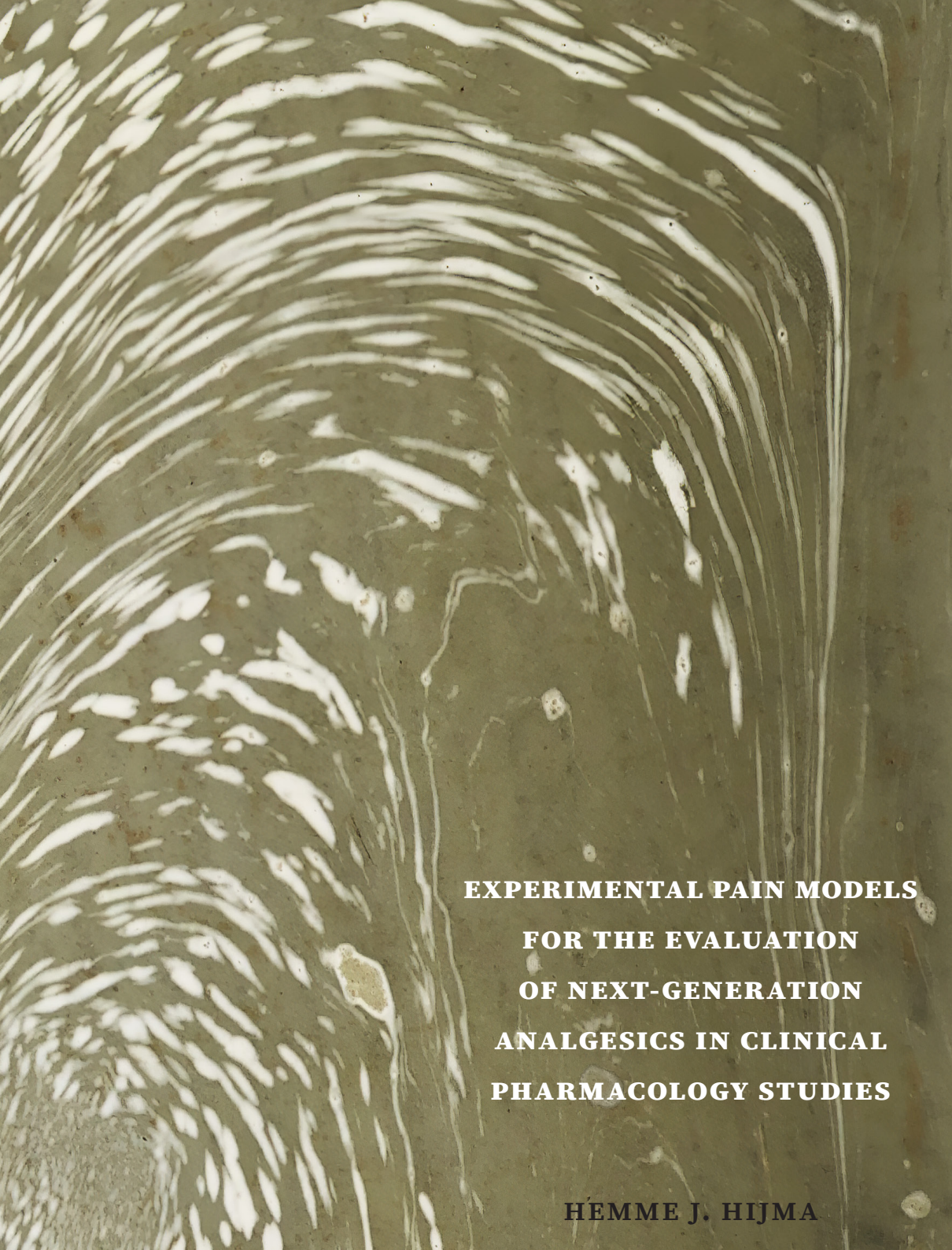
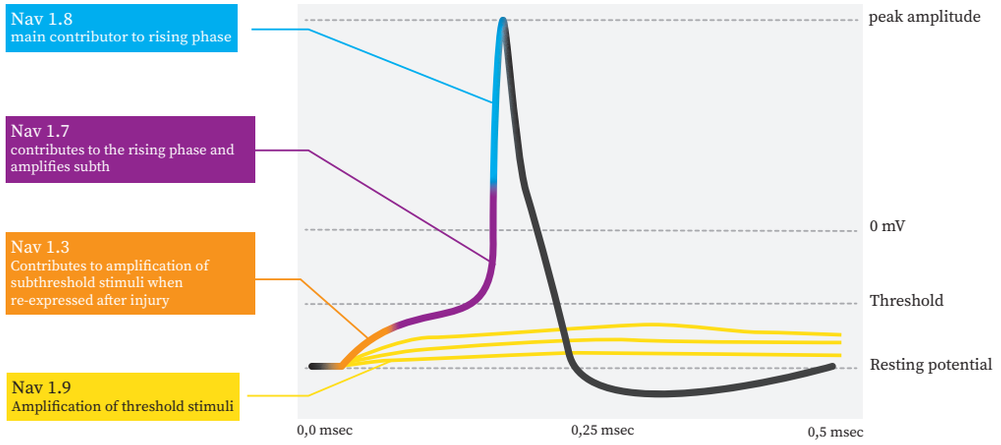


EXPERIMENTAL PAIN MODELS FOR THE EVALUATION OF NEXT-GENERATION ANALGESICS IN CLINICAL PHARMACOLOGY STUDIES

HEMME J. HIJMA

Illustration of unique role of various Na_V channels in action potential generation.
(Adapted from [40])



EXPERIMENTAL PAIN MODELS FOR THE EVALUATION OF NEXT-GENERATION ANALGESICS IN CLINICAL PHARMACOLOGY STUDIES

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CHAPTER 1

Introduction: Analgesic drug development: proof-of-mechanism and proof-of-concept in early phase clinical studies

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ABSTRACT

Effective treatment for many pain disorders is still lacking, which is due to the complexity of pain in general and of the underlying pathology of many pain syndromes. This results in most investigational analgesic drugs failing to reach registration; either due to lack of efficacy, or due to the drug's adverse effect profile. To increase the number of analgesics that reach the patient, it is essential to carefully and rationally plan the clinical development program. By including proof-of-mechanism (POM) and/or proof-of-concept (POC) methods in early-phase clinical drug studies, the analgesic drug developer will be better informed about the key characteristics of the studied drug, which will aid in making crucial decisions during the development process. Here, we describe the top 10 currently most developed analgesic drug classes, link them mechanistically to appropriate methods to demonstrate POM and POC in early-phase clinical trials, and include pros and cons of each of the methods described. Lastly, we discuss how each analgesic drug class requires a tailored experimental approach for proper evaluation of POM and POC, and how this can contribute to an efficient and question-based approach in early-phase analgesic drug research.

INTRODUCTION

Pain, while being one of the most common symptoms for which patients seek medical attention, is in terms of available treatment one of the main therapeutic areas in which little progress has been achieved: a mere 59 compounds have been registered for the treatment of pain between 1960 – 2010, with only two-thirds of those being an analgesic. [1] Where major breakthrough discoveries including opioids and acetylsalicylic acid have been discovered decades ago, most first-line therapies currently available still lack either long-term effectiveness (e.g., prolonged use of opioids increases sensitivity to pain (i.e. hyperalgesia (**Figure 1**)), instead of providing pain relief) or have a poor risk-benefit profile (e.g., systemic administration of lidocaine reduces pain but simultaneously induces cardiac arrest). One of the main challenges preventing more analgesics successfully entering the market, is the complexity and multimodality of the underlying pathology of pain. To tackle this, and thus increase the number of analgesics actually reaching the patients, it is needed to understand and evaluate the signal processing dysfunction causing a patient's pain symptoms, rather than developing drugs based on clinical symptoms alone. [2]

Adopting the conventional approach wherein only pharmacokinetics (PK), safety and tolerability are considered main objectives in Phase I/II of the development, leaves essential questions on a drug's actual effects unanswered till late, or even post-approval, which may result in multi-million dollars ill-invested on ineffective drugs, or having severe public health consequences. [3] Instead, by evaluating proof-of-mechanism (POM) and proof-of-concept (POC) early-on in development, the developer is well-informed when making go/no-go decisions. While PK, safety and tolerability assessment unmistakably are important, it should be accompanied by study objectives answering key questions regarding the drug's properties: whether the study drug reaches the target site and if so, if it has its intended pharmacological effect (i.e. POM), or enabling trials with models resembling the (pain) condition(s) the drug is aimed to treat (i.e. POC). [4] Here, we use the term POC for demonstrating analgesic effects, either in patients with pain or in healthy subjects using experimental models to evaluate pain thresholds, as proposed by Campbell et al. [5] We do realize that the term 'POC' is also often used for the first signs of clinical effects in the target population, but believe that in the context of analgesic drug development, it is fair to consider demonstrating effects on

pain thresholds – if the evoked pain test reflects a process involved in the relevant target population with clinical pain – as POC of having analgesic properties. For all biomarkers* that reflect target engagement more 'proximal', i.e. closer to the mechanism of the compound, here we use the term POM, which includes tests of target engagement (binding of the drug to its (receptor-)target), assessed at the body location targeted (e.g., synovial fluid sampled from the knee), and also tests that clearly link to the drugs- pharmacological properties (e.g., pupillometry for μ-opioid receptor (MOR) agonists, see section **Opioids**). [6] Human experimental pain studies are valuable assets to establish POC in early-phase analgesic drug development, and together with POM assessments may provide the drug developer important evidence to help make pivotal decisions on dose selection, which patient (sub)populations to target, and/or evade unnecessary investments in compounds that otherwise were poised to fail later-on. [4,5] In the case of testing first-in-class drugs, it may be that applicable models for both POM and POC are lacking, which may justify not trying to prove mechanism or – in case of analgesics – not demonstrating effects on pain thresholds and directly entering testing in patients, but should never lead to testing neither, and leaving questions unanswered. [3]

Here, we list the top 10 currently most developed analgesic drug classes and link them mechanistically to applicable methods for evaluating POM and POC in early-phase clinical trials, including pros and cons for each method described. We review how experimental studies fit into analgesic drug development, in an effort to contribute to an efficient and question-based approach.

MATERIALS AND METHODS

The Clarivate Analytics Integrity- and Biopharm Insight databases were used as sources to uncover which analgesic drugs are currently in development. [7,8] Both databases aggregate data from various sources including scientific journals, conference papers, statements of regulatory agencies, company websites and clinical trial websites such as clinicaltrials.gov. See **Appendix A** for details on the searches performed. A short term representing the drug class that aligned with the compound's main mechanism

* A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. [163]

of action was added manually for reporting. If the mechanism of action was not listed, details were searched on the respective manufacturer's website or related business news. If still no details could be obtained, the compound was allocated to the group 'undisclosed'. Listings from both databases were collated and duplicate entries removed.

The top 10 currently most developed drug classes, excluding 'undisclosed', was used for further reporting. Respective methods chosen to evaluate a class's POM and POC have been determined using expert knowledge, with claims supported by literature available in the public domain. An overview of the dosing regimen used in each trial listed is available in **Appendix B**.

RESULTS

Top 10 analgesic compound classes currently in early-phase development

The main mechanism of action was identified for 426 compounds, of the 508 unique entries included in total. **Figure 2**. displays the top 10 most developed analgesic drug classes, which is comprised of 270 (~53% of total enlisted) compounds. Refer to **Appendix C** for the complete list.

The majority of entries identified (n=83) target opioid receptors (**Figure 2**). Most of these opioidergic drugs, as well as those belonging to the non-steroidal anti-inflammatory drug (NSAID)-class (n=47), are therapies or combinations developed using a new drug delivery strategy (e.g., abuse deterrent, prodrug or administration route) rather than classified as a novel drug entity. Voltage-gated sodium channel (Na_V) inhibitors (n=43), the third most developed class, in addition to consisting of marketed drugs with updated drug formulation (e.g., lidocaine patches), includes a substantial amount of novel, selectively targeting drugs (e.g., selective $Na_V1.7$ and $Na_V1.8$ inhibitors). Further within-class details are discussed on a per-class basis in the remainder of this article.

Methods to evaluate POM and POC per drug class

OPIOIDS Opioids have been widely available for decades and serve as the main therapy of choice for severe pain indications, albeit suffering from a high abuse risk and severe adverse effects (AEs) when

administered at higher doses. Opioids such as morphine achieve pain relief mainly by targeting μ-opioid receptors (MORs), which are abundantly present throughout the human body both peripherally and in the central nervous system, resulting in the wide range of pain indications that opioids can treat. Notable AEs, such as addiction and (fatal) respiratory depression following opioid (over)dosing, however, are also attributable to that same (μ-opioid) receptor. [9] Approximately a third of the opioids listed therefore not only target MORs, but also (ant)agonize the δ- and/or κ-opioid receptors (DOR and KOR, respectively), of which buprenorphine is an example. The KOR, similar to MOR, is abundantly present throughout the body, whereas DOR expression is limited to the brain's basal ganglia and neocortical regions. [10]

METHODS FOR EVALUATING POM

The mechanism of action of opioids, and tests proving those principles, have been well-described over the years. Potency, efficacy and action duration of μ-opioid receptor agonism may be evaluated by assessing miosis using pupillometry, which in addition to confirming POM in humans (i.e. extent of target engagement of MORs), in parallel serves as translational biomarker as MOR-agonism also induces miosis in rabbits and dogs. [6,11] Pupillometry has been used extensively for characterizing the effects of many (experimental) opioid drugs, including fentanyl, naltrexone and buprenorphine, and selectivity of the KOR antagonist LY2456302. [11,12] While easily implementable, it has been debated if opioid-induced miosis is a peripheral effect (as it follows activation of the pupillary sphincter muscle, see e.g., Rollins et al., 2014) rather than affecting the central nervous system (CNS). [6,11] In addition, the method does require specific equipment and analysis methods. The latter also holds true for experimental Functional Magnetic Resonance Imaging (FMRI) studies, in which hemodynamic responses associated with neuronal activation are measured in the brain's pain matrix, following nociceptive stimulation. [13] As such, oral morphine was found to significantly affect brain areas where opioidergic receptors are predominant after heat stimulation using a contact heat evoked potential stimulator. [14]

Classical opioids, as said before, suffer from a high frequency of AEs, which are hypothesized to be absent in the opioids that are currently being developed. Proxies to evaluate the risk-benefit profile early-on in development are therefore also of importance. The most suitable model

depends on the drug's potency, administration route and dose. Applicable methods for MOR-like side effects – or absence thereof – include the dynamic end-tidal forcing technique to study effects on ventilation/respiratory depression, [15] abuse potential of the drug defined as changes on a drug-liking Visual Analogue Scale (VAS), [16] and/or motility of bowel as a measure for opioid-induced constipation by determining gastrointestinal transit times. [17] All mentioned tests directly link to MOR effects and can be tested in sequential fashion with POC tests (see below), but – except for the easily adoptable drug-liking VAS – require specific tools and expertise, limiting their use.

METHODS FOR EVALUATING POC

The cold pressor test, an evoked pain test using cold pain to measure pain thresholds, is primarily used to demonstrate analgesic effects of (MOR-) opioids both in an experimental context [12,18], and clinical context, as the cold pressor test allows for diagnosing fibromyalgia or opioid-induced hyperalgesia (OIH). [19] The model's superiority in detecting analgesia lies in its tonic stimulus, which evokes the opioidergic-linked endogenous central pain inhibiting system [20,21]. See **Table 1** for a detailed description of the method. The effects of potent opioids such as fentanyl have been further characterized by a battery of distinctive nociceptive tests, with changes apart from those noted on the cold pressor test, also reported for heat and electrical pain thresholds; corroborating the broad applicability of opioids as analgesics. [18,22] To assess KOR agonism, visceral pain thresholds induced by a multi-modal esophageal probe may be used, as shown by Arendt-Nielsen et al. and through the suggested role of the KOR in the visceral pain system. [23,24] To note, experimental pain tests require specialized tools and training which limit their applicability. Also, evoked pain thresholds are subject to a relatively high inter-individual variability, which is likely related to the tests' subjective outcome variable (i.e., reporting of when a pain threshold is reached). To counter this, two (or more) period cross-over study designs are often used, which allows comparing treatment effects within a single individual.

For POC studies in clinical pain, i.e., trials assessing the first signal of treatment efficacy in a well-defined patient subpopulation, the dental impaction pain model (including third molar surgery) has been most widely used and found particularly useful for assessing dose ranging and profiling of (novel) opioids, and NSAIDs. [25]

NSAIDS NSAIDs act mainly by targeting the cyclooxygenase (COX) enzymes (COX-1 and -2), that are responsible for inducing fever and inflammatory pain through prostaglandin E_2 (PGE_2) synthesis. [26] Given the side effects induced by classic (non-selective) NSAIDs such as gastrointestinal bleeds attributable to COX-1 inhibition, drug developers turned to selective inhibition of COX-2. While initially praised for their expected efficacy and safety through target specificity, the selective COX-2 inhibitors were later found to induce significant cardiovascular side effects, leading to discontinuation of (the development of) most COX-2 selective inhibitors. [27] Drugs currently being developed and belonging to this class are primarily non-selective COX inhibitors based on marketed NSAIDs, but novel due to their formulation, or by being combined with another drug and developed as a single treatment.

METHODS FOR EVALUATING POM

One of the challenges with COX inhibitors related to proving their pharmacological effects, is the mismatch between drug plasma concentrations and exerted analgesic and/or AEs in inflammatory disease states, which is likely related to the complex pathophysiology of inflammation. [28] Evaluation of (other) biomarkers based on the drug's proposed action mechanism is therefore advised for e.g., calculating dosing regimens. Examples include PGE_2 and thromboxane B2 level determination. For subtype-selective drugs, the IC_{80} of COX-2 (i.e. concentration of drug needed to inhibit COX-2 by 80%) versus effective concentrations at COX-1 can be used for proof-of-specificity. [28] Evaluation of these markers may not be available in a routine laboratory, which then requires assay set-up and additional funds.

METHODS FOR EVALUATING POC

For analgesics intended to treat inflammatory pain, the ultraviolet B (UVB)-induced hyperalgesia model, also referred to as the 'sunburn model', is primarily used as a readout for POC. [13] UVB promotes inflammation through increased production of various cytokines and prostaglandins originating from the affected keratinocytes. [29,30] See **Table 1** for a detailed description of the method. The model has shown robust responses to NSAIDs such as ibuprofen [18,31,32]. Alternatively, freeze injury may be utilized to evoke local hyperalgesia lasting over three days that, in combination with the Von Frey hair filament assessment, has been

shown to be responsive to NSAIDs administered both topically and systemically. [33,34] While yielding robust results with relatively little variability compared to other experimental pain models, application of these models may lead to post-inflammatory hyperpigmentation marks on the study participants' skin, lasting months or even years. [13,35] In addition, studies utilizing the UVB model are hampered by the need for a more homogenous study population, as irradiation levels needed to induce hyperalgesia are only safe in lighter-skinned individuals. [35]

Together with the dental impaction pain model (as mentioned in the section **Opioids – Methods for evaluating POC**), bunionectomy surgery has been used to evaluate an NSAID's efficacy in patients suffering from acute pain, in the POC setting. Others include the joint replacement and soft tissue surgery models, although the former two (dental- and bunionectomy model) yield higher assay sensitivity. [36]

Na_V INHIBITORS Of the human Na_V channels discovered, four ($Na_V1.3$, $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$) have been found to be primarily present on nociceptors. [37] Each of these has unique properties and plays a key role in the generation and/or propagation of action potentials (**Figure 3**). [37,38] As such, $Na_V1.7$ is a key contributor to the initial rising phase of the action potential, but may also amplify subthreshold stimuli, being a low activation channel as $Na_V1.3$ and $Na_V1.9$. $Na_V1.3$ is primarily involved following axotomy and other forms of peripheral nerve injury. [39] Where $Na_V1.3$ and $Na_V1.7$ have fast gating kinetics (i.e., opening and closing of the channel), these properties are for $Na_V1.9$ ultra-slow, also in comparison to $Na_V1.8$. The latter is a high activation threshold channel that acts during the later rising phase to support high frequency firing (i.e., hyperexcitability). [40]

First generation, non-selective Na_V inhibitors have been one of the most widely used class of analgesics in the clinic for decades. Alike opioids, these also suffer from a poor risk-benefit profile, as exemplified by lidocaine to which we alluded in the introduction. [41,42] To this end and following the discovery of $Na_V1.3$, $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$'s contribution to pain signal initiation and propagation, subtype selective Na_V inhibitors are currently being developed to treat acute and neuropathic pain disorders. Most selective inhibitors that came up in our search target either $Na_V1.7$ or $Na_V1.8$. While theoretically a promising target for analgesics, $Na_V1.3$-subtype specific inhibitors are investigated to a limited

extent, likely because $Na_V1.3$ is highly homologous to other sodium channels (up to 85% for $Na_V1.2$). [43] Development of $Na_V1.9$-selective drugs is precluded by the inability to express the channel in heterologous systems, which is needed to study protein structure and function. [44,45]

Currently registered therapies for this drug class include the first-generation anticonvulsants phenytoin and carbamazepine, which are used primarily in the treatment of trigeminal neuralgia and as third-line therapies for other forms of neuropathic pain.

METHODS FOR EVALUATING POM

With Na_V inhibitors primarily acting on action potential firing, the nerve excitability threshold tracking technique may yield detailed information on channel selectivity and amplitude of drug effects on peripheral nerves. [46] This measurement produces information on physiological conditions, the state of ion channels involved in nerve excitation, as well as on the functionality of energy-dependent pumps. It allows for the identification of exposure levels needed for state- and frequency- dependent block of sodium channels. [47] Threshold tracking is generally used to assess motor neuron excitability in e.g., amyotrophic lateral sclerosis patients. It can, however, also be used to assess sensory neuron function, and can be considered POM given it is a distinct readout for the pharmacological effects of Na_V inhibition. [47,48] The non-selective Na_V inhibitors lidocaine, mexiletine and tetrodotoxin have been characterized using this technique. [49–51]

To measure central effects induced by Na_V inhibitors, drug effects can be evaluated using transcranial magnetic stimulation (TMS). TMS may be utilized either by recording TMS-evoked electroencephalographic (EEG) potentials (TEPs), or evoked electromyographic (EMG) responses. See **Table 1** for a detailed description of the method. TEP P180, a late-phase potential controlled by axonal excitability, has shown negative responsiveness (i.e. decreases) to the Na_V inhibitors lamotrigine and carbamazepine, [52,53] whereas motor thresholds as measured by EMG responses were increased following lacosamide and carbamazepine administration. [54] Both threshold tracking and TMS are non-invasive and utilized routinely in experimental studies, but are considered complex both to execute and to analyze generated data. Evaluation of excitability following TMS as POM moreover may may not be applicable for the selective Na_V inhibitors, as they act mostly on ion channels present on peripheral nerves

or dorsal root ganglia (DRG; i.e. compounds targeting $Na_V1.7$ or $Na_V1.8$) (**Figure 3**), while TMS can be used to measure CNS/cortical excitability.

By exposing hyperexcited induced pluripotent stem cell-derived sensory neurons, obtained from patients with inherited erythromelalgia (IEM), to the selective $Na_V1.7$ inhibitor PF-05089771, Cao et al. have presented a method to confirm POM of Na_V inhibitors in a lab-based experiment. It has been proposed that this method may have broader utility than in IEM, e.g., also in other pain conditions of which hyperexcitability is the underlying cause. [55]

METHODS FOR EVALUATING POC

Prior to the reports from the two studies included in this thesis (**Chapter 2** and **3**), no published data were available that reported positive effects of selective Na_V inhibitors on human experimental pain models in a healthy subject population. Preclinical work showed that the selective $Na_V1.8$ inhibitor A-803467 reduced thermal and mechanical hyperalgesia, and attenuated neuropathic pain in multiple preclinical readouts, [56–59] whereas the selective $Na_V1.7$ inhibitor PF-05089771 attenuated sensations of burning pain in patients with diabetic neuropathy. [60] The heat pain test with and without the capsaicin model to induce hyperalgesia and burning sensations may thus be applicable yet noting is was not sensitive to selective Nav1.8 inhibition in two studies (**Chapter 2** and **3**). In another study, the selective $Na_V1.8$ inhibitor VX-150 significantly reduced pain in two studies in patients with acute pain (in patients that underwent bunionectomy surgery, and in patients with knee osteoarthritis (OA)) and small fiber neuropathic pain respectively. [61,62] Stated POC studies in patients therefore are suitable to evaluate (selective) Na_V inhibitors, but we also propose that the cold pressor pain test may establish POC for such compounds considering the above results. The cold pressor pain test namely is sensitive to neuropathic pain treatments as pregabalin and mexiletine, [32], **Chapter 4**] and serves as a readout of TRPM8-mediated cold pain sensations through its interplay with $Na_V1.8$. [63,64] Rationale for this test is discussed in more detail in **Section 1** of this thesis. As an alternative to performing a study with pain models, a POC study in patients with trigeminal neuralgia is proposed based on positive findings of two Na_V inhibitors in this population. Both carbamazepine and more recently selective $Na_V1.7$ inhibitor vixotrigine (BIB074, formerly raxatrigine) proved to be efficacious in this population. [65]

CANNABINOIDS Fueling an ever-growing trend, [66] both the natural *cannabis sativa* L. (cannabis) and cannabis-derived cannabinoids are amongst the currently most developed drugs, with an estimated sale value of 1.9 billion in 2020 in the Unites States alone. [67] Cannabinoids, apart from acting on the cannabinoid-1 and -2 receptor (CB1 and CB2, respectively), [68] may relieve pain by acting on serotonin (5-HT) receptors [69] and transient receptor potential (TRP) channels including the TRPV and -A subtypes. [70] (**Figure 4**) Selective CB2 receptor agonists are of specific interest for drug developers given their observed efficacy in a range of preclinical inflammatory and neuropathic pain models, whilst mitigating psychotropic effects attributed to activation of central CB1 receptors. [71] For cannabinoids, adequate biomarkers largely depend on the receptor targeted, and dose used. Given their action is so distinct, we here define three cannabinoid subgroups for which POM and POC options are discussed: those primarily targeting CB1 and CB2 receptors, those primarily inhibiting fatty acid amide hydrolase (FAAH), and cannabidiol (CBD).

It is important to note that while multiple CB2 agonists and FAAH inhibitors are reported in our search, no recent trials were found on evaluation of these class subtypes in clinical pain. Despite promising preclinical evidence, the CB2 agonist GW842166 was discontinued following its failure to demonstrate meaningful analgesia in patients with acute dental pain, and the FAAH inhibitor PF-04457845 failed to relieve pain in OA patients, although these are reports of almost a decade ago. [72,73] PF-04457845, however, recently was shown to reduce cannabis withdrawal symptoms in men, suggesting that FAAH inhibitors possibly may be better suitable as a treatment for indications other than pain. [74]

METHODS FOR EVALUATING POM

When the drug is a ligand for both CB1 and CB2, e.g., in the case of tetrahydrocannabinol (THC)-formulations or cannabis-based formulations containing THC, motivation and attention-based cognitive tests are most applicable to evaluate POM at low doses, whereas high doses affect blood pressure, heart rate and subjective feeling (e.g., VAS feeling high or evaluation of psychotomimetic feelings). [75] The latter two, effect on heart rate and feeling high, also serve as POM biomarkers for selective CB1 agonists. [76] Lack of observed effects on these proxies may therefore be beneficial for POM of a CB2-selective analgesic. While evidently more costly, radiotracer positron emission tomography (PET) imaging studies, often used

for POM, may provide more detailed information on the availability and (drug) occupancy of CB1 (using e.g., PET tracer [^{18}F]MK-9470) and CB2 (using e.g., [^{11}C]NE4), in the brain. [77,78]

A POM approach for cannabinoid drugs primarily inhibiting FAAH, on the other hand, is through assessment of endocannabinoid levels, with specific focus on anandamide levels as they increase upon FAAH inhibition. [79] FAAH inhibition can be measured using a fluorescence assay. A striking example in which such a POM approach proved to be essential, is the infamous BIA 10–2474 (Bial) trial in which a novel FAAH inhibitor was tested. While safety, tolerability, PK and FAAH inhibition were evaluated, only the former three were used for dose escalation decisions. When the crucial data on the FAAH inhibitory effects had been taken into consideration, it could probably have prevented the death of a healthy volunteer and irreversible brain damage in four other healthy study participants. [80] In the case of CBD, the main non-psychoactive component of cannabis, investigators have difficulty showing POM, as CBD apart from having low affinity for CB1 and CB2, also acts on a plethora of other receptors throughout the body. CBD therefore links to many diseases and (neuro)protective properties. [81] While early-phase studies in the context of CBD and pain are scarce, a possible, yet costly and complex, approach is by evaluating striatal activation during a verbal memory task using FMRI. CBD has been found to augment, and THC attenuate, the striatal activation, therefore this may be utilized for differentiation of CBD from THC-based drugs. [82]

METHODS FOR EVALUATING POC

POC read-outs for CB1/CB2 ligands are complex, as translation between healthy volunteer- and patient studies has been difficult: despite theoretical evidence, THC administered in two distinct experimental pain studies with healthy subjects, induced hyperalgesia rather than analgesia. [83,84] Therefore, testing within a well-chosen patient subpopulation seems more appropriate. For example, pressure pain thresholds, but not spontaneous or electrical pain, assessed in fibromyalgia patients have been found reactive to THC administration, [85] as were pain scores reported by patients with multiple sclerosis. With the latter, it is important to take temporal effects into account when designing such a study, given effects can take weeks to develop. [86]

Genotyping may allow for POC evaluation of a FAAH inhibitory drug: alterations in sensitivity to cold pain are associated with FAAH polymor-

phisms in lower back- and postoperative pain conditions. [87,88] The latter is an expensive approach, and only applicable to a limited patient population. Alternatively, assessing EEG readouts from laser evoked potentials (LEPs) generated on capsaicin-treated skin, may be suitable. Schaffler et al. demonstrated that, in subjects with a confirmed hyperalgesic response to capsaicin, the FAAH inhibitor ASP8477 reduced sensitization, demonstrated by a decrease in LEP N2-P2 peak-to-peak amplitudes compared to placebo. [89]

For CBD, preclinical evidence has established a POC role for the UVB-induced hyperalgesia model (**Table 1**), as CBD reduces keratinocyte-mediated inflammation, and potentially protects keratinocytes against UVB irradiation. [90,91] While there plausibly is a role for experimental pain models in characterizing CBD's analgesic effects, given the beneficial effects from CBD reported by chronic pain patients, [92] there is little to no clinical evidence available in the public domain other than the cited experimental pain study in fibromyalgia patients, where no analgesic effects attributable to CBD could be demonstrated. [85]

NMDA MODULATOR N-methyl-D-aspartate receptor (NMDA) antagonists in a clinical setting have shown robust efficacy in treating (opioid-induced) hyperalgesia, neuropathic pain syndromes and pain following opioid tolerance. [93] Primarily represented by ketamine and methadone, this drug class relieves pain by blocking the excitatory signal at the NMDA receptor, typically induced by binding of glycine and glutamate to their respective receptors. [94] Changes in glutamatergic neurotransmission, however, may also induce notable CNS-side effects, which have led to the recreational abuse of these drugs and failure of many novel NMDA antagonists during development. [95]

METHODS FOR EVALUATING POM

TMS (see **Na_V inhibitors – methods for evaluating POM**) may be used to evaluate effects of NMDA antagonists on motor cortex excitability. Previously, ketamine was found to increase the motor cortex responses, and memantine to significantly affect its plasticity. [96,97] When planning to include TMS for evaluating NMDA receptor modulation, one – apart from the cons mentioned in the previous section – should be aware of a delay in effects, in the case that the to-be-tested drug has prolonged action characteristics. [96]

METHODS FOR EVALUATING POC

NMDA antagonists such as ketamine have been profiled in an experimental setting on a variety of pain paradigms. The (cutaneous) heat pain test and, to a lesser extent, the (cutaneous) electrical pain test most adequately display ketamine's analgesic potential. [18,98] The thermal grill test, during which warm and cold stimuli are applied simultaneously to the skin to evoke a paradoxical pain sensation, is suitable to confirm activation of the glutaminergic- rather than the endogenous opioid system, as ketamine reduced paradoxical pain intensity whereas the opioid-receptor antagonist naloxone did not. [99] Recently, NMDA receptor antagonists have been suggested as potential treatments for central sensitization, [100] which was positively evaluated in an experimental setting using the freeze injury hyperalgesia model (also see. **NSAIDs – Methods for evaluating POC**). [95]

NERVE GROWTH FACTOR (NGF) MODULATOR The interaction of NGF with tropomyosin kinase A (TrkA) – which is highly expressed in the DRG – has been found a key step in the sensitization of nociceptors. [101] Antagonists are therefore expected – and developed – as a treatment for chronic pain with specific focus on inflammatory conditions. [102] Development of this class was temporarily halted by the US Food and Drug Administration following reports that anti-NGF antibodies caused rapid joint destruction in patients with OA. [102] More recent data, however, suggest that lower dose anti-NGF antibodies may have a more favorable risk-benefit profile. [101] Approximately half of the NGF-compounds enlisted are anti-NGF antibodies, the other half TrkA-selective inhibitors.

