of EMG activity from the biceps femoris muscle, the active recording electrode was placed under the skin overlying the biceps

femoris muscle, the reference electrode was placed under the skin just below the stifle joint and the ground electrode was placed under the skin covering the hock.

EMG activity was recorded using the same settings as described for the evoked potential recording. Stimulus intensity was 0.5 mA and recordings were continued until two trials without visible withdrawal reflexes of the stimulated hind paw were obtained.

2.6 Data and Statistical analysis

For the stimulus response characteristics, the three recordings obtained per stimulus intensity were averaged per animal. After averaging, data extracted for analysis included the latency of the N2-P2 complex (Fig. 1A) and the peak-to-peak amplitude of the N2-P2 complex. The N1-P1 and N3-P3 complex (Fig. 1A) were not analysed on account of the less consistent representation both between and within dogs. Since at 0.0 mA no responses could be recorded, peak latencies and amplitudes were not analysed for this intensity. The latencies and peak-to-peak amplitudes of the N2-P2 complex recorded at 0.2, 0.5 and 1.0 mA were analysed using a repeated measurements analysis of variance (RM-ANOVA) with repeated factor “intensity”.

For the determination of conduction velocity, grand average waveforms of the recordings following either proximal or distal stimulation were made and the latency shifts of the observed peaks (N1, P1, N2, P2, N3 and P3) were calculated, by subtracting the latency obtained after proximal stimulation from the latency obtained after distal stimulation. Subsequently, the conduction velocity of the peripheral fibres involved in the generation of the peaks was calculated by dividing the average distance between the two stimulation sites by the latency shifts of the peaks. No further statistical analysis was used.

For the determination of possible EMG artefact, cortical responses obtained during trials with EMG activity of the hind paw were visually compared with cortical responses obtained during trials without EMG activity. No further statistical analysis was used.

3. Results

3.1 Behavioural reactions

The stimulations were tolerated well by all dogs. Withdrawal of the stimulated hind paw and lip licking were the only behavioural signs observed. These behavioural signs were observed to increase in both frequency and intensity with increasing stimulus intensities.

3.2 General waveform characteristics

The most consistent complex that could be recorded was the N2-P2 complex (Fig 1A). This complex, observed in the 80-140 ms latency range, was found to be very consistent both within, as well as between, dogs. Next to the N2-P2 complex, two other complexes could be observed in a large number, but not all, of the recordings. The first of these complexes, N1-P1, was a small complex in the 55-80 ms latency range. The second, N3-P3, was a large complex in the 200-350 ms latency range. Since the N2-P2 was the only complex observed in all recordings, data analysis of the stimulus response characteristics was performed on this complex only.

3.3 Intensity response characteristics

Figure 1A shows a representative example of a SEP recorded at a stimulus intensity of 1.0 mA. N2 to P2 peak-to-peak amplitudes showed a significant increase with increasing stimulus intensity (RM-ANOVA, $F_{2,14}$=8.145, P=0.005) (Fig. 1B). The latency of N2 showed a significant decrease with increasing stimulus intensities (RM-ANOVA, $F_{2,14}$=5.449, P=0.018) (Fig. 1C). The latency of P2 showed a trend towards a significant decrease with increasing stimulus intensities (RM-ANOVA, $F_{2,14}$=3.489, P=0.055) (Fig.1C).

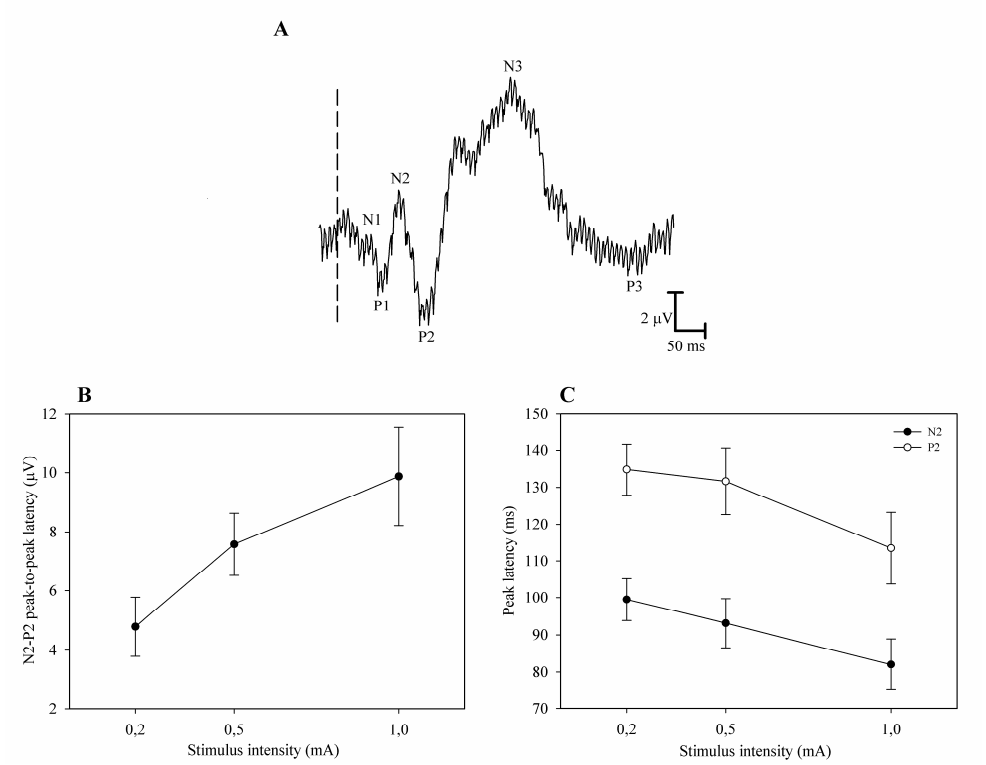

Figure 1. Stimulus intensity response characteristics. A) Representative example of the SEP recorded at a stimulus intensity of 1.0 mA. The dotted vertical line indicates the stimulus onset, the characters N and P followed by the numbers 1, 2 or 3 indicate the peaks as defined in the text. B) N2-P2 peak-to-peak amplitude in relation to stimulus intensity. Shown are means ± SEM, n = 8 per intensity. C) Peak latency of both N2 (filled symbols) and P2 (open symbols) in relation to stimulus intensity. Shown are means ± SEM, n = 8 per intensity.

3.4 Determination of conduction velocities

Figure 2 shows the grand average waveforms of the responses obtained after proximal and distal stimulation. A latency shift (Table 1) within the A-delta fibre range of 4-30 m/s (Bromm and Lorenz, 1998) was calculated for all peaks except the N3 peak.

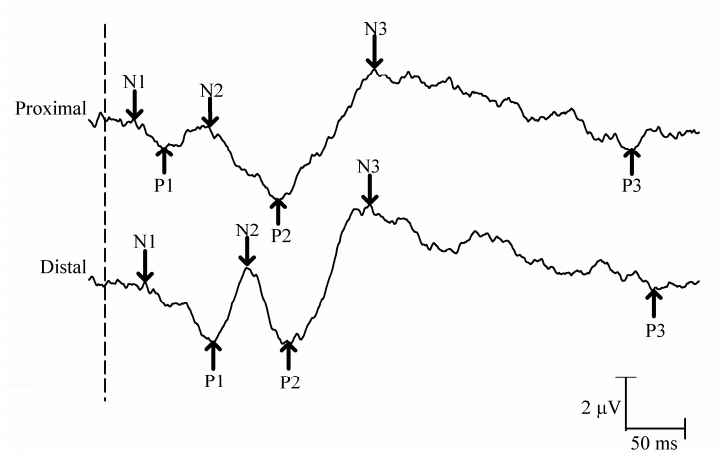

Figure 2. Grand average SEP waveforms used to calculate conduction velocities. The waveforms were obtained after proximal (upper trace) and distal (lower trace) stimulation The dotted vertical line indicates stimulus onset, whereas the arrows indicate the peak latencies of the N1, P1, N2, P2, N3 and P3.

Table 1. Calculated conduction velocities based on the shift in latency of the peaks after proximal and distal stimulation and the distance between the stimulation sites.

Peak	Latency after distal stimulation (ms)	Latency after proximal stimulation (ms)	Latency shift (ms)	Average distance between proximal and distal stimulation site (cm)	Calculated conduction velocity (m/s)
N1	49	39	10		17.5
P1	106	72	34		5.1
N2	134	102	32	17.5	5.5
P2	171	162	9		19.5
N3	238	241	-3		-58.3
P3	480	457	23		7.6

3.5 Determination of possible EMG artefact

In all 4 dogs tested, the EMG response recorded from the biceps femoris muscle gradually disappeared within 3-9 recordings, while the SEPs recorded from the scalp did not change over subsequent recordings. Figure 3 shows a representative example of two recordings in

one dog. It is clearly visible how the EMG response is completely abolished in subsequent recordings while the SEP remains unchanged.

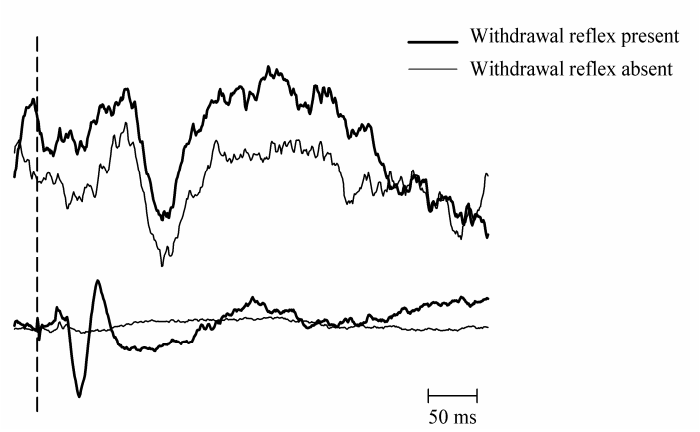

Figure 3. SEPs in relation to the EMG of the stimulated hind paw. Shown are recordings of the SEP (upper traces) and the EMG recorded from the stimulated hind paw (lower traces) during presence (bold lines) and absence (plain lines) of the withdrawal reflex. The dotted vertical line indicates the stimulus onset.

4. Discussion

The present study indicates that intra-epidermal electrical stimulation can be successfully applied to record nociception-related SEPs in awake dogs: 1) the calculated conduction velocities indicate that the SEPs primarily represent a nociceptive response, 2) the stimulus response characteristics of the N2-P2 complex indicate that recording SEPs allows for quantitative analysis of nociception, 3) no EMG artefact in the SEPs was found and 4) the SEP waveforms showed obvious similarities with nociception-related SEPs recorded in man and monkeys (Beydoun et al., 1997; Inui et al., 2002b). These results suggest that data generated with this model can provide useful information for both animal and human pain research.

SEPs, used to study nociception, must be mediated by afferent nerve fibres involved in nociception, i.e. A-delta and/or C-fibres (Bromm and Lorenz, 1998). The mediating fibre type can be determined by calculating its conduction velocity, based on the latency shift of

the peaks of the response, when using a fixed recording site and both a proximal and distal stimulation site (Shaw et al., 1999; Tran et al., 2001; Inui et al, 2002b). In the present study, the results obtained with this approach indicated that the SEPs were mediated by A-delta nociceptive fibres, since the conduction velocity for all peaks with the single exception of N3, fell within the A-delta fibre range, i.e. 4-30 m/s (Bromm and Lorenz, 1998).

The conduction velocity data do, however, demonstrate some peculiarities that deserve further evaluation. Firstly, for the P1 and N2, a conduction velocity slower than for the P2 was observed (5-5.5 m/s vs 19.5 m/s), while the P1 and N2 appeared earlier in the signal. Peaks appearing earlier in the signal may likely be considered to be mediated by faster conducting fibres. However, due to central processing in the brain, the final responses recorded from the scalp may appear at another latency than expected on basis of the conduction velocity alone (Ploner et al., 2006). Secondly, for the N3 peak, a negative conduction velocity was calculated. The N3 peak can be considered to have an endogenous origin (Bromm and Lorenz, 1998) making it more susceptible to variations in attention level, consequently leading to a greater variability in the latency shift (Beydoun et al., 1993; Ohara et al., 2004b; Wang et al., 2004). This might explain why no latency shift within the A-delta range was found for this peak.

In this study, a maximum stimulus intensity of 1.0 mA was chosen since higher stimulus intensities could lead to co-activation of non-nociceptive fibres by the intra epidermal electrical stimulation method (Inui et al., 2002b). Furthermore, since good signal qualities were obtained at 0.5 mA, increasing the stimulus intensity above 1.0 mA, would not increase signal quality but would potentially increase the unpleasantness for, and thus impair the welfare of, the animals studied. Finally, in man, signals were successfully recorded at stimulus intensities of 0.19 to 0.22 mA (Inui et al., 2002b) suggesting that there is no need to increase the stimulus intensities above 1.0 mA.

In this canine model, increasing stimulus intensities resulted in higher peak-to-peak amplitudes and shorter latencies of the N2-P2 complex, which is in agreement with studies in man (Carmon et al., 1978; Beydoun et al., 1993; Kanda et al., 2002; Ohara et al., 2004a). The peak-to-peak amplitudes of SEPs recorded from vertex after noxious stimulation have been shown to positively correlate with the subjective perception of the stimuli in both humans and rats (Carmon et al., 1978; Kakigi et al., 2000; Leffaucher et al., 2001; Kanda et al., 2002; Ianetti et al., 2004; Ohara et al., 2004a; Stienen et al., 2006; van Oostrom et al.,

2007). Therefore, the graded response character of the N2-P2 complex can be used to quantify nociception. For example, analgesic drug-induced changes in nociception might be evaluated by studying the amplitude reduction after administration of the drug (Buchsbaum et al., 1981; Chapman et al., 1982; Beydoun et al., 1997; Schaffler et al., 1987; Arendt-Nielsen et al., 1990a; 1990b; 1991; Nielsen et al., 1991; Meier et al., 1993; Lorenz et al., 1997a; 1997b). However, effects other than the intrinsic analgesic drug effects, such as a reduction in attention due to sedative actions, must be taken into account. A reduction in attention has been shown to reduce the amplitudes of SEPs (Beydoun et al., 1993; Ohara et al., 2004b; Wang et al., 2004). Although these reductions are actually accompanied by a reduction in conscious pain perception in humans (Carmon et al., 1978; Beydoun et al., 1993; Ohara et al., 2004b), they can not be regarded as intrinsic analgesic effects of the drug administered. To differentiate between the intrinsic analgesic effects and sedative effects of a drug, the recording of SEPs can be combined with the recording of auditory evoked potentials (AEPs) (Meier et al., 1993; Lorenz et al., 1997a; 1997b). AEPs indicate the vigilance level of the subject and as such can indicate whether the changes in SEPs are (in part) related to sedation or to true intrinsic analgesic effects of the drug administered. In dogs, recording of AEPs has been shown in a set-up similar to the present study (Murrell et al., 2004). Therefore, when studying analgesic drug effects using the present model, AEPs can be recorded in conjunction with the SEPs to exclude possible sedative effects, leaving only the true analgesic effects to be assessed.

The presence or absence of the hind paw withdrawal reflex induced no difference between SEPs recorded. Therefore, the SEPs obtained in this study fully reflect the supraspinal neuronal activity and not EMG activity from nocifensive reflexes. This is in accordance with a previous study, in which SEPs were recorded in monkeys following laser stimulation (Beydoun et al., 1997).

The general waveform of the SEPs recorded in this dog model shows striking similarities to the pain/nociception related evoked potentials in man (Inui et al., 2002b) and monkeys (Beydoun et al., 1997). In man, the N2-P2 complex, occurring in the 150-300 ms latency range, is often found to be the only consistent complex. Taking into account the smaller distance between the stimulation and recording sites in dogs, the latency range of the N2-P2 complex found in this study (80-140 ms) can be considered comparable to the one documented in man. Similar to our findings, the N2-P2 complex in human studies is

often but inconsistently preceded by a small N1-P1 complex and followed by a large slow N3-P3 complex.

Noxious electrical stimulation is considered to potentially co-activate non-nociceptive fibres, resulting in responses that are not fully nociception-specific. In contrast, laser stimulation is considered to selectively activate A-delta and C nociceptive fibres, leading to responses selectively related to nociception (Kakigi et al., 2000). From ethical and practical points of view, the choice for laser stimulation in animal models leaves room for further discussion and improvement. Firstly, laser studies in man regularly report the mandatory use of eye protection for both the experimenters and the subjects under investigation (Beydoun et al., 1993; Tran et al., 2001; Kanda et al., 2002; Ohara et al., 2004a; 2004b). However, studies using laser stimulation in awake animals do not report such protection for the animals (Shaw et al., 1999), even when protection for the experimenters is reported (Beydoun et al., 1997). This suggests that the eyes of the animals were not protected against the high intensity laser light, which can lead to severe retinal damage (Barkana and Belkin, 2000). Secondly, in animals, skin burns might result from unintentionally high laser stimulus intensities since animals can not directly indicate the nociceptive threshold intensity. Thirdly, the animals are easily stressed by the firm fixation or anaesthesia needed to immobilize the animal to be able to consistently aim the laser beam at the stimulus site. Fourthly, laser stimulation involves rather expensive equipment and the need of specific knowledge on the correct and safe use of the equipment. Combined, the use of an alternative to laser stimulation should be considered when studying nociception in animal models. Intra-epidermal electrical stimulation provides such an alternative, as indicated by the present and previous studies (Inui et al., 2002a; 2002b; 2003a; 2003b; 2006; Wang et al. 2004; Ogino et al. 2005, Katsarava et al., 2006). This specific type of electrical stimulation was shown to preferentially activate A-delta nociceptive fibres. It is not accompanied by any damaging side effects. The stimulation electrode can be fixed to the animal, consequently requiring minimal restraint. And the equipment needed for this type of stimulation is much cheaper and can be used without specialized knowledge.

In conclusion, the present data show that 1) nociception related SEPs can be recorded from the vertex in awake dogs, using intra-epidermal electrical stimulation, 2) the signals can be used to quantify nociception, 3) the signals are devoid of EMG artefact and 4) the signals show similarities with pain-related evoked potentials recorded in man and monkeys.

Therefore, it is concluded that intra-epidermal electrical stimulation can be successfully used to study nociception in animal models.

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Neurophysiologic assessment of sedative and analgesic effects of constant rate infusion of dexmedetomidine in a dog model.

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Abstract

In this study the sedative and analgesic effects of dexmedetomidine were neurophysiologically characterized in an acutely instrumented dog model. Auditory- and somatosensory evoked potentials (AEPs and SEPs respectively) were recorded before, during and after a constant rate infusion (CRI) of saline or dexmedetomidine (1.0, 3.0, 5.0 µg/kg bolus, followed by 1.0, 3.0, 5.0 µg/kg/h CRI, respectively) in eight beagle dogs. Both AEPs and SEPs were evaluated using the rate-dispersion-factor (RDF). Blood plasma samples were taken during infusion of dexmedetomidine to determine its plasma concentrations. The results showed a significant reduction of the AEP at doses of 1.0 µg/kg/h and higher and a significant reduction of the SEP at doses of 3.0 µg/kg/h and higher. Both the AEP and the SEP were not further reduced at doses greater than 3.0 µg/kg/h, however a slower return towards baseline values was observed at the higher doses. Dexmedetomidine plasma levels during infusion were 0.533 +/- 0.053 ng/ml, 1.869 +/- 0.063 ng/ml and 4.017 +/- 0.385 ng/ml (mean +/- SEM) for doses 1.0, 3.0 and 5.0 µg/kg/h, respectively. Based on these results it can be concluded that 1) dexmedetomidine has sedative as well as analgesic effects that can be quantitatively described using neurophysiologic parameters, 2) analgesia is achieved at higher plasma levels than those needed to achieve sedation and 3) it is suggested that increasing plasma levels above 1.869 ng/ml does not enhance the magnitude of the sedative and analgesic effects, but does prolong its duration of action.

1. Introduction

Dexmedetomidine is the dextro-optical isomer, and active component, of the racemic mixture medetomidine. It is a potent α2-adrenoceptor agonist and is used for its sedative and analgesic properties (Sinclair 2003; Paris and Tonner, 2005).

The sedative and analgesic effects of dexmedetomidine have been studied in different species, including rats, cats, dogs and man (Idänpään-Heikkilä et al., 1994; Guo et al., 1996; Hayashi et al., 1996; Bol et al., 1999; Ansah et al., 2000; Ebert et al., 2000; Hall et al., 2000; Xu et al., 2000; Kuusela et al., 2000; 2001; Talke et al., 2003; Angst et al., 2004 Cortinez et al., 2004; Sanders et al., 2005; Pascoe et al., 2006;). Clear sedative effects at low doses and clear sedative as well as analgesic effects at higher doses have been reported, based upon the results obtained by using verbal reporting (man) or reflex testing (animal models). However both verbal reporting and reflex testing are susceptible to the sedative effects of dexmedetomidine, thus potentially impairing the proper determination of the analgesic effects. In man, the profound sedation induced by the higher doses of dexmedetomidine prohibits the subject under investigation to report pain. Consequently, the analgesic effects of higher doses of dexmedetomidine are difficult to investigate (Ebert et al., 2000; Angst et al., 2004; Cortinez et al., 2004). The studies using nocifensive reflex testing in animal models, all report distinct analgesic effects after administration of high doses of dexmedetomidine (Idänpään-Heikkilä et al., 1994; Guo et al., 1996; Hayashi et al., 1996; Bol et al., 1999; Ansah et al., 2000; Xu et al., 2000; Kuusela et al., 2000; 2001; Talke et al., 2003; Pascoe et al., 2006). However, the validity of the data on analgesic effects of dexmedetomidine, obtained with nocifensive reflex testing in animal models, can be debated. First, these reflexes are more likely to be indicative of spinal (anti)nociception than of conscious pain perception and analgesia, since they have been demonstrated in decerebrate animals (Danneman et al., 1994; Thorn et al., 1994; Weng and Schouwenborg, 1998), and consequently do not necessarily involve higher brain structures mandatory for pain perception. Second, due to the muscle relaxant properties of dexmedetomidine the effectors of the anti-nociceptive reflexes, the skeletal muscles, are inhibited (Farber et al., 1997; Weinger et al., 1989). This may readily lead to an overestimation of the analgesic effect of dexmedetomidine, since absence or reduction of the reflex under these circumstances might be due to muscle relaxation rather than analgesia (Bergadano et al., 2006). Taken together, a more in-depth evaluation of the proper, systemic analgesic effect

of dexmedetomidine is needed in order to establish the true extent of its analgesic properties.