METHODS FOR EVALUATING POM

For compounds developed to treat localized (inflammatory) pain conditions – such as NGF antibodies to treat knee OA –, distribution to, and availability of the drug in the target tissue is key. Demonstration of this is feasible by performing synovial fluid sampling. [103] TrkA, however, is not highly expressed in blood cells and therefore does not allow for testing of target engagement in blood. Alternatively, in the case where a TrkA inhibitor is studied, skin biopsies can be utilized for studying inhibition of NGF-induced TrkA phosphorylation *ex vivo* . [104]

METHODS FOR EVALUATING POC

TrkA receptors, apart from modulation of various receptors such as TRPV1 through expression in the DRG, are also available on mast cells. NGF, through TrkA, therefore induces a pro-inflammatory response with increase of e.g., histamines, 5-HT and NGF, resulting in a positive feedback loop. [105] The UVB-induced hyperalgesia model (**Table 1**) induces a (local) inflammatory response which, amongst others, results in increased NGF levels, [106] and applicability for POC as previously described. [31] To note, while the capsaicin-induced hyperalgesia model theoretically may also be suitable, given it induces sensitization and local inflammation through TRPV1 and mast cell activation, [107–109] to the best of the authors' knowledge no clinical evidence is publicly available to substantiate the use of this model in the context of POC for this compound class.

5-HT MODULATORS 5-HT mediates pleiotropic behavioral effects including mood and anxiety through a family of 14 different receptor subtypes. Additionally, 5-HT plays a complex part in both hyperalgesic and analgesic states, dependent on the receptor (sub)type targeted and action site, with choice of the descending inhibitory pathways in the CNS, the trigeminal system, or afferent nerve fibers (**Figure 4**). [110] Various 5-HT subtype-selective (5-HT1B,-1D,-1F,-2B) modulators are currently developed, of which approximately half are to treat migraine or other headache syndromes.

METHODS FOR EVALUATING POM

To assess blood-brain-barrier penetrability of the drug, cerebrospinal fluid may be sampled by performing a lumbar puncture. Proper POM studies are lacking for subtype selective 5-HT modulators; however, subclass-related AEs reported so-far may guide POM evaluation. As such, triptans (5-HT1B/1D agonists) are found to induce vasoconstrictive effects and chest tightness. While vasoconstriction can only be assessed *in vitro*, using e.g., isolated arteries obtained from explanted hearts following cardiac transplantation, [111] evaluation of chest tightness is part of the clinical evaluation. The 5-HT1F selective agonist lasmiditan dose-dependently induces dizziness, which can be evaluated using a VAS. [112]

METHODS FOR EVALUATING POC

Receptor hypersensitivity and deficits in the 5-HT descending pain inhibitory pathway following low 5-HT levels have generally been accepted to play a pivotal role in migraine pathophysiology and central sensitization. [113] Using experimental models such as quantification of the conditioned pain modulation (CPM) response, effects of 5-HT selective drugs on central pain systems may be evaluated for POC, although clinical evidence for this approach is still limited and the test itself difficult to execute. [5,114] It is therefore suggested to evaluate pain thresholds in a multimodal test battery that apart from CPM also evaluates heat pain thresholds, as it has been demonstrated that both pain detection and tolerance thresholds are sensitive to 5-HT function following acute tryptophan depletion. [115]

It is noteworthy to mention that, while cilostazol, Calcitonin Gene Related Peptide- and Isosorbide-5-mononitrate-induced headache models do induce migraine-like attacks and are established experimental tests, they fail to respond to the 5-HT agonist sumatriptan in healthy volunteers, which makes these models unsuitable for POC of this specific drug class. [116–118] Rather, the Pituitary Adenylate Cyclase-Activating Polypeptide 38 (PACAP38)-induced headache model can be used, as pretreatment with sumatriptan attenuated headache induced by PACAP38 in a double-blind cross-over setting. [119]

TRPV1 MODULATORS Primarily known for the hot burning sensations caused by capsaicin, the active component of chili peppers, agonists of the TRPV1 channel – abundantly expressed on nociceptive c-fibers (**Figure 4**) – induce hyperalgesia in low concentrations, while overstimulation of that same receptor relieves pain through (temporary) nerve ablation. Antagonism of TRPV1 is of interest for analgesic drug developers as well, following reporting of positive effects in preclinical inflammatory- and cancer pain models. [120] First-generation antagonists, however, induced hyperthermia and impaired noxious heat sensation in many study participants. Development of this class of drugs was therefore initially halted. [121] Apparently it is possible to circumvent this problem, as at least seven later-generation compounds have recently progressed in the clinic without displaying these unwanted effects. [122] TRPV1 modulators have mostly been developed to treat neuropathic pain.

METHODS FOR EVALUATING POM

The relationship between capsaicin, vasodilation and flare is common knowledge for years, and extensively studied as POM. [123] Laser Doppler perfusion imaging, although requiring expensive machinery, is a reliable method to assess macrovascular changes in the skin including capsaicin-induced flare, and is proposed to evaluate mediators of neurogenic inflammation, such as TRPV1 modulators. [124]

METHODS FOR EVALUATING POC

For drugs developed as TRPV1 antagonists, capsaicin-induced hyperalgesia serves as an excellent experimental pain model. In low to medium concentrations (i.e. up to 3%), capsaicin administered either intradermally or topically has been successfully used for decades as a TRPV1-receptor-mediated challenge. [125] Readouts for this challenge include heat- and mechanical pain thresholds, but also effects on capsaicin-induced flare as shown in an early-phase drug study previously. [126] While extensively used, large inter-individual variability is reported for the response to capsaicin. [5] As such, the administration route (intradermal or topical) and test procedure employed (e.g., re-heating of treated area) have been correlated to enhancement of capsaicin-induced sensitization. [127]

More recent advancements in evaluating TRPV1 agonism include assessing changes in nociceptive detection thresholds following intra-epidermal electrical stimulation, which allows for temporally discriminating altered peripheral, versus altered central pain processing mechanisms. [128] The technique, however, is currently still in development and has therefore not yet been applied widely.

Notwithstanding the usefulness of capsaicin to demonstrate POM and POC, translation of positive preclinical data to clinical efficacy has been especially hard for TRPV1 antagonists. No clinically significant effects could be observed for AZD1386 on the Western Ontario and McMaster Universities pain scale, in a POC study in patients with chronic pain from knee OA. [129] Noteworthy, AZD1386 did significantly decrease pain intensity versus placebo in the same study. It has been proposed that by excluding NSAID-sensitive patients – who presumably have an inflammatory component to their pain – study outcomes were negatively influenced, suggesting that patient selection may influence POC study outcomes. [130] In a different study assessing dental pain, AZD1386 elicited significant analgesia, although effects were very short-lived (up to 1 h). [131] Another antagonist,

GRC-6211, while reported to be selective, highly potent and yield good bioavailability across various preclinical models, was discontinued after a clinical trial in OA pain was suspended. For further suggested reading on this topic, see the comprehensive review of e.g., Kort and Kym, 2012. [129]

CALCIUM MODULATORS Calcium channels present on peripheral nerve fibers are responsive to a variety of noxious stimuli. Upon activation, action potential propagation is increased due to an increased calcium influx. Voltage-gated calcium channels at central nerve terminals subsequently propagate pain signals through increased release of glutamate (**Figure 4**). [132] Calcium channel modulators – of which the gabapentioids are by far the most prescribed – inhibit this signal, resulting in their usefulness as treatments for (neuropathic) pain disorders. [133]

METHODS FOR EVALUATING POM

Similar to NMDA antagonists and Na_V inhibitors, the role of calcium channels in membrane excitability makes evaluation of altered excitability following TMS a viable biomarker, although the exact affected parameters vary between the calcium channel inhibitors administered. [134] Previously, it has been shown that intracortical excitability is a Gamma-aminobutyric acid (GABA)-controlled process, involving the interneuronal circuits in the motor cortex. As such, gabapentin prolonged cortical silent periods and the short intracortical inhibition, in addition to reducing intracortical facilitation, whereas in contrast losigamone (a sodium and calcium channel inhibitor without neurotransmitter properties) increased motor thresholds without affecting intracortical excitability, thereby demonstrating specificity of the mentioned cortical excitability parameters for calcium channel modulators. [135–138]

METHODS FOR EVALUATING POC

The analgesic effects of gabapentinoids have been profiled in experimental studies and have demonstrated nociceptive effects in multiple evoked pain tests, including a multimodal test battery. Oral doses of 300 mg pregabalin have shown robust and reproducible effects on pressure- and cold pressor pain thresholds. [32] Results for secondary hyperalgesia to pinprick and allodynia to brushing following topical capsaicin application vary, while noting that the allodynia assessment did produce more robust results. [139]

GABA MODULATORS As the chief inhibitory neurotransmitter, GABA reduces neuronal excitability throughout the central nervous system. Interestingly, many non-selective GABA-ergic compounds including benzodiazepines have evident pharmacological effects, of which analgesia is not one. This may be because significant adverse effects such as sedation precede the drug's antinociceptive effects. [140], As GABA(A) subunits α2 and α3 have been linked to pain relief, while sedation has been attributed to GABA(A) subunit α1, GABA-ergic drug developers seek subtype specificity. Compounds listed in this class either target GABA(A) subunits α2, α3 and/or α5 and aim to treat neuropathic pain, or target GABA(B) for chronic and osteoarthritis pain relief. [141,142]

METHODS FOR EVALUATING POM

For GABA modulators, POM partly depends on demonstrating subtype selectivity, which can be obtained by discriminating the observed pharmacological effects against those observed from non-selective GABA-ergic drugs, including sedation. A selection of neuropsychological and neurophysiological tests may be used to differentiate effects from e.g., α2 and α5-selective-drugs to those from non-selective GABA(A) agonists. A VAS measuring alertness to assess sedation, a test to quantify swaying of the body as proxy for postural imbalance, a VAS measuring 'feeling high' to evaluate a drugs' abuse potential, and effects on saccadic eye movement using a computer-based eye tracking system, have all repeatedly been used to prove GABA(A) selectivity. [143–145] While VAS scales are cheap and easily adoptable, the body sway test and saccadic eye movement tasks do need specific (computer) equipment and trained staff.

For demonstrating GABA(A) versus GABA(B) selectivity as an extension of POM, TMS (**Table 1**) experiments may be useful. Using multiple GABA-ergic drugs alprazolam (a classical positive allosteric modulator (PAM) at α1, α2, α3, and α5 subunit-containing GABA(A) receptors), diazepam (classical non-selective benzodiazepine), zolpidem (PAM at α1 GABA(A) subunits only) and baclofen (GABA(B) agonist)), an amplitude increase of the N45 potential has been shown to display GABA(A)-selectivity, whereas a decreased amplitude of the N100 potential showed GABA(B)-selectivity. [146] Oscillatory changes following single-pulse TMS are feasible as readouts also: opposite effects have been demonstrated for GABA(A)- and GABA(B)-ergic compounds on α-band-synchronization measured in the stimulated sensorimotor cortex and lateral frontal cortex. [147]

METHODS FOR EVALUATING POC

While available literature on experimental pain studies with GABA-selective drugs is scarce, the analgesic profile of a partial α2/α3/α5-selective GABA(A) agonist has previously been characterized: pressure-evoked and cold pressor- evoked pain thresholds were inhibited in a similar fashion to 300 mg pregabalin. [148] Another study, testing the benzodiazepines clonazepam and clobazam, reported analgesia in a capsaicin-pressure cuff algometry challenge, further indicating a possible role of pressure pain as a proxy for GABA-ergic analgesic drug effects. [149] It must be noted, however, that clonazepam and clobazam are not regarded as analgesics in general clinical use, their effectiveness in pain being limited to the clinical study setting. [150]

DISCUSSION

In the present article, we have listed the analgesic drug classes that are currently most developed, and have mechanistically linked them to biomarkers suitable for use in early-phase drug studies, to aid in efficient and question-based analgesic drug development. For proper evaluation of POM and POC, each drug class requires a tailored experimental approach. A few methods including TMS, the capsaicin- and UVB-induced hyperalgesia models and the cold pressor evoked pain test were found to be more widely applicable across drug classes.

POM and POC, as we defined these terms in the introduction may not align with how they are commonly used by the scientific community. POC, while here describing experimental models to evaluate pain thresholds in healthy volunteers or patients, is often also regarded as the first signal of clinical efficacy within the relevant target patient population. Although we added the reporting of effectiveness in a healthy population to POC – as it proves the analgesic potential of a compound –, such studies (i.e. positive POC trials performed in healthy volunteers) do not warrant omitting POC studies in an applicable patient population. Evoked pain tests, while preferably mechanistically linked to administered drug and target patient population, only induce pain that is short-lived, neglecting the more chronic, and emotional aspects that coexist in patients experiencing pain. Rather, results from healthy volunteer POM/POC studies allow for an improved and often leaner, therefore more cost-efficient design of successive trials in patients. POM and POC studies therefore serve as a translational step between preclinical experiments and studies in

the relevant patient populations seeking to find the first signal of clinical efficacy. By confirming the active concentration range in a POM or POC study, fewer dose levels need to be evaluated in patients rendering these studies more (cost-)efficient. Moreover, (first-in-human) single or multiple ascending dose (SAD, MAD) studies that are a mandatory part of any drug's development trajectory, often can be enabled with POM and/or POC models. Important information on the drug's characteristics including pharmacokinetic-pharmacodynamic (PK-PD) relationships – especially of interest when evaluating wide dose ranges as during SAD or MAD studies – can then be generated, at little additional cost.

Due to the complexity of pain and its underlying mechanisms, a wide variety of analgesics with ever-increasing specificity are currently being developed. The classes discussed here represent those mostly developed, although it must be noted that compounds within a particular class may still vary substantially, for example due to (sub)type selectivity or route of administration. As such, $Na_V1.7$ contributes differently to analgesia than $Na_V1.8$ does; therefore, arguably other, even more specific biomarkers may be superior in evaluating compounds targeting either channel. The encompassing commentary nonetheless holds true: each technique mentioned does provide a firm handhold for assessing POM and/or POC, and that development of each unique compound needs a tailor-made approach.

While it is thus important to evaluate proxies aligning with the proposed mechanism of action, it is equally important to not narrow the study objectives unnecessarily. By testing multimodally – i.e., in addition to evaluating the desired endpoint, also include models each representing a distinct (pain) pathophysiology to evaluate effects other than expected – an (analgesic) effect profile can be created. [18] Multimodal testing in general does not significantly increase subject burden or study costs, yet provides increased knowledge on the drug's putative mechanism of action, and therefore confidence to make pivotal decisions about the compound's future. [13] This argument, however, only applies to analgesics of which the exact mechanism of action is linked to the suggested test. If there is no scientific rationale behind e.g., a NSAID possibly affecting electrical pain thresholds, it would be futile to add this method to a POC study. Yet even when there is an evident rationale to use a specific test, the relationship between the experimental model and the disease it mimics often is not fully elucidated. E.g., it is rational to use the UVB-induced

inflammatory hyperalgesia model to evaluate a TrkA inhibitor's potential to treat inflammatory pain, although the correlation between the model (increased NGF levels following UVB exposure), and the disease state that it mimics (NGF upregulation in synovial fluid in patients with osteoarthritis) is not fully known. It must therefore be noted that, while POM and POC studies do bridge the gap between preclinical research and studies in patient (sub)populations, positive results generated in such trials do not guarantee a drug's efficacy in a patient population. Two examples are the CB2 agonist GW842166 and the FAAH inhibitor PF-04457845, as discussed in section **Cannabinoids** of this chapter.

Also in a therapeutic area such as pain, research and technology has reached unprecedented levels that allow for meticulous assessment of a compound's (dose-dependent) effects. By incorporating tests such as those mentioned into early-phase trials, success – or failure – of a novel drug may be confirmed rather sooner than later. Moreover, with the change to personalized medicine and target selectivity, drugs developed to treat a multitude of conditions from the start are in decline. Instead, highly selective drugs treating well-chosen patient subpopulations are being developed. POC, but especially POM trials will aid in a crucial aspect related to this change. By incorporating methods that evaluate the drug's mechanism of action accurately, POM studies can confirm target selectivity that may be unachievable using POC experimental models alone. Therefore, results obtained for POM, but also POC – or preferably combined –, can help determine the optimal dose and patient (sub)population to target in the following development phase(s) – and aid in increasing the number of treatments reaching patients.

Aims and outline of this thesis

Continuing efforts are made to expand and further improve our knowledge on pain signaling and effective treatment of pain. One is by developing and validating new methods for early-phase clinical drug studies that have improved accuracy or improved resemblance to clinical pathophysiology, and may so improve the evaluation of a drug's mechanism of action and analgesic potential. The other is by actually testing novel compounds that are proposed to have a superior clinical utility, using methods that we believe to be appropriate for evaluating their POM and/or POC. For all types of pain but especially within the field of neuropathic pain, there is

still much to be gained, as illustrated by the large unmet medical need and limitedly efficacious drug treatments that are currently available. [151]

Neuropathic pain is defined by the International Association for the Study of Pain (IASP) as "pain that arises as a direct consequence of a lesion or diseases affecting the somatosensory system". [152] A key contributor to the chronification of neuropathic pain is central sensitization, which may manifest clinically as hyperalgesia (also see **Figure 1**), a symptom non-existent in healthy individuals. Models that can induce hyperalgesia and tools that can reliably assess altered functioning following induction, are sought-after as they may aid in examining the potential of (novel) analgesics as neuropathic pain treatment. Hyperalgesia – in experimental context – may be induced peripherally (i.e., increasing responsiveness to stimuli locally by increasing nociceptor sensitivity at the affected area), or centrally (i.e., increasing responsiveness to stimuli by increasing sensory neuron excitability at the dorsal horn and thalamus; **Figure 4**). [125] Hyperalgesia models that are suitable for use in early-phase drug studies can be an important asset for improved POC of the analgesic drug classes described here in **Chapter 1**. To be 'suitable for use', we applied the general criteria for usability of a biomarker, as described previously:

- The model should induce a clear, consistent response across studies, and across drugs from the same class
- A clear response to therapeutic doses must be observed
- Dose (concentration)-response relationship can be demonstrated (if the study design allows for this)
- There should be a plausible relationship between the model, the pharmacology of the tested drug class and the disease pathophysiology. [153]

As convincing evidence in favor of hyperalgesia models with respect to the above criteria is limited, further research is warranted. The main objectives of this thesis therefore were to evaluate applicable tools for profiling the effects of (novel) analgesics using hyperalgesia models and other established nociceptive tests (**Section I**), and explore other tools that may even better predict an analgesic's effects in healthy volunteers (**Section II**).

Section I

In this first section, we assessed the validity of a panel of nociceptive and hyperalgesia models in context of the assessment of analgesic effects of (novel) NAv inhibitors. We tested a novel and selective NAv1.8 inhibitor, VX-150, in a dedicated POC two-way cross-over study and reported our findings in **Chapter 2**. In **Chapter 3** the safety, tolerability and nociceptive test results of a first-in-human study with NAv1.8-selective inhibitor VX-150 are described. To further study how NAv inhibition modulates nociceptive processing, in **Chapter 4** we tested the two registered, non-selective, NAv inhibitors lacosamide and mexiletine using a nociceptive test battery and UVB-induced hyperalgesia model.

Section II

Next, we evaluated a selection of models on their potential to induce hyperalgesia in healthy subjects. **Chapter 5** describes results from a clinical study in which we studied the suitability of the human endotoxemia to induce inflammatory hyperalgesia. **Chapter 6 and 7** discuss how depriving healthy subjects from sleep induces sex-dependent enhanced pain sensitivity, and report that different readouts may be applicable. In **Chapter 8**, we investigated whether we could improve our existent topical capsaicin (cream) formulation with an updated (ethanolic solution) formulation by testing its potential to induce peripheral and central sensitization.

The main findings of this thesis are summarized and discussed in **Chapter 9**, which also includes general conclusions and recommendations on the use of experimental models in early-phase analgesic drug development.

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Table 1 Key POC methods often used in early-phase analgesic drug development.

Method	Details
TMS	**METHOD** A non-invasive, painless technique to stimulate the brain. Through a coil which is kept on the head of the subject, a magnetic pulse is applied to the brain. This magnetic pulse induces an electric field and electrical currents in the cortex, which in turn, if large enough, depolarize neurons and initiate action potentials. Because the focus of the magnetic field lies just beneath the coil, TMS activates a brain area in the superficial cortical layers of only a few centimeters in diameter. [154,155]
	PRO'S TMS provides the opportunity to assess cortical excitability, which can be regarded as a measure of how easily neurons and cortical networks are activated by the magnetic pulse. [154]
	CON'S Expensive method requiring specific knowledge to be performed and analyzed. Results are subject to operator-variability, and found effects may be difficult to interpret mechanistically.
	CLINICAL TRANSLATION widely used as supportive diagnostic tool for a variety of neurological diseases. [156]
UVB-induced hyperalgesia	**METHOD** First, the minimal dose of UVB needed to induce erythema (MED, in mJ/cm^2) is determined for each subject individually. Subsequently, 18-24h prior to planned test days, 2x or 3x MED is applied on healthy skin, to induce inflammatory hyperalgesia. Readouts include heat pain ratings using a thermode, or mechanical allodynia surrounding affected site using e.g., Von Frey filaments (i.e., secondary allodynia).[13]
	PRO'S Low inter- and intra-subject variability, stable hyperalgesia for 36 h
	CON'S Induction of post-inflammatory hyperpigmentation lasting 6 months (2xMED dose) up to multiple years (3xMED dose). Requires a more homogenous study population as irradiation levels needed to induce hyperalgesia are only safe in lighter-skinned individuals. [35]
	CLINICAL TRANSLATION Agreement between drug effects reported in this model, and trials in patients suffering from burn injury and postoperative pain. [157]
Capsaicin-induced hyperalgesia	**METHOD** Low concentration capsaicin is either topically applied and absorbed for a brief period (e.g., 30 min) or injected intradermally, to induce transient burn-like sensations on and around the treated area/location. The same readouts can be evaluated as mentioned for the UVB-induced hyperalgesia model.
	PRO'S Method that is easy to use, highly customizable and selectively links to TRPV1 agonism. The sensitizing effects after topical application are considered mild with effects approximately lasting a day. Both the topical and intradermal model induce reproducible primary heat sensitizationX. [158] Intradermal injection exerts reproducible effects on the area surrounding the area of application (i.e. secondary area).
	CON'S Subject burden for the intradermal model is high, as the injection induces a near-maximal pain sensation (rating of ~9/10). [159] After topical application, the response on the secondary area is highly variable both between subjects and within the same subject. [127,160]
	CLINICAL TRANSLATION Used as model to induce secondary hyperalgesia. In combination with mechanical stimulation, drug efficacy for this model is associated with effective treatment of trigeminal neuralgia and renal colic. [157] High concentrations are indicated in postherpetic neuralgia through temporary denervation that may last up to months. [13]
Cold pressor pain test	**METHOD** Variations of this test are available, all involving submerging (a part of) the hand or foot in cold water. Cold pressor test methodology often used (Eckhart et al., Jones et al.): A blood pressure cuff is inflated to 20 mmHg below resting-diastolic pressure, after which the ipsilateral hand is submerged in a cold water bath. Pain is rated using a rating scale (e.g., VAS) until pain tolerance or 120s is reached, whichever comes first. Subjects are instructed to remove their hand, the cuff deflates at that same time. [161,162]
	PRO'S broadly applicable, easily adoptable test that may also be used to induce the CPM response.
	CON'S temperature of the water, and ability of equipment to maintain set limit (i.e. by circulating water) results in evident differences between studies, limiting translatability of the model. [13]
	CLINICAL TRANSLATION used for diagnosing fibromyalgia or opioid-induced hyperalgesia [19]

CPM: conditioned pain modulation, MED: minimal erythema dose, TMS: transcranial magnetic stimulation, UVB: ultraviolet B, VAS: visual analogue scale

Figure 1 High-level illustration of definitions of allodynia and hyperalgesia With gradually increasing intensity of a pain stimulus, a normal pain response is expected to increase following a sigmoid curve, as described on the right part of the Figure. Allodynia is defined as perceiving a stimulus as painful where it normally would not be perceived as such, this is defined as allodynia. (blocked area under the left sigmoid curve) E.g., a stroke with a brush or feather that produces a painful sensation. Hyperalgesia is defined as having an increased sensitivity to a painful stimulus, that normally would also perceived as painful (striped area under the left sigmoid curve). E.g., a blow with a hammer that was rated with a pain intensity of 3 out of 10, where the pain typically would be rated as 1 out of 10.

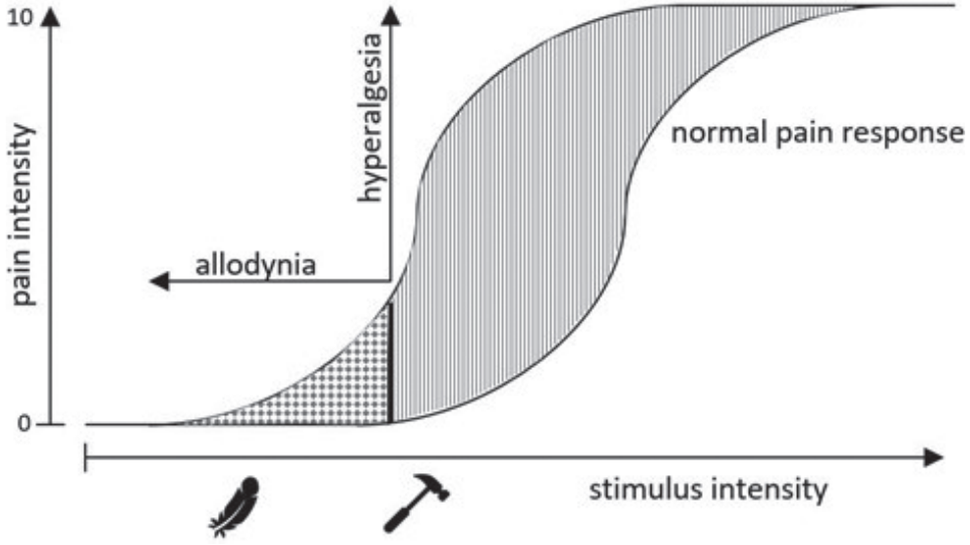
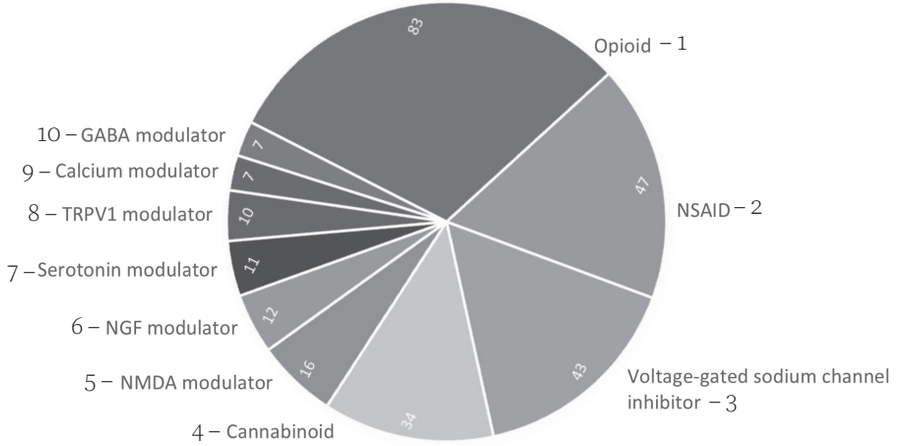


Figure 2 Top 10 analgesic drug classes currently in early phases of drug development (until the therapeutic exploratory phase (phase I/II)) Numbers represent number of unique compounds currently in development, per respective class.