Recording of evoked potentials can be considered a better and more valid alternative than reflex testing to assess sedative and analgesic effects of anaesthetic drugs in animal models. Auditory- and pain-related evoked potentials (AEPs and SEPs respectively) recorded from the scalp 1) provide insight in the activity of the central nervous system at the supra spinal level in response to auditory and painful stimuli respectively (Sims and Moore, 1984; Bromm and Lorenz, 1998; Nayak and Roy, 1998; Huang et al., 1999; Pypendop et al., 1999; Kakigi et al., 2000; Shi et al., 2004; Murrell et al., 2004; 2005), 2) and more specifically from the vertex, are indicative of the perception of the respective stimuli applied (Suzuki and Taguchi 1965; Davis and Zerlin, 1966; Davis et al., 1968; Kaskey et al., 1980; Kakigi et al., 2000; Kanda et al., 2002, Stienen et al., 2006; van Oostrom et al., 2007;), 3) are not influenced by concomitant muscle relaxant effects of drugs investigated (Wiederholt and Iragui-Madoz, 1977; Miyazato et al., 1999) and 4) by their nature, provide a method of assessment without the necessity to verbally report on sedation or pain.

To overcome these possible confounding factors in nocifensive reflex testing due to sedation and/or muscle relaxation, in the present study the sedative and analgesic effects of constant rate infusion (CRI) of dexmedetomidine (0.0, 1.0, 3.0 and 5.0 µg/kg/h) were investigated, using AEPs and SEPs recorded from the vertex in a dog model. Plasma concentrations of dexmedetomidine during infusion were determined in order to correlate the sedative and analgesic effects of dexmedetomidine with its corresponding plasma levels.

2. Materials and Methods

2.1 Animals

Animal care and experimentation were performed in full accordance with the protocols approved by the Science Committee and the institutional Animal Experimentation Committee (Utrecht University, Utrecht, The Netherlands).

Eight Beagle dogs (7 male, 1 female) found to be healthy upon clinical examination, in age ranging between 3-9 years, and weighing 12.9 ± 1.5 kg (mean ± SD) were studied. The dogs were housed individually and allowed daily exercise, outdoors in groups.

Prior to the experiments, the dogs were accustomed to the experimental surroundings and procedures. Food, but not water, was withheld 12 hrs prior to all recording sessions.

2.2 General experimental outline

Dogs were placed in a specially designed hammock, from which the hind paws protruded towards the ground, resulting in a stable and relaxed manner of restraint. Catheters (Vasofix® 18G, B. Braun Melsungen AG, Melsungen, Germany) were placed in both cephalic veins. The sites at which the recording and stimulation electrodes were placed, were shaven and the sites for the recording electrodes were anesthetized locally (0.3 ml lidocaine s.c., lidocaine HCl B. Braun 2%, B. Braun Melsungen AG, Melsungen, Germany). Subsequently, the recording electrodes (stainless steel needle electrodes 15.0 x 0.7 mm, 13L60, Dantec Medical, Skovlunde, Denmark) were placed at the vertex (i.e., at the midline of the scalp halfway between nasion and inion; active), at the left ear-flap (reference) and at the nape of the neck (ground) and connected to a bio-electric amplifier (Bio-electric amplifier AB 601-G, Nihon Kohden, Tokyo, Japan).

The stimulation electrode to deliver the noxious stimuli for recording of the SEPs was placed at the right metatarsus and connected to a Grass stimulator (Model S-88, Grass Medical instruments, Quincy, Mass., USA).

Ear plugs of an in-ear system (Eartone 3A insert earphones, Cabot Corp, Indianapolis, Ind.) to deliver the auditory clicks for recording of the AEPs were placed bi-aurally in the external ear canal.

After checking correct functioning of all equipment, sessions started with recordings of 5 AEPs and 5 SEPs in alternating sequence (baseline). After baseline recordings, a blood sample was taken and CRI of dexmedetomidine or saline was started. 30 minutes after the start of the CRI another 5 AEPs and 5 SEPs were recorded in alternating sequence. During CRI, 5 blood samples were taken; one after each SEP recording. After collecting the last blood sample, the CRI was terminated. 30 minutes after terminating the CRI, the last 5 AEPs and 5 SEPs were recorded in alternating sequence.

All dogs were subjected to 4 recording sessions during which they received either dexmedetomidine (1.0, 3.0 or 5.0 µg/kg/h) or saline. For an overview of the time-line, sequence and details of the events during the sessions see table 1 and the descriptions below.

Table 1. Overview of the timeline of the experiment. AEP means recording of an auditory evoked potential. SEP means recording of a somatosensory evoked potential. Numbers 1-15 indicate the recording trial. Sample means collection of a blood sample.

	Baseline						CRI						Post infusion				
	1 AEP SEP	2 AEP SEP	3 AEP SEP	4 AEP SEP	5 AEP SEP sample	Start infusion 30 minutes waiting period	6 AEP SEP sample	7 AEP SEP sample	8 AEP SEP sample	9 AEP SEP sample	10 AEP SEP sample	Stop infusion 30 minutes waiting period	11 AEP SEP	12 AEP SEP	13 AEP SEP	14 AEP SEP	15 AEP SEP
Time (h:m)	0:00	0:10	0:20	0:30	0:40		1:20	1:35	1:50	2:05	2:20		3:00	3:10	3:20	3:30	3:40

2.3 Drug administration

Dexmedetomidine (Dexdomitor, Orion Corporation, Espoo, Finland) was administered by CRI, preceded by a loading dose, via the catheter in the left cephalic vein, using a syringe pump (Graseby Anaesthesia Pump 3500, Graseby Medical LTD, Watford Herts, United Kingdom).

Doses used were i) 1.0 µg/kg bolus given over 1 minute, followed by 1.0 µg/kg/h CRI, ii) 3.0 µg/kg bolus given over 1 minute, followed by 3.0 µg/kg/h CRI and iii) 5.0 µg/kg bolus given over 1 minute, followed by 5.0 µg/kg/h CRI. During control sessions, saline was infused. Volumes across doses were kept constant at 1.0 ml/kg (bolus) and 1.0 ml/kg/h (CRI).

2.4 Recording of AEPs and SEPs

Both AEPs and SEPs were recorded from the vertex, using the electrode at the left ear-flap and the electrode at the nape of the neck as reference and ground respectively.

AEPs were recorded as described by Murrell et al. (2004, 2005). 512 clicks of 80 dB sound pressure level and 0.2 ms in duration were given bi-aurally with a stimulus repetition rate of 3 Hz. AEPs were recorded with a sampling frequency of 10 kHz using dedicated

software build on a Labview platform (AD) (Labview 7.2, National Instruments Netherlands B.V., Woerden, The Netherlands). The signals were band-pass filtered between 15 and 300 Hz using an additional 50 Hz notch filter and amplified 20,000 times. For each AEP, 512 subsequent data-epochs of 150 ms were averaged, using a delay of 25 ms.

SEPs were recorded as described by van Oostrom et al. (2007). 32 noxious stimuli using intra-epidermal electrical stimulation (Inui et al., 2002a), of 0.5 mA and 2 ms in duration were given on the lateral aspect of the right metatarsus with a stimulus repetition rate of 0.2 Hz. SEPs were recorded with a sampling frequency of 10 kHz, using dedicated software build on the Labview platform. Signals were band-pass filtered between 1.59 and 40 Hz and amplified 20,000 times. For each SEP, 32 subsequent data-epochs of 600 ms were averaged, using a delay of 100 ms.

In previous studies, the intra-epidermal electrical stimulation (presently used to record the SEPs) has been shown to preferentially activate A-delta nociceptive fibres, leading to distinct pinprick sensations, and consequently yielding responses related to nociception rather than to tactile or mixed sensations. (Inui et al. 2002a; 2002b; 2003a; 2003b; 2006; Wang et al. 2004; Ogino et al. 2005, Katsarava et al., 2006; van Oostrom et al., 2007)

2.5 Blood plasma collection and dexmedetomidine plasma level analysis

Blood for plasma level analysis of dexmedetomidine was withdrawn from the catheter in the right cephalic vein. A sample of 5 ml was collected after taking a 5 ml pre-sample to avoid contamination with saline used to flush the catheter after each sample withdrawal. Blood samples were collected in EDTA coated tubes and directly stored on ice. Within 60 minutes after collection, the blood samples were centrifuged at 1300g and 4^0 C for 15 minutes. After centrifuging the plasma was separated from the cell fraction and stored at -20^0 C.

Dexmedetomidine plasma concentrations were analysed using liquid chromatography tandem mass spectrometry (CRST Bioanalytics, Turku, Finland). The lower limit of quantification was 0.020 ng/ml. The average between-run precision was 3.4% and the average accuracy at different quality control sample concentrations was between 92.5 and 101.1%. On account of cost considerations only the 5 samples taken during the infusion period were analyzed.

2.6 Data and statistical analysis

Calculations were performed with the aid of Microsoft Excel 2003. Statistical analysis was performed with Sigmastat 2.0. Differences were considered to be significant when $P<0.05$.

Both AEPs and SEPs were evaluated using the Rate Dispersion Factor (RDF). The RDF is an expression of the overall shape of the evoked potential waveform in the latency range studied. It is obtained by averaging the absolute differences between all pairs of subsequent sampled data points y_k in a specified latency range from x to m (see equation 1) (Mantzaridis and Kenny, 1997; Haberham et al., 2000; Stienen et al., 2004; van Oostrom et al., 2007). Both decreases in amplitude and increases in latency of the evoked potential components, decrease the value of the RDF, whereas both increases in amplitude and decreases in latency of the evoked potential components increase the value of the RDF.

$$\text{Equation 1}: \text{RDF} = \frac{1}{m - x} \sum_{k=x+1}^{m} \left| y_k - y_{k-1} \right|$$

For the AEPs, the RDFs were calculated for the complete latency range post-stimulus (125 ms) based on previous studies (Haberham et al., 2000; Mantzaridis and Kenny, 1997). For the SEPs, the RDFs were calculated for the latency range (x to m) of the N2-P2 complex (see figure 1). This complex occurs in the 88-135 ms latency range and is previously described to be specifically related to processing of noxious stimuli (van Oostrom et al., 2007). The latency range (x to m) of the N2-P2 complex was determined in the *average waveform* of the 5 *baseline* recordings for each dog and each recording session separately. Subsequently the RDFs of the individual signals for that session were calculated using this latency range (x to m). As such, for both the AEP and SEP, there was no need of determining peaks in individual signals, leading to a highly objective way of analyzing the signals, since determining peaks in individual signals is prone to confounding errors especially when signals are weak (e.g. during infusion of higher doses of dexmedetomidine).

For each animal and recording session separately, the RDFs of all individual signals were calculated as a percentage of the mean of the RDF values of the baseline recordings, which was set at 100%. The RDF percentage changes were statistically analyzed using a

two-way repeated measures analysis of variance (2-way RM-ANOVA) with repeated factors "time" and "treatment" followed by *post-hoc* analysis when appropriate.