GABA: gamma-aminobutyric acid; NaV: voltage-gated sodium channel; NMDA: N-methyl-D-aspartate receptor; NGF: Nerve Growth Factor; NSAID: non-steroidal anti-inflammatory drug; TRPV1: transient receptor potential cation channel subfamily V member 1.

Figure 3 Illustration of unique role of various Na_V channels in action potential generation. (Adapted from [40]) *(full color version of this illustration on inside of the cover)*

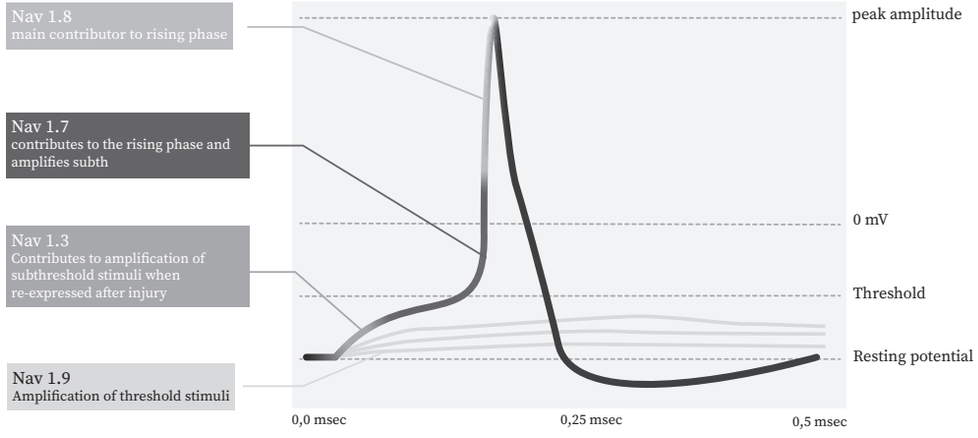
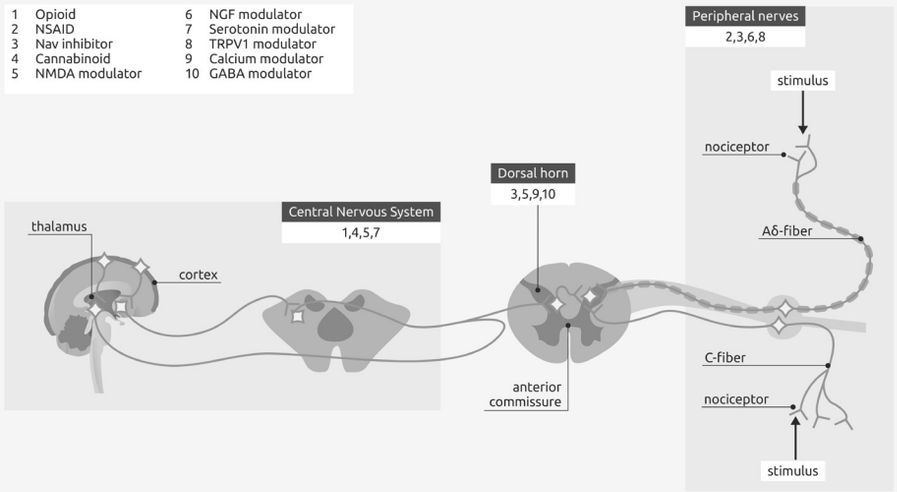


Figure 4 Schematic overview of primary target location per analgesic drug class. Drug classes are described with numbers, legend in top left corner describes which number links to which class. The pain pathway is described as having three distinctive target locations: the central nervous system, dorsal horn, and peripheral nerves. While specific drug classes may target multiple sites to a lesser extent as well, for sake of reasoning only the main target locations are linked to a specific drug class.



GABA: gamma-aminobutyric acid; NaV: voltage-gated sodium channel; NGF: Nerve Growth Factor; NMDA: N-methyl-D-aspartate receptor; NSAID: non-steroidal anti-inflammatory drug; TRPV1: transient receptor potential cation channel subfamily V member 1.

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[SUPPLEMENTARY MATERIAL AVAILABLE ONLINE AT THE PUBLISHER'S WEBSITE]

SECTION I

CHAPTER 2

A Phase 1, Randomized, Double-Blind, placebo-controlled, crossover study to evaluate the pharmacodynamic effects of vx-150, a highly selective $Na_V1.8$ inhibitor, in healthy male adults

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ABSTRACT

OBJECTIVE To evaluate the analgesic potential, safety, tolerability and pharmacokinetics of VX-150, a pro-drug of a highly selective $Na_V1.8$ inhibitor, in healthy subjects.

DESIGN This was a randomized, double-blind, placebo-controlled, crossover study in healthy subjects.

SUBJECTS Twenty healthy male subjects with an age of 18-55, inclusive, were enrolled. Eligibility was based on general fitness, absence of current or previous medical conditions that could compromise subject safety and a training assessment of pain tolerance across pain tests, to exclude highly tolerant individuals that could compromise the ability to detect analgesic responses. All dosed subjects completed the study.

METHODS Subjects were randomized 1:1 to 1 of 2 sequences receiving a single VX-150 dose and subsequently placebo, or vice versa, with at least 7 days between dosing. A battery of pain tests (pressure, electrical stair, (capsaicin-induced) heat and cold pressor) was administered pre-dose and repetitively up to 10 h post-dose, with blood sampling up to 24 h post-dose. Safety was monitored throughout the study. Data were analyzed with a repeated measures mixed-effects model.

RESULTS VX-150 induced analgesia in a variety of evoked pain tests, without affecting subject safety. Significant effects were reported for cold pressor and heat pain thresholds. Maximum median concentration for the active moiety was 4.30 ug/mL at 4 h post-dose.

CONCLUSION Results of this proof-of-mechanism study are supportive of the potential of VX-150, a highly selective $Na_V1.8$ channel inhibitor, to treat various pain indications.

INTRODUCTION

Pain is a protective mechanism designed to prevent tissue injury, but when persisting beyond its usefulness pain results in one of the most common and incapacitating chronic disorders for which patients seek medical attention. Although a variety of treatment options are available, current pharmacological therapies suffer from poor efficacy, or a high risk of adverse events (AEs). [1] For example, systemic lidocaine (a non-selective sodium channel inhibitor) may effectively reduce pain, but its utility is limited because of prominent side effects when given at dose levels that are required for pain relief. [2,3] Opioids, although prominently and ever increasingly used in the treatment of pain, have a high abuse liability. Annual death rates due to opioid overdose were approximately 47,000 deaths in the US in 2018 and were estimated to between 10,000-20,000 in Europe in 2014. [4,5] – Moreover, with long term use, opioids induce pain (i.e. hyperalgesia) instead of providing the intended pain relief.

The limited treatment options currently available – especially for patients suffering from chronic pain – and growing awareness of the risks that are associated with the standards of care, underscore the need for new pharmacological treatment options to manage pain. Certain subtypes of the voltage-gated sodium channels (Na_Vs), which facilitate electrical signaling in neurons, [6] have been identified as potential targets for selective analgesic drugs aimed to provide pain relief without unwanted side effects. The role these channels play in normal physiology, in pathological states arising from mutations in sodium channel genes and animal models, and of the pharmacology of known sodium channel modulating agents, together indicate that $Na_V1.3$, $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$ can play critical roles in pain signaling. [7–9] Of these Na_V subtypes, $Na_V1.8$ is a sensory neuron-specific channel with preferential expression in the dorsal root ganglion, and trigeminal ganglion neurons. [10] $Na_V1.8$ is highly expressed on nociceptors where it mediates pain sensation and chronic pain. [11] As such, $Na_V1.8$ gain-of-function mutations are thought to directly cause chronic pain in patients with painful small fiber neuropathy. [12–14] Moreover, $Na_V1.8$ has been found to quickly recover from inactivation and exhibit a more depolarized voltage-dependency of (in-) activation compared with other named subtypes, [15] highlighting its

involvement in repetitive firing and neuronal excitability [11] and so to central sensitization and chronification of pain. Inhibiting $Na_V1.8$ has been found to result in analgesia, [16,17] a finding which supported the channel as a pharmacological target and showed that selective $Na_V1.8$ inhibitors may have the potential to treat pain where the primary mechanism for pain is nociceptor hyperexcitability.

VX-150 is an orally bioavailable prodrug that rapidly converts into its active moiety, which is a highly selective inhibitor for $Na_V1.8$ relative to the other sodium channel subtypes (>400-fold) and being developed for the treatment of pain. To investigate the analgesic potential of novel compounds, such as VX-150, in early-phase trials with healthy volunteers, evoked pain tests may be included in the design. A variety of different pain tests related to different mechanisms that are involved in clinical pain have been developed to inform the investigator on the analgesic potential of a new investigational product. A comprehensive battery of different pain tests has been developed at our institution, which allows measurement of different mechanisms involved in nociception in an integrated manner and in a fixed and repeated fashion over-time. [18] Previously, this pain test battery has been used to show analgesic potential – and lack thereof – of a variety of analgesic compounds including certain Na_V inhibitors. [19,20] This study evaluated the analgesic potential of VX-150 in healthy males, a placebo-controlled cross-over fashion and reports effects of VX-150 in a multitude of endpoints. As literature suggests that pain perception of women may change across the menstrual cycle phase [21–23] we limited our study to men only to reduce variability and increase the chance of demonstrating a treatment effect in a phase 1 trial setting.

MATERIALS AND METHODS

The study was conducted at the Centre For Human Drug Research (CHDR), and executed in accordance with the Declaration of Helsinki (1964, amended most recently in 2008) of the World Medical Association and the Guideline for Good Clinical Practice. Prior to start of the procedures, the study received Medical Ethics Committee approval (from Stichting Beoordeling Ethiek Biomedisch Onderzoek (BEBO), Assen, The Netherlands). The study was registered under ToetsingOnline number NL63609.056.17 and EudraCT number 2017-003557-42.

Design

This was a phase 1, randomized, double-blind, placebo-controlled, two-way crossover study to evaluate the analgesic effects of VX-150 in healthy adult male subjects (**Figure 1**). A randomized crossover design was chosen to enhance the power to detect treatment differences by reducing the variability, which is lower when a within-subject comparison is used than the between-subject variability of a parallel arm study. Male subjects with an age of 18-55, inclusive, were screened for general fitness and current or previous medical conditions that could put the subject at risk or bias study results (e.g. neurological, mental and/or cardiovascular disease, (chronic) pain, significant allergies, malignancies or conditions affecting drug absorption). All participants voluntarily provided written informed consent prior to any of the study assessments. Any information, including illustrations, are as anonymized as far as possible to comply with privacy regulations.

Twenty male subjects were enrolled in a 1:1 ratio to one of the two treatment sequences (i.e. ten subjects per sequence) to receive a single dose of VX-150 or placebo, in two treatment periods (**Figure 1**). A washout period of at least 7 days was used between the two periods. Screening procedures were within 28 days before admission to the clinical research unit on Day -1 of the first treatment period; a safety follow-up visit 5-9 days after the last dosing day completed study participation. Both treatment periods consisted of an in-house period of two nights and one full study day each. Blood sampling for pharmacokinetics (PK) and a panel of pain tests as described below, was performed on Day 1 in both treatment periods.

Study drug VX-150 and placebo administration procedures

During each treatment period, a single dose of VX-150 1250 mg or placebo was administered as a capsule in the morning of Day 1 in fasted state. Compliance to dosing was performed with a hand-and-mouth check. The 1250 mg dose was chosen based on previous studies with VX-150, where it was found safe and well-tolerated. Results of those studies also indicated that maximum pharmacodynamic effects were expected to be observed for the current study when using this dose. (unpublished data)

Study procedures – safety

Safety evaluations included adverse event monitoring, clinical laboratory assessments, clinical evaluation of vital signs, standard 12-lead electrocardiograms (ECGs), and physical examinations.

Study procedures – pharmacodynamic (PD)

During each treatment period, nociceptive (pain) detection and tolerance thresholds were evaluated repeatedly over time using a validated battery of evoked pain models, with the following sequence: pressure pain test, electrical stair pain test (1), cold pressor pain test, electrical stair pain test (2), heat pain test on untreated skin, and heat pain test on capsaicin-treated skin. The heat pain test on capsaicin-treated skin and untreated skin were switched prior to dosing to allow for the pre-dose heat pain test on capsaicin-treated skin to be performed 30 minutes after capsaicin administration while keeping remainder of the sequence intact (capsaicin model described further along this section). Prior to enrollment and as part of the screening procedures, subjects received a training session in order to minimize learning effects and to exclude any subjects from study participation indicated to be too sensitive or tolerable to the tests. The latter was defined as achieving tolerance at more than 80% of the maximum input intensity for the cold pressor-, electrical-, or pressure pain test. Subjects were allocated to a separate room without any form of distraction, where they were asked to sit in a chair. For all but the thermal and capsaicin test (details further along this section), subjects were given an electronic visual analogue scale (eVAS) slider to hold, with which they could indicate their current perceived pain intensity. The eVAS ranged from 0-100. 0 defined 'no pain', eVAS > 0 the Pain Detection Threshold (PDT), and eVAS = 100 the Pain Tolerance Threshold (PTT; 'worst pain tolerable'). When PTT was reached, the test automatically stopped and immediately relieved subjects from their pain. Per measurement, eVAS versus time was used to calculate the Area Above the eVAS pain Curve (AAC; for the cold pressor pain test) or Area Under the eVAS pain Curve (AUC; for the pressure-, electrical stair pain test and CPM). In both treatment periods, the complete test sequence was performed twice pre-dose, and at 1, 2, 4, 7 and 10 h post-dose.

ELECTRICAL STIMULATION PAIN TEST The method of electrical stimulation is based on that of Arendt-Nielsen et al, and used in previous studies using the same integrated pain test battery. [19,24–26] The test has been shown to primarily assess nociception generated from the Aδ- and C-sensory afferent fibers, which pass nociceptive signals from the periphery to the spinal cord. The Aδ-fibers conduct the signal relatively rapidly, causing the sharp localization of pain and the rapid spinal response which is perceived during a transcutaneous electrical stimulus.[27]

Two electrodes (Ag-AgCl) were positioned on clean (if needed, scrubbed) skin on the left tibial bone. Location of the first electrode was 100 mm distal the caudal end of the patella; the second electrode 135 mm directly underneath the first. Resistance between the electrodes was less than 2 kΩ. The single (stair) stimuli that were given (10-Hz tetanic pulse, duration of 0.2 ms) were controlled by a computer-controlled constant current stimulator. Intensity of the current increased from 0 mA in steps of 0.5 mA/s. Pain intensity was measured using the eVAS, until pain tolerance level (PTT), or the maximum output of 50 mA was reached.

PRESSURE PAIN TEST The method to induce pressure pain in this study, has been shown to primarily assess nociception generated from the muscle with minimal contribution by cutaneous nociceptors, [28] and is based on methods previously described.[29]

A constant pressure, increasing with of 0.5 kPa/s (controlled by an electro-pneumatic regulator (ITV1030-31F2N3-Q, SMC Corporation, Tokyo, Japan), Power1401mkII analogue–to-digital converter and Spike2 software (CED, Cambridge, UK)) was forced on the gastrocnemius muscle using an 11 cm wide tourniquet cuff (VBM Medizintechnik GmbH, Sulz, Germany). Pneumatic pressure increased until the subject indicated his PTT, or maximum pressure of 100 kPa was achieved, at which point the device released pressure to the tourniquet.

COLD PRESSOR PAIN TEST For the cold pressor test, an extremity (in this study a hand) is submerged into cold water. This assessment is used in clinical studies to investigate cardiovascular responses, nociception, opioid-induced hyperalgesia, and to induce a conditioned pain modulation response (CPM, previously termed diffuse noxious inhibitory control (DNIC)-like effects, see 2.4.4.).[30,31] The method used here is based

on methods described previously. [19,32,33] In summary, the subject was asked to put his non-dominant hand into a water bath with circulating water (minimal depth 200 mm) at 35 ± 0.5°C for 2 min. At 1 min 45 sec, a blood pressure cuff on the upper arm – placed there before start of the test – inflated to 20 mmHg below resting diastolic pressure, to minimize return of warm blood to the hand. At 2 min, the subject moved their non-dominant hand from the warm water bath into a similar sized bath with circulating water at 1.0 ± 0.5°C. Subjects were instructed to indicate their PDT (first change in sensation from cold non-painful to painful), increase in pain intensity and PTT (cold sensation is no longer tolerable) using the eVAS slider. When PTT was reached or when the non-dominant hand was in the 1.0°C water for 120 s, subjects removed their hand from the water, at which point the blood pressure cuff would also deflate. Time to reach PDT and PTT, or time limit of 120 s, was used for analysis.

CONDITIONED PAIN MODULATION (CPM) Effects of VX-150 on the descending inhibitory control pathway were evaluated using the CPM paradigm.[30] By calculating the difference of pain detection- and pain tolerance thresholds of the electrical stair pain test directly after the cold pressor pain test, minus the electrical stair pain detection and tolerance thresholds prior to the cold pressor pain test, a possible modulatory effect was quantified.

APPLICATION OF CAPSAICIN 1% CREAM; CAPSAICIN-INDUCED PAIN TEST AND HEAT PAIN TEST Capsaicin 1% cream was used as a model for cutaneous heat sensitization, by selectively agonizing the transient receptor potential cation channel subfamily V member 1 (TRPV1) channel. [34–36]

Capsaicin 1% cream, produced according to the Formulary of Dutch Pharmacists (Formularium der Nederlandse Apothekers, FNA), was applied during screening procedures, to evaluate whether subjects were hyperresponsive to the cream, and in both treatment periods applied 60 min prior to study drug administration. A 3×3 cm surface on the dominant volar forearm was used for the application of 1% capsaicin cream, after which the area was covered by a cotton gauze. The non-dominant volar forearm served as a non-stimulated control (i.e. not treated with capsaicin). 30 min post-application, the cream was wiped off towards the center of the application site.

Immediately after and subsequently at given time points, heat PDTs were determined on the capsaicin-treated skin (on dominant volar forearm), as well as on normal (non-stimulated) skin (on non-dominant volar forearm). To evaluate these PDTs, a thermode (Q-Sense, Medoc, Israel) with a contact area of 3 cm×3 cm was placed on a marked area on the subject's non-dominant volar forearm, and on a marked area on the subject's dominant volar forearm (on which capsaicin was applied). Starting at 32°C, the temperature of the thermode increased by 0.5°C/s until the subject perceived the stimulus as painful (PDT) or when a temperature of 50°C was reached. PDT was recorded by the subject pushing a button on the hand-held feedback control. The average of a triplicate measurement was used for analysis.

Study procedures – PK

Plasma PK parameters were assessed for the active moiety of VX-150 and its major circulating metabolite. Blood was sampled before dosing (0 h (hours)), and at 0.5, 1, 1.5, 2, 3, 4, 5, 7, 10, 12, and 24 (Day 2) h after dosing in both treatment periods.

Statistical considerations and analysis

SAMPLE SIZE The size of twenty subjects was chosen based on known variability in the cold pressor and capsaicin PD assessments [19,26] and was considered sufficient to meet the objectives of the study. For a one-sided significance level of 0.05, there was at least 80% power to detect a standard effect size of 0.6.

STATISTICAL ANALYSIS Demographic and PK data are presented as mean ± standard deviation (SD). Analyses of plasma concentration versus time data for the active moiety of VX-150 and its circulating metabolite were determined using standard noncompartmental methods.

The period baseline value was defined as the average of the non-missing pretreatment measurements for all pain tests, except the capsaicin-induced pain test. For the capsaicin-induced pain test, the second predose assessment served as baseline given there was no capsaicin applied prior to this assessment taking place.

The change from period baseline in each primary endpoint was analyzed as a dependent variable with a repeated measures mixed model,

with sequence, period, treatment, time point within period, and treatment by time point interaction as fixed effects, and subject nested within sequence as a random effect. Denominator degrees of freedom for the F-test for fixed effects were estimated using the Kenward-Roger approximation. The least squares (LS) means and the 95% confidence interval (95% CI) of treatment difference at each post-dose time points are given. For the secondary endpoints, a summary of raw values and change from period baseline values were provided at each scheduled time point by treatment group and overall using descriptive statistics.

To calculate the effect size – defined as the estimate of difference between the VX-150 and placebo contrast – over the whole period, all repeatedly measured parameters were analyzed with a mixed effects model with treatment, time and treatment by time as fixed factors and subject, subject by treatment and subject by time as random factors and the (average) period baseline measurement as covariate. The Kenward-Roger approximation was used to estimate denominator degrees of freedom and model parameters were estimated using the restricted maximum likelihood method. The cold pressor variables AAC, PDT and PTT, and the Pressure PDT and PTT are log transformed before analysis due to their log-normal distribution. Results are back transformed and expressed as percentage difference for the estimated difference between treatments.
The effect size calculation was performed post-hoc in order to compare study results to previous studies with the same pain test battery at an exploratory level.[19,20,26] All statistical inferences and p-values were also exploratory. Therefore, no multiplicity adjustment was performed for any of the PD analyses.

RESULTS

Baseline characteristics

Twenty male subjects were enrolled and completed all study assessments. Mean age was 27.9 (8.6) years, most (70%) were of Caucasian descent and mean body mass index was 23.18 (2.77). Demographic and baseline characteristics are given in **Table 1**.

Pharmacodynamic results

PRIMARY ENDPOINTS Significant effects of VX-150 were observed on the cold pressor- and heat pain test (**Table 2**). For the cold pressor test PTT, LS mean changes from baseline were substantially higher in the VX-150 1250 mg treatment group compared to placebo from 2 through 10 h post-dose; the largest treatment effect was observed at 4 h post-dose, although also significantly differed at 2, 4, 7, and 10 h post-dose (LS mean difference for 95% confidence interval (LSM 95% CI), placebo versus VX-150 per timepoint, at 1h: (-1.92, 5.31); 2h: (0.8, 20.12); 4h: (12.74, 42.72); 7h: (8.43, 32.33) and at 10h: (8.43, 32.33)). For heat PDTs on untreated skin ('normal heat PDTs'), thresholds in the placebo group were consistently lower compared to VX-150 at each time point, but only significantly differed at 2 and 10 h post-dose (**Table 2**) (LSM 95% CI, placebo versus VX-150 per timepoint, at 1h: (-0.4751, 1.0208); 2h (0.0806, 1.5764); 4h: (-0.0716, 1.4242); 7h: (-0.0234, 1.4724); and at 10h: (0.1822, 1.7070)). No significant effects were reported for capsaicin-induced PDT, or electrical stimulation-, pressure- or CPM PTT.

SECONDARY ENDPOINTS For the electrical stimulation- and cold pressor pain test, PDTs were higher after VX-150 treatment compared to placebo at each timepoint, but did not greatly differ (**Table 2**). CPM and pressure PDT results did not evidently differ between treatments.

TREATMENT EFFECT OVER 10 H (EFFECT SIZE ANALYSIS) Cold pressor PTT displayed the largest effect size of 53.7% when comparing VX-150 to placebo when analyzed over the full time course of 10 h, followed by pressure PTT (6.76%), electrical stair PTT (2.76%), capsaicin heat PDT (1.81%) and heat pain PDT (1.6%) (**Figure 2**, **Table 3**). Effects were significant for both cold pressor PTT and AAC (PTT: *p<0.001,* estimate of difference (ED): 53.7%, 95% CI: 24.9 – 89.2%, AAC: *p=0.002*, ED: 43.7%, 95% CI: 16.2 – 77.3%), as well as heat pain PDT (*p=0.01* 95% CI: 0.16 – 1.23) (**Table 3**). Results for other endpoints were not significant.

SAFETY Overall, VX-150 was well tolerated with no significant findings during study execution. AEs that were reported were evaluated to be mild or moderate in severity, incidence was comparable in subjects receiving placebo or VX-150 treatment, none led to study discontinuation. Most

reported AEs were headache and catheter site pain. There were no clinically meaningful changes or trends in laboratory (chemistry, hematology, coagulation, and urinalysis) values, vital sign measurements, or ECG. Two subjects (10%) while in the VX-150 treatment group received an analgesic as concomitant medication (ibuprofen and paracetamol) to treat headache. In both cases, the medication was administered well after the last pain test was performed.

Pharmacokinetic results

Mean plasma concentration-time profiles of the active moiety and its major circulating metabolite after the administration of single oral doses of 1250 mg VX-150 are presented in **Figure 3**, related parameters found in Supplementary **Table S1**. After having increased to its peak concentration at 4.30 µg/mL at 4 h post-dose, VX-150 gradually decreased afterwards – describing a PK profile common for a capsule formulation and in line with results from earlier studies evaluating the PK of VX-150 as capsule formulation (unpublished data).

DISCUSSION

This study was performed to evaluate the analgesic potential of a single dose of VX-150 in a panel of pain tests in healthy adult male subjects. Overall, VX-150 demonstrated an analgesic response at up to 10 h for a subset of pain tests without displaying any notable adverse effects, thereby favoring $Na_V1.8$ inhibitor VX-150 as a potential treatment for pain.
Despite the fact that selective Na_V inhibitors have been considered as an important possible alternative for opioids in pain treatment, [6,8] they have yet to live up to that promise. As such, multiple studies could not report analgesic effects for various selective Na_V inhibitors. [6,20,37] This study is the first to report significant analgesic effects of a selective $Na_V1.8$ inhibitor in a human experimental pain study, favoring the use of selective $Na_V1.8$ inhibitors as analgesics. Here, we show that VX-150 primarily influenced cold pressor pain thresholds, most likely by indirectly modulating transient receptor potential subfamily M, member 8 (TRPM8) activity. This non-selective ion channel is present on both Aδ- and C-fibers, where it is activated by cooling agents such as menthol and cold temperatures as during the cold pressor pain test. [38,39] TRPM8-mediated pain

sensation is through increased calcium influx of voltage gated calcium channels following activation of $Na_V1.8$. When blocked by VX-150 pain relief is achieved. The interplay between TRPM8 and $Na_V1.8$ has previously been described in both models of sensory neurons and breast cancer. [40,41] Although $Na_V1.8$ is not directly affected by heat, it is essential for propagation of and sustaining the pain signal that follows activation of heat-sensitive TRPV1 and -3 channels, explaining the significant effects reported for the heat pain test, and suggestive (non-significant) effects over time for capsaicin-induced hyperalgesia. [42–44] Effects of VX-150 on capsaicin-induced pain thresholds were also expected, as capsaicin induces an inflammatory-like hyperalgesia that only can be attenuated by blocking tetrodotoxin-resistant channels such as $Na_V1.8$. [45] Given the sample size of the study, the limited effect size and variability observed within our test results (**Table 2**) may have prevented the VX-150-treated group from differing significantly from placebo (**Figure 2**). These assumptions also hold true for the electrical pain-, CPM, and pressure pain paradigms, as no significant effects could be noted for either test. While reasons are speculative, plausibly the absence of effects on the electrical pain test – which induces pain by activating nerves directly, bypassing the sensory nerve endings – may be due to the test not specifically activating nociceptors, therefore is not modulated by alterations in $Na_V1.8$ signaling.[24] For CPM, it may be that VX-150 has an insufficient role in the inhibitory descending pain pathway, but – as stated – may in an equal chance be attributed to individual subject variability, when noting that CPM is particularly influenced by this.[30,46] For pressure pain, the tolerance increase observed in the placebo group up to 5h post-dose (**Figure 2**) may have diminished the treatment effect reported for VX-150. It, however, may also be worthwhile considering that a different mechanical pain test (e.g., assessment of secondary mechanical allodynia surrounding the capsaicin-treated skin using Von Frey filaments) may have been more applicable, given there is preclinical evidence available describing a link between $Na_V1.8$ and mechanical allodynia in relation to neuropathic and inflammatory pain models, but not in regard to solely assessing pressure pain, as reported here. [47,48] It must thus be noted that, a priori, we did not expect VX-150 to influence all pain tasks – as such, no (analgesic) drug is expected to influence all tests we included. Rather, the integral combination of evoked pain models is used to profile the analgesic effects, and magnitude of observed effects, for each compound specifically. This

allows for benchmarking of tested drugs as briefly touched upon in the introduction section, discussed in more detail previously[19,26] and in the last paragraph of the discussion section, hereunder.

Following the discovery of $Na_V1.7$-deficiency underlying insensitivity to pain, [49] $Na_V1.8$ has been studied as an analgesic target for conditions where the mechanism is related to peripheral nociceptor hyperexcitability. Nonclinical studies have reported that $Na_V1.8$ inhibitors in addition to reversing cerebellar deficits in a rodent model of multiple sclerosis, showed potential to treat multiple pain conditions including neuropathic and inflammatory. [50] Specifically, the $Na_V1.8$ inhibitor A-803467 attenuated mechanical and thermal hyperalgesia in diabetic rats, reduced neuropathic pain in the L5/L6 spinal nerve injury model, in the chronic constriction injury of sciatic nerve model and in the capsaicin-induced secondary mechanical allodynia model, as well as reduced thermal hyperalgesia in the Complete Freund's adjuvant model for inflammatory pain [51–54]. Here, we used an integral nociceptive test battery to confirm preclinical results and characterize the analgesic profile of VX-150, in an effort to bridge the gap to later-phase clinical trials. For the latter, each pain modality was plotted against the observed treatment effect size over the full 10 h time course (**Figure 4**, **Table 3**). Again, the most pronounced effect was observed in the cold pressor pain test, a model also found sensitive to neuropathic pain treatments as pregabalin and mexiletine. [26], **Chapter 4**] Recently, two clinical proof-of-concept trials were completed in which the efficacy of VX-150 was evaluated for two pain phenotypes. Not only did VX-150 relieve acute pain in patients that underwent bunionectomy surgery, it also reduced pain ratings in 46 patients with chronic pain caused by small fiber neuropathy. [55,56] Both studies align with results here, i.e. the rapid onset of analgesia (acute pain) and most pronounced results in the cold pressor pain test (a model sensitive to certain neuropathic pain treatments).

To the best of our knowledge, this study is the first to report analgesic effectiveness of a selective Na_V inhibitor in an experimental pain study with healthy volunteers. Previously, we were not able to show any effects of a selective $Na_V1.7$ inhibitor (PF-05089771) using the same pain test battery. [20] While we cannot be certain, this plausibly may be due to either one, or a combination, of the following reasons. Both compounds, although termed similar, represent a different class (i.e., $Na_V1.7$ vs $Na_V1.8$ inhibitors). $Na_V1.7$ is thought to act as a threshold channel, whereas the

contribution of $Na_V1.8$ to signal conductance lies with repetitive firing and neuronal excitability; [11,57] thereby arguably resulting in distinctive effects when either channel is inhibited. Results of the $Na_V1.7$ inhibitor study in that sense could, for example, have benefitted from having included the TRPV1-sensitizing capsaicin model, when noting that in a later-phase clinical trial PF-05089771 significantly reduced burning pain sensations in diabetic neuropathy patients (DNP); suggesting a link between $Na_V1.7$ and TRPV1 on the peripheral nociceptor terminals. [58] Furthermore, the dose, potency and/or the extent of blood-brain-barrier penetration of the two compounds can significantly differ, thereby resulting in the discrepancy of results discussed here.

The results of the study that we report here must be read with the following considerations. First, as literature suggests that pain perception of women may change across the menstrual cycle phase [21–23] we limited our study to men only to reduce variability and increase the chance of demonstrating a treatment effect in phase 1 setting. Whether effects on pain thresholds are exerted in both women and men remains to be seen, however, this is very likely in view of the identical role of $Na_V1.8$ in nociceptive nerve function in men and women. The electrical stair test following the cold pressor test was used to observe possible effects of the conditioned pain modulation (CPM) response. Heat PDTs were quantified after this second electrical stair test (see section **Study procedures – pharmacodynamic (PD)**), to increase logistical feasibility of including both two baseline pain test sequences with application of capsaicin, pre-VX-150 administration. The heat pain test therefore may have been influenced by an ongoing CPM response. The potential bias on heat PDTs – if present at all – will however have been limited given CPM effects are generally only short-lived. [30,59–62] In addition, the effect of VX-150 on pain was quantified in a controlled setting in which pain tests were always performed in the same order; therefore, affecting all results equally during each crossover period. Unadjusted multiple testing was performed to assess VX-150's temporal effect (primary analysis, **Table 2**) and the size of its total analgesic effect (**Table 3**). While we acknowledge the increased risk of reporting erroneous inferences, the effect size analysis was performed as an add-on to allow for comparing study results presented here to others using the same pain test battery, [19,20,26,63] and was deemed reasonable given the experimental nature of the study.

Experimental pain studies are of major importance for the investigator as obtained results may aid in decision making during the early phases of drug development. By repeatedly testing a fixed sequence of distinctive pain modalities over time, valuable data are collected that can inform on the active dose range and analgesic profile, as now for VX-150 and previously for a variety of other compounds with distinctive mechanisms of action. [18,19,63–65] Evoked pain models can thus provide confidence in proceeding with a compound to the next trial phase, or help evade questions on whether the right dose and/or patient population has been chosen, later-on in the development. For VX-150, the substantial response on the cold pressor PTT from 2 h up until the last time point at 10 h post-dose with an over-time effect size of 53.7% (**Figure 4**), informs on robust acute analgesic effects; outperforming 300 mg pregabalin and 3μg/kg fentanyl – both well-known analgesics for treating neuropathic- and acute pain, respectively – on the same test (effect size of treatment versus placebo of 46.4 and 17.1%, respectively). [19] Combined with previous work, the translatability of $Na_V1.8$ models from non-clinical, to experimental pain studies and eventually to clinical stage seems to up the ante in the search for novel selective non-opioid analgesics.

CONCLUSION

VX-150 induced analgesia in a variety of evoked pain tests, without affecting subject safety. Results of this proof-of-mechanism study are therefore supportive of the analgesic potential of VX-150, a highly selective $Na_V1.8$ channel inhibitor.

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Table 1 Demographic and other baseline characteristics, represented as mean (± SD) of total subject set.

Demographic category	Number (N = 20)
SEX, N (%)	
Male	20 (100.0)
AGE (YEARS)	
Mean (SD)	27.9 (8.6)
RACE, N (%)	
Caucasian	14 (70.0)
Black or African American	3 (15.0)
Asian	1 (5.0)
Mixed	1 (5.0)
Other	1 (5.0)
WEIGHT (KG)	
Mean (SD)	74.6 (10.3)
HEIGHT (CM)	
Mean (SD)	179.3 (6.6)
BMI (KG/M^2)	
Mean (SD)	23.18 (2.77)

BMI: Body Mass Index. Cm: centimeters, Kg: kilograms. SD: standard deviation.

Table 2 Primary analysis for pain thresholds.

		Pain test endpoints			
		PDT		PTT	
Pain modality		Placebo	VX-150	Placebo	VX-150
Capsaicin (°C)	Baseline	36.45 ±2.25	35.69 ±2.09	NA	
	1 h	39.71 ±2.59	39.91 ±2.86) (-0.56, 2.48)		
	2 h	40.17 ±2.55	40.32 ±3.17 (-0.73, 2.55)		
	4 h	40.14 ±2.86	40.84 ±3.09 (-0.19, 3.12)		
	7 h	40.98 ±2.83	41.41 ±2.90 (-0.42, 2.82)		
	10 h	41.23 ±2.54	41.53 ±3.12 (-0.53, 2.64)		
Heat (°C)	Baseline	44.63 ±2.81	44.50 ±2.32	NA	
	1 h	44.09 ±2.84	44.16 ±2.33 (-0.48, 1.02)		
	2 h	43.7 0±3.35	***44.39 ±2.65 (0.08, 1.58)***		
	4 h	43.58 ±3.14	44.15 ±2.87 (-0.07, 1.42)		
	7 h	43.32 ±3.20	43.94 ±2.63 (-0.02, 1.47)		
	10 h	43.16 ±3.67	***44.07 ±3.20 (0.18, 1.71)***		

(Table continues on next page)

		Pain test endpoints			
		PDT		PTT	
Pain modality		Placebo	VX-150	Placebo	VX-150
Cold pressor (s)	Baseline	6.86 ±4.83	6.09 ±3.82	21.00 ±12.19	21.48 ±11.60
	1 h	8.12 ±6.26	7.02 ±4.68	21.17 ±11.06	23.43 ±11.87 (-1.92, 5.32)
	2 h	6.53 ±4.73	8.37 ±8.02	23.21 ±13.36	***34.78 ±25.92 (0.80, 20.12)***
	4 h	6.56 ±4.19	7.52 ±7.98	20.46 ±11.53	***48.63 ±37.19 (12.74, 42.72)***
	7 h	6.22 ±5.18	8.27 ±7.71	18.52 ±11.71	***40.59 ±32.19 (8.43, 32.33)***
	10 h	6.05 ±5.35	10.05 ±13.68	20.00 ±12.75	***40.89 ±32.45 (6.16, 33.03)***
Electrical (mA)	Baseline	7.56 ±4.86	7.41 ±3.88	18.45 ±7.05	18.66 ±7.39
	1 h	6.98 ±4.28	7.68 ±5.20	18.71 ±7.62	18.60 ±7.64 (-1.90, 1.29)
	2 h	7.77 ±4.05	8.09 ±5.90	19.09 ±7.12	19.39 ±7.89 (-1.61, 1.78)
	4 h	8.28 ±4.27	9.02 ±6.75	18.38 ±6.58	19.66 ±8.77 (-1.11, 3.14)
	7 h	7.91 ±4.22	9.27 ±6.17	18.93 ±7.20	20.54 ±8.03 (-1.00, 3.62)
	10 h	8.62 ±5.62	9.29 ±6.69	20.46 ±8.33	21.24 ±7.61 (-2.36, 3.21)

(Table continues on next page)

(Continuation Table 2)

		Pain test endpoints			
		PDT		PTT	
Pain modality		Placebo VX-150		Placebo VX-150	
Pressure (kPa)	Baseline	21.27 ±13.49	22.66 ±12.78	48.31 ±19.81	47.42 ±15.37
	1 h	23.63 ±16.95	22.82 ±13.76	49.97 ±18.25	48.31 ±17.27 (-7.55, 5.32)
	2 h	24.82 ±15.15	24.70 ±17.10	52.46 ±20.80	53.04 ±21.66 (-4.00, 7.62)
	4 h	24.79 ±16.40	25.38 ±18.49	53.48 ±18.11	55.03 ±22.07 (-3.87, 9.65)
	7 h	22.83 ±14.89	25.02 ±18.60	48.92 ±16.99	53.92 ±21.10 (-1.17, 11.63)
	10 h	22.35 ±14.18	25.70 ±17.90	49.28 ±19.97	54.86 ±24.78 (-3.11, 15.72)
CPM (mA)	Baseline	0.44 ±1.44	0.21 ±2.51	0.64 ±1.64	0.72 ±1.29
	1 h	1.38 ±3.57	1.05 ±2.21	1.19 ±1.55	0.86 ±1.36 (-1.72, 0.84)
	2 h	0.94 ±2.56	1.24 ±2.83	0.81 ±1.60	1.50 ±2.14 (-0.78, 1.80)
	4 h	0.13 ±2.62	1.01 ±2.36	1.14 ±1.51	0.82 ±1.94 (-1.61, 0.96)
	7 h	1.16 ±2.29	0.71 ±3.11	1.46 ±1.79	1.07 ±1.99 (-1.63, 0.93)
	10 h	0.95 ±1.34	1.20 ±2.77	1.14 ±1.56	1.44 ±2.39 (-1.14, 1.45)

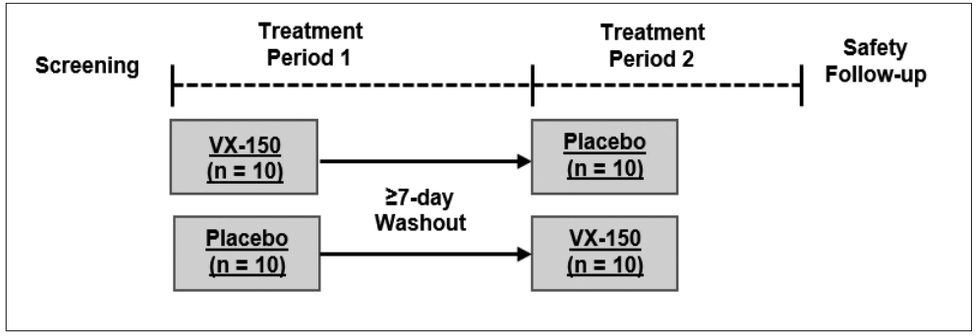
Values represent mean ±SD. Least square mean difference for 95% confidence interval (LSM 95% CI) are presented between brackets in the VX-150 column for the primary endpoints (i.e. capsaicin PDT, heat PDT, cold pressor PTT, electrical PTT, pressure PTT and CPM PTT). Only descriptive analysis was performed for the other (secondary) endpoints. Bold marked numbers denote at which timepoints LSM 95% CI between the placebo and VX-150 group excluded 0, and represent the treatment that was favored (e.g. if in the right column, the interval favored VX-150). °C: degrees Celsius, CPM: conditioned pain modulation, h: hour, kPa: kilopascal, mA: milliampere, PDT: pain detection threshold, PTT: pain tolerance threshold, s: second, SD: standard deviation.

Table 3 PainCart evoked pain model results. Effect size analysis from pre-dose up until 10 h post-dose.

Pain test modalities (contrast placebo versus VX-150)						
	Capsaicin	Heat	Cold pressor	Electrical	Pressure	CPM
PDT	0.728 °C *(p=0.07)* (-0.07 – 1.53)	**0.694 °C *(p=0.01)*** (0.16 – 1.23)	14.8 % *(p=0.488)* (-23.9 – 73.0)	0.88 mA *(p=0.137)* (-0.31 – 2.07)	-12.3 % *(p=0.154)* (-27.2 – 5.6)	-0.147 mA *(p=0.692)* (-0.94 – 0.65)
PTT			**53.7 % *(p< 0.001)*** (24.9 – 89.2)	0.53 mA *(p = 0.428)* (-0.84 – 1.89)	3.2 % *(p=0.557)* (-7.7 – 15.4)	0.080 mA *(p=0.748)* (-0.439 – 0.60)
AAC/AUC			**43.5 % *(p=0.002)*** (16.2 – 77.3)	-61.80 mA*% *(p=0.333)* (-192.47 –68.87)	-197.28 % *(p=0.445)* (-728.62 – 334.05)	24.38 mA*% *(p=0.4098)* (-36.86 – 85.62)

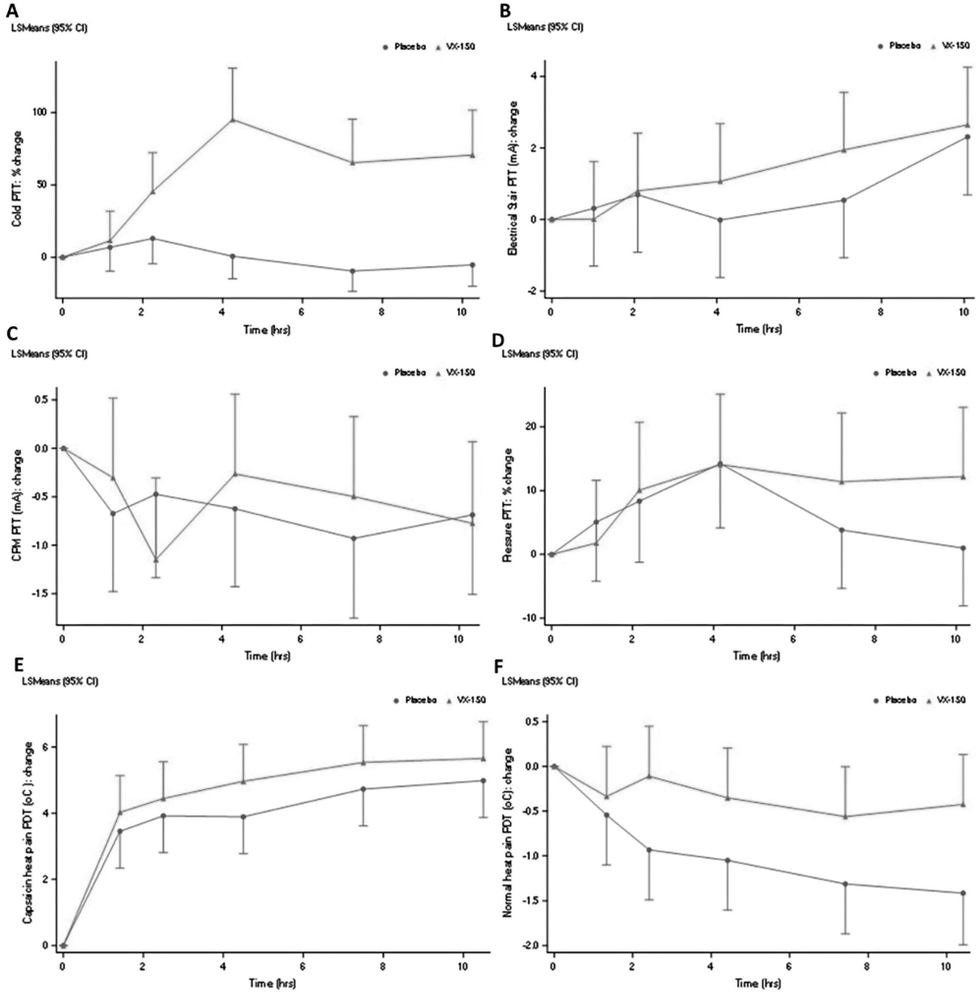
Numbers represent estimates of the difference, next to the p-value which is displayed in italic. Lower and upper limit of 95% confidence interval are shown between parentheses. Values are presented in % for tests of which the data were log transformed (i.e. cold pressor- and pressure pain test), otherwise in the unit in which they were measured. Bold values denote nominal significance ($p < 0.05$). Estimates >0 favor VX-150, estimates <0 favor placebo. °C: degrees Celsius, CPM: conditioned pain modulation paradigm, AAC/AUC: area above/under the eVAS pain curve, eVAS: electronic Visual Analogue Scale, mA: milliampere, PDT: pain detection threshold, PTT: pain tolerance threshold.

Figure 1 Study design Twenty subjects were randomized and equally allocated to one of the two treatment sequences.



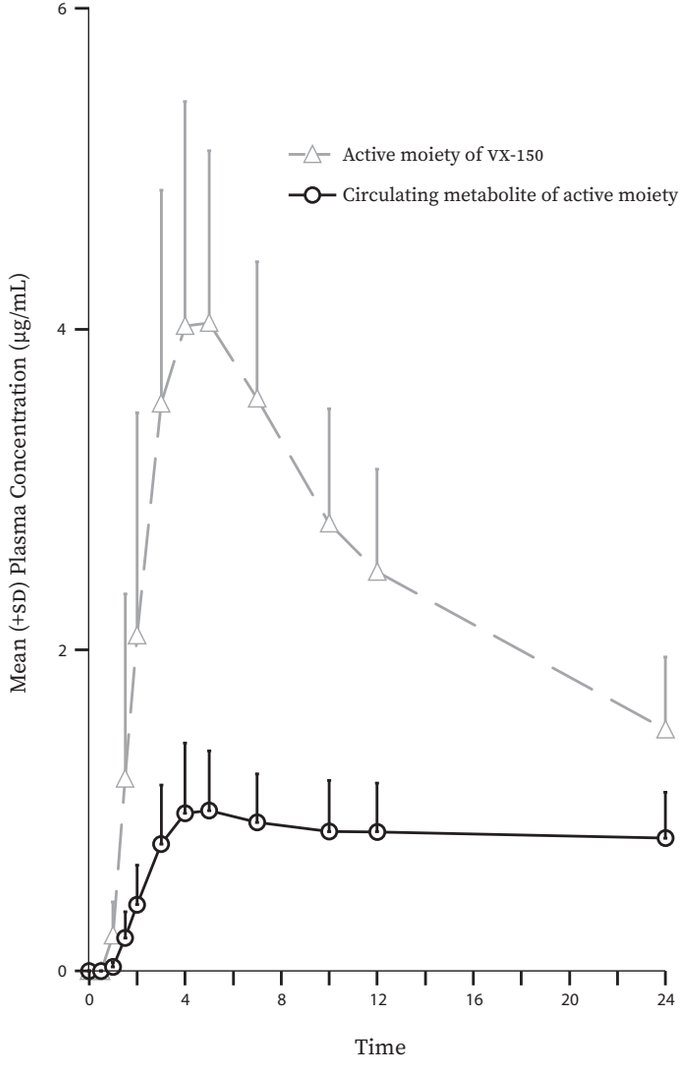
n = number of subjects.

Figure 2 Primary PainCart test endpoints, presented as change from baseline in percentages (%) Baseline has been defined as the average of two pre-dose measurements of that occasion, except for the capsaicin-induced PDT. For this test, the second pre-dose assessment served as baseline given there was no capsaicin applied prior to this assessment taking place. Values on y-axis represent the least square means change and the 95% confidence interval, time is described in hours on the x-axis.



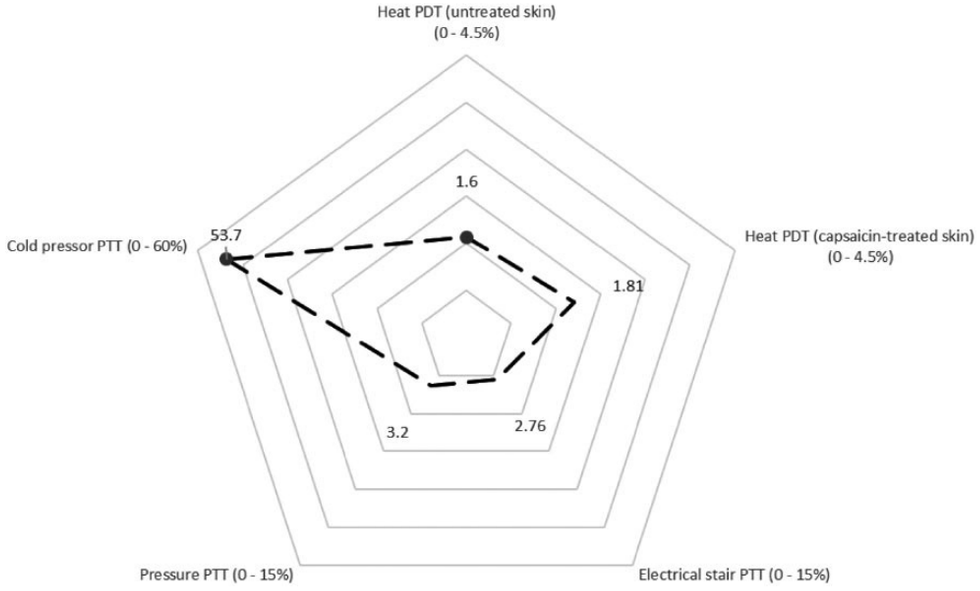
a: cold pressor PTT; b: electrical stair PTT; c: CPM PTT; d: pressure PTT; e: heat PDT on capsaicin-treated skin ('capsaicin heat PDT'); f: heat PDT on untreated skin ('normal heat PDT'). °C: degrees Celsius, CPM: conditioned pain modulation, mA: milliamperes, PDT: pain detection threshold, PTT: pain tolerance threshold.

Figure 3 PK results Mean concentration of VX-150's active moiety, and of its major circulating metabolite (in µg/mL, on x-axis) following single oral doses of 1250 mg VX-150 over time (in hours, on y-axis). Data represented on a linear scale.



µg: micrograms, h: hours, mL: milliliter, SD: standard deviation.

Figure 4 Analgesic profile of 1250 mg VX-150 Visualization of the effect size of VX-150 per pain modality, defined as the ED between the least square means of the contrast placebo – VX-150. Round markers for heat pain PDT and cold pressor PTT indicate a significantly different treatment effect of VX-150 compared to placebo over the complete time course of pre-dose up until 10 h post-dose ($p < 0.05$). Percentage ranges provided in parentheses reflect the range of responses reported across a battery of analgesics summarized in an earlier report of this profile model; except for the cold pressor PTT, which had to be increased from 0-50% to 0-60% to reflect the larger effect size of VX-150 observed in this study. [19] For cold pressor PTT and pressure PTT, the ED as included in table 3 was used as the data for these endpoints were log-transformed for analysis and therefore already presented in %. For other endpoints, as those were not log transformed, the ED was divided by the first least square mean of the contrast (i.e. of placebo) and multiplied by 100 allowing the effect size to be reported as percentages as well.



ED: estimate of difference, PDT: pain detection threshold, PTT: pain tolerance threshold.

[SUPPLEMENTARY MATERIAL AVAILABLE ONLINE AT THE PUBLISHER'S WEBSITE]

CHAPTER 3

A Phase 1, Randomized, Double-blind, Placebo-Controlled, Single- and Multiple dose Escalation Study Evaluating the Safety, Pharmacokinetics, and Pharmacodynamics of VX-128, a Highly Selective $Na_V1.8$ inhibitor, in Healthy Adults

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ABSTRACT

Selective inhibition of certain voltage-gated sodium channels (Na_Vs), such as $Na_V1.8$, is of primary interest for pharmacological pain research and widely studied as a pharmacological target due to its contribution to repetitive firing, neuronal excitability and pain chronification. VX-128 is a highly potent and selective $Na_V1.8$ inhibitor that was being developed as a treatment for pain. We evaluated the safety, tolerability and pharmacokinetics of VX-128 in healthy subjects in a single- and multiple-ascending-dose (SAD, MAD) first-in-human study. Pharmacodynamics were evaluated in the MAD-part using a battery of evoked pain tests. Overall, single doses of VX-128 up to 300 mg were well tolerated, although AE incidence was higher in subjects receiving VX-128 (41.7%) compared to placebo (25.0%). After multiple dosing of up to 10 days, skin rash events were observed at all dose levels (up to 100 mg once daily, QD), in 5 of 26 (19.2%) subjects, including one subject receiving VX-128 (100 mg QD) who had an SAE of angioedema. A trend in pain tolerance were observed for cold pressor- and pressure pain, which was dose-dependent for the latter. VX-128 was rapidly absorbed (median time to maximum plasma concentration (T_{max}) between 1-2 hours) with a half-life of approximately 80 hours at 10mg QD, and approximately 2-fold accumulation ratio after 10 and 30mg QD. Although VX-128, when given in a multiple dose fashion, resulted in early study termination due to tolerability issues, effects were observed on multiple pain tests that may support further investigation of $Na_V1.8$ inhibitors as pain treatments.

INTRODUCTION

Voltage-gated sodium channels (Na_Vs) – and inhibition of these channels specifically – have been a main area of interest for pharmacological pain research in the last decades. Currently, Na_V inhibitors are among the most investigated drugs classes in early-phases of the trajectory (i.e. up to clinical trial phase IIA) – only surpassed by the opioid, and non-steroidal anti-inflammatory drug classes. [1] Pain relief by Na_V inhibitors has been indicated through blocking of the $Na_V1.3$, $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$ subtypes, while blocking of other Na_V subtypes (e.g. $Na_V1.5$, which is predominantly present in cardiac muscle) leads to unwanted (cardiac) side effects. For example, the first-generation non-selective Na_V inhibitor lidocaine is effective in reducing pain and widely used as a topical agent; however, its systemic use is limited given the high risk of cardiac adverse effects at doses required for alleviating pain. [2–4]

To reduce side effects associated with broad inhibition of Na_V subtypes while increasing long-term efficacy, pharmacological research shifted to selectively inhibiting pain-facilitating channels, such as $Na_V1.8$: a sensory neuron-specific channel preferentially expressed on the dorsal root ganglion (DRG) and trigeminal ganglion neurons that has been found to play a critical role in pain signaling. [5,6] Specifically, gain-of-function mutations in the $Na_V1.8$ gene – which alter $Na_V1.8$ channel properties in a proexcitatory manner and so increase DRG neuron excitability – have been reported to cause chronic pain in patients with painful small fiber neuropathy. [7–9] Furthermore, $Na_V1.8$ contributes to repetitive firing and neuronal excitability, as $Na_V1.8$ rapidly recovers from inactivation and has a more depolarized voltage-dependency of (in)activation when compared to other Na_Vs. Evidence from *in vitro* studies indicate excitation of $Na_V1.8$ is therefore involved in the development of peripheral sensitization, eventually leading to central sensitization and pain chronification, [6,10] whereas inhibition of $Na_V1.8$ was shown to block this activity leading to analgesia *in vitro*. [11,12] These findings combined demonstrate the potential of $Na_V1.8$ as a pharmacological target for the treatment of pain, specifically when related to nociceptor hyperexcitability.

Based on the above, VX-128, an orally bioavailable, highly potent and selective $Na_V1.8$ inhibitor was developed. We evaluated the safety, tolerability and pharmacokinetics (PK) of VX-128 in healthy subjects in a single- and multiple-ascending-dose (SAD, MAD) first-in-human study. Phar-

macodynamics (PD) were additionally evaluated in the MAD-part using an integrated battery of evoked pain tests. [13–16]

MATERIALS AND METHODS

Overall study design

This was a two-part first-in-human (FIH) study to evaluate the safety and tolerability, PK and PD of VX-128 in healthy adults. Both parts (A and B) had a randomized, double-blind, placebo-controlled parallel-group design; part A evaluated VX-128 in single ascending doses (SAD), and part B in multiple ascending doses (MAD). Dose escalation was based on a review of the available safety-, tolerability- and PK data from (the) preceding cohort(s).

The study was performed at the Centre For Human Drug Research (CHDR, Leiden, The Netherlands), in accordance with the Declaration of Helsinki of 1975, its amendments and the Guideline for Good Clinical Practice. Approval was received from Medical Review and Ethics Committee Stichting Beoordeling Ethiek Biomedisch Onderzoek (Stichting BEBO, Assen, The Netherlands) before study start. The study was registered under ToetsingOnline number NL63609.056.17 and EudraCT 2017-003557-42.

DESIGN PART A – SAD Four cohorts of 8 subjects each were randomized in a 3:1 ratio to receive VX-128 or placebo as oral suspension under fasted conditions on Day 1. Subjects were admitted to the clinical research unit (CRU) on Day -1, received a single dose of VX-128 or placebo on Day 1, and discharged on Day 5. Safety assessments (12-lead and continuous ECGs, vital signs, safety laboratory testing, and physical examinations (PE)) and PK blood sampling were conducted throughout the study. Each subject completed his or her study participation with a safety follow-up visit 7-10 days after study drug dosing.

DESIGN PART B – MAD Three cohorts (B1-B3) of 12 subjects, each randomized in a 5:1 ratio to receive VX-128 or placebo as an oral suspension, were admitted to the CRU on Day -1, dosed with VX-128 or placebo on Days 1 up to and including 10, and discharged from clinic on Day 14. Pain thresholds were measured using a panel of evoked pain tests (section **Pain test procedures**, below) on Day 1 (all cohorts) and on Day 10 (only

Cohort B2). Safety assessments (12-lead ECG, safety laboratory testing, PE and vital signs), and the Columbia-Suicide Severity Rating Scale (C-SSRS) were carried out throughout the study and evaluated for any trends or abnormalities. Plasma PK was sampled throughout the study (section **Study procedures – PK**, below). Subjects completed study participation with a safety follow-up visit 7-10 days after the last study drug administration.