The results of the dexmedetomidine plasma level analysis were presented as ng/ml and analysed using a 2-way RM-ANOVA with repeated factors "time" and "treatment" followed by *post-hoc* analysis when appropriate.

3. Results

3.1 Effects of dexmedetomidine infusion on AEPs

Figure 1 (panel A) shows the grand average waveforms of the AEPs recorded during CRI of dexmedetomidine. Peaks that are consistent with findings in previous studies on AEPs recorded in dogs are denoted by the labels P0, Na, Pa, Nb and Pb (Sims and Moore, 1984; Nayak and Roy, 1998; Huang et al., 1999; Pypendop et al., 1999; Murrell et al., 2004; 2005). It is clearly shown how increasing doses of dexmedetomidine decrease the amplitudes of the peaks and increase the latencies.

The RDF changes calculated for the AEPs recorded during the baseline, infusion and post-infusion period for the four sessions (saline control, 1.0, 3.0 and 5.0 µg/kg/h dexmedetomidine) are shown in figure 2 (panel A). It is clearly visible how infusion of dexmedetomidine dose dependently decreases the RDF of the AEP and how the RDF returns to baseline values after the infusion is terminated.

The 2-way RM-ANOVA showed a significant time×dose interaction for the RDF percentage change ($F_{42,294}$= 4.033, P< 0.001). *Post-hoc* analysis showed significant time effects for doses 1.0, 3.0 and 5.0 µg/kg/h and significant dose effects for time-points 6-11 and 13. For details of the statistics see table 2.

3.2 Effects of dexmedetomidine infusion on SEPs

Figure 1 (panel B) shows the grand average waveforms of the SEPs recorded during CRI of dexmedetomidine. Peaks consistent with a previous study on SEPs recorded in dogs, are denoted by the labels N1, P1, N2, P2, N3 and P3 (van Oostrom et al., 2007). The latency-range (*x* to *m*) for which the RDF is calculated is denoted by the dotted vertical lines. The RDF changes, calculated for the SEPs, recorded during the baseline, infusion and post-infusion period for the four sessions (saline control, 1.0, 3.0 and 5.0 µg/kg/h

dexmedetomidine), are shown in figure 2 (panel B). It is clearly visible how infusion of dexmedetomidine dose dependently decreases the RDF of the SEP and how the RDF returns to baseline values after the infusion is terminated.

The 2-way RM-ANOVA showed a significant time×dose interaction for the RDF percentage change ($F_{42,294}$= 1.693, P= 0.007). *Post-hoc* analysis showed significant time effects for doses 3.0 and 5.0 µg/kg/h and significant dose effects for time-points 6-9. For details of the statistics see table 3.

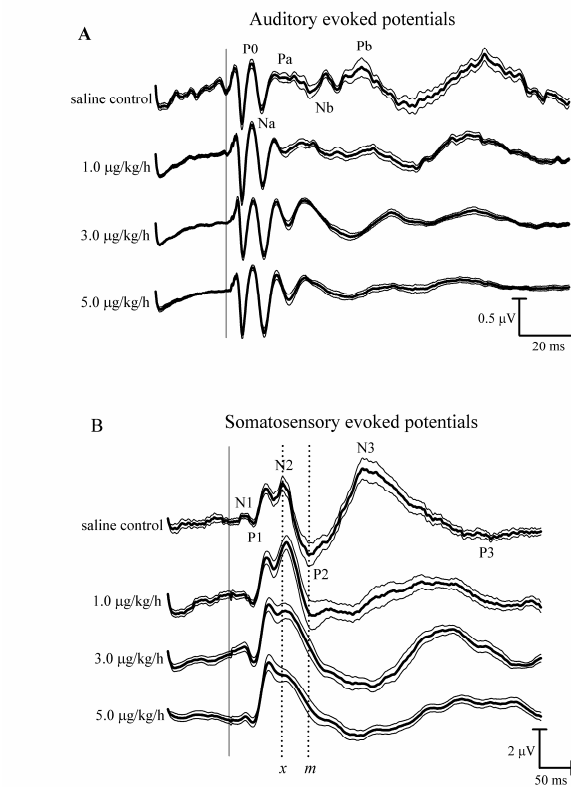

Figure 1. Grand average AEP and SEP waveforms during infusion. Grand mean (bold lines) ± SEM (plain lines) waveforms of the AEPs (panel A) and SEPs (panel B) recorded during infusion of saline and the different doses of dexmedetomidine. n = 40 for each dose. The solid vertical lines indicate the stimulus onset, whereas the dotted vertical lines in panel B indicate the latency range (x to m) for which the RDF is calculated. Specific peaks consistent with findings in previous studies are indicated with the characters N and P followed by the characters a and b and/or numbers 0, 1, 2 or 3 as defined in the text.

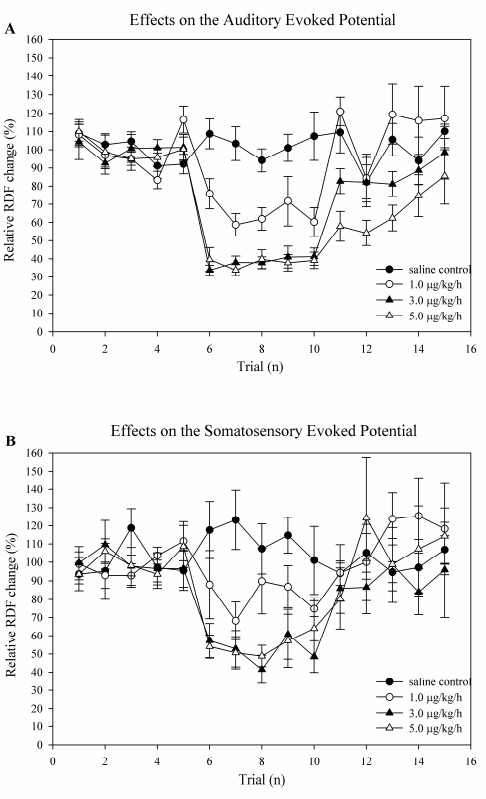

Figure 2. RDFs calculated for the AEP and SEP before, during and after dexmedetomidine CRI. Data are presented as mean relative RDF ± SEM, *n* = 8, calculated for the AEP (panel A) and SEP (panel B). Trial 1-5, 6-10 and 11-15 are recorded during baseline, CRI and post CRI respectively.

Table 2. AEP statistics. Shown are the statistical procedures and outcomes for the *post-hoc* statistical analysis of the changes in the RDF calculated for the AEP. These statistical procedures followed the initial 2-way RM-ANOVA after a significant time*dose interaction was found, see text.

Test	Significance found for	Significant effects (P<0.05) found with Tukey multiple comparisons
1-way RM-ANOVA repeated factor "time"	Dose 1.0 ug/kg/h $F_{14,98}$ = 6.092 P < 0.001	Time-point 1 vs 7, 8, 10
		Time-point 5 vs 7, 8, 10
		Time-point 11 vs 7, 8, 9, 10
		Time-point 13 vs 7, 8, 9, 10
		Time-point 14 vs 7, 8, 9, 10
		Time-point 15 vs 7, 8, 10
	Dose 3.0 ug/kg/h $F_{14,98}$ = 14.113 P < 0.001	Time-point 1 vs 6, 7, 8, 9, 10
		Time-point 2 vs 6, 7, 8, 9, 10
		Time-point 3 vs 6, 7, 8, 9, 10
		Time-point 4 vs 6, 7, 8, 9, 10
		Time-point 5 vs 6, 7, 8, 9, 10
		Time-point 11 vs 6, 7, 8, 9, 10
		Time-point 12 vs 6, 7, 8, 9, 10
		Time-point 13 vs 6, 7, 8, 9, 10
		Time-point 14 vs 6, 7, 8, 9, 10
		Time-point 15 vs 6, 7, 8, 9, 10
	Dose 5.0 ug/kg/h $F_{14,98}$ = 15.522 P < 0.001	Time-point 1 vs 6, 7, 8, 9, 10, 11, 12, 13, 14
		Time-point 2 vs 6, 7, 8, 9, 10, 11, 12, 13
		Time-point 3 vs 6, 7, 8, 9, 10, 11
		Time-point 4 vs 6, 7, 8, 9, 10, 11, 12
		Time-point 5 vs 6, 7, 8, 9, 10, 11, 12, 13
		Time-point 14 vs 6, 7, 8, 9, 10
		Time-point 15 vs 6, 7, 8, 9, 10
1-way RM-ANOVA repeated factor "dose"	Time-point 6 $F_{3,21}$ = 34.042 P < 0.001	Dose 0.0 vs 1.0, 3.0, 5.0
		Dose 1.0 vs 3.0, 5.0
	Time-point 7 $F_{3,21}$ = 27.558 P < 0.001	Dose 0.0 vs 1.0, 3.0, 5.0
		Dose 1.0 vs 5.0
	Time-point 8 $F_{3,21}$ = 28.510 P < 0.001	Dose 0.0 vs 1.0, 3.0, 5.0
		Dose1.0 vs 3.0, 5.0
	Time-point 9 $F_{3,21}$ = 14.592 P < 0.001	Dose 0.0 vs 3.0, 5.0
		Dose 1.0 vs 3.0, 5.0
	Time-point 10 $F_{3,21}$ = 20.137 P < 0.001	Dose 0.0 vs 1.0, 3.0, 5.0
	Time-point 11 $F_{3,21}$ = 13.264 P < 0.001	Dose 0.0 vs 5.0
		Dose 1.0 vs 3.0, 5.0
	Time-point 13 $F_{3,21}$ = 5.516 P = 0.006	Dose 0.0 vs 5.0
		Dose 1.0 vs 5.0

Table 3. SEP statistics. Shown are the statistical procedures and outcomes for the *post-hoc* statistical analysis of the changes in the RDF calculated for the SEP. These statistical procedures followed the initial 2-way RM-ANOVA after a significant time*dose interaction was found, see text.

Test	Significance found for	Significant effects (P<0.005) found with Tukey multiple comparisons
1-way RM-ANOVA repeated factor "time"	Dose 3.0 $F_{14,98}$ = 3.409 P < 0.001	Time-point 2 vs 8, 10
	Dose 5.0 $F_{14,98}$ = 4.311 P < 0.001	Time-point 12 vs 6, 7, 8, 9, 10
		Time-point 15 vs 6, 7, 8
1-way RM-ANOVA repeated factor "dose"	Time-point 6 $F_{3,21}$ = 4.519 P = 0.014	Dose 0.0 vs 3.0, 5.0
	Time-point 7 $F_{3,21}$ = 7.852 P = 0.001	Dose 0.0 vs 1.0, 3.0, 5.0
	Time-point 8 $F_{3,21}$ = 6.376 P = 0.003	Dose 0.0 vs 3.0, 5.0
	Time-point 9 $F_{3,21}$ = 3.868 P = 0.024	Dose 0.0 vs 3.0, 5.0

3.3 Dexmedetomidine plasma concentrations

Figure 3 shows the dexmedetomidine plasma concentrations during the infusion period. Statistical analysis showed a significant increase in the dexmedetomdine plasma level with increasing doses of dexmedetomidine CRI and no time effects (time×dose n.s.; time n.s.; dose $F_{2,14}$= 20.716, P< 0.001). *Post-hoc* analysis showed a significant difference between all treatments (Tukey HSD P< 0.05).

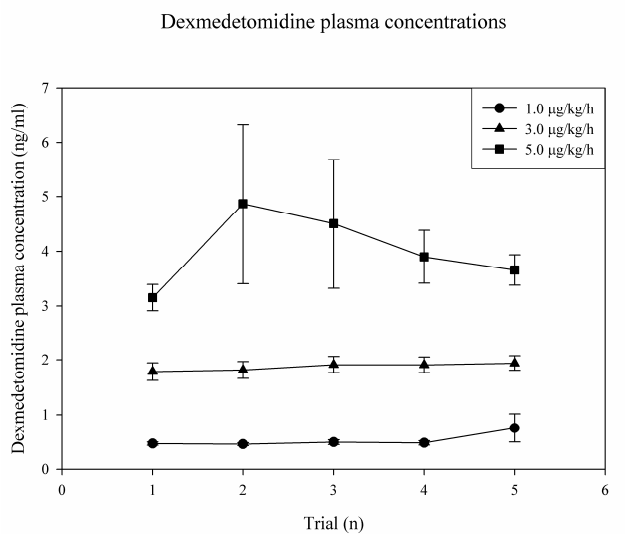

Figure 3. Dexmedetomidine plasma concentrations. Shown are the plasma concentrations (mean ± SEM, *n* = 8) of dexmedetomidine reached during CRI of the three different doses per recording trial.

4. Discussion

The present study clearly describes how sedative and analgesic effects of dexmedetomidine are documented by a significant dose dependent reduction of both the AEP and the SEP in an acutely instrumented dog model, without the introduction of possible limiting factors such as inability to report pain due to sedative effects or absence of nocifensive reflexes due to muscle relaxant effects of the drug.

The AEP recorded from vertex has been successfully used to study the sedative effects of different anaesthetic drugs in different species, including man, rats and dogs (Mantzaridis and Kenny, 1997; Jensen et al., 1998; Nayak and Roy, 1998; Pypendop et al., 1999; Haberham et al. 2000; Murrell et al., 2004; 2005). These studies consistently found a decrease in the amplitudes and an increase in the latencies of the AEP following the administration of anaesthetic drugs. In the present study, similar effects were found during infusion of dexmedetomidine, thus characterizing the sedative effects of this drug. The data, as presented in figure 2, clearly show a dose dependent reduction of the RDF during infusion of dexmedetomidine. A significant reduction from baseline of the RDF calculated

for the AEP was found during dexmedetomidine infusion at the dose of 1.0 µg/kg/h and higher, indicating that clear sedative effects of dexmedetomidine can already be expected at this low dose.

Similar to the use of the AEP, the SEP has been successfully used to study analgesic effects of different analgesic drugs in different species, including man, rats and monkeys (Buchsbaum et al., 1981; Chapman et al., 1982; Schaffler et al., 1987; Arendt-Nielsen et al., 1990a; 1990b; 1991; Nielsen et al., 1991; Beydoun et al., 1997; Stienen et al., 2004; 2005; 2006). These studies consistently documented a decrease in the amplitudes and an increase in the latencies of the SEP following the administration of an analgesic drug. In the present study, such changes in amplitude and latency were also found during CRI of dexmedetomidine, showing the analgesic effects of this drug. The data presented in figure 2 clearly demonstrate the dose dependent reduction of the RDF during dexmedetomidine infusion. A significant reduction of the RDF calculated for the SEP was found for doses 3.0 µg/kg/h and 5.0 µg/kg/h. Although infusion of 1.0 µg/kg/h does reduce the RDF this effect was not found to be significant. Combined these data indicate that clear analgesic effects can be expected at doses of 3.0 µg/kg/h and higher.

The present study is the first study using AEPs and SEPs to investigate the sedative and analgesic effects of dexmedetomidine. These signals allow direct insight in the activity of the central nervous system after applying arousing (e.g. auditory or noxious) stimuli and consequently reflect to what extent the stimuli are perceived (Davis and Zerlin, 1966; Suzuki and Taguchi 1965; Davis et al., 1968; Kaskey et al., 1980; Sims and Moore, 1984; Bromm and Lorenz, 1998; Nayak and Roy, 1998; Huang et al., 1999; Pypendop et al., 1999; Kakigi et al., 2000; Kanda et al., 2002; Shi et al., 2004; Murrell et al., 2004, 2005; Stienen et al., 2006; van Oostrom et al., 2007). As such, changes in AEPs and SEPs can be used to study a potential reduction in the perception of auditory and noxious stimuli (i.e. sedation and analgesia, respectively) without the need of a verbal or behavioural read-out parameter. The latter parameters are easily attenuated by effects other than analgesia. For example, the verbal pain response to noxious stimuli has been shown to be attenuated by the sedative effects of dexmedetomidine (Ebert et al., 2000; Angst et al., 2004; Cortinez et al., 2004), whereas the behavioural responses might be attenuated by its sedative and/or muscle relaxant effects (Weinger et al., 1989; Farber et al., 1997; Bergadano et al., 2006). These effects may lead to invalid interpretation and conclusions with respect to the

analgesic quality of drugs such as dexmedetomidine, i.e. it can be mistakenly interpreted that dexmedetomidine provides effective analgesia, while in fact the drug primarily provides effective sedation and muscle relaxation, rather than effective analgesia. By using the recording of AEPs and SEPs the present data are not influenced by such effects.

Upon comparing the sedative and analgesic effects documented in the present study, it can be suggested that the sedative effects are found at lower doses than the analgesic effects. This finding provides neurophysiological support for the findings from previous studies on dexmedetomidine in man and rats, reporting significant sedative effects at doses not providing significant analgesic effects (Bol et al., 1999; Ebert et al., 2000; Angst et al., 2004). Furthermore, the results from the 3.0 and 5.0 µg/kg/h CRI suggest that there is a ceiling effect for both the sedative and analgesic effects during dexmedetomidine infusion, since the sedative and analgesic effects at doses of 3.0 and 5.0 µg/kg/h did not differ. These results provide further evidence for reports suggesting that increasing the doses of dexmedetomidine above a certain level does not provide stronger sedative and analgesic effects (Jaakola et al., 1991; Hall et al., 2000). It has been reported that higher doses of alpha-2 agonists do not further contribute to the sedative or analgesic effects but do increase the duration of its actions (Sinclair, 2003). The present data on the AEP support this with regard to the duration of the sedative effects. The return to baseline of the AEP was significantly slower with increasing doses of dexmedetomidine. For the SEPs there was no indication of a slower return to baseline values at higher doses of dexmedetomidine. However, it can not be excluded that the highest dose used in this study (5.0 µg/kg/h) might not have been high enough to induce prolonged analgesic actions. Furthermore, the differences in duration of the analgesic actions between the different doses might have occurred during the 30 minutes interval after stopping the infusion, during which no SEPs were recorded.

The changes in AEPs and SEPs in the present study demonstrate the presence of significant sedative effects without significant analgesia following CRI of dexmedetomidine at an infusion rate of 1.0 µg/kg/h, with concurrent dexmedetomidine plasma concentrations of 0.533 ± 0.053 ng/ml (mean ± s.e.). Sedative as well as analgesic effects were seen at the infusion rates of 3.0 and 5.0 µg/kg/h corresponding with dexmedetomidine plasma concentrations of 1.869 ± 0.063 and 4.017 ± 0.385 (mean ± s.e.) respectively. The relationship between the dexmedetomidine plasma concentrations and the

present effects on the AEPs and SEPs are in line with previous studies. In rats (Bol et al., 1999), cats (Ansah et al., 2000), dogs (Kuusela et al., 2000) and man (Ebert et al., 2000; Angst et al., 2004, Cortinez et al., 2004), sedative effects are reported at plasma concentrations of 2.13 ng/ml, 9.2 ng/ml, 5.5 ng/ml and 0.7 ng/ml respectively. However, the data interpretation and final conclusions depend strongly on the respective study designs and the parameters chosen to define and quantify sedation. The latter argument holds true for the analgesic effects as well. In man, analgesic effects were reported at plasma concentrations as low as 0.6 ng/ml (Cortinez et al., 2004) whereas others did not find any analgesic effect at plasma concentrations up to 1.23 ng/ml (Angst et al., 2004). These differences might be ascribed for a large part to the different types of pain models used: cold pressure test vs. heat and electrical pain, respectively.

The present data support the view that increasing the dose of dexmedetomidine above a certain level (3.0 µg/kg/h in this study) does not further contribute to the sedative and analgesic actions of the drug. However, in both man and dogs, the dose-dependent cardiovascular side effects are described, with increasing doses of dexmedetomidine (Bol et al., 1999; Ebert et al., 2000; Sinclair, 2003; Pascoe et al., 2006). In clinical setting it might therefore be preferred to use the lowest dose possible, since 1) increasing the dose leads to more profound cardiovascular side-effects, 2) increasing the dose may lead to longer durations of the effects making adequate regulation of the effects more difficult and 3) increasing the dose above a certain level does not necessarily augment its analgesic and sedative effects.

In conclusion this study shows clear sedative and analgesic effects for dexmedetomidine CRI. Sedation is apparent at already low dose administration whereas higher doses are needed to provide analgesia. It is suggested that higher doses than those used in the present study, do not improve the sedative and analgesic effects, but do increase the duration of the actions.

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9

Summary and preface to the general discussion

This thesis focuses on the investigations into the validity and applicability of the somatosensory evoked potential recorded from the vertex after noxious/nociceptive stimulation (the Vx-SEP) as parameter to study (anti)nociception in rats and dogs.

Chapter 2 describes the development of a specific Pavlov fear-conditioning paradigm, which allows for studying the relationship between the Vx-SEP and the emotional unpleasantness of the stimuli applied to evoke the Vx-SEP, as experienced by the animal. This study demonstrated that it is possible to record SEPs in conjunction with fear-conditioning and to determine the unpleasantness experienced by the animal using the duration of freezing behaviour.

Chapter 3 describes the initial investigation in an effort to demonstrate whether a parameter of nociception, in this case the Vx-SEP, is directly related to the unpleasantness of noxious/nociceptive stimuli applied. To this aim, the paradigm described in **chapter 2** was used. In this study, the unpleasantness of the noxious/nociceptive stimuli was modulated by the use of the analgesic drug fentanyl. The resulting data showed that fentanyl had dose-dependent parallel effects on both the Vx-SEP amplitude and the duration of freezing behaviour. Based on this finding, it was concluded that the Vx-SEP is indicative of the unpleasantness of the noxious/nociceptive stimuli experienced by the animal, and therefore might be considered a parameter of animal pain rather than just nociception.

Chapter 4 describes a further study into the relationship between the Vx-SEP and unpleasantness of stimuli. In contrast to **chapter 3**, the unpleasantness of the noxious/nociceptive stimuli in the SEP fear-conditioning paradigm was modulated by using different stimulus intensities, rather than anaesthetic drugs, to avoid possible confounding drug factors. The data corroborated earlier findings that a positive relation between the Vx-SEP amplitude and the duration of freezing behaviour exists. Based on these findings, excluding the possible confounding drug factors, it was concluded that the Vx-SEP is indicative of the unpleasantness of the noxious/nociceptive stimuli.