Participants

Healthy males (parts A and B) and females of non-childbearing potential (only part A) aged 18-55, inclusive, underwent screening procedures prior to enrollment. Key criteria that were evaluated for eligibility were overt healthiness and that subjects had no present or past medical conditions that could put the subject's safety in jeopardy, or influence study outcomes (e.g. history of or current cardiovascular, mental or neurological disorders, (chronic) pain, significant allergies, malignancies or any conditions affecting drug absorption). Written informed consent was obtained from all study participants prior to any assessment taking place. Subjects were allowed to participate in only one cohort of one study part.

A training session with the pain test battery (section **Pain test procedures**, below) was part of screening procedures, to minimize learning effects, as well as to exclude any subjects indicating to be too sensitive or tolerable to the included tests. The latter was defined as being tolerant to more than 80% of the maximum input intensity for the either the pressure-, electrical- or cold pressor pain test.

Study drug VX-128, and study drug administration procedures

VX-128 is a potent and selective orally bioavailable molecule that targets the $Na_V1.8$ sodium channel (details on the potency and selectivity of VX-128 undisclosed by sponsor request).

In the morning of dosing days, a single dose of VX-128 was administered as an oral suspension with 240mL of water in fasted state. A taste-masking agent was provided prior and after dosing. Doses administered in part A were 10, 40, 120 or 300 mg; in part B 10, 30 or 100 mg based on the maximum recommended starting dose determined from preclinical toxicity studies performed in monkeys, being the most sensitive species

(not published). No dose above 100mg once daily (QD) was tested in part B due to the study being terminated prematurely (details in section **Results – Safety and tolerability**).

Study procedures – safety

Subject safety was evaluated on an on-going basis by adverse event monitoring, clinical laboratory assessments, clinical evaluation of vital signs, standard 12-lead ECGs, and physical examinations.

Study procedures – PK

Blood plasma was sampled to evaluate VX-128 concentration time profiles. Samples in Part A (SAD) were collected pre-dose on Day 1 (0 h), and at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12, 24 (Day 2), 36 (Day 2), 48 (Day 3), 72 (Day 4), and 96 h (Day 5) post-dose. Samples in part B (MAD) were collected pre-dose on Day 1, (0 h), and at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8 and 12 h post-dose. Samples were collected before the next administered dose on Days 2, 5, 6, 7, 8, and 9. On Day 10, samples were collected pre-dose and at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12, 24 (Day 11), 36 (Day 11), 48 (Day 12), 72 (Day 13), and 96 h (Day 14) after the final dose (that was given on Day 10).

Study procedures – pharmacodynamic (PD)

PAIN TEST PROCEDURES A detailed description of all pain test procedures is provided in a related article. [17]

In brief, analgesic effects were measured twice pre-dose, at baseline, and at 1h, 2h, 4h, 7h and 10h post-dose using an evoked pain tests battery in a fixed sequence: electrical stair pain test (1), pressure pain test, cold pressor pain test, electrical stair pain test (2), heat pain test on untreated skin, and heat pain test on capsaicin-treated skin. The latter two tests (heat pain on capsaicin- and heat pain on untreated skin) were switched pre-dose, ensuring that the pre-dose heat pain test on capsaicin-treated skin was performed 30 minutes after application of the capsaicin, and whilst keeping the remainder of the test sequence intact [details on the capsaicin application hereunder].

For all assessments except the heat pain tests, subjects were asked to hold an electronic visual analogue scale slider (eVAS slider), to indicate

their current perceived pain intensity. The eVAS ranged from 0-100. eVAS at 0 was defined as 'no pain', eVAS > 0 as the Pain Detection Threshold (PDT), and eVAS = 100 as the Pain Tolerance Threshold (PTT): 'worst pain tolerable'. When PTT was reached, the test automatically stopped, thereby immediately relieving the subject from their pain.

Heat PDTs were determined on the capsaicin-treated skin (on dominant volar forearm), as well as on normal (non-stimulated) skin (on the non-dominant volar forearm), and recorded by the subject pushing a button on the hand-held feedback control. The average of a triplicate measurement was used for further analysis of heat PDTs.

APPLICATION OF CAPSAICIN 1% CREAM (MAD PART ONLY) Capsaicin 1% cream was used as cutaneous heat sensitization model, by selectively agonizing the transient receptor potential cation channel subfamily V member 1 (TRPV1) channel. [18,19] Capsaicin 1% cream was applied during screening to confirm subjects were not hyperresponsive to the cream, and was applied for 30 minutes, starting 60 min prior to study drug administration on a 3×3 cm area on the dominant volar arm. The non-dominant volar forearm served as a non-stimulated control. Further details of the capsaicin model used may be found in our related article. [17]

Statistical considerations and analysis

RANDOMIZATION Both study parts were double-blind; subjects were randomly assigned to treatments. The randomization code was produced by a qualified randomization vendor (Cytel Inc., Waltham, MA, USA), and approved by a designated unblinded biostatistician who was not part of the study execution team.

SAMPLE SIZE No formal sample size calculations were performed. Parts A and B enrolled eight and 12 subjects per ascending dose, respectively. This is a typical sample size for a FIH study in healthy subjects.

PK AND PD ANALYSIS Safety, demographic and PK data are presented as mean ± standard deviation (SD) unless stated otherwise. PK parameters for VX-128 were determined using standard non-compartmental methods.

For PD results, the baseline value was defined as (the average of) the non-missing pretreatment measurements for all pain tests. Only descriptive statistics were reported. Numbers represent mean [±standard deviation (SD)], unless stated otherwise.

RESULTS

Baseline characteristics

In part A, 80 individuals were screened so that 32 male subjects were randomized. Eight subjects received placebo; six subjects per dose level received VX-128 10, 40, 120, or 300 mg. Subjects not enrolled were mostly excluded based on hypertension, illicit drug use, abnormal clinical chemistry results or logistical or personal reasons (e.g., change in personal or clinical planning). In Part B, 93 individuals were screened resulting in 31 male subjects that were randomized. Five subjects received placebo, ten subjects received VX-128 10mg QD, ten subjects 30 mg QD, and six subjects received VX-128 100 mg QD. Primary reasons for exclusion of subjects in part B were reporting to have too high tolerance to pain tasks at screening, hypertension, abnormal clinical chemistry results, illicit drug use or logistical reasons.

Demographics and other subject characteristics were generally similar in both parts (i.e. SAD and MAD) and in study cohorts (**Table 1**). Mean subject age for SAD and MAD was 28.6 (±8.9) years and 32.1 (±10.5) years, respectively. In both study parts, approximately 87% were White.

Safety and tolerability

SAD VX-128 administered as a single dose was generally well tolerated up to the highest evaluated dose (300 mg). AEs in subjects who received VX-128 were generally mild; mild AEs occurred in eight subjects (33.3% of those dosed with VX-128). Moderate AEs occurred in two subjects (8.3%). The most common AE was headache and only occurred in subjects who received VX-128 (37.5%, **Table 2**). AE incidence was higher in subjects receiving VX-128 compared to those receiving placebo (n=10 (41.7%) versus n=2 (25%), respectively). One subject had a minimally prolonged QT interval 4.5 h post VX-128 300 mg administration (447 to 460 ms) which was mild in severity and resolved without intervention or sequelae. Overall, there were no clinically meaningful changes in laboratory results, vital signs, or ECGs.

Of the subjects that were administered VX-128, three received paracetamol orally post-study drug administration to treat malaise (~36 h post-VX-128 10mg administration), myalgia (~87 h post-VX-128 40 mg administration) or influenza (~152 h post-VX-128 40 mg administration). These AEs occurred in one individual each.

MAD VX-128 administered as multiple doses was generally well-tolerated, with the exception of the occurrence of rash events in 5 of 26 (19%) subjects who received VX-128. The occurrence of rash led to treatment discontinuation in 2 subjects who received 100 mg QD of VX-128. The clinical study was subsequently terminated early due to tolerability issues. AEs in subjects that received VX-128 were generally mild and occurred in 18 subjects (69.2% of those receiving VX-128; **Table 3**). The most common AEs reported were headache (in n=9 subjects, 34.6%), and somnolence and dizziness (n=4, 15.4% each). AE incidence in the VX-128 group was lower than in the placebo group [VX-128: n=18 (69.2%), placebo: n=4 (80%)]. There were no clinically meaningful changes in laboratory results, vital signs, ECGs, or evidence of suicidal thoughts based on the C-SSRS.

Five subjects (19.2%), after QD dosing of a week or more with VX-128 (all dose levels) had rash-related AEs: rash papular (n=2), toxic skin eruption (n=2), and rash maculo-papular (n=1), and resulted in discontinuation of two subjects receiving the highest tested dose (100 mg) on Day 8. Refer to **Table S1** for details on these AEs. One subject discontinued due to toxic skin eruption, the other due to toxic skin eruption and dyspnea which was followed by an SAE of angioedema on Day 9. The SAE resolved the following day; while the subject continued to receive oral cetirizine until 13 days after the last study drug dose. Biopsies of this subject's skin eruptions were taken on Days 8 and 9 and both showed superficial dermatitis with eosinophilic granulocytes. Another subject in part B had an episode of hyperventilation and was hospitalized, which was therefore classified as an SAE. This subject, however, was found to have been administered with placebo after randomization code release.

Skin and subcutaneous tissue disorders of two subjects that received 10 mg VX-128 were treated with topical cooling cream on Day 12; triamcinolone was additionally administered topically on the skin of to one of these subjects on Day 13 to treat eczema. Topical cooling cream was applied

to one subject dosed with 100 mg VX-128 QD to treat skin and subcutaneous tissue disorders on Day 8; whom also received paracetamol for pain around biopsy place that day, and for headache on Day 11. The same subject received intravenous clemastine to treat angioedema on Day 9, and oral cetirizine to treat allergic symptoms on Days 10-19. A different subject receiving 100 mg VX-128 received intravenous clemastine as treatment for skin and subcutaneous tissue disorders on Day 9, and oral paracetamol-caffeine to treat headache on Day 11.

Pharmacokinetic results

PK parameters of VX-128 in the SAD part were evaluated on Day 1, in the MAD-part on Day 1 and Day 10. Mean plasma concentration-time profiles of VX-128 in plasma after single and multiple oral doses are displayed in **Figure S1**. PK parameters are found in **Table 5**. The PK of VX-128 after multiple oral doses on Day 10 were similar to the profile observed after single doses of VX-128 in the SAD-part.

As a single dose, VX-128 was rapidly absorbed: peak plasma concentrations (median T_{max}) ranged from 1 to 2 h and increased with higher doses. The highest exposure of VX-128 was observed at the 300 mg dose, which resulted in a mean peak plasma concentration (i.e. C_{max}) of 1020 ng/mL. C_{max} of VX-128 following a single dose appeared to increase dose proportionally over the 10 mg to 300 mg dose range. The mean exposure (i.e. area under the concentration versus time curve from the time of dosing to the last measurable concentration: $AUC_{0\text{-last}}$) ranged between 756 and 23800 ng·h/mL; the mean terminal half-life ($T_{1/2}$) ranged between 52 and 71 h – both which also increased with higher doses.

In the MAD-part, PK parameters of the highest dose level (100 mg) were not evaluable on Day 10 due to premature study termination (see section **Safety and tolerability**, above). The highest exposure was observed in the 100 mg QD dose level on Day 1, yielding a mean C_{max} of 531 ng/mL and mean $AUC_{0\text{-}24h}$ of 5030 ng·h/mL. Mean $T_{1/2}$ after 10 days of VX-128 10 mg QD was approximately 80 h, and after 30 mg QD 87 h. The mean accumulation ratio for AUC0-24h of VX-128 on Day 10 was 2.3-fold after 10 mg and 30 mg QD dosing.

PD results (MAD part only)

On day 1, cold pressor PTT and pressure PTT increased at all doses compared to placebo, at each timepoint (i.e. up until 10 h post-dose) (**Figure 1**, **Table S2**). The trend of effect observed on pressure PTT was dose-dependent. On day 10, a similar trend towards an effect of VX-128 30mg versus placebo was observed for cold pressor PTT and pressure PTT.

No effect of VX-128 was observed on the PDT endpoints for the cold pressor, electrical stimulation, pressure pain, CPM, capsaicin-induced and thermal pain tests, or on the PTT endpoints for the electrical stimulation pain test and CPM (**Figure 1**, **Table S2**).

DISCUSSION

This study evaluated the safety, tolerability, pharmacokinetic and pharmacodynamic effects of VX-128 in healthy subjects. VX-128 was rapidly absorbed and its PK after multiple oral doses on Day 10 was similar to that after single oral doses in Part A. C_{max} of VX-128 following a single dose appeared to increase dose proportionally. After multiple dosing of up to 10 days, skin rash events were observed, at all dose levels (up to 100 mg once daily, QD), in 5 of 26 (19.2%) of subjects including one subject receiving VX-128 (100 mg QD) who had an SAE of angioedema. The clinical study was subsequently terminated early due to tolerability issues. Although only descriptive statistics were performed, the pharmacodynamic results suggest VX-128 may be a potent analgesic, as there were dose-dependent increases in pressure pain-, and increases in cold pressor pain thresholds.

The occurrence of skin rash observed after multiple dosing may represent an allergic reaction to the administered compound(s) or to one or more of its (unknown) metabolites however, there is no evident link with $Na_V1.8$, or to Na_V inhibition. No reports are available providing an exact frequency of non-selective Na_V inhibitors inducing skin rash, although certain cases are known. Specifically, mild skin rash has been reported following administration of non-selective Na_V inhibitors phenytoin and mexiletine, and after multiple dosing of selective $Na_V1.7$ inhibitor PF-05089771 at higher dose levels. [20–22] The comparable incidence of skin rash between all evaluated multiple dose levels of VX-128 suggests that the occurrence is not exposure-related. Although the structural characteristics of VX-128 are not publicly available, we were not able to find an

evident link between the $Na_V1.8$ class and rash-AEs, suggesting it may be a compound-related rather than a class effect.

Although not statistically tested, we observed VX-128-related effects on nociceptive thresholds. No test was primarily targeted *a priori*; the study was exploratory in nature. In addition, little evidence is available on effects of Na_V inhibitors on experimental pain tests: in studies with registered drugs such as lidocaine, mexiletine and lacosamide limited and variable analgesic effects were observed. [23–25] We therefore used a multimodal test battery to evaluate the effects of VX-128 on distinctive types of evoked pain. Increases in PDT and PTT from baseline are indicative of analgesic effects, which we observed in cold pressor PTT and pressure PTT following VX-128 treatment on Day 1 (10, 30, and 100 mg QD) and Day 10 (30 mg QD). The analgesic effects of VX-128 were most evident at the 100 mg dose. Effects on the cold pressor- and pressure pain models link to the mechanism of action of VX-128. *In vitro* studies showed that $Na_V1.8$ is able to rapidly recover from inactivation, demonstrating its involvement in repetitive firing, neuronal excitability and so in neuropathic pain conditions where nociceptor hyperexcitability is the underlying mechanism. [6,10,26] The cold pressor task interplays, amongst others, with $Na_V1.8$ via the transient receptor potential subfamily member 8 channel. [17] We previously reported significant effects on cold pressor PTT of a different $Na_V1.8$ inhibitor, VX-150, in a similar study in healthy males. [17] Suggestive effects on pressure PTT of VX-128 correlates to results of a preclinical study with $Na_V1.8$-deficient mice – both mechanical and thermal pain were reduced in that model. [27] Interestingly, the $Na_V1.8$ inhibitor VX-150, affected cold pressor PTT and heat PDT, but not pressure PTT in the previous study. While this discrepancy is not fully understood, it is of interest to note that VX-150 is a prodrug, distinct from VX-128, with a different level of selectivity for $Na_V1.8$.

Previously, we demonstrated statistically significant analgesic effects with VX-150 on the same pain test battery. [17] That study was performed with an adequately powered two-way cross-over design, in contrast to the MAD part of current study, in which analgesic effects were evaluated in parallel and not powered for determination of statistically significant differences. In any proof-of-concept/mechanism study, but especially in (evoked) pain studies with healthy volunteers where the outcome measure is based on personal perception, cross-over designs are deemed superior given the low inter-subject variability. [28,29] However, in this

study it may be appreciated that VX-128 seemed to influence pressure PTT in a dose-dependent manner, and cold pressor PTT consistently (**Figure 1**). In a parallel-designed SAD/MAD trial primarily assessing a drug's safety and tolerability profile, pain test results may display the first signs of analgesic activity based on a dose-dependent increase of pain thresholds; as reported here. Alternatively, a stand-alone cross-over pain study, such as the VX-150 study, [17] can statistically assess the analgesic potential with a dose selected for this purpose. Including evoked pain tasks in early-phase studies with healthy volunteers thus may serve two distinct goals of equal importance and interest.

While there is evident interest in developing selective Na_V inhibitors as non-opioid alternative pain treatment, preclinical findings have not often been confirmed in the clinic. [30,31] Both this study and that of VX-150, highlight the importance of proof-of-pharmacology studies in early-phase clinical research. Repetitively performing fixed sequences of distinctive pain tests over time, provides valuable data on the analgesic profile and the active dose range, as presented here (**Figure 1**) and previously for various compounds with distinctive mechanisms of action. [14,16,17] Experimental pain studies also support dose selection and patient selection for a subsequent proof-of-concept trial. [1,32] Even when deciding not to proceed with a particular compound, results may help in designing future studies testing drugs with a similar mechanism of action.

The current study has several limitations. The MAD part only included men in order to reduce test variability, and, as suggested in literature, that pain perception of women changes throughout the menstrual phase. [33–35] The conclusions therefore are limited to men, while noting that nociceptive functioning of $Na_V1.8$ is identical in men and women and therefore plausible that VX-128 would induce similar effects in women. As the study focused on safety and tolerability, the design was not powered to detect analgesic effects. The trial was also halted prematurely resulting in an incomplete dataset, therefore, the analgesic effects discussed are not statistically tested and only suggestive. The (second) electrical stair pain task, which followed after the cold pressor pain task, was included to quantify the conditioned pain modulation (CPM) response. Heat PDTs were evaluated after this second electrical test, to allow for two baseline (i.e., pre-dose) pain test battery sequences to be performed in combination with 30 minutes of capsaicin application. Therefore, heat PDTs plausibly were influenced through a persistent CPM response. However, we

expect that the bias on study results – if present at all– is limited as (1) pain tasks were performed in the same sequence throughout the study and (2) the duration of a CPM response is generally only brief. [36–40]

CONCLUSION

The $Na_V1.8$ inhibitor VX-128, despite having led to skin rash and one subject with angioedema after multiple dosing and thereby halting the reported study for tolerability issues, induced analgesic effects on cold pressor- and pressure pain thresholds, warranting further investigation of $Na_V1.8$ inhibitors for the treatment of pain.

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Table 1 Subject baseline characteristics, both study parts.

	SAD Placebo N = 8	SAD 10 mg N = 6	SAD 40 mg N = 6	SAD 120 mg N = 6	SAD 300 mg N = 6	MAD Placebo N = 5	MAD 10 mg qd N = 10	MAD 30 mg qd N = 10	MAD 100 mg qd N = 6
SEX, N (%)									
Male	8 (100)	6 (100)	6 (100)	6 (100)	6 (100)	5 (100)	10 (100)	10 (100)	6 (100)
AGE (YEARS)									
Mean (SD)	32.1 (11.4)	25.7 (2.3)	31.7 (9.8)	29.8 (10.6)	22.7 (2.4)	30.2 (8.7)	30.7 (9.9)	33.7 (11.7)	33.3 (12.7)
RACE, N (%)									
White	7 (87.5)	5 (83.3)	5 (83.3)	5 (83.3)	6 (100.0)	5 (100.0)	8 (80.0)	9 (90.0)	5 (83.3)
Black or African American	1 (12.5)	0	0	1 (16.7)	0	0	2 (20.0)	0	0
Asian	0	1 (16.7)	0	0	0	0	0	0	1 (16.7)
Other	0	0	1 (16.7)	0	0	0	0	1 (10.0)	0
WEIGHT (KG)									
Mean (SD)	73.3 (10.2)	84.8 (16.6)	85.5 (8.8)	77.7 (4.9)	85.3 (14.6)	76.5 (16.5)	77.8 (14.6)	76.7 (13.3)	77.0 (13.4)
HEIGHT (CM)									
Mean (SD)	180.5 (8.4)	184.3 (9.7)	180.4 (8.0)	179.8 (4.2)	180.5 (9.8)	175.1 (6.4)	178.1 (8.3)	177.7 (5.6)	180.5 (9.0)
BMI (KG/M^2)									
Mean (SD)	22.42 (2.34)	24.91 (3.97)	26.43 (3.74)	24.03 (1.26)	26.11 (3.91)	24.98 (5.32)	24.39 (3.27)	24.46 (5.29)	23.65 (3.97)

BMI: Body Mass Index, N: number of subjects, SD: standard deviation

Table 2 Adverse Events in At Least 2 Subjects, Part A (SAD).

	Placebo[a] N = 8 n (%)	10 mg N = 6 n (%)	40 mg N = 6 n (%)	120 mg N = 6 n (%)	300 mg N = 6 n (%)	VX-128 total N = 24 n (%)	Total N = 32 n (%)
Number of AEs (Total)	3	19	4	8	3	34	37
Subjects with any AEs	**2 (25.0)**	**4 (66.7)**	**2 (33.3)**	**2 (33.3)**	**2 (33.3)**	**10 (41.7)**	**12 (37.5)**
SUBJECTS WITH AES BY RELATIONSHIP							
Not related	1 (12.5)	0	0	1 (16.7)	0	1 (4.2)	2 (6.3)
Unlikely related	1 (12.5)	0	0	0	1 (16.7)	1 (4.2)	2 (6.3)
Possibly related	0	4 (66.7)	2 (33.3)	1 (16.7)	1 (16.7)	8 (33.3)	8 (25.0)
Related	0	0	0	0	0	0	0
SUBJECTS WITH AES BY SEVERITY							
Mild	2 (25.0)	3 (50.0)	1 (16.7)	2 (33.3)	2 (33.3)	8 (33.3)	10 (31.3)
Moderate	0	1 (16.7)	1 (16.7)	0	0	2 (8.3)	2 (6.3)
Severe	0	0	0	0	0	0	0
Life threatening	0	0	0	0	0	0	0
Subjects with SAEs	0	0	0	0	0	0	0
Subjects with AEs leading to death	0	0	0	0	0	0	0
SYSTEM ORGAN CLASS[b] PREFERRED TERM							
Nervous system disorders	0	4 (66.7)	2 (33.3)	1 (16.7)	2 (33.3)	9 (37.5)	9 (28.1)
Headache	0	2 (33.3)	0	1 (16.7)	2 (33.3)	5 (20.8)	5 (15.6)
Tension headache	0	2 (33.3)	2 (33.3)	0	0	4 (16.7)	4 (12.5)
General disorders and administration site conditions	1 (12.5)	3 (50.0)	1 (16.7)	0	0	4 (16.7)	5 (15.6)
Fatigue	0	2 (33.3)	0	0	0	2 (8.3)	2 (6.3)

(Table continues on next page)

(Continuation Table 2)

	Placebo[a] N = 8 n (%)	10 mg N = 6 n (%)	40 mg N = 6 n (%)	120 mg N = 6 n (%)	300 mg N = 6 n (%)	VX-128 total N = 24 n (%)	Total N = 32 n (%)
Musculoskeletal and connective tissue disorder	1 (12.5)	0	1 (16.7)	1 (16.7)	0	2 (8.3)	3 (9.4)
Myalgia	1 (12.5)	0	1 (16.7)	0	0	1 (4.2)	2 (6.3)

AE: adverse event; n: number of subjects with data; N: number of subjects in the analysis set; SAE: serious adverse event

Note: When summarizing number of events, a subject with multiple events within a category was counted multiple times in that category. When summarizing number and percentage of subjects, a subject with multiple events within a category was counted only once in that category.

a Placebo was the pooled placebo from each cohort.

b PTs were provided only for adverse events that occurred in ≥2 subjects from any treatment group. A subject with multiple events within an SOC or PT was counted only once within the SOC or PT.

Table 3 Adverse Events in At Least 2 Subjects, Part B (MAD).

	Placebo[a] N = 5 n (%)	10 mg QD N = 10 n (%)	30 mg QD N = 10 n (%)	100 mg QD N = 6 n (%)	VX-128 total N = 26 n (%)	Total N = 31 n (%)
Number of AEs (Total)	21	22	12	40	74	95
Subjects with any AEs	**4 (80.0)**	**6 (60.0)**	**6 (60.0)**	**6 (100.0)**	**18 (69.2)**	**22 (71.0)**
SUBJECTS WITH AES BY RELATIONSHIP						
Not related	2 (40.0)	1 (10.0)	0	0	1 (3.8)	3 (9.7)
Unlikely related	0	0	5 (50.0)	2 (33.3)	7 (26.9)	7 (22.6)
Possibly related	2 (40.0)	5 (50.0)	1 (10.0)	2 (33.3)	8 (30.8)	10 (32.3)
Related	0	0	0	2 (33.3)	2 (7.7)	2 (6.5)
SUBJECTS WITH AES BY SEVERITY						
Mild	4 (80.0)	6 (60.0)	6 (60.0)	5 (83.3)	17 (65.4)	21 (67.7)
Moderate	0	0	0	1 (16.7)	1 (3.8)	1 (3.2)
Severe	0	0	0	0	0	0
Life threatening	0	0	0	0	0	0
AEs leading to treatment discontinuation	0	0	0	2 (33.3)	2 (7.7)	2 (6.5)
Subjects with SAEs	1 (20.0)	0	0	1 (16.7)	1 (3.8)	2 (6.5)
Subjects with AEs leading to death	0	0	0	0	0	0
SYSTEM ORGAN CLASS[b] PREFERRED TERM						
Nervous system disorders	2 (40.0)	5 (50.0)	3 (30.0)	4 (66.7)	12 (46.2)	14 (45.2)
Headache	1 (20.0)	4 (40.0)	2 (20.0)	3 (50.0)	9 (34.6)	10 (32.3)
Somnolence	1 (20.0)	1 (10.0)	1 (10.0)	2 (33.3)	4 (15.4)	5 (16.1)
Dizziness	0	3 (30.0)	0	1 (16.7)	4 (15.4)	4 (12.9)
Gastrointestinal disorders	2 (40.0)	1 (10.0)	1 (10.0)	4 (66.7)	6 (23.1)	8 (25.8)
Nausea	0	1 (10.0)	0	2 (33.3)	3 (11.5)	3 (9.7)

(Table continues on next page)

(Continuation Table 3)

	Placebo[a] N = 5 n (%)	10 mg QD N = 10 n (%)	30 mg QD N = 10 n (%)	100 mg QD N = 6 n (%)	VX-128 total N = 26 n (%)	Total N = 31 n (%)
Abdominal discomfort	2 (40.0)	0	0	0	0	2 (6.5)
Injury, poisoning, and procedural complications	1 (20.0)	2 (20.0)	1 (10.0)	3 (50.0)	6 (23.1)	7 (22.6)
Procedural pain	1 (20.0)	1 (10.0)	0	2 (33.3)	3 (11.5)	4 (12.9)
Skin and subcutaneous tissue disorders	1 (20.0)	2 (20.0)	2 (20.0)	2 (33.3)	6 (23.1)	7 (22.6)
Skin rash (maculo-) papular	0	2 (20.0)	1 (10.0)	0	3 (11.5)	3 (9.7)
Toxic skin eruption	0	0	0	2 (33.3)	2 (7.7)	2 (6.5)
General disorders and administration site conditions	1 (20.0)	2 (20.0)	0	3 (50.0)	5 (19.2)	6 (19.4)
Fatigue	0	1 (10.0)	0	1 (16.7)	2 (7.7)	2 (6.5)
Medical device site dermatitis	0	1 (10.0)	0	1 (16.7)	2 (7.7)	2 (6.5)
Musculoskeletal and connective tissue disorders	1 (20.0)	2 (20.0)	1 (10.0)	2 (33.3)	5 (19.2)	6 (19.4)
Back pain	0	2 (20.0)	1 (10.0)	0	3 (11.5)	3 (9.7)
Myalgia	1 (20.0)	0	1 (10.0)	1 (16.7)	2 (7.7)	3 (9.7)

AE: adverse event; n: number of subjects with data; N: number of subjects in the analysis set; QD: daily; SAE: serious adverse event

Note: When summarizing number of events, a subject with multiple events within a category was counted multiple times in that category. When summarizing number and percentage of subjects, a subject with multiple events within a category was counted only once in that category.

a Placebo was the pooled placebo from each cohort.

b PTs were provided only for adverse events that occurred in ≥2 subjects from any treatment group. A subject with multiple events within an SOC or PT was counted only once within the SOC or PT.

Table 4 PK results part A (SAD) and B (MAD).