Chapter 5 describes a study in which the anaesthetic profile, i.e. the sedative and analgesic actions, of constant rate infusion (CRI) of dexmedetomidine in the rat was characterised. To this aim, auditory evoked potentials (AEPs) and Vx-SEPs were recorded before during and after CRI of dexmedetomidine. The results showed that dexmedetomidine dose dependently suppressed the AEP and the SEP. The AEP was suppressed at lower doses (4.0 µg/kg/h) than the Vx-SEP (10.0 µg/kg/h). These data indicate that dexmedetomidine CRI in the rat can provide both sedation and analgesia, however analgesia will only occur at doses that provide significant sedation.

Chapter 6 describes the investigation into the possible amnesic effects of dexmedetomidine following noxious/nociceptive stimulation. As it was established in **chapters 3 and 4** that the Vx-SEP is indicative of the perception of the noxious/nociceptive stimuli, the Vx-SEP in this study was used to monitor the perception of sensory input in the Pavlov fear-conditioning paradigm. It was shown that dexmedetomidine dose dependently reduced the duration of freezing behaviour in conjunction with a parallel reduction in Vx-SEP amplitude. Two conclusions could be drawn from these results, 1) the data further supported the Vx-SEP being indicative of the perception of noxious/nociceptive stimuli and 2) dexmedetomidine attenuates the formation of a CS-US association, and consequently memory formation, only at doses that suppress sensory perception. These data clearly suggest that the alpha-2 adrenergic agonist dexmedetomidine applied in this model, does possess intrinsic amnesic properties.

Chapter 7 describes a technique to record Vx-SEPs in awake dogs, following intra-epidermal electrical stimulation. The data obtained showed that 1) the signals obtained increased in amplitude and decreased in latency with increasing stimulus intensities 2) the signals primarily represented A-delta fibre activity, 3) the signals were not contaminated by EMG artefacts and 4) the signals show similarities with Vx-SEPs recorded in man and monkeys after laser stimulation. Based on these findings, it was concluded that it is possible to record Vx-SEPs related to nociception after intra-epidermal electrical stimulation in awake dogs.

Chapter 8 describes a study in which the anaesthetic profile, i.e. the sedative and analgesic actions, of constant rate infusion (CRI) of dexmedetomidine in the dog was characterised. To this aim, AEPs and Vx-SEPs were recorded before during and after CRI of dexmedetomidine. The results showed that dexmedetomidine dose dependently suppressed the AEP and the Vx-SEP. The AEP was suppressed at lower doses (1.0 µg/kg/h) than the Vx-SEP (3.0 µg/kg/h). These data indicate that dexmedetomidine CRI in the dog can provide both sedation and analgesia, however analgesia can only be expected at doses that provide significant sedation.

In summary, this thesis shows that 1) Vx-SEPs are indicative of the unpleasantness of noxious/nociceptive stimuli in animals, similar to the situation in man, 2) the Vx-SEP can be recorded in different animal species and 3) the Vx-SEP can be used as a tool to study the antinociceptive/analgesic effects of clinically used anaesthetic drugs.

10

General discussion

1. General applicability of SEPs to study (anti)nociception

The studies described in this thesis, indicate that 1) somatosensory evoked potentials recorded from the vertex after nociceptive stimulation (Vx-SEP) are indicative of the unpleasantness of the stimulation experienced by the animal and therefore might be indicative of animal pain rather than just nociception, 2) Vx-SEPs can be successfully recorded in different animal species and therefore can be used to study (anti)nociception in different animal species and 3) evoked potentials can be successfully used to study the anaesthetic profile of drugs in different animal species. In conclusion, the recording of Vx-SEPs can be a valuable addition to the more regularly used parameters of acute nociception in animal models, such as the tail-flick and the paw withdrawal response (Stanley and Paice, 1997; Le Bars et al., 2001; Hogan, 2002), in assessing the efficacy of analgesic drugs and to study mechanisms of (anti)nociception.

The recording of Vx-SEPs may have important added value over the traditional parameters used to study (anti)nociception in animal models. Firstly, Vx-SEPs can monitor effects on the nociceptive system independent from the site of action of these effects, since they reflect the activity within the final station of the nociceptive system, i.e. the cerebral level (Bromm and Lorenz, 1998). In contrast, the traditionally used nocifensive reflexes primarily assess the spinal processing of sensory stimuli, neglecting the higher central nervous areas (Le Bars et al., 2001).

Secondly, Vx-SEPs are not influenced by muscle relaxant effects of drugs administered (Wiederholt and Iragui-Madoz, 1977). In contrast, the final output of the traditionally used nocifensive reflexes depends on the motor system of the subject studied and is therefore subject to drug effects on the motor system, independent from effects on the nociceptive system (Le Bars et al., 2001). For example, muscle relaxant drugs can effectively block a nocifensive reflex while the perception of the nociceptive stimuli is still fully intact. As discussed in Chapter 8, the latter phenomenon might be a problem in assessing the anti-nociceptive effects of for example alpha-2 adrenoceptor agonists, since these drugs have been shown to have muscle relaxant properties (Weinger et al., 1989; Sinclair, 2003). Since, Vx-SEPs are not influenced by muscle relaxant effects an alpha-2 adrenoceptor agonist effect on the Vx-SEPs strongly suggests a primary effect on the nociceptive system.

Thirdly, in man, Vx-SEPs correlate with the subjective perception of the stimuli applied (Kakigi et al., 2000; Kanda et al., 2002). The present thesis shows that this also

applies to animals (Chapter 3, 4 and 6). Since the Vx-SEP correlates with the unpleasantness of the stimuli applied, it can be considered a measure of animal pain rather than just nociception.

Finally, when specific protocols for recording of Vx-SEPs, as described in this thesis, are used, subsequent recordings yield comparable results. In contrast, nocifensive reflexes are prone to significant learning effects (Le Bars et al., 2001), i.e. animals learn to respond earlier and more vigorously upon repeated testing, making repeated testing within animals more difficult.

When applied to study (anti)nociception, the recording of Vx-SEPs has some limitations, which require further attention in the (near) future.

Firstly, the recording and interpretation of Vx-SEPs to study (anti)nociception, is not as easy applicable as nocifensive reflex testing. Recording of Vx-SEPs requires the use of specialized equipment such as stimulators, bio-electrical amplifiers, special recording and analyzing software, necessitating special knowledge in these fields. Furthermore, a straight forward interpretation of the Vx-SEP and changes thereof is difficult, since the exact underlying neuronal mechanisms of the Vx-SEP signal and therefore its definite meaning have not been fully elucidated as yet. However, much progress is made in elucidating these neuronal processes underlying the Vx-SEPs, making the final interpretation and meaning of the signals more straight forward (Garcia-Larrea et al., 2003; Dowman et al., 2007). In addition, with the development of more powerful computers and better amplifiers, the technique of Vx-SEP recording becomes more and more straightforward. At present commercially available software for recording and analyzing the signals can be obtained (for example see: www.brainproducts.com). In the near future, recording of Vx-SEPs to study (anti)nociception will become a basic tool used regularly in clinical neurology, similar to the recording of non-nociception related somatosensory evoked potentials (Kakigi et al., 2005).

Secondly, at present the recording of SEPs in the small rodents used in analgesiometric studies is frequently, quite invasive, with the animals being instrumented with permanently placed EEG recording electrodes. This drawback might be overcome by the development of “mini electrocaps”, which can be temporarily placed over the head of a small rodent, making invasive surgery unnecessary. In larger species, such as dogs (this thesis chapter 7

and 8) and monkeys (Beydoun et al., 1997), it is already possible to record Vx-SEPs using non-invasive techniques.

Thirdly, the whole procedure involving the recording of Vx-SEPs can be time consuming and involves repeated nociceptive stimulation, which impairs animal welfare. Dedicated averaging and signal extracting techniques, however, make it possible to study Vx-SEPs using a small number of stimuli or even a single stimulus (Purves and Boyd, 1993; Merzagora et al., 2004; 2007; Mayhew et al., 2006)

Fourthly, at present, most studies using Vx-SEPs primarily assess acute nociception induced by nociceptive stimuli applied at the skin. Therefore, the value of Vx-SEPs in studying chronic pain, pain from deeper tissues/viscera and temporal and spatial summation of pain is not fully clear as yet. However, evoked potentials recorded after nociceptive stimulation of the viscera, to study visceral pain have recently been reported (Murrell et al., 2007). Furthermore, hyperalgesic states due to chronic pain have been shown to be reflected in increased Vx-SEP amplitudes in comparison with normal control levels (Shi et al., 2004). Also, the use of multiple stimulation electrodes has been successfully applied to study spatial summation (Chen et al., 2002; Katsarava et al., 2007). Combined, these studies indicate that Vx-SEPs might as well be useful to study other pain states than acute nociception of the skin.

Finally, under specific circumstances, such as hypnosis, inhalation of low doses of isoflurane and special stimulation procedures, the Vx-SEPs have been shown not to correlate with the subjective perception of the stimuli, indicating that the Vx-SEPs are not under all circumstances indicative of (anti)nociception. (Meier et al., 1993; Treede et al., 2003; Roth et al., 1996; Chen et al., 2000). However, in the majority of studies, the signals correlate very well with the subjective perception of the stimuli and effects of analgesic drugs can be objectively assessed (Kakigi et al., 2000; Kanda et al., 2002).

In conclusion and despite minimal drawbacks, the recording of Vx-SEPs has a number of important advantages over nocifensive reflex testing, its shortcomings can be overcome in the (near) future and therefore, recording of Vx-SEPs must become the method of choice to study (anti)nociception in animal models.

2. SEPs in relation with the perception of nociceptive stimuli in animals

Many people agree that in animal models the terms nociception and anti-nociception instead of the terms pain and analgesia must be used, since it is considered impossible to show that animals experience the stimuli applied as emotionally unpleasant. In order to speak of pain, it is necessary to show that the subject studied experiences an emotional unpleasantness since pain is defined as *"An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage"* (Mersky et al., 1979).

The fact that animals cannot verbally communicate the unpleasantness of pain, and consequently prohibit it from being quantitatively evaluated, is the argument generally used to apply the term nociception instead of pain in animals. However, this line of reasoning must be considered a misconception of the emotional perception in animals. Moreover, it principally violates the definition of pain as worded by the IASP, since the definition continues in the following way: "*Note: The inability to communicate verbally does not negate the possibility that an individual is experiencing pain and is in need of appropriate pain-relieving treatment."*

Furthermore, it can be argued that the ability to verbally report unpleasantness is in no way a more valid argument to speak of pain than just using the rule of analogy to assume that an individual senses unpleasantness. When a person verbally reports pain, this report is subjective. At all times, the receiver of this verbal report has to compare it with his/her own experience, following the principles of an analogous perception, to be able to fully value the reported response. The fact that the latter evaluation is also possible by observing nocifensive reactions of animals, underscores that it must be possible to speak of pain in animals instead of nociception. The IASP definition on pain indicates that pain is indeed deduced from reports and/or observations which we subsequently compare with our own experiences and therefore there should be no difference between verbal or behavioural responses, since it continues as follows: *"Notes: Pain is always subjective. Each individual learns the application of the word through experiences related to injury in early life. Biologists recognize that those stimuli which cause pain are liable to damage tissue. Accordingly, pain is that experience we associate with actual or potential tissue damage. It*

is unquestionably a sensation in a part or parts of the body, but it is also always unpleasant and therefore also an emotional experience. Experiences which resemble pain but are not unpleasant, e.g., pricking, should not be called pain. Unpleasant abnormal experiences (dysesthesias) may also be pain but are not necessarily so because, subjectively, they may not have the usual sensory qualities of pain. Many people report pain in the absence of tissue damage or any likely pathophysiological cause; usually this happens for psychological reasons. There is usually no way to distinguish their experience from that due to tissue damage if we take the subjective report. If they regard their experience as pain and if they report it in the same ways as pain caused by tissue damage, it should be accepted as pain. This definition avoids tying pain to the stimulus. Activity induced in the nociceptor and nociceptive pathways by a noxious stimulus is not pain, which is always a psychological state, even though we may well appreciate that pain most often has a proximate physical cause."