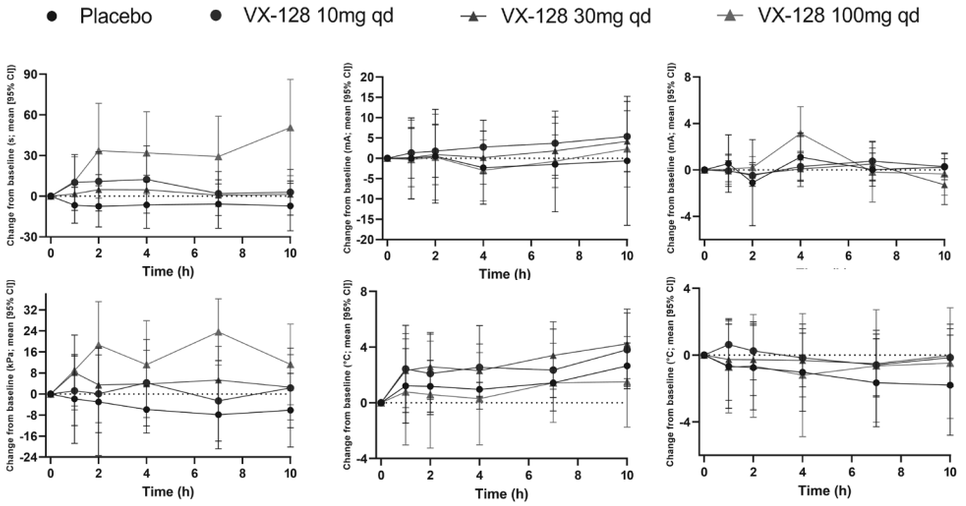
Parameter	VX-128 Dose						
	SAD 10 mg (N = 6)	SAD 40 mg (N = 6)	SAD 120 mg (N = 6)	SAD 300 mg (N = 6)	MAD 10 mg qd (N = 10)	MAD 30 mg qd (N = 10)	MAD 100 mg qd (N = 6)
DAY 1							
t_{max} (h)[a]	1.00 (0.50, 2.00)	1.50 (1.00, 2.00)	1.75 (1.00, 4.00)	2.00 (1.50, 3.00)	1.00 (1.00, 3.00)	1.00 (1.00, 2.00)	1.50 (1.00, 3.00)
C_{max} (ng/mL)	57.4 (47)	189 (29)	545(29)	1020 (21)	67.2 (27)	221 (23)	531 (29)
$AUC_{0\text{-}24h}$ (ng×h/mL)	756 (33)	3350 (24)	10400 (33)	23800 (24)	522 (15)	1630 (21)	5030 (17)
$t_{1/2}$ (h)	52.0 (36)	70.5 (36)	60.0 (23)	70.7 (42)	NA	NA	NA
DAY 10							
t_{max} (h)[a]	NA				1.25 (1.00, 2.10)	1.00 (1.00, 2.02)	ND
C_{max} (ng/mL)					95.4 (27)	316 (26)	
$AUC_{0\text{-}24h}$ (ng×h/mL)					1210 (31)	3870 (27)	
$t_{1/2}$ (h)					80.8 (41)	87.1 (65)	

Mean (CV%) is presented unless stated otherwise. $AUC_{0\text{-}24h}$: AUC from the time of dosing to 24 hours; C_{3h}: concentration determined at 3 hours after dosing on Day 1; C_{max}: maximum observed plasma concentration; CV%: coefficient of variation; N: number of subjects in the analysis set; NA: not applicable; NCA: non-compartmental analysis; ND: not determined; PK: pharmacokinetic; qd: daily; $t_{1/2}$: terminal half-life; t_{max}: time of maximum plasma concentration.

Note for plasma PK: NCA was not done for Cohort at 100 mg qd on Day 10, as subjects did not complete dosing as planned due to premature study termination.

a Median (minimum, maximum) is presented for t_{max}

Figure 1 Selection of evoked pain test results – change from baseline. A. Mean (95% CI) Cold Pressor Pain Test results: Pain Tolerance Threshold on Day 1; B. Mean (95% CI) Electrical Stimulation pain test: Pain Tolerance Threshold on Day 1; C. Mean (95% CI) Conditioned pain modulation: Pain Tolerance Threshold on Day 1; D. Mean (95% CI) Pressure Pain test: Pain Tolerance Threshold on Day 1; E. Mean (95% CI) Capsaicin-induced pain test: Pain Detection Threshold on Day 1; F. Mean (95% CI) Thermal pain test (on control/untreated skin): Pain Detection Threshold on Day 1 Effects of placebo (n=5), VX-128 10 mg QD (n=10), VX-128 30 mg QD (n=10) and VX-128 100 mg QD (n=10) on selected evoked pain test endpoints determined on Day 1 of study part B. Descriptive statistical analysis was performed; data are represented as means with 95% CI. Effects of VX-128 were noted for cold pressor PTT at the highest tested dose (100 mg QD) and suggestive dose-dependent effects of VX-128 for pressure pain PTT. *(full color version of this illustration on inside of the cover)*



A. Cold Pressor PTT; B. Electrical Stimulation PTT; C. Conditioned Pain Modulation PTT; D. Pressure PTT; E. Capsaicin-induced PDT; F: Thermal PDT (on control/untreated skin). Abbreviations: °C: degrees Celsius, CI: confidence interval, h: hour(s), kPa: kilopascal, mA: milliamperes, n= sample size, PDT: pain detection threshold, PTT: pain tolerance threshold, s: seconds, SD: standard deviation.

[SUPPLEMENTARY MATERIAL AVAILABLE ONLINE AT THE PUBLISHER'S WEBSITE]

CHAPTER 4

Comparative analgesic activity of two distinct sodium channel inhibitors, mexiletine and lacosamide, in healthy subjects

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ABSTRACT

BACKGROUND Selective voltage-gated sodium channels (Na_V) inhibitors are being developed for the treatment of chronic pain. Early-phase clinical studies that include evoked pain tests in their design may bridge the gap between nonclinical results and studies in patients with chronic pain. This study was performed to expand our knowledge on the mechanism by which Na_V inhibitors affect evoked pain tests in healthy subjects, and how they can be utilized in the development trajectory of (selective) Na_V inhibitors.

METHODS This was a randomized, double-blind, double-dummy, placebo-controlled, three-way crossover study. Eighteen healthy males received mexiletine 333mg, lacosamide 300mg and placebo on three visits, separated by at least seven days. Evoked pain tests (pressure-, electrical stair-, electrical burst-, cold pressor test, heat pain test on UVB-treated and untreated skin), short-form McGill Pain Questionnaires, intra-epidermal stimulation (IES) and safety assessments were performed at different timepoints on each study day.

RESULTS Mexiletine significantly increased cold pressor pain tolerance (Estimate of Difference (ED): 10.5%; p=.03), and altered affective cold pain perception (ED: -0.13, p=.04). Mexiletine did not affect other pain tests or IES; lacosamide did not produce any significant analgesic effects. Both drugs were well-tolerated without safety concerns.

CONCLUSIONS We profiled two non-selective Na_V inhibitors and show that only the cold pressor test was sensitive to effects of a single dose of 333mg of mexiletine. Results presented here may be used for benchmarking of other Na_V inhibitors, in particular when Na_V-1.8 inhibition is involved.

INTRODUCTION

In recent years, a plethora of new mechanisms have been found that may play a role in induction of analgesia. One is targeting voltage-gated sodium-selective ion channels (Na_V), that function by propagating, regulating and/or elongating the action potential of excitable nerve or muscle cells. [1] Of the nine subtypes discovered, $Na_V1.3$, $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$ – primarily found on peripheral nerves and dorsal root ganglia – are particularly important in the pathophysiology of pain, and have been linked to inflammatory- and neuropathic pain syndromes. [2–4] Each of these four subtypes have unique biophysical characteristics, and a role in action potential generation and pain signalling. [5]

Na_V inhibition has been explored as therapeutic option for providing pain relief which, amongst others, has led to the discovery of the well-known non-selective Na_V inhibitor lidocaine. Non-selective systemic inhibition of Na_V channels, however, may lead to significant (cardiovascular) side effects, limiting the applicability of this drug class. Analgesic research thus has moved to selective inhibition of $Na_V1.7$, $Na_V1.8$ and to a lesser extent $Na_V1.3$, $Na_V1.9$ subtypes as target for pain relief without inducing notable adverse effects. [2] Compounds that made it to the human testing, however, have withheld their clinical potential up to now. [6] Nonetheless, drug developers remain evidently interested in this drug class: of all analgesics currently in early-phase development, Na_V inhibitors are the second most developed, only following opioids. [7]

Human experimental pain studies in healthy volunteers may bridge the gap from promising nonclinical results, to studies in patients and investigational products reaching the market. [8] A broad range of different nociceptive tests, each mimicking a distinct (clinical) pain mechanism, has been described and can be used to determine the analgesic potential of (investigational) drugs. At the Centre for Human Drug Research (CHDR), a comprehensive battery of evoked pain tasks has been developed for early-phase clinical drug studies, that allows the characterization of an analgesic effect profile in repeated fashion over-time. [9] By testing registered drugs – for which the action mechanism is well-described – on this battery, a library of analgesic profiles has been created that allows benchmarking novel investigational analgesics with putative or unknown mechanisms of action. [10] Previously, we described the analgesic potential – and sometimes lack thereof – of a variety of analgesics, including certain Na_V inhibitors, using this nociceptive test battery. [11–13]

To further expand our knowledge on the mechanism by which Na_V inhibitors affect nociceptive tests, and how such tests can be utilized in the development of novel (selective) Na_V inhibitors in early-phase development, this study evaluated the analgesic profile of two distinct Na_V channel inhibiting drugs which have been described to be of use in the treatment of (neuropathic) pain. We profiled the analgesic effects of therapeutic dose levels of mexiletine and lacosamide, in a three-way cross-over, placebo-controlled fashion.

METHODS

The study was conducted at the Centre for Human Drug Research, Leiden, The Netherlands, after having received approval of the Medical Ethics Committee 'Stichting Beoordeling Ethiek Biomedisch Onderzoek', Assen, The Netherlands, and in accordance with the Helsinki Declaration of 1975, as revised in 1983. Study results reported here were part of a trial of which results related to a distinctly different objective, namely determining effects on peripheral nerve excitability threshold tracking, have been reported elsewhere. [14] The trial was prospectively registered in the Netherlands Trial Register (NTR number NL7327).

Study participants and design

This was a randomized, double-blind, double-dummy, placebo-controlled, three-way crossover study in healthy male volunteers. All subjects voluntarily provided written informed consent prior to any study assessment taking place. Male individuals aged 18 to 45, inclusive, were screened for general fitness, previous or currently ongoing medical conditions that could jeopardize the subject's safety and/or influence study results (e.g. (chronic) pain, significant allergies, cardiovascular, neurological and/or mental disease). Subjects with a darker skin (i.e. Fitzpatrick skin type IV, V or VI), widespread acne, freckles, tattoos or scarring on the back (see section **Evoked pain test battery** for rationale), smokers and those (with a history of) abusing drugs and/or alcohol were excluded from participation. Subjects were asked to refrain from tobacco- or nicotine products from 1 month prior to dosing, and from medication and dietary supplements from 14 days before first dose. Alcohol use was

prohibited from 24 h before start of study until the final visit and caffeine from 24 h before each dose until 2 days afterwards. Strenuous physical exercise was not allowed from 48 hours before each study day until the end of that visit.
A pain test training session was part of the screening procedures prior to enrolment, to minimize learning effects and exclude volunteers from participating that reported to be too sensitive, or indicating to be too tolerable to the tests. The latter has been defined as achieving tolerance above 80% of the maximum input for the electrical-, pressure-, or cold pressor pain test during screening (section **Evoked pain test battery**, below).

Eighteen male subjects were enrolled to each receive mexiletine, lacosamide and placebo in randomized order, on three separate study visits, with each visit separated by a washout period of at least seven days from the next. Screening procedures were within 42 days of the first drug administration. A safety follow-up visit, scheduled 7-10 days after the last dose, completed study participation.

Each study visit consisted of one full day (Day 1), and an evening of admittance scheduled the preceding day (i.e. evening of Day -1), which included re-assessment of eligibility in an abbreviated manner and induction of erythema by UVB (see section **Evoked pain test battery**, below). Blood was sampled to assess safety and pharmacokinetic (PK) parameters, and a sequence of nociceptive tests (details in section **Study procedures – pharmacodynamic (PD)**) was performed on Day 1. Assessments were performed at approximately the same time; meals were provided at set times to circumvent a possible influence of diurnal rhythm and/or food.

PK results have been reported elsewhere. [14]

Study drug and placebo administration procedures

Lacosamide (3 film-coated tablets of 100 mg Vimpat, UCB Pharma S.A.) and mexiletine (2 hard capsules of 167 mg Namuscla, Lupin Europe GmbH) were over-encapsulated to ensure double-blind double-dummy drug administrations. Subjects were allocated at random to receive all three treatments over three separate study visits: one visit receiving lacosamide 300 mg and placebo capsules; one visit receiving mexiletine 333 mg and placebo capsules; and one visit receiving only placebo capsules.

Study procedures – pharmacodynamic (PD)

EVOKED PAIN TEST BATTERY During each treatment period, a fixed sequence of evoked pain tasks was performed to evaluate nociceptive detection and tolerance thresholds: twice pre-dose to serve as baseline, and at 3.5 h and 6 h post-dose. The battery of tests included in this study, has been validated for use in early phase analgesic drug studies; each test and its applicability has been described in detail previously. [9–12,15,16]

In brief, each subject was assigned to a separate room without any form of distraction. Here, they were asked to sit comfortably in a chair, and during all tests but the heat pain test (see next paragraph) were given an electronic visual analogue scale (eVAS) slider to hold and to indicate their current perceived pain intensity with. The slider ranges from 0 to 100; with 0 being defined as having 'no pain', slider > 0 defining the Pain Detection Threshold (PDT), and 100 defining the Pain Tolerance Threshold (PTT; 'worst pain tolerable'). Upon reaching the PTT, or maximum duration or intensity that was defined to be safe, the test automatically stopped and so immediately relieved subjects from pain.

The ultraviolet B (UVB)-hyperalgesia 2 times minimal erythema dose (2MED) model was included as a proxy for inflammatory pain, by inducing a localized erythemic response on the subject's upper back 18 h prior to each study drug administration. This method has been described in detail previously – the erythema is known to produce stable hyperalgesia to heat for over 36 hours post-UVB-irradiation, but also to cause post-inflammatory hyperpigmentation (PIH) lasting years if not administered in acceptable doses. [17,18] Only lighter skinned subjects (i.e. Fitzpatrick skin type I-III) were therefore eligible to be screened. At screening, the minimal dose to evoke erythema (MED) was assessed on skin on the upper back, following procedures as described previously. [17,18] Only subjects with a MED below 355mJ/cm^2, as determined to be within safe limits, [17] were exposed to 2MED during remainder of the study and were subject to the UVB-induced heat pain test. At timepoints as described for the evoked pain tests above, a heat thermode (QSense, Medoc, Israel) was placed on the UVB-irradiated area and gradually increased in temperature until a subject indicated to start detect pain, which was defined as the UVB-induced heat PDT. The average of a triplicate measurement was used for further analysis. Skin on the upper back contralateral to that of where that study visit erythema was induced, was used for determining heat PDTs on normal skin.

The following fixed sequence was used throughout the study: heat pain test on normal skin, heat pain test on UVB-irradiated skin (if applicable, see above), tourniquet pressure pain task, electrical burst pain task, electrical stair pain task (#1), cold pressor pain task, and electrical stair pain task (#2). By calculating the difference observed in the pain detection- and pain tolerance thresholds of the electrical stair pain test directly after the cold pressor pain test, versus the electrical stair pain detection and tolerance thresholds observed prior to the cold pressor pain test, a possible modulatory (conditioned pain modulation, CPM) response was quantified. To evaluate the affective and sensory components of the pain perceived, the Dutch version of the short-form McGill Pain Questionnaire (SF-MPQ) was performed following the pressure-, electrical burst-, electrical stair- and cold pressor pain task. [19,20]

INTRA-EPIDERMAL STIMULATION (IES) IES was applied using a flexible electrode consisting of 5 inter-connected micro-needles, each protruding 0.5 mm from the surface of the electrode. These micro-needles protrude through the stratum corneum of the skin, but do not penetrate the epidermis and are therefore considered non-invasive. This superficial intrusion in the epidermis permits preferential activation of superficial Aδ-fibers, [21,22] and a previous validation study showed that stimulation using this electrode resulted in a sharp pricking sensation. [23] Estimation of the nociceptive detection threshold, psychometric slope and the habituation of detection probability over time for single- and double-pulse intra-epidermal electric stimuli may be used to characterize peripheral and central changes of the nociceptive system. [24–27]

Three stimulus types were used: 1) a stimulus consisting of one cathodic square-wave electrical current pulse with a pulse width of 0.21 ms; 2) a stimulus consisting of one cathodic square-wave electrical current pulse with a pulse width of 0.42 ms; and 3) a stimulus consisting of two cathodic square-wave electrical current pulses with a pulse width of 0.21 ms and an inter-pulse interval of 10 ms. Refer to e.g. van den Berg et al. for further details on the IES test including safety measures. [28]

Study procedures – safety

Assessments to evaluate subject safety included adverse event (AE) monitoring, clinical laboratory assessments (i.e. blood chemistry and blood

haematology panels, and urinalysis), standard 12-lead electrocardiograms (ECGs), continuous Holter-ECG assessment throughout Day 1, clinical evaluation of vital signs and physical examinations.

Statistical considerations and analysis

No formal sample size calculation was performed. A sample of 18 in crossover setting was in line with previous studies using the described pain test battery and resemble a typical early phase experimental pain study. This sample size allowed to evaluate the distinct objectives reported elsewhere, [14] and to compare results reported here to others with a similar study design.

Effects observed for each treatment (placebo versus lacosamide, and placebo versus mexiletine) on pain thresholds were calculated using a mixed model analysis of covariance (ANCOVA), using baseline as covariate. Baseline has been defined as the average of the two pre-dose measurements. Period, time, treatment, and treatment by time were included as fixed factors; subject, subject by time and subject by treatment used as random factors. Statistical significance was defined as $p < 0.05$. Values represent mean (±standard deviation (SD)), unless stated otherwise.

RESULTS

Subject characteristics

Subject characteristics are included in **Table 1**. Eighteen healthy male subjects were enrolled and completed all study assessments. The mean age was 25.2 (4.7) years, all were of white race and had a mean body mass index (BMI) of 23.5 (3.1). Eight subjects had a MED below 355 mJ/cm2 and were thus eligible to be subject to the UVB-induced hyperalgesia tests.

Pharmacodynamic results

EVOKED PAIN TESTS Results are presented in **Table 2**, **3** and **Figures 1**, **2**. A single dose of mexiletine 333mg induced significant analgesic effects on cold pressor PTT (Estimate of Difference (ED): 10.5%, 95% confidence interval (CI): 0.8 – 21.1 %, p = .03), and affective pain perception as noted on the SF-MPQ (ED: -0.13, p = .04). Cold pressor PDTs were consistently increased following mexiletine administration compared to placebo, but did not significantly differ (ED: 23%, 95% CI: -1.1 – 52.9 % p = .06).

A single dose of lacosamide 300mg did not produce any statistically significant analgesic effects. The heat PDT on normal skin was higher after administration of lacosamide than after administration of placebo, but this difference did not reach statistical significance (ED: -1.6%, 95% CI: -0.0 – 3.2 % p = .05).

A post-dose increase compared to baseline for electrical burst- and stair PTTs was observed in all treatment arms (mexiletine, lacosamide and placebo). Effects on these parameters among treatments were comparable and did not significantly differ. In contrast, for heat PDT on UVB-irradiated skin a post-dose decrease compared to baseline was observed in all treatment arms. Effects on this endpoint were comparable between treatments and did not significantly differ.

IES No significant effects of mexiletine 333mg or lacosamide 300mg were noted on the (nociceptive) detection thresholds, psychometric slopes or detection probability habituation of any of the three stimuli tested (**Table 2**, **3**).

Safety

Overall, mexiletine and lacosamide were well tolerated without any significant safety concerns. A total of 28 AEs in 14 subjects were reported following mexiletine administration, of which 25 AEs were deemed at least possibly related to mexiletine administration and the majority was dizziness (n=11, 61.1%). Following lacosamide administration, a total of 20 AEs in 11 subjects were observed, of which 15 AEs were found to be possibly related to lacosamide administration; most frequently dizziness (n=5, 27.8%) and somnolence (n=4, 22.2%). No clinically relevant changes were noted in any other safety assessment.

DISCUSSION

In the current study we evaluated the analgesic profile of two distinct marketed and voltage-gated sodium channels, mexiletine and lacosamide, using an nociceptive test battery. Mexiletine induced significant analgesia on cold pressor PTT, but not on other pain tests. Lacosamide

did not significantly influence any nociceptive test. Both analgesics were well-tolerated; PK results were in line with previous reports (PK results of this study published elsewhere). [14, 29,30]

Mexiletine is an anti-arrhythmic Na_V inhibitor and oral analogue of lidocaine that has been available for over 40 years, with renewed interest as it received orphan drug designation and EU authorization (as Namuscla) for the treatment of myotonic disorders in 2018. [31,32] Apart from reducing contractility of heart and muscle cells through inhibition of Na_Vs, mexiletine induces analgesia by non-selectively exerting effects on Na_Vs located on peripheral nerves. [33] Data also show that mexiletine effectively inhibits $Na_V1.8$ in human neuroblastoma cells. [34] Mexiletine is only to a limited extent prescribed as treatment for neuropathic pain, of which cold pain is a frequent symptom. [35,36] $Na_V1.7$ and 1.8 both are found to contribute to cold pain – $Na_V1.8$ dominates (pain) perception in cold temperatures where $Na_V1.7$ plays a crucial role in $Na_V1.8$-negative neurons. A significant reduction of cold pressor PTT by mexiletine thus aligns mechanistically, and aligns with previous work in which we reported significant effects of a selective $Na_V1.8$ inhibitor on the same cold pain task (**Figure 1**). [37] No effects of mexiletine were noted on other pain tasks, including the electrical burst test (**Figure 1**). The electrical burst paradigm induces temporal summation, a phenomenon suggested to play a role in neuropathic pain and to be responsive to effects of drugs used for the treatment of neuropathic pain. Previously, Wallace et al. were also unsuccessful in observing effects of mexiletine on capsaicin-induced allodynia, another method suggested to mimic symptoms of neuropathic pain. [38]

Lacosamide is an anticonvulsant and non-selective Na_V inhibitor used for treating partial-onset seizures and neuropathic pain. There is evidence that lacosamide binds to fast-inactivated $Na_V1.7$ with slower binding kinetics than the 'classical' Na_V inhibitors (e.g., carbamazepine). [39] Although lacosamide is efficacious in preclinical neuropathic pain models, [40] we did not observe any significant effects of a single dose of 300 mg in this study. Others also reported mostly discouraging clinical results of lacosamide as analgesic: no effects were observed on UVB- and capsaicin-induced allodynia in a different human experimental pain study, and only limited efficacy was reported for lacosamide as neuropathic pain- and fibromyalgia treatment following a systematic review. [41,42] Recently, however, it was proposed that the efficacy of lacosamide

as analgesic may depend on the genetic makeup of the patient, as the drug proved to be effective in treating $Na_V1.7$-related small fibre neuropathy at a dose of 200 mg twice daily. [43] This may, at least partly, explain our negative findings. Based on all above considerations, it also suggests that lacosamide might not be the best model drug for evaluating the sensitivity of the pain tasks to analgesics in general, and to selective $Na_V1.7$ inhibition in particular. Alternatively, the pain tests used here may be less sensitive to effects of $Na_V1.7$ inhibition as the tests were also unsuccessful in showing effects of a selective $Na_V1.7$ inhibitor previously. [12] In contrast, we were able to show that 300 mg of lacosamide decreased motor and sensory median nerve excitability using threshold tracking. This indicates that efficacious concentrations were reached at the nerves, and makes it less likely that negative results on the pain tasks were caused by insufficient drug levels at the intended site of action. [14] While evoked pain models may not be suitable to quantify the pharmacodynamic effects of analgesics that preferentially inhibit $Na_V1.7$, future early-phase studies testing such ligands may benefit from including nerve excitability threshold tracking as a biomarker. [14] Adopting such a method will allow drug developers to evaluate proof-of-mechanism early-on, although noting that it remains valuable to employ a model to study the actual analgesic effects (i.e., proof-of-concept). [7]

In addition to these drug-specific findings, we observed an evident – and based on our previous experience unexpected – increase in electrical pain tolerance thresholds post-dose in all groups, including placebo. (**Figure 1**). We have not observed a notable placebo response in previous studies with this pain task battery. [9,10] Theoretically, the threshold tracking test, with which the excitability of electrically stimulated peripheral nerves can be studied, that was also performed in this study, [14] may have influenced the electrical pain tasks reported here. The pre-dose baseline threshold tracking task (performed at the wrist of the dominant arm) was scheduled after the electrical stair- and burst paradigms (performed on skin overlying tibia bone of left leg), but due to logistical reasons had to be scheduled before the pain tasks at 3.5 h and 6 h post-dose. As both measurements utilize electrical stimuli and as part of the threshold tracking procedure may be considered slightly painful by some participants, the threshold tracking task may have (partly) influenced the evident change from baseline in all groups. A possible mechanism for this is through induction of the endogenous inhibitory pain pathway, i.e.,

CPM response. CPM can influence multiple pain modalities and is not limited to electrical pain. [44] Given that pain intensity of threshold tracking test is low, that no other task performed before the electrical pain tests showed a similar tolerance increase across all treatment groups (i.e., heat pain test on normal and on UVB-irradiated skin; pressure pain test), and given that the CPM response only influences pain thresholds for a limited time (within 10 minutes), [44] we think it is unlikely that the threshold tracking measurement influenced pain thresholds through CPM.

Results reported here are to be read with the following considerations. We only included male volunteers in an effort to reduce variability and so increase odds to demonstrate statistically significant effects of the drugs studied. This decision was based on the fact that our study was exploratory in nature and previous work indicated that pain perception in women may change across the menstrual cycle. [45–47] This limits the conclusions that are drawn here to males, although noting that there is no literature available indicating that sex influences Na_V-related pain signal processing. The UVB hyperalgesia model was tested in eight of the total 18 subjects as the UVB model was only performed on light-skinned individuals for which the MED is deemed safe (see details in section **Evoked pain test battery**). Although this model has relatively little test variability compared to the other models used, [17] related results thus should be interpreted with caution given the low number of subjects evaluated for this outcome.

Experimental pain studies in healthy volunteers are an important step between promising preclinical research and patient studies: they allow for evaluation of effect size, dose selection and which patient (sub)population to target in case analgesic effects are observed early-on in drug development. Here, we evaluated two distinct non-selective Na_V inhibitors and showed that mexiletine significantly reduced cold pain, supporting previous findings on significant effects of two selective $Na_V1.8$ inhibitors on this task. [37,48] The lack of effect for the partially $Na_V1.7$-mediated inhibitor lacosamide could indicate that these pain tasks are not as sensitive to (selective) $Na_V1.7$ inhibition, but could also be related to lacosamide's limited analgesic efficacy. [42] Studying various analgesics using similar evoked pain tests allows for profiling and benchmarking of their effects against other drug classes profiled previously. As such, we determine from this study that, at this exposure, mexiletine's analgesic effect on cold pain (effect size cold pressor PTT: 10.5%; **Figure S1**) is in range with the

effects of the opioid fentanyl (17.1%), yet modest in comparison to the selective alpha-2δ ligand pregabalin (46.4%) and the selective $Na_V1.8$ inhibitor VX-150 (53.7%). [9,37]

In conclusion, we profiled the analgesic effects of two non-selective Na_V inhibitors using a multimodal nociceptive test battery and show that, with the doses used, only the cold pressor test was sensitive to effects of mexiletine. Results presented here may be used to benchmark the Na_V inhibitor drug class, in particular when Na_V-1.8 inhibition is involved.

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Table 1 Subject characteristics.

Demographic category	Number (N = 18)
SEX, N (%)	
Male	18 (100%)
Age (years)	
Mean (SD)	25.2 (4.7)
RACE, N (%)	
White	18 (100%)
WEIGHT (KG)	
Mean (SD)	79.8 (12.7)
HEIGHT (CM)	
Mean (SD)	184.0 (7.8)
BMI (KG/M^2)	
Mean (SD)	23.5 (3.1)
FITZPATRICK SKIN TYPE	
Type II	13 (72.2%)
Type III	5 (27.8%)
MED	
251 mJ/cm^2	2 (11.1%)
355 mJ/cm^2	6 (33.3%)
>355 mJ/cm^2	10 (55.5%)

Values represent mean (±SD) of total subject set, unless stated otherwise. BMI: Body Mass Index. cm: centimetres, kg: kilograms. MED: minimal erythema dose, mJ: millijoule, SD: standard deviation.

Table 2 Results of primary evoked pain task endpoints – mexiletine.

Mexiletine 333 mg – placebo over 7 h						
				95% CI		
	LSM Mexiletine	LSM Placebo	ED	Lower	Upper	p-value
HEAT PDT						
untreated skin	44.38 °C	44.61 °C	-0.5%	-2.1%	1.1%	0.52
UVB-*inflamed skin*	40.99 °C	41.19 °C	-0.5%	-2.9%	1.9%	0.67
ELECTRICAL STAIR						
PDT	6.61 mA	6.33 mA	4.6%	-11.3%	23.3%	0.58
PTT	17.81 mA	17.45 mA	2.1%	-6.6%	11.5%	0.64
MPQ – affective	0.49	0.52	-0.04	-0.14	0.06	0.45
MPQ – sensory	1	1.02	-0.02	-0.11	0.08	0.24
ELECTRICAL BURST						
PDT	2.07 mA	1.99 mA	3.8%	-16.9%	29.7%	0.74
PTT	8.44 mA	8.35 mA	1.1%	-10.1%	13.8%	0.85
MPQ – affective	0.41	0.45	-0.04	-0.11	0.03	0.28
MPQ – sensory	0.94	0.97	-0.02	-0.11	0.06	0.6
PRESSURE						
PDT	16.11 kPa	16.46 kPa	-2.2%	-14.4%	11.9%	0.74
PTT	41.70 kPa	41.77 kPa	-0.2%	-9.8%	10.5%	0.97
MPQ – affective	0.21	0.27	-0.06	-0.15	0.04	0.22
MPQ – sensory	0.79	0.8	-0.01	-0.09	0.07	0.86
COLD PRESSOR						
PDT	5.13 s	4.17 s	23%	-1.1%	52.9%	0.06
PTT	16.93 s	15.32 s	10.5%	0.8%	21.1%	***0.03***
MPQ – affective	0.42	0.55	-0.13	-0.26	-0.01	***0.04***
MPQ – sensory	0.89	0.92	-0.03	-0.13	0.07	0.53
CPM						
PDT	-0.33 mA	-0.77 mA	0.44 mA	-0.43 mA	1.32 mA	0.31
PTT	-0.39 mA	-0.62 mA	0.23 mA	-0.31 mA	0.78 mA	0.39
IES – SINGLE 0.21MS						
Slope	13.86 mA^{-1}	12.47 mA^{-1}	11.1%	-11.4%	39.5%	0.35
DT	0.46 mA	0.50 mA	-7.7%	-25.5%	14.3%	0.45
Habituation	-0.03 $stim^{-1}$	-0.03 mA/s	0.002 mA/s	-0.01 mA/s	0.01 mA/s	0.73
IES – SINGLE 0.42MS						
Slope	28.92 mA^{-1}	30.12 mA^{-1}	-4.0%	-23.2%	20.0%	0.71
DT	0.24 mA	0.21 mA	15.6%	-8.7%	46.4%	0.22
Habituation	-0.02 $stim^{-1}$	-0.02 mA/s	-0.01mA/s	-0.02mA/s	0 mA/s	0.11
IES – DOUBLE 0.21MS						
Slope	20.32 mA^{-1}	18.95 mA^{-1}	7.2%	-14.0%	33.6%	0.52
DT	0.31 mA	0.33 mA	-3.3%	-19.6%	16.3%	0.71
Habituation	-0.03 $stim^{-1}$	-0.03 mA/s	-0 mA/s	-0.01 mA/s	0.01 mA/s	0.76

(Table continues on next page)

Values are presented in % for tests for which the data were log-transformed, for CPM the data are presented in the unit in which they were measured. Those in bold and italic denote significant treatment effects ($p < 0.05$). Estimates >0 are in favour of mexiletine, negative values in favour of placebo. LSM describe mean values per treatment. °C: degrees Celsius; CI: confidence interval; CPM: conditioned pain modulation paradigm; ED: estimate of difference; h: hour; LSM: least square means; mA: milliampere; MPQ: affective and sensory component ('-affective' and '-sensory' endpoint, respectively) of the short form McGill Pain Questionnaire; (P)DT: (pain) detection threshold; PTT: pain tolerance threshold; $stim^{-1}$: per stimulus (i.e., change of the detection probability per applied stimulus, which measures the habituation); UVB: ultraviolet B.