In conclusion, it seems odd that the terms pain and analgesia are not used in conjunction with animal models researching pain and/or nociception. The most likely explanation can possibly be found in the fact that it is never previously investigated whether parameters used in animal models of "nociception" can be correlated to a state of emotional unpleasantness (i.e. "pain") in animals.

This thesis describes novel studies that apply an innovative approach to investigate whether a specific parameter of animal "nociception", i.e. the Vx-SEP, correlates with the emotional unpleasantness of the stimuli applied and as such can be regarded as a parameter of animal pain. The application of the newly developed SEP fear-conditioning paradigm, as described in Chapter 2, has shown, as presented in Chapter 3, 4 and 6, that the Vx-SEP indeed correlates with the subjective perception of nociceptive stimuli and as such can be regarded as a measure of animal pain and not just nociception. The fact that in (most chapters of) this thesis the terms (anti)nociception instead of pain and analgesia are used, is due to the fact that the reviewers of international journals are as yet reluctant to accept the terms pain and analgesia in reports on animal studies.

Although the SEP fear-conditioning paradigm as described in Chapter 2 is a novel and promising approach to show that parameters of nociception in animals can in fact be regarded as parameters of animal pain, several drawbacks of this model must be considered.

Firstly, the freezing behaviour, used as the golden standard to demonstrate that the animal experienced the stimuli as unpleasant, is interpreted by humans to be indicative of unpleasantness experienced by the animal during the training phase. The validity of this assumption is not supported by direct evidence, due to a lack of direct communication with animals as such. However, it has been shown that freezing behaviour is mediated by the amygdala (Maren, 2001). This indicates that freezing behaviour is related to intense emotional feelings and highly likely represents the emotional unpleasantness experienced during the training session of the fear-conditioning paradigm (Fanselow and Bolles, 1979; Anagnostaras et al., 2000).

Secondly, the CS-US association, as described in Chapters 3, 4 and 6, can potentially be influenced by mechanisms other than changes in unpleasantness of the US. For example, a change in CS perception will also attenuate the CS-US association and as such, decrease freezing behaviour. Also, amnesic effects of drugs administered can cause a disruption of CS-US formation, leading to a reduction in freezing behaviour. In both situations the decrease in freezing behaviour might potentially be falsely interpreted as a decrease in unpleasantness of the US, while in fact the US still induced a level of unpleasantness similar to the control situation. However, in Chapter 4 of this thesis, the correlation between the Vx-SEP and the duration of freezing behaviour has been shown without these possible drug-induced confounding co-factors.

Finally, a positive correlation does not necessarily mean a causal relationship. The Vx-SEP amplitude might increase with stimulus intensities and decrease with analgesic drug administration parallel to, but independently from the subjective perception of the stimuli applied. In fact, it has been suggested that evoked potentials reflect the state of the sensory pathway rather than the subjective perception (Treede et al., 2003). However, although there is a possibility that the changes in Vx-SEP amplitudes are not causally related to changes in pain-perception, the positive relation between the Vx-SEP and the duration of freezing behaviour as found in chapters 3, 4 and 6 still qualify the Vx-SEP a valid and useful indicator of the extent to which nociceptive stimuli are perceived. Furthermore, it is highly unlikely that the Vx-SEP, which reflects the activity within the nociceptive pathways after nociceptive stimulation, is causally unrelated to the sensory perception. What else than the activity within the central nervous system can finally initiate a conscious perception or feeling?

In conclusion, the fact that animals can not verbally report pain (in human language) does not necessarily exclude that they do experience nociceptive stimuli as unpleasant. Therefore, using the term pain, when working with animals is not by definition inappropriate. In fact, the definition of pain by the IASP suggests that using the term pain when speaking of a situation in animals is equal appropriate as using the term pain when speaking of a situation in man. This thesis showed that it is possible to correlate the Vx-SEP with the unpleasantness of the stimuli applied to evoke the signal as such making it a parameter of animal pain, rather than just nociception.

3. SEPs in different species

It is suggested that in mammals, including man, the pain system has remained its original organisation throughout evolution (Garcia-Larrea et al., 2003). Therefore, processing of nociceptive stimuli is considered to be similar in different mammal species. Across species, it can therefore be expected that recording of Vx-SEPs yields comparable signals with comparable origin, characteristics and comparable applicability with respect to studying (anti)nociception.

The present thesis and previous work shows that Vx-SEPs can be recorded in different species, including man, monkeys, dogs, sheep and rats (Beydoun et al., 1997; Morris et al., 1997; Kakigi et al., 2005; Stienen et al., 2004; 2005). When comparing these signals across species, multiple similarities can be found. Firstly, for all species the main characteristics of the signals are similar, i.e. Vx-SEPs increase in amplitude with increasing stimulus intensities and dose-dependently decrease after administration of analgesic drugs.

Secondly, the waveforms of Vx-SEPs recorded in man, monkeys, dogs and sheep occur in similar latency ranges and share interesting similarities. The latter are characterized by an initial sharp and large deflection around 100 ms (the N2-P2 complex in Chapters 7 and 8 of this thesis) and followed by later slower and less defined waves, mostly lower in amplitude (N3-P3 complex in Chapters 7 and 8 of this thesis).

Finally, for both man and rats it has been shown that the signals correlate with the subjective perception of the stimuli applied (Kanda et al., 2002; Ianetti et al., 2004, Chapter 3, 4 and 6 of this thesis). It is most likely that this also accounts for the signals recorded in the other species, however, this needs further future investigation.

In conclusion, Vx-SEPs recorded in different species show interesting similarities, indicating that processing of nociceptive stimuli follows a common mechanism across species. This supports the possibility of combining the results from studies executed in different species, including man, to finally and fully elucidate the underlying neuronal mechanisms of nociception.

4. Final conclusions

This thesis shows that, 1) Vx-SEPs in animals correlate with the subjective perception of the nociceptive stimuli applied, similar to the situation in man. 2) Vx-SEPs can be recorded in different species and 3) Vx-SEPs can be used to study anti-nociceptive effects of anaesthetic drugs. Combined, the Vx-SEP can be regarded as a valuable tool in pain research and moreover an important addition to the regularly used animal models to study nociception, based on behavioural parameters. The introduction of Vx-SEPs as the principal data source for studying the neuronal activity within the nociceptive system, in an effort to understand the exact neuronal mechanisms underlying nociception/pain perception in both animals and man, principally differs from the traditional, behaviour-based, approach. Consequently, this new SEP-based approach might produce a new area of results generated in pain research, ultimately leading to the definitive and strongly needed understanding of the intriguing phenomenon, pain.

By studying possible changes in the Vx-SEP when simultaneously modulating special neuro-anatomical areas within the central nervous system, the role of these neuro-anatomical areas in nociception can be further elucidated. As such, the entire neuronal pathway involved in nociception can be functionally characterized, leading to a more in-depth understanding of nociception and pain in man and animals. Furthermore, if specific neuro-anatomical areas can be shown to be involved in nociception, these areas can be regarded as possible targets in developing 1) specifically targeted and more reliable ways of monitoring nociception and pain, 2) specifically targeted, reliable and safe anaesthesia and 3) specifically targeted and adequate treatment of clinical pain.

Therefore, a multidisciplinary approach in which neurophysiological, anaesthesiological, behavioural and neuro-anatomical knowledge are combined, should be the approach to come to the final and definite understanding, monitoring and treatment of nociception and pain.

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11

Summary in Dutch

(Samenvatting in het Nederlands)

Dit proefschrift beschrijft een serie experimenten ter bepaling van de validiteit en toepassingsmogelijkheden van de somatosensory evoked potential gemeten ter hoogte van de vertex na nociceptieve stimulatie (de Vx-SEP) als parameter voor (anti)nociceptie in de rat en hond.

Hoofdstuk 2 beschrijft een methode om de relatie tussen de Vx-SEP en de onplezierige perceptie van nociceptieve stimuli in dieren te kunnen onderzoeken. Deze methode maakt gebruik van een paradigma waarbij Pavlov angst-conditionering en het meten van Vx-SEPs geïntegreerd wordt. De resultaten van deze studie laten zien dat het mogelijk is om Vx-SEPs te meten in een dier tijdens Pavlov angst-conditionering, terwijl tegelijkertijd de mate van onplezierigheid van de nociceptieve stimuli bestudeerd kan worden aan de hand van de duur van het zogenaamde freezing gedrag van het dier.

Hoofdstuk 3 beschrijft, door toepassing van het paradigma zoals beschreven in **hoofdstuk 2**, het verband tussen de Vx-SEP en de feitelijke mate van onplezierigheid van de toegediende nociceptieve stimuli. In deze studie, werd de onplezierigheid van de nociceptive stimuli gemoduleerd door het analgetisch farmacon fentanyl. Fentanyl zorgde voor een dosis afhankelijk, en parallel, effect op zowel de Vx-SEP amplitude als de duur van het freezing gedrag getoond door het dier. Op basis van deze resultaten werd geconcludeerd dat de Vx-SEP een indicator is voor de mate van onplezierigheid van nociceptive stimuli in dieren en daarom mogelijk gezien kan worden als een parameter voor pijn in dieren en niet slechts van (anti)nociceptie.

Hoofdstuk 4 beschrijft een tweede studie om de relatie tussen de Vx-SEP en de mate van onplezierigheid van nociceptive stimuli te onderzoeken. In tegenstelling tot **hoofdstuk 3** werd de onplezierigheid van de toegediende stimuli niet gemoduleerd door het toedienen van een analgetisch farmacon, maar door het variëren van de intensiteit van de stimuli. Op deze manier werden eventueel ongewenste farmacon invloeden voorkomen. De resultaten bevstigen de eerdere bevindingen die een positieve relatie tussen de Vx-SEP en de mate van onplezierigheid van de nociceptive stimuli lieten zien. Op basis van deze resultaten werd de conclusie dat de Vx-SEP een parameter is voor de onplezierigheid van nociceptieve stimuli nader onderbouwd.

Hoofdstuk 5 beschrijft een studie naar het anesthesiologische profiel, d.w.z. de sedatieve en analgetische eigenschappen, van continue infusie van dexmedetomidine in de rat. Hiertoe werden auditory evoked potentials (AEPs) en Vx-SEPs gemeten, voor, tijdens en na infusie van dexmedetomidine. De resultaten lieten zien dat dexmedetomidine een dosis afhankelijke onderdrukking van zowel de AEP als de Vx-SEP geeft. De AEP werd onderdrukt bij een lagere dosering (4.0 µg/kg/h) dan de Vx-SEP (10.0 µg/kg/h). Op basis van deze data kan worden geconcludeerd dat continue infusie van dexmedetomidine in de rat zowel sedatie als analgesie geeft, maar dat analgesie alleen kan worden verwacht in combinatie met duidelijke sedatie.