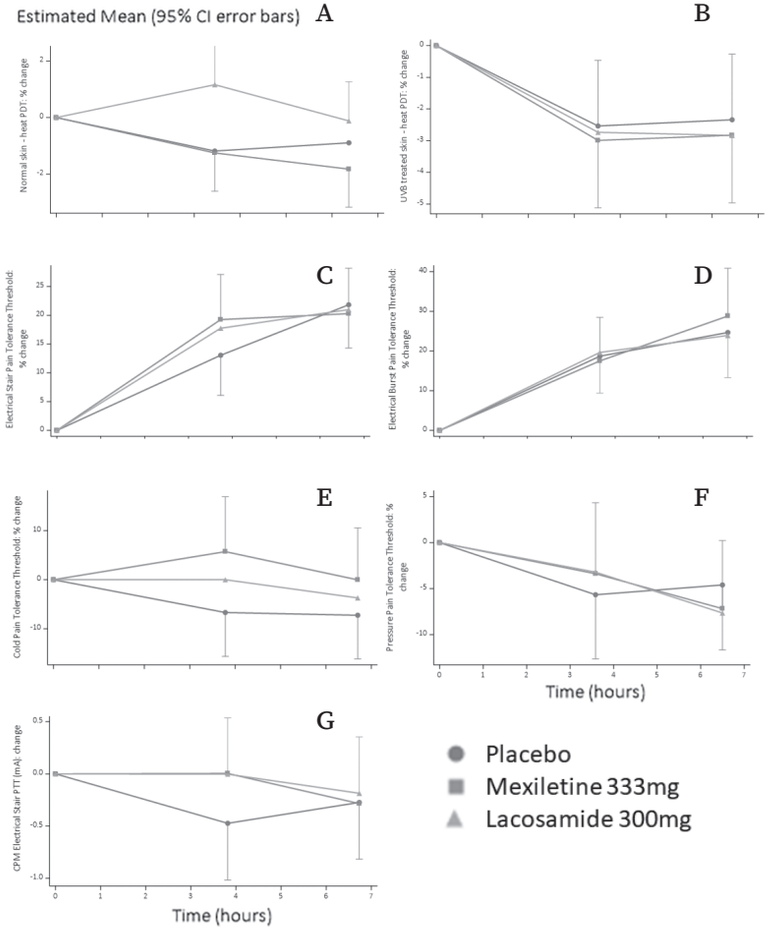
Table 3 Results of primary evoked pain task endpoints – lacosamide.

Lacosamide 300 mg – placebo over 7 h						
				95% CI		
	LSM Lacosamide	LSM Placebo	ED	Lower	Upper	p-value
HEAT PDT						
untreated skin	45.31 °C	44.61 °C	1.6%	-0.0%	3.2%	0.05
UVB-*inflamed skin*	41.04 °C	41.19 °C	-0.4%	-2.7%	2.0%	0.75
ELECTRICAL STAIR						
PDT	6.75 mA	6.33 mA	6.7%	-9.7%	26.1%	0.43
PTT	17.75 mA	17.45 mA	1.7%	-6.9%	11.1%	0.70
MPQ-affective	0.45	0.52	-0.07	-0.17	0.03	0.16
MPQ – sensory	0.97	1.02	-0.06	-0.15	0.04	0.24
ELECTRICAL BURST						
PDT	1.96 mA	1.99 mA	-1.5%	-21.1%	22.8%	0.89
PTT	8.35 mA	8.35 mA	0.1%	-11.1%	12.7%	0.99
MPQ-affective	0.45	0.45	0	-0.08	0.07	0.94
MPQ-sensory	0.95	0.97	-0.02	-0.1	0.07	0.66
PRESSURE						
PDT	16.90 kPa	16.46 kPa	2.7%	-10.2%	17.4%	0.69
PTT	41.63 kPa	41.77 kPa	-0.3%	-10.0%	10.3%	0.95
MPQ-affective	0.29	0.27	0.02	-0.07	0.12	0.63
MPQ-sensory	0.79	0.8	-0.01	-0.09	0.07	0.75
COLD PRESSOR						
PDT	4.62 s	4.17 s	10.8%	-10.9%	37.8%	0.34
PTT	16.16 s	15.32 s	5.5%	-3.8%	15.6%	0.25
MPQ-affective	0.51	0.55	-0.04	-0.16	0.09	0.55
MPQ-sensory	0.92	0.92	0	-0.1	0.1	0.97
CPM						
PDT	-0.20 mA	-0.77 mA	0.57 mA	-0.30 mA	1.32 mA	0.19
PTT	-0.34 mA	-0.62 mA	0.28 mA	-0.27 mA	0.83 mA	0.31
IES-SINGLE 0.21MS						
Slope	11.86 mA^{-1}	12.47 mA^{-1}	-4.9%	-24.6%	19.9%	0.66
DT	0.55 mA	0.50 mA	10.3%	-11.7%	37.8%	0.37
Habituation	-0.03 $stim^{-1}$	-0.03 mA/s	0 mA/s	-0.01mA/s	0.01mA/s	0.96
IES-SINGLE 0.42MS						
Slope	25.78 mA^{-1}	30.12 mA^{-1}	-14.4%	-32.0%	7.7%	0.17
DT	0.25 mA	0.21 mA	21.9%	-3.6%	54.2%	0.21
Habituation	-0.02 $stim^{-1}$	-0.02 mA/s	-0 mA/s	-0.01 mA/s	0.01 mA/s	0.43
IES-DOUBLE 0.21MS						
Slope	20.32 mA^{-1}	18.95 mA^{-1}	-7.8%	-26.4%	15.6%	.47
DT	0.37 mA	0.33 mA	14.4%	-5.6%	38.7%	0.16
Habituation	-0.03 $stim^{-1}$	-0.03 mA/s	-0 mA/s	-0.01 mA/s	0.01 mA/s	0.93

Values are presented in % for tests for which the data were log-transformed, for CPM the data are presented in the unit in which they were measured. Those in bold and italic denote significant treatment effects ($p < 0.05$). Estimates >0 are in favour of lacosamide, negative values in favour of placebo. LSM describe mean values per treatment. °C: degrees Celsius; CI: confidence interval; CPM: conditioned pain modulation paradigm; ED: estimate of difference; h: hour; LSM: least square means; mA: milliampere; MPQ: affective and sensory component ('-affective' and '-sensory' endpoint, respectively) of the short form McGill Pain Questionnaire; (P)DT: (pain) detection threshold; PTT: pain tolerance threshold; $stim^{-1}$: per stimulus (i.e., change of the detection probability per applied stimulus, which measures the habituation); UVB: ultraviolet B.

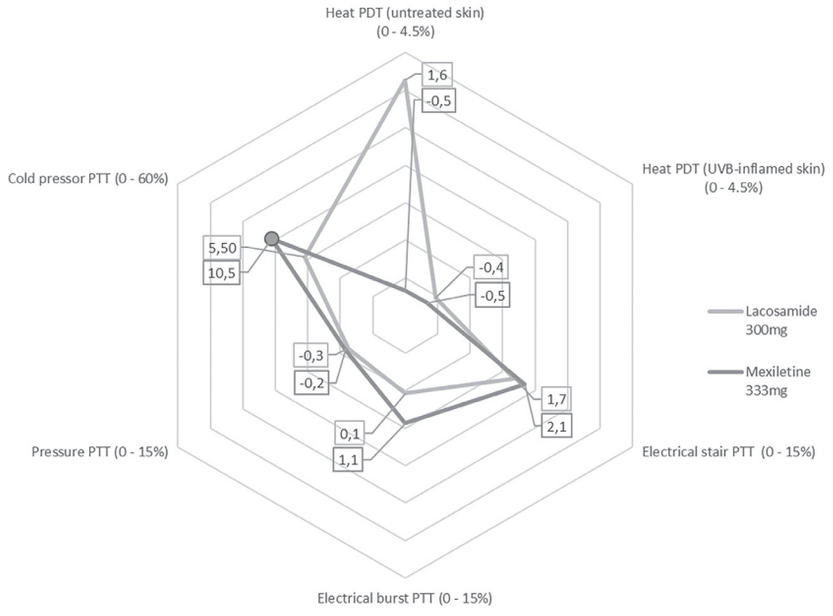
(Table continues on next page)

Figure 1 Evaluation of pain thresholds following mexiletine, lacosamide or placebo administration. Effects of single doses of placebo, Mexiletine 333mg and Lacosamide 300 mg (n=18) on selected evoked pain test endpoints. Data are represented as estimated means with 95% CI. a) heat PDT on untreated skin b): heat PDT on UVB-inflamed skin, c) electrical stair (single stimulus) PTT, d) electrical burst (repeated stimulus) PTT, e) cold pressor PTT, f) pressure PTT, g) Conditioned Pain Modulation PTT.



°C: degrees Celsius, 95% CI: 95% confidence interval, CPM: Conditioned Pain Modulation, mg: milligrams, n: number of subjects, PDT: pain detection threshold, PTT: pain tolerance threshold.

Figure 2 Analgesic profile of mexiletine and lacosamide. Effect size of mexiletine 333mg and lacosamide 300mg on the primary pain task endpoints, visualized using the ED between the least-squares means of the contrast mexiletine/lacosamide – placebo. Negative values thus favour placebo. The round marker indicates the significant effect on cold pressor PTT of mexiletine compared to placebo, over the full time course ($p < 0.05$). Percentage ranges included between parentheses reflect the range of effect size reported across a battery of analgesics summarized in earlier reports. [9,37] The EDs as included in Table 2 and 3 were used, as the data for these end points were log-transformed for analysis thus already presented in percentages.



ED: estimate of difference, PDT = Pain Detection Threshold, PTT = Pain Tolerance Threshold, UVB: ultraviolet B.

4

SECTION II

CHAPTER 5

Challenging the challenge: a randomized controlled trial evaluating the inflammatory response and pain perception of healthy volunteers after single-dose LPS administration, as a potential model for inflammatory pain in early-phase drug development.

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ABSTRACT

BACKGROUND AND AIMS Following an infection, cytokines not only regulate the acute immune response, but also contribute to symptoms such as inflammatory hyperalgesia. We aimed to characterize the acute inflammatory response induced by a human endotoxemia model, and its effect on pain perception using evoked pain tests in two different dose levels. We also attempted to determine whether combining a human endotoxemia challenge with measurement of pain thresholds in healthy subjects could serve as a model to study drug effects on inflammatory pain.

METHODS AND RESULTS This was a placebo-controlled, randomized, cross-over study in 24 healthy males. Twelve subjects were administered a bolus of 1 ng/kg LPS intravenously, and twelve 2 ng/kg LPS. Before days of placebo/LPS administration, subjects completed a full study day without study drug administration, but with identical pain threshold testing. Blood sampling and evoked pain tests (electrical burst and -stair, heat, pressure, and cold pressor test) were performed pre-dose and at frequent intervals up to 10 h post-dose. Data were analysed with a repeated-measures ANCOVA. For both dose levels, LPS induced an evident acute inflammatory response, but did not significantly affect any of the pain modalities. In a post-hoc analysis, lowering of pain thresholds was observed in the first 3 hours after dosing, corresponding with the peak of the acute inflammatory response around 1-3 h post-dose.

CONCLUSION Mild acute systemic inflammation, as induced by 1 ng/kg and 2 ng/kg LPS intravenous administration, did not significantly change pain thresholds in this study. The endotoxemia model in combination with evoked pain tests is not suitable to study acute inflammatory hyperalgesia in healthy males.

INTRODUCTION

The experience of pain, a main symptom in virtually any medical condition, can dramatically decrease a patient's quality of life [1] and has been linked to many pathogenic mechanisms. [2] Tissue injury or (chronic) inflammatory conditions may result in the exaggerated response to certain noxious stimuli, i.e. hyperalgesia – a well-known feeling when affected by an infectious disease, such as the common cold or influenza. [3, 4] A major underlying mechanism of inflammatory hyperalgesia is the release of various soluble mediators, including bradykinin, sensitization-inducing cytokines (e.g. interleukin -1β, -6 and -8 (IL-1β, IL-6, IL-8) and tumor necrosis factor-alpha (TNF-α)). [5-10] While playing a key role in the regulation of the immune response, [11] persistent elevation of these cytokines is known to contribute to nerve-inflammation and pathologic pain, and has been linked to diseases such as osteoarthritis, rheumatoid arthritis and inflammatory bowel disease. [7, 12, 13]

Inflammation itself can be divided in the acute phase (processes at site of inflammation: increase in blood flow, vascular permeability, fluids, leucocytes and inflammatory mediators as listed above) and the chronic phase (recruitment of specific humoral and cellular response, and in cases development of autoimmune conditions). [14] To mimic the former, lipopolysaccharide (LPS) may be used to evoke a controlled acute immune response by activation of the Toll-like receptor 4 (TLR4). In rodents, administration of LPS drives an acute immune response, resulting in altered heat and mechanical pain thresholds. [15-21] Similarly, in humans, intravenous administration of LPS evokes an acute immune response reflected by increased levels of circulating cytokines, and is dose-dependent. [22-24] By combining this human endotoxemia model with evoked pain tests, effects of LPS on multiple pain thresholds have been shown. Following low dose (0.4 and 0.6 ng/kg) LPS administration, significantly reduced pressure- and visceral pain thresholds [25-28] and altered subjective pain ratings [25] were reported between 1 to 3.5 h post-dose. These hyperalgesic effects, although not significant, showed a trend up to 6 h for pressure pain thresholds following a 0.8 ng/kg LPS-dose. [26] A high intravenous LPS dose (2 ng/kg) significantly reduced cutaneous (pressure), heat, electrical and cold pressor pain thresholds at 2 h post-dose. [29, 30] However, in most cases the sample size was small and/or the study design unequipped to compensate for the substantial variability of experimental pain tests. [31-33] Moreover, most previous work on inflammatory

hyperalgesia only separately studied the relationship between LPS-dose and pain response, or the time course of the pain response, or the effect of acute inflammation on a few pain modalities, and never all in a controlled and integrated fashion.

The current study was designed to systematically evaluate the effect of an acute systemic inflammatory challenge on pain thresholds in healthy male volunteers, and to validate the combination of experimental endotoxemia with evoked pain tests as a proxy for inflammatory hyperalgesia in early-phase clinical drug studies, if robust effects were to be found. An acute inflammatory response was induced by intravenous administration of LPS. Cytokine and stress hormone responses were frequently monitored over time. In parallel, a validated battery of pain tests, the PainCart, was performed at set times throughout the day. PainCart previously has been validated and used to show the analgesic profile of a wide variety of compounds. [34-37] Two different LPS doses (1 ng/kg and 2 ng/kg bodyweight) were used to evaluate possible dose-dependency. A sample of 12 subjects per dose level (i.e. 24 in total) was chosen to reflect a cohort in a typical phase 1 drug study. We hypothesized, based on prior research as discussed above, that LPS administered to 12 healthy males, could induce robust inflammatory hyperalgesia in an adequately controlled setting. If so, the endotoxemia model combined with evoked pain tests would be of use as a model in early-phase drug testing.

METHODS

The study was conducted at the Centre For Human Drug Research (CHDR), according to the Declaration of Helsinki of 1975, its amendments and the Guideline for Good Clinical Practice. The study dossier and protocol received Medical Ethics Committee approval prior to initiation of the clinical phase (Medical Ethics Committee: Stichting Beoordeling Ethiek Biomedisch Onderzoek, Assen, The Netherlands). The study was registered under ToetsingOnline number NL65264.056.18 and under ISRCTN number 13923422.

Study design

This was a double-blind, cross-over, placebo-controlled study in healthy male volunteers receiving a single intravenous dose of LPS or placebo (see for schematic overview **Figure 1**). Male subjects aged 18-55, inclusive,

were medically screened for general fitness, previous exposure to LPS, and for medical conditions which could create risk for the subject or bias study results (e.g. history of sepsis, cardiovascular disease, acute or chronic pain conditions, previous syncope or malignancies). Medication use (both prescription and over the counter) was prohibited. All participants provided written informed consent prior to any study assessments, and their privacy rights were observed throughout. Being a study exploratory of nature, only men were included in the study.

The study evaluated the effects of two LPS doses: 1 ng/kg and 2 ng/kg, in two separate groups of 12 subjects. The two groups were tested in subsequent order (i.e. first the 1 ng/kg dose group, thereafter the 2 ng/kg dose group). Subjects were randomized to one of two different treatment arms. Per dose level, eleven subjects were allocated to treatment arm A (occasion 1: no treatment; occasion 2: placebo administration; occasion 3: LPS-administration), and one subject was allocated to treatment arm B (occasion 1: no treatment; occasion 2: LPS administration; occasion 3: placebo administration). This disbalanced study design was selected since an intravenous LPS challenge cannot be repeatedly performed within one volunteer: LPS induces an innate memory response, regulated at the cellular and epigenetic level, that may last for months. [38-40]. Data from a placebo administration day following LPS administration therefore may be biased and is considered to be not reliable for further analysis (see also section **Statistical analysis**, below) By adopting a disbalanced randomization scheme of 11:1 per dose level, the amount of non-biased data was optimized whilst being able to maintain a double-blind design. Other options, such as a single-blind or open-label design, were not considered valid alternatives due to the high subjectivity and corresponding nocebo effects, which experimental pain models are subject to.

At pre-defined time points throughout the day, blood for quantification of the inflammation and stress markers was sampled and the PainCart test battery, as described below and illustrated in **Figure 2**, was performed. Blood was sampled in occasions 2 and 3, PainCart was performed in all three occasions.

Intravenous LPS challenge

Intravenous LPS challenges were only performed on occasion 2 and 3. Subjects received 1 ng/kg (cohort 1) or 2 ng/kg (cohort 2) *E. Coli*-purified LPS (GMP-grade from Lot#94332B4, List Biological Laboratories Inc. CA,

USA), or placebo (0.9% NaCl), administered as a 2-minute infusion. To ensure that subjects would stay adequately hydrated, additionally glucose/saline was infused (2.5% glucose/0.45% sodium chloride) starting 2 h (hours) prior to LPS/placebo administration, until 6 h afterwards.

Study assessments – blood-based markers

For analysis of various cytokine concentrations, cortisol and C reactive protein (CRP), blood was collected in Natrium Heparin tubes and analyzed using electrochemiluminescence (cytokines analyzed with the Meso Scale Discovery, Rockville, Maryland, USA, with the following Lower limits of quantification (LLOQ): IL-1 receptor antagonist (IL-1ra): 91.6 pg/mL, IL-1β: 0.280 pg/mL, IL-6: 1.49 pg/mL, IL-8: 1.21 pg/mL, IL-10: 0.666 pg/mL, TNF-α: 0.720 pg/mL); cortisol and CRP analyzed using Cobas8000 e602; Roche Diagnostics, with the following LLOQ's: cortisol: 70 nmol/L and CRP: 0.3 mg/L. For the analysis of bradykinin, kallikrein, cortisol and prostaglandin E_2 (PGE_2), blood was sampled in K2EDTA tubes. Bradykinin, kallikrein and PGE_2 were analyzed using ELISA (bradykinin and PGE_2: Abcam, Cambridge, UK; bradykinin LLOQ: 187 pg/mL, and PGE_2 LLOQ: 39.1 pg/mL, kallikrein: R&D Systems, Abingdom, UK with LLOQ: 46.9 pg/mL).

Study assessments – pain tests

On each occasion, nociceptive (pain) detection and tolerance thresholds were measured repeatedly using a fixed sequence battery of pain tests before (at -1 h, and 0 h) and after (2, 4, 8 and 10 h) LPS administration. (**Figure 2**) Tests were performed as described earlier [34, 36] using the following sequence: pressure, electrical burst, electrical stair (1), cold pressor, electrical stair (2), and heat pain test. A training session was part of the screening procedures to reduce any possible learning effects, as well as to exclude any subjects indicating intolerable to pain tests, or achieving tolerance at more than 80% of the maximum input intensity for the cold pressor-, electrical-, or pressure pain test. Assessments were performed with the subject sitting comfortably in a chair, leg raised, in a quiet room that was fitted with ambient lighting. Each subject was assigned to a separate room to minimize any distraction.

HEAT PAIN ASSESSMENT To determine primary hyperalgesia to heat, thermal pain detection thresholds (PDTs) were measured with a

thermode (Medoc QSense, Israel, contact area: 30mm × 30mm), that was placed on the subject's volar forearm. After start of the test, the thermode gradually increased in temperature from 32 °C with 0.5 °C/s, until the subject perceived the stimulus as painful (PDT), or if a temperature of 50 °C was reached. The subject indicated his PDT by pushing the button on the hand-held feedback control. The average of a triplicate measurement was used for further analysis.

PRESSURE PAIN ASSESSMENT An 11 cm wide tourniquet cuff (VBM Medizintechnik GmbH, Sulz, Germany) was placed over the subject's gastrocnemius muscle. The tourniquet was controlled by an electro-pneumatic regulator (ITV1030-31F2N3-Q, SMC Corporation, Tokyo, Japan), Power1401mkII analogue–to-digital converter and Spike2 software (CED, Cambridge, UK). During the test, the subject indicated his pain intensity using an electronic Visual Analogue Scale (eVAS)-slider, with 0 and 100 defined as 'no pain' and 'worst pain tolerable', respectively. eVAS > 0 was used as PDT. The pressure evoked by this cuff constantly increased with a rate of 0.5 kPa/s until the subject indicated his Pain Tolerance Threshold (PTT – eVAS to 100), or if 100 kPa was reached.

COLD PRESSOR PAIN ASSESSMENT The subject placed his non-dominant hand into a water bath (minimal depth of 200 mm) at 35 ± 0.5 °C, for 2 minutes. After 1.45 minutes, a blood pressure cuff that was placed on the upper arm, was inflated to 20 mmHg below resting diastolic pressure, to limit warm blood returning to the non-dominant hand. After 2 minutes, the subject changed his hand from the first water bath directly into a similar sized water bath, with a temperature of 1.0°C. Using the eVAS slider, the subject was instructed to indicate his PDT, the increase in pain intensity and PTT. When the time limit of 120 s, or PTT (eVAS-slider to 100) was reached, the subject removed his hand from the water bath. Simultaneously, the blood pressure cuff was deflated. The time (in seconds) the subject needed to reach PDT, and to reach PTT (or the time limit of 120 s) was used for analysis.

ELECTRICAL STIMULATION ASSESSMENT (ELECTRICAL BURST AND STAIR) On clean skin overlying the left tibial bone near the caudal end of the patella, two electrodes (Ag-AgCl) were placed. For the stair test, sole stimuli (10 Hz tetanic pulse with a duration of 0.2 ms) were administered by a constant current stimulator. Current intensity increased

from 0 mA to a maximum of 50 mA, in steps of 0.5 mA/s. For the burst test, each single stimulus (train of five, 1 ms square wave pulses repeated at 200 Hz) was repeated five times with a frequency of 2 Hz at the same current intensity with a random interval of 3 to 8 s between the repetitions. Current intensity increased identical to the stair test. For both tests, PDT was determined as eVAS > 0; PTT as eVAS = 100 or if 50 mA was reached.

CONDITIONED PAIN MODULATION (CPM) A possible drug effect on the centrally acting descending inhibitory control pathway, was measured using the conditioned pain modulation (CPM) response, which was quantified by calculating the difference of pain detection and pain tolerance threshold of the electrical stair pain test directly after the cold pressor pain test, minus the electrical stair pain detection and tolerance thresholds prior to the cold pressor pain test. [37]

Measures for safety monitoring

The day before each test day (i.e. Day -1 for each occasion) subjects were confined to the clinic and eligibility confirmed by an abbreviated screening of medical history, vital signs and safety laboratory results. During study days, subjects were monitored for overall well-being, as well as any possible adverse events, by clinical staff. Vital signs including temperature were measured at 4 h and whenever deemed necessary. Subjects reported back to clinic 7±2 days after last dosing for a safety follow-up visit.

Statistical analysis

Data are presented as mean ±standard deviation (SD). eVAS versus time was used to calculate the Area Above the eVAS pain Curve (AAC; for the cold pressor pain test) or Area Under the eVAS pain Curve (AUC; for the pressure-, electrical burst- and stair pain test and CPM). Parameters were initially analyzed without transformation, but as the data suggested otherwise, log-transformation was applied. Log-transformed parameters were back-transformed after analysis allowing results to be interpreted as percentage change. To establish whether significant treatment effects could be detected, all repeatedly measured parameters were analyzed with a mixed model analysis of covariance (ANCOVA) with treatment, time and treatment by time as fixed factors and subject, subject

by treatment and subject by time as random factors and the (average) baseline measurement as covariate. The Kenward-Roger approximation was used to estimate denominator degrees of freedom and model parameters were estimated using the restricted maximum likelihood method. Contrasts were calculated between LPS versus placebo (occasion 3 vs occasion 2, only for data from subjects receiving LPS in occasion 3), LPS versus no treatment (occasion 2 or 3 vs occasion 1 – all subjects) and placebo versus no treatment (occasion 2 or 3 vs occasion 1 – all subjects). For all parameters included in the analysis, contrasts were calculated for a time window ranging from pre-dose up until 10 hours post-LPS-administration. A sample size of 12 subjects per cohort was based on previous crossover studies investigating similar objectives, and on the feasibility of including the proposed model in early-phase pharmacological studies. [25, 27, 28, 41]

RESULTS

Baseline characteristics

24 subjects were enrolled and finished the complete study. Apart from these 24 subjects, two subjects did participate in occasion 1, but stopped participation before being dosed in the occasion 2 for personal reasons, and were therefore replaced. Although the protocol allowed for flexibility in when subjects returned to clinic (i.e. 7-21 days between occasion 1 and 3), all subjects but one (due to personal circumstances) reported to the clinic once a week (e.g. every Monday). Mean age was 30.8 ±9.5 years, mean body mass index was 23.8 ±2.3 m^2; most subjects (58.3%) were Caucasian. Further baseline characteristics can be found in **Table 1**. Although there were a few treatment-emergent adverse events observed around the projected E_{max} (around 2 h post-dose, see next section), such as chills or short and transient nausea symptoms, this was not reflected in out-of-range vital signs or other clinically significant safety findings.

Inflammatory response to LPS

All markers were assessed from pre-dose up until 10 hours post-LPS administration. For IL-6, IL-8 and TNF-α in both dose levels (1 ng/kg and 2 ng/kg), a time-dependent and significant increase was observed shortly

after LPS administration in comparison to placebo. Peak concentrations for TNF-α were observed at 1 h post-dose (peak concentration 1 ng/kg dose: 66.49 pg/mL; 2 ng/kg dose: 249.35 pg/mL); for IL-1b, IL-6, IL-8 and IL-10 peaks were observed at 2 h post-dose (IL-1b 1 ng/kg dose: 0.39 pg/mL; IL-1b 2 ng/kg dose: 1.10 pg/mL; IL-6 1 n/kg dose: 65.55 pg/mL; IL-6 2 ng/kg dose: 200.88 pg/mL; IL-8 1 ng/kg dose: 195.96 pg/kg; IL-8 2 ng/kg dose: 515.83 pg/mL; IL-10 1 ng/kg dose: 15.25 pg/mL; IL-10 2 ng/kg: 42.41 pg/mL). After peaking, concentrations of all these markers rapidly decreased, and approached baseline values again at 10 h post-dose (**Figure 3-a**, **-b**, **-c** and **-d**). For IL-1b and IL-10, no statistical testing could be performed given most results from the placebo occasion were below LLOQ, as expected.

IL-1ra concentrations increased shortly after LPS exposure compared to placebo, yet also remained vastly elevated: between 2-10 h post-dose >57% of the samples at the 1 ng/kg dose level, and >93% of the samples at the 2 ng/kg dose level were above the upper limit of quantification of 2930 pg/mL. CRP concentrations showed a delayed response to LPS, by increasing from 4 h onwards without a tendency to decrease at our last measured time point (at 10 h post-dose; concentration 1 ng/kg dose: 7.15 mg/mL; 2 ng/kg dose: 10.07 mg/mL) (**Figure 3-d**).

Measures for inflammatory hyperalgesia

PRIMARY ANALYSIS Overall, LPS administration did not significantly alter pain thresholds over time, i.e. PDT, PTT and/or AUC endpoints from pre-dose up until 10 h post-dose, as shown in **Table 2** and **Figure 4**. Baseline values, summary graphs and the statistical table may be found in **Appendix A**, **B** and **C.1**, respectively. For both dose levels, LPS decreased heat pain PDT and electrical stair PTT between 1 and 2 h post-administration (**Figure 4-a** and **b**), although the contrast with the no treatment- or placebo occasion was not significant when analyzed for the full time profile (0-10 h post-dose, **Table 2**). Lowering of pain thresholds around 2 hours post-administration were also observed for pressure pain PTT and electrical burst PTT after 2 ng/kg LPS administration (**Figure 4-f** and **j**).