Hoofdstuk 6 beschrijft een onderzoek naar de mogelijk amnestische effecten van dexmedetomidine na nociceptieve stimulatie. Aangezien in **hoofdstuk 3 en 4** werd aangetoond dat de Vx-SEP een parameter is voor de perceptie van nociceptieve stimuli, werd de Vx-SEP in deze studie gebruikt om de perceptie van sensorische input tijdens de Pavlov angst-conditionering te bepalen. Dexmedetomidine had in deze studie een dosis afhankelijk en parallel effect op de amplitude van de Vx-SEP en de duur van het freezing gedrag getoond door het dier. Op basis van deze resultaten werden twee conclusie getrokken. 1) De resultaten onderbouwen nogmaals de eerdere bevindingen die een positieve relatie tussen de Vx-SEP en de mate van onplezierigheid van de nociceptive stimuli lieten zien..2) Dexmedetomidine geeft alleen een suppressie van een CS-US associatie, en dus van geheugenvorming, bij doseringen die de perceptie van sensorische input aantasten. Deze resultaten suggereren nadrukkelijk dat de alpha-2 adrenerge agonist dexmedetomidine geen intrinsieke amnestische eigenschappen bezit.

Hoofdstuk 7 beschrijft een techniek om Vx-SEPs in wakkere honden te kunnen meten na intra-epidermale electrische stimulatie. De resultaten van deze studie laten zien dat 1) de gemeten signalen toenemen in amplitude en dalen in latentietijd bij toenemende stimulussterkte, 2) de signalen meest waarschijnlijk zijn gerelateerd A-delta vezel activiteit, 3) er geen artefact van spieractiviteit in de signalen aanwezig is en 4) de signalen overeenkomsten vertonen met signalen die gemeten zijn in mensen en apen. Op basis van deze resultaten werd geconcludeerd dat het mogelijk is om bruikbare Vx-SEPs gerelateerd aan nociceptie te meten in de wakkere hond na intra epidermale electrische stimulatie.

Hoofdstuk 8 beschrijft een studie naar het anesthesiologische profiel, d.w.z. de sedatieve en analgetische eigenschappen, van continue infusie van dexmedetomidine in de hond. Hiertoe werden AEPs en Vx-SEPs gemeten, voor, tijdens en na infusie van dexmedetomidine. De resultaten lieten zien dat dexmedetomidine een dosis afhankelijke onderdrukking van zowel de AEP als de Vx-SEP geeft. De AEP werd onderdrukt bij een lagere dosering (1.0 µg/kg/h) dan de Vx-SEP (3.0 µg/kg/h). Op basis van deze data kan worden geconcludeerd dat continue infusie van dexmedetomidine in de hond zowel sedatie als analgesie geeft, maar dat analgesie alleen kan worden verwacht in combinatie met duidelijke sedatie.

Samengevat laat dit proefschrift zien dat 1) de Vx-SEP een indicator is voor de mate van onplezierigheid van nociceptieve stimuli in dieren, zoals dat ook in mensen het geval is, 2) de Vx-SEP in verschillende diersoorten gemeten kan worden en 3) de Vx-SEP kan worden toegepast om de analgetische effecten van klinisch toegepaste anesthetica te bestuderen.

Dankwoord

Ik zet mijn bril en helm op en klik mijn schoenen in de pedalen. Er is geen weg meer terug, nu stilvallen betekent omvallen.
Ik heb goed gegeten, ben in goede conditie en heb een goede moraal. Kortom, een goede basis om deze rit binnen de tijd uit te rijden. Er zijn tal van dingen die mis kunnen gaan, maar beren op de weg zijn schaars in Nederland. Kop omlaag en stampen. Af en toe bijschakelen, een jump maken en weer op straf tempo door. Dorst lessen, honger stillen, neus ophalen. Schelden op alles wat me irriteert, inclusief mezelf.
Ik draai linksaf de weg tot de wetenschap op, het blijkt een bijna rechte weg met een paar kleine oneffenheden. Het gaat voorspoedig, een enkele keer een lekke band en eenmaal een ketting die het begeeft.
Daar is het bord, langzaam beginnen de letters zich af te tekenen, snel komt het dichterbij, ik kan het al lezen, even afzien nog, ik ga het halen, ik passeer het bord binnen de tijd, ik heb het gehaald, ik ben er: “De Uithof”.

Met dank denk ik terug aan:
De mensen die mij hebben leren fietsen.
De ploegleider die mij contracteerde.
De sponsoren.
De agenten die een oogje dichtknepen wanneer ik door rood fietste.
De mensen die een hekel aan mij hadden vanwege mijn bij tijd en wijle ronduit asociale weggedrag.
De mensen die mij aanmoedigden langs de kant van de weg.
De mensen bij wie ik in het wiel mocht zitten bij (forse) wind tegen.
De mensen voor wie ik kopwerk mocht doen.
De mecaniciens die ervoor zorgden dat alles bleef rollen.
De mensen met nuttige tips en trucs op fietsgebied.
De mensen van de ravitaillering.

Wanneer je jezelf herkent in meerdere personen hierboven dan kan dat kloppen en hoogstwaarschijnlijk zegt dat ook iets over de mate van bijdrage aan het succesvol doorlopen en afronden van dit promotietraject. Wanneer je het bovenstaande stuk wel hebt gelezen, maar jezelf niet herkent, moet je het nog maar eens lezen en nu wat meer je fantasie gebruiken, niet alles zo letterlijk nemen, je staat er wel degelijk tussen!

Zo,….en nu eerst eens afstuderen……..

Acknowledgements

I put on my glasses and helmet and click my shoes onto the pedals. There is no way back, stopping know means to topple over.
I ate well, my condition is good and my moral is high. In other words this is a good basis to finish this stage before the time limit. Lots of thing can go wrong, but *there aren't to many bears on the dutch roads*. Head down and plodding along. Shift gear, making a jump and continuing at high speed. Quench the thirst, appeasing the hunger, sniffing. Reviling all irritating things, including myself.
I turn left to the *weg tot de wetenschap* (road to science) it turns out to be an almost straight road with only a few irregularities. Everything is going prosperously, a few flat tires and one broken chain. I can see the sign, the characters start to appear, I can read it now, going deep for just a little bit longer, I am going to make it, I pass the sign, I made it, I am there: "*De Uithof*".

I will gratefully remember the following persons:
The people who taught me how to cycle.
The team leader who contracted me.
The sponsors.
The coppers who looked the other way when I jumped the traffic lights.
The people who were irritated by my bad driving habits.
The supportive people along the roadside.
The people who had me in tow when going against the wind.
The people who used my slipstream.
The mechanics keeping the cycle sound.
The people with helpful tips and tricks related to cycling.
The people of the provisionment.

When you recognise yourself in more than one of the persons described above you could be right. This is probably indicative of the relative contribution you made to the successful finishing of this PhD.
When you did read the text above but did not recognise yourself in one of the persons described, you should read the text again and use your imagination a little more. Do not take it literally, you are certainly there!

Well that is about it,..…..lets finally finish vet school ……


FOKKE & SUKKE
SNAPPEN ER NIKS VAN
Help de proefdieren de wereld uit !!!
WAT HEBBEN WE FOUT GEDAAN??
RGvT

Curriculum Vitae

Hugo van Oostrom was born on January 26th, 1981 in Amsterdam, the Netherlands. He grew up in Baambrugge, The Netherlands, where he went to elementary school and spent a happy youth with his brother Rogier and parents Han & Gerty.

From 1993 till 1999 he went to secondary school, RSG Broklede, in Breukelen, The Netherlands, which he finished *cum laude*. This enabled him to directly start with the study of Veterinary Medicine at the Faculty of Veterinary Medicine, Utrecht, The Netherlands. There he fell in love at first sight with his beautiful girlfriend Natasja.

During an anaesthesiology lecture in his fourth year at the Faculty of Veterinary Medicine, Prof. Dr. L.J. Hellebrekers observed his interest for the field of anaesthesiology and his potential to become a fine researcher. It was therefore, that he was invited to do one year of research following a special tenure kindly financed by the Faculty. The research concerned the use of somatosensory evoked potentials to objectively quantify pain perception in animals and was supervised by the talented and inspiring researcher Dr. P.J. Stienen.

During this one year of research, Hugo was asked to prolong his work as a researcher to complete a full PhD before officially graduating as a veterinarian. Apparently he responded positively.

Hugo is now passing through the clinical rotations necessary to graduate as a veterinarian in 2009. After graduating as a veterinarian, Hugo hopes to obtain an internship followed by a residency, in order to become a specialist in Veterinary Anaesthesiology.

In his daily life, besides studying and doing research, Hugo likes eating and cooking, going to the gym, cycling, playing and listening music, restoring classic cars, watching movies and sleeping.


FOKKE & SUKKE
VERZINNEN IETS BETERS
PROEFDIEREN? NERGENS VOOR NODIG!!
IN NEDERLAND ZIJN TOCH GENOEG STUDENTEN MET GELDZORGEN??
F
RGvT

Curriculum Vitae

Hugo van Oostrom werd geboren op 26 januari, 1981 te Amsterdam. Hij groeide op in Baambrugge waar hij naar de basisschool ging en een gelukkige jeugd doormaakte samen met zijn broer Rogier en ouders Han & Gerty.

Hij doorliep van 1993 tot 1999 de middelbare school RSG Broklede, te Breukelen, welke hij *cum laude* afrondde. Dit stelde hem in staat direct door te stromen naar de opleiding diergeneeskunde aan de Faculteit Diergeneeskunde, te Utrecht. Daar werd hij op het eerste gezicht verliefd op zijn mooie vriendin Natasja.

Tijdens een college anesthesiologie in het vierde studiejaar, merkte Prof. Dr. L.J. Hellebrekers zijn interesse voor de anesthesiologie en zijn potentie om een goede onderzoeker te worden op. Dat was de reden dat hij werd gevraagd om een jaar onderzoek te doen in het kader van een speciaal traject dat financieel mogelijk werd gemaakt door de Faculteit. Het onderzoek had betrekking op het gebruik van somatosensory evoked potentials om pijn perceptie in dieren objectief vast te kunnen stellen en werd begeleid door de talentvolle en inspirerende onderzoeker Dr. P.J. Stienen.

Tijdens dit jaar onderzoek werd Hugo gevraagd om zijn werkzaamheden als onderzoeker te verlengen om zo een volledig promotie traject te doorlopen voordat hij zou afstuderen als dierenarts. Blijkbaar heeft hij hier positief op gereageerd.

Hugo doorloopt op dit moment de co-schappen welke verplicht zijn om af te kunnen studeren als dierenarts in 2009. Hugo hoopt, nadat hij is afgestudeerd als dierenarts, een roulantschap gevolgd door een opleidingsplaats tot specialist veterinaire anesthesiologie te bemachtigen.

Naast studeren en onderzoek doen, houdt Hugo in het dagelijkse leven van eten en koken, fitnessen, wielrennen, muziek maken en luisteren, klassieke auto’s restaureren, films kijken en slapen.


FOKKE & SUKKE
OFFEREN ZICH WEL WEER OP
OK OK....
HIER ZIJN WEER TWEE PROEFDIEREN !!
HA, LEKKER!
RGvT