A significant effect of 2 ng/kg LPS was observed for the CPM AUC endpoint (versus placebo; ED: -71.04%, 95% CI: -139.76 – -2.33%). **Figure 4-k** and **l** show that CPM, although unaffected by LPS at 1 ng/kg, is decreased (i.e. lower endogenous inhibition) around 2 hours post-administration.

Thereafter, CPM rapidly returns around baseline at 4 h, before decreasing until last measured time point at 10 h post-administration.

Cold pressor PTT and AAC, electrical burst PDT and pressure pain PDT were significantly reduced by 1 ng/kg LPS compared to placebo (cold pressor PTT: ED: -15.8%, 95% CI: -25.7 – -4.7% and AAC: ED: -14.9%, 95% CI: -27.2 – -0.6%, electrical burst pain test PDT ED: -15.8%, 95% CI: -28.3 – -1.1% and pressure pain test PDT (ED: 23.7%, 95% CI: 3.6 – 47.7%). All these PainCart modalities simultaneously showed an LPS effect versus no treatment (pressure pain test PDT: ED: 41.3%, 95% CI: 18.9 – 68.0%) or placebo effect versus no treatment (cold pressor test PTT: ED: 18.9%, 95% CI: 6.1 – 33.3%; cold pressor test AAC: ED: 16.5%, 95% CI: 1.3 – 33.9%; electrical burst pain test PDT: ED: 30.1%, 95% CI: 12.9 – 50.0%) (**Table 2**).

POST-HOC ANALYSIS OF PAINCART RESULTS (PRE-DOSE UP UNTIL 6 H POST-DOSE) As with the primary analysis, LPS administration did not significantly alter pain thresholds over time, i.e. PDT, PTT and/or AUC endpoints from pre-dose up until 6 h post-dose, as shown in **Table 3** (statistical table in **Appendix C.2**). No dose-dependency was observed.

In the 1 ng/kg cohort, no significant effect of LPS versus placebo were found. Effects of LPS versus no treatment were found for the pressure PDT (ED: 43.2%, 95% CI: 17.5 – 74.5%); and CPM PDT (ED 1.99%, 95% CI: 0.46 – 3.51%). Placebo differed significantly from no treatment for cold pressor PTT and AAC (PTT: ED: 22.8%, 95% CI: 9.0 – 38.5%; AAC: ED: 18.8%, 95% CI: 2.7 – 37.4%) and for pressure pain PDT (ED: 18.1%, 95% CI: 8.0 – 60.6%).

In the 2 ng/kg cohort, significant effect of LPS versus placebo, and LPS versus no treatment were only found for the electrical stair PTT and AUC endpoints (versus placebo: PTT: ED: -9.5%, 95% CI: -17.0 – -1.2%; AUC: ED: 197.68, 95% CI: 31.80 – 361.55%; versus no treatment: PTT: ED: -9.6%, 95% CI: -16.9 – -1.6%; AUC: ED: 157.08%, 95% CI: 1.81 – 312.35%). No effects were observed in the placebo versus no treatment contrast.

Stress hormone response

Cortisol levels significantly increased after both 1 ng/kg and 2 ng/kg LPS administration, peaking at 3 h post-dose and gradually returning to baseline afterwards (**Figure 5-a**). No time-dependent fluctuations were observed in the placebo groups. LPS administration did not substantially alter bradykinin or PGE_2 levels (**Figure 5-b**,**c**). As half of the results were below LLOQ, kallikrein concentrations were not interpretable.

DISCUSSION

The goal of this study was to evaluate the effect and dose-dependency of an acute LPS-driven inflammatory response on pain perception using evoked pain tests. We did not observe significant pain threshold lowering in 12 healthy male subjects per dose level in a highly controlled setting – correcting for treatment-, placebo-, and long-term carry-over-effects, when analyzed over a 10 h period.

The underlying mechanism of inflammatory hyperalgesia is through the activation of the primary afferent nociceptors following increased release of sensitization-inducing mediators such as TNF-α, IL-1β, and bradykinin: molecules which are known to induce thermal and mechanical hyperalgesia. [6, 42, 43] Increased expression of these mediators is a downstream effect of the enhanced production of PGE_2, which in turn is caused by activation of cyclooxygenase-1 or -2 by a pro-inflammatory stimulus. [44, 45] LPS drives this response by activation of the TLR4 that, apart from being located on inflammatory cells, is also found on the dorsal root ganglia, dorsal root horn, Schwann cells and neuraxial glia. Based on these physiological mechanisms, and literature showing a link between LPS-induced acute inflammation and reduction in pain thresholds in both rodents and humans, the current study was performed. Adding to available reports, we have set-up our trial to study the dose-effect relationship between LPS and pain perception using comprehensive battery of evoked pain tests, and have evaluated these over-time. Here, LPS indeed induced a clear acute inflammatory response at both dose levels (1 ng/kg and 2 ng/kg), but this did not translate to significant effects on pain thresholds. A few isolated significant contrasts were observed, but no evident dose-dependent effects were found over the full-time course (pre-dose up to 10 h post-dose). However, when looking at the profiles for both doses in more detail – using a post-hoc analysis to assess pain thresholds from pre-dose to 6 h post-dose –, significant effects were reported for the cold pressor- and electrical stair pain test. Hyperalgesic effects were most pronounced two hours after dosing, and seem to correspond with the acute inflammatory response peak.

Previous human endotoxemia studies evaluating pain perception are inconclusive on potential sex-related differences. [27, 28] To exclude for a potential effect of gender, we limited our study to men. Although the selected LPS dose for our study was low (1 ng/kg and 2 ng/kg), approximately

50% of the subjects reported effects as feeling cold and/or sick. These clinical symptoms, though inevitably related to LPS exposure, may have interfered with (heat) pain testing and treatment blinding. We mitigated bias as much as possible by use of a double-blind design, allocation of subjects to separate testing rooms during study days, and standardized sequence and timing of pain tests. The electrical stair PTT immediately after the cold pressor test was used to quantify the conditioned pain modulation (CPM) response. Heat PDT's were quantified after the post-cold pressor electrical stair (see **Figure 2**) and may therefore possibly have been influenced through an ongoing CPM response (**Figure 2**). However, the possible bias – if at all present – will have been limited, as CPM is typically only short-lived [46-50] and because effects of LPS on pain thresholds were determined in a controlled fashion in which heat PDTs were always determined in the same order; they will therefore have been equally affected during each cross-over occasion. Our study included a sample size of 12 subjects per cohort. Using a Minimal Detectable Effect Size (MDES) calculation and results from the 12 subjects in the 2 ng/kg cohort, for heat PDT with a power of 80%, we could have detected a mean difference of 0.87 °C assuming a SD of differences of 0.98 °C; and for pressure PTT a mean difference of 16.3 kPa assuming a SD of differences on a 0.17 log scale. A size of 12 subjects per group was chosen to reflect a typical phase I drug study cohort, aligning with our study objective to validate the model for use in such a study.

Others have previously reported significant effects of intravenous LPS administration on nociception and pain tolerance thresholds, which contrasts with our findings. For example, de Goeij et al. showed that the inflammatory response following a 2 ng/kg intravenous LPS challenge significantly influenced the thermal, pressure and cold pressor pain test 2 h after the challenge. [29] It is important to note, however, that this 27 subject-study was performed in a non-cross-over fashion. In another study, Janum et al. demonstrated the effects of 2 ng/kg LPS on thermal pain and mechanical pain at 2 h post-dose, and on mechanical pain up to 6 h post-dose. [30] While noting that LPS-induced hyperalgesia was majorly reported for mechanical pain assessed with a methodology different from ours (i.e., a handheld algometer to measure pain sensitivity with, versus a tourniquet cuff to measure pain sensitivity (PDT) and tolerance (PTT) with) and so possibly contributes to the discrepancy between study outcomes, the current study has several key advantages over both de Goeij and Janum's

work. First, this study was designed to control for the substantial inter- and intra-subject variability of evoked pain tests, which is key for clinical pain research. [31-33] This contrasts with earlier studies that did not use a cross-over study design. [26, 28, 29] Moreover, as described in the methods sections, LPS has a long-term effect on the innate immune response, a factor that has not been taken into account in the other experimental pain studies of Wegner et al., de Goeij et al. and Karshikoff et al., [26-29, 51] yet has been mitigated in the current study. Finally, the majority of previous studies assessed LPS-induced hyperalgesia solely at one time point, [25, 27-29] or at a maximum of three time points, [26] and were performed around the E_{max} of LPS (2-3 h post-dose). Although results of Wegner et al. hint that effects are subtle after 3 h and non-significant at 6 h post-dose, the exact temporal relationship between the inflammatory response and hyperalgesia is not known. Only assessing specifically around the E_{max} is therefore a suboptimal experimental approach. In the current study, extensive time courses (pre-dose up to 10 h) were generated for cytokine and pain responses, allowing the full integration of both, and showing that the hyperalgesic effects of LPS are more subtle and time constrained (only briefly around 2 h post-dose) than assumed thus far.

Nonetheless, preclinical work also reported positive results that are discrepant from ours. [15-21] The reason for this may be two-fold. Apart from translatability issues, i.e., the fact that pre-clinical models often cannot be confirmed in other animal- or clinical models due to substantial inter-species variability, [52, 53] the LPS dose given to mice plausibly induced a more severe acute inflammatory response – yielding more pronounced clinical symptoms and therefore potential effects on pain thresholds –, when compared to the dose we administered to humans. We consciously did not exceed an LPS dose of 2 ng/kg knowing that notable flu-like symptoms would hamper execution of the (pain) tests, and that high-dose LPS administration may result in severe side effects such as (fatal) cardiac issues, sepsis and renal and/or kidney injury. [54-56] LPS doses, such as the ones we used, that induce a significant cytokine response with a mild adverse effect profile therefore might not be sufficient to evidently alter pain thresholds in humans. The marked cortisol response as shown in **Figure 5**-a may be causative for this lack in response. Elevated cortisol levels namely can increase pain thresholds when pain itself is not the 'stressor' [57-59] and so may have diminished the cytokine-driven hyperalgesia.

Despite thus being suitable for studying inflammatory pain-targeting compounds in rodents, the endotoxemia model cannot be used in humans for the same purpose: only subtle, non-significant effects of LPS on pain perception were observed over time. To evaluate if a more concise time window around the T_{max} of LPS would produce significant hyperalgesic effects, and so confirm positive findings from Wegner et al.'s work with a non-crossover design but similar timeframe, [26] we additionally performed a post-hoc analysis (**Table 3**). Now assessing pain thresholds from pre-dose up until 6 h post-LPS administration, this analysis showed significant effects for LPS vs placebo on electrical burst PTT (in 2 ng/kg cohort) and cold pressor PTT (in 1ng/kg cohort), but still no thermal- or mechanical hyperalgesia was observed. Given that a response on the latter two was expected based on human physiology, as outlined in the beginning of this chapter, we believe that we can conclude that no clear, dose-dependent and reproducible effect of LPS-induced endotoxemia on evoked pain thresholds were observed. The endotoxemia model is therefore not suitable for use in adequately controlled early-phase studies testing analgesics. Continuously infusing LPS as suggested by Kiers et al., [60] as alternative means to create a valid inflammatory hyperalgesia model, is also not a solution. Although the immune response will be extended, and thereby plausibly will induce more pronounced hyperalgesia, Kiers et al. also reported more pronounced and less transient flu-like symptoms, both during and after continuous infusion. In an experimental pain study such adverse effects would make execution of the study unfeasible, as discussed in the previous paragraph. Our conclusion does not relate to the validity of the human endotoxemia model for early-phase drug studies involving different mechanisms of action (i.e., anti-inflammatory), or other scientific settings.

CONCLUSION

Mild acute inflammation, as induced by 1 ng/kg and 2 ng/kg LPS administration, does not significantly change evoked pain thresholds in healthy male subjects. The endotoxemia model in combination with evoked pain tests is therefore not suitable to study drug effects on acute inflammatory hyperalgesia in healthy males.

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Table 1 Subject baseline characteristics.

Total subjects	24
AGE (YEARS)	
Mean (SD)	30.8 (9.5)
Range	19 – 52
GENDER	
Male	100%
ETHNICITY	
Caucasian	58.3%
Mixed	12.5%
Black/African	12.5%
Asian	8.3%
Other	8.3%
HEIGHT (CM)	
Mean (SD)	179 (6.9)
Range	168.5 – 190.4
WEIGHT (KG)	
Mean (SD)	76.4 (9.7)
Range	58.2 – 94.5
BMI (KG/M^2)	
Mean (SD)	23.8 (2.3)
Range	20.1 – 27.9

BMI: Body Mass Index

Table 2 PainCart evoked pain model results; pre-dose up until 10 h post-dose.

	Contrast for 1 ng/kg LPS dose			Contrast for 2 ng/kg LPS dose		
Modality	**LPS vs placebo**	**LPS vs none**	**placebo vs none**	**LPS vs placebo**	**LPS vs none**	**placebo vs none**
HEAT						
PDT	-0.4 *(p=0.486)* (-1.03 – 0.51)	-0.13 *(p=0.721)* (-0.87 – 0.62)	0.11 *(p=0.757)* (-0.65 – 0.88)	0.18 *(p=0.545)* (-0.44 – 0.81)	-0.22 *(p=0.441)* (0.80 – 0.36)	0.30 *(p=0.286)* (-0.88 – 0.27)
COLD PRESSOR						
PDT	3.5 *(p=0.750)* (-17.2 – 29.5)	29.1 *(p=0.057)* (-0.8 – 68.1)	26.9 *(p=0.075)* (-2.6 – 65.3)	20.5 *(p=0.232)* (-12.3 – 65.7)	13.8 *(p=0.305)* (-35.9 – 15.8)	**28.3** ***(p=0.0292)*** (-46.6 – -3.7)
PTT	**-15.8** ***(p=0.010)*** (-25.7 – -4.7)	0.8 *(p=0.8873)* (-10.1 – 13.0)	**18.9** ***(p=0.005)*** (6.1 – 33.3)	0.2 *(p=0.976)* (-14.6 – 17.7)	2.4 *(p=0.766)* (-13.3 – 21.0)	3.7 *(p=0.657)* (-12.2 – 22.4)
AAC	**-14.9** ***(p=0.044)*** (-27.2 – -0.6)	0.2 *(p=0.979)* (-12.9 – 15.2)	**16.5** ***(p=0.034)*** (1.3 – 33.9)	3.8 *(p=0.657)* (-12.8 – 23.6)	1.1 *(p=0.892)* (14.2 – 19.2)	1.3 *(p=0.865)* (-16.2 – 16.1)
ELECTRICAL STAIR						
PDT	-7.2 *(p=0.498)* (-26.2 – 16.7)	-3.3 *(p=0.747)* (-22.2 – 20.1)	5.4 *(p=0.710)* (-14.8 – 30.4)	11.3 *(p=0.399)* (-33.7 – 18.8)	-8.9 *(p=0.463)* (-29.8 – 18.1)	2.0 *(p=0.874)* (-21.3 – 32.2)
PTT	-5.0 *(p=0.252)* (-13.3 – 4.1)	-2.7 *(p=0.503)* (-10.4 – 5.8)	2.5 *(p=0.545)* (-5.7 – 11.3)	-7.4 *(p=0.059)* (-14.6 – 0.3)	-6.0 *(p=0.118)* (-13.1 – 1.7)	2.3 *(p=0.552)* (-5.4 – 10.6)
AUC	50.0 *(p=0.717)* (-239.19 – 339.19)	134.19 *(p=0.293)* (-127 – 395.39)	89.28 *(p=0.476)* (-169.69 – 348.26)	123.47 *(p=0.119)* (-35.09 – 282.04)	79.47 *(p=0.279)* (-69.29 – 228.23)	-47.69 *(p=0.507)* (-195.07 – 99.70)
ELECTRICAL BURST						
PDT	**-15.8** ***(p=0.037)*** (-28.3 – -1.1)	12.2 *(p=0.124)* (-3.4 – 30.3)	**30.1** ***(p=0.001)*** (12.9 – 50.0)	0.8 *(p=0.960)* (-26.9 – 38.9)	11.1 *(p=0.474)* (-17.6 – 49.8)	12.1 *(p=0.436)* (-16.8 – 51.0)
PTT	3.7 *(p=0.568)* (-9.1 – 18.1)	1.5 *(p=0.794)* (-9.7 – 14.0)	-2.6 *(p=0.650)* (-13.4 – 9.6)	-9.6 *(p=0.165)* (-21.8 – 4.6)	-4.4 *(p=0.495)* (16.5 – 9.4)	6.9 *(p=0.315)* (-6.6 – 22.5)
AUC	-4.29 *(p=0.920)* (-92.90 – 84.33)	-11.98 *(p=0.772)* (-96.71 – 72.76)	-19.92 *(p=0.620)* (-102.07 – 62.23)	39.59 *(p=0.325)* (-42.61 – 121.80)	-0.06 *(p=0.999)* (-76.12 – 76.0)	-44.84 *(p=0.233)* (-120.92 – 31.24)

(Table continues on next page)

	Contrast for 1 ng/kg LPS dose			Contrast for 2 ng/kg LPS dose		
Modality	**LPS vs placebo**	**LPS vs none**	**placebo vs none**	**LPS vs placebo**	**LPS vs none**	**placebo vs none**
PRESSURE						
PDT	**23.7** ***(p=0.022)*** (3.6 – 47.7)	**41.3** ***(p<0.001)*** (18.9 – 68.0)	18.1 *(p=0.060)* (-0.8 – 40.5)	6.2 *(p=0.419)* (-8.8 – 23.6)	3.0 *(p=0.675)* (-10.9 – 19.1)	-2.2 *(p=0.752)* (-15.3 – 12.9)
PTT	5.5 *(p=0.522)* (-10.5 – 24.2)	5.1 *(p=0.526)* (-10.5 – 23.3)	1.9 *(p=0.811)* (-13.2 – 19.6)	-2.6 *(p=0.622)* (-12.7 – 8.7)	-10.8 *(p=0.058)* (-20.8 – 0.4)	-9.1 *(p=0.116)* (-19.5 – 2.6)
AUC	123.79 *(p=0.987)* (-221.28 – 468.86)	38.09 *(p=0.862)* (-324.06 – 400.24)	-6.63 *(p=0.991)* (-365.29 – 352.02)	248.34 *(p=0.194)* (-137.84 – 634.52)	299.26 *(p=0.296)* (-283.15 – 881.68)	151.13 *(p=0.593)* (-429.42 – 731.67)
CPM						
PDT	0.46 *(p=0.421)* (-0.71 – 1.63)	1.06 *(p=0.070)* (-0.1 – 2.21)	0.91 *(p=0.101)* (-0.19 – 2.02)	0.30 *(p=0.556)* (-0.74 – 1.34)	0.70 *(p=0.159)* (-0.29 – 1.69)	0.58 *(p=0.220)* (-0.38 – 1.54)
PTT	0.49 *(p=0.233)* (-0.35 – 1.34)	-0.23 *(p=0.568)* (-1.09 – 0.62)	0.72 *(p=0.085)* (-1.54 – 0.11)	0.08 *(p=0.956)* (-0.37 – 0.53)	0.33 *(p=0.347)* (-0.80 – 0.13)	-0.32 *(p=0.696)* (-0.78 – 0.14)
AUC	-72.57 *(p=0.185)* (-183.38 – 38.25)	30.43 *(p=0.575)* (-141.70 – 80.85)	26.55 *(p=0.611)* (-80.62 – 133.73)	**-71.04** ***(p=0.043)*** (-139.76 – -2.33)	-47.78 *(p=0.171)* (-117.69 – 22.12)	5.13 *(p=0.881)* (-64.91 – 75.16)

Numbers represent estimates of the difference (in %), next to the p-value which is displayed in italic. Lower and upper limit (in %) of 95% confidence interval are shown between parentheses. AAC: area above the eVAS pain curve, AUC: area under the eVAS pain curve, CPM: conditioned pain modulation paradigm, eVAS: electronic Visual Analogue Scale, LPS: Lipopolysaccharide, PDT: pain detection threshold, PTT: pain tolerance threshold. Estimates >0 favor the first mentioned condition (i.e. LPS in LPS vs placebo contrast), estimates <0 favor the second condition (i.e. placebo in LPS vs placebo contrast).

Table 3 PainCart evoked pain model results; pre-dose up until 6 h post-dose. Numbers represent estimates of the difference (in %), next to the p-value which is displayed in italic. Lower and upper limit (in %) of 95% confidence interval are shown between parentheses.

	Contrast for 1 ng/kg LPS dose			Contrast for 2 ng/kg LPS dose		
Modality	**LPS vs placebo**	**LPS vs none**	**placebo vs none**	**LPS vs placebo**	**LPS vs none**	**placebo vs none**
HEAT						
PDT	-0.52 *(p=0.188)*(-1.304 – 0.274)	-0.41 *(p=0.273)* (-0.18 – 0.35)	0.09 *(p=0.805)* (-0.69 – 0.88)	-0.16 *(p=0.638)* (-0.87 – 0.54)	-0.41 *(p=0.217)* (-1.06 – 0.25)	0.15 *(p=0.643)* (-0.79 – 0.50)
COLD PRESSOR						
PDT	4.4 *(p=0.739)* (-19.5 – 35.3)	29.4 *(p=0.092)* (-4.3 – 75.1)	26.5 *(p=0.125)* (-6.7 – 71.6)	6.4 *(p=0.728)* (-26.2 – 53.5)	-5.8 *(p=0.721)* (-33.0- 32.5)	-13.6 *(p=0.0384)* (-38.5 – 21.4)
PTT	-19.3 *(p=0.003)** (-29.1 – -8.1)	-0.4 *(p=0.947)* (-11.7 – 12.3)	**22.8** ***(p=0.002)*** (9.0 – 38.5)	-7.1 *(p=0.34)* (-20.6 – 8.6)	2.1 *(p=0.795)* (-13.4 – 20.4)	9.8 *(p=0.254)* (-6.9 – 29.3)
AAC	-18.2 *(p=0.019)** (-30.3 – -4.1)	-3.3 *(p=0.743)* (-15.5 – 13.0)	**18.8** ***(p=0.023)*** (2.7 – 37.4)	-3.4 *(p=0.708)* (-20.0 – 16.7)	3.0 *(p=0.736)* (-13.9 – 23.2)	6.2 *(p=0.495)* (-11.1 – 26.8)
ELECTRICAL STAIR						
PDT	-13.6 *(p=0.252)* (-33.1 – 11.6)	-7.0 *(p=0.543)* (-26.9- 18.3)	10.1 *(p=0.414)* (-13.2 – 39.7)	-23.1 *(p=0.089)* (-43.4 – 4.4)	-20.0 *(p=0.108)* (-39.2 – 5.3)	3.3 *(p=0.809)* (-21.4 – 35.7)
PTT	-4.7 *(p=0.337)* (-13.9 – 5.5)	-4.2 *(p=0.349)* (-12.7 – 5.1)	1.5 *(p=0.748)* (-7.5 – 11.3)	**-9.5** ***(p=0.027)*** (-17.0 – -1.2)	**-9.6** ***(p=0.021)*** (-16.9 – -1.6)	0.1 *(p=0.977)* (-8.0 – 8.9)
AUC	38.3 *(p=0.787)* (-257.12 – 333.72)	157.94 *(p=0.233)* (-110.28 – 426.16)	110.30 *(p=0.397)* (-155.74 – 376.34)	**197.68** ***(p=0.022)*** (31.80 – 361.55)	**157.08** ***(p=0.048)*** (1.81 – 312.35)	-32.75 *(p=0.664)* (-186.45 – 120.95)

(Table continues on next page)

	Contrast for 1 ng/kg LPS dose			Contrast for 2 ng/kg LPS dose		
Modality	**LPS vs placebo**	**LPS vs none**	**placebo vs none**	**LPS vs placebo**	**LPS vs none**	**placebo vs none**
ELECTRICAL BURST						
PDT	-17. *(p=0.064)* (-31.8 – 1.2)	1.4 *(p=0.882)* (-15.7 – 22.0)	17.9 *(p=0.071)* (-1.4 – 41.1)	-1.4 *(p=0.934)* (-30.3 – 39.5)	6.7 *(p=0.686)* (-22.8 – 47.5)	11.6 *(p=0.493)* (-19.1 – 54.0)
PTT	3.2 *(p=0.635)* (-10.1 – 18.5)	-0.1 *(p=0.991)* (-11.7 – 13.1)	-3.7 *(p=0.536)* (-15.1 – 9.1)	-13.2 *(p=0.070)* (-26.6 – 1.2)	-7.5 *(p=0.274)* (-19.8 – 6.7)	6.3 *(p=0.388)* (-7.9 – 22.8)
AUC	-1.22 *(p=0. 979)* (-97.23 – 94.79)	-4.52 *(p=0.920)* (-96.06 – 87.03)	-8.74 *(p=0.843)* (-97.91 – 80.44)	62.0 *(p=0.155)* (-25.13 – 149.13)	26.43 *(p=0.508)* (-54.37 – 107.22)	-34.75 *(p=0.384)* (-115.4 – 45.89)
PRESSURE						
PDT	12.3 *(p=0.259)* (-8.6 – 37.9)	**43.2** ***(p<0.001)*** (17.5 – 74.5)	**18.1** ***(p=0.008)*** (8.0 – 60.6)	6.8 *(p=0.467)* (-10.9 – 27.9)	8.5 *(p=0.344)* (-8.7 – 29.1)	3.6 *(p=0.676)* (-12.6 – 22.9)
PTT	3.9 *(p=0.650)* (-12.5 – 23.4)	6.7 *(p=0.429)* (-9.7 – 26.2)	1.9 *(p=0.550)* (-11.1 – 24.2)	-4.0 *(p=0.481)* (-14.7 – 8.0)	-4.9 *(p=0.394)* (-15.5 – 7.1)	-3.3 *(p=0.567)* (-14.1 – 8.9)
AUC	81.74 *(p=0.814)* (-221.28 – 468.86)	20.47 *(p=0.950)* (-648.83 – 689.77)	-114.24 *(p=0.727)* (-783.11 – 554.63)	295.75 *(p=0.152)* (-115.85 – 707.35)	41.15 *(p=0.840)* (-376.34 – 458.63)	-159.87 *(p=0.433)* (-575.95 – 256.21)
CPM						
PDT	1.22 *(p=0.121)* (-0.33 – 2.76)	**1.99** ***(p=0.011)*** (0.46 – 3.51)	0.69 *(p=0.363)* (-0.91 – 2.18)	0.56 *(p=0.441)* (-0.88 – 2.00)	0.95 *(p=0.164)* (-0.40 – 2.31)	0.45 *(p=0.489)* (-0.85 – 1.76)
PTT	0.62 *(p=0.243)* (-0.43 – 1.67)	-0.12 *(p=0.835)* (-1.23 – 1.00)	-0.96 *(p=0.080)* (-2.05 – 0.12)	0.06 *(p=0.862)* (-0.65 – 0.77)	0.01 *(p=0.977)* (-0.66 – 0.68)	-0.04 *(p=0.911)* (-0.73 – 0.65)
AUC	-68.54 *(p=0.275)* (-194.26 – 57.18)	-59.83 *(p=0.360)* (-190.42 – 80.76)	36.71 *(p=0.565)* (-90.93 – 164.34)	-69.33 *(p=0.133)* (-160.44 – 21.79)	-62.93 *(p=0.164)* (-152.21 – 26.35)	2.36 *(p=0.958)* (-85.74 – 90.47)

AAC: area above the eVAS pain curve, AUC: area under the eVAS pain curve, CPM: conditioned pain modulation paradigm, eVAS: electronic Visual Analogue Scale, LPS: Lipopolysaccharide, PDT: pain detection threshold, PTT: pain tolerance threshold. Estimates >0 favor the first mentioned condition (i.e. LPS in LPS vs placebo contrast), estimates <0 favor the second condition (i.e. placebo in LPS vs placebo contrast).

Figure 1 Flow of study visits per group (n=12). Screening and inclusion assessments could be performed up to 42 days prior to the first study day (occasion 1). Upon inclusion, subjects were assigned to one of the two treatment arms as indicated (stratified n=11 and n=1) and were admitted to the clinical unit three times in total, with a time window of 7-21 days between the first and last date of admittance (i.e. between occasion 1 and occasion 3). Participation was concluded with a safety follow-up visit, 5-9 days after the last dosing performed in occasion 3.

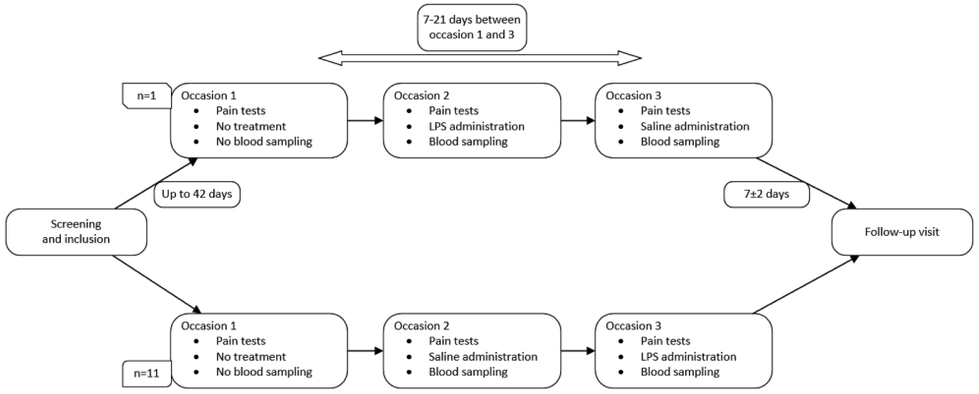


Figure 2 Sequence and timing of performed pain tests (PainCart) with respective endpoints.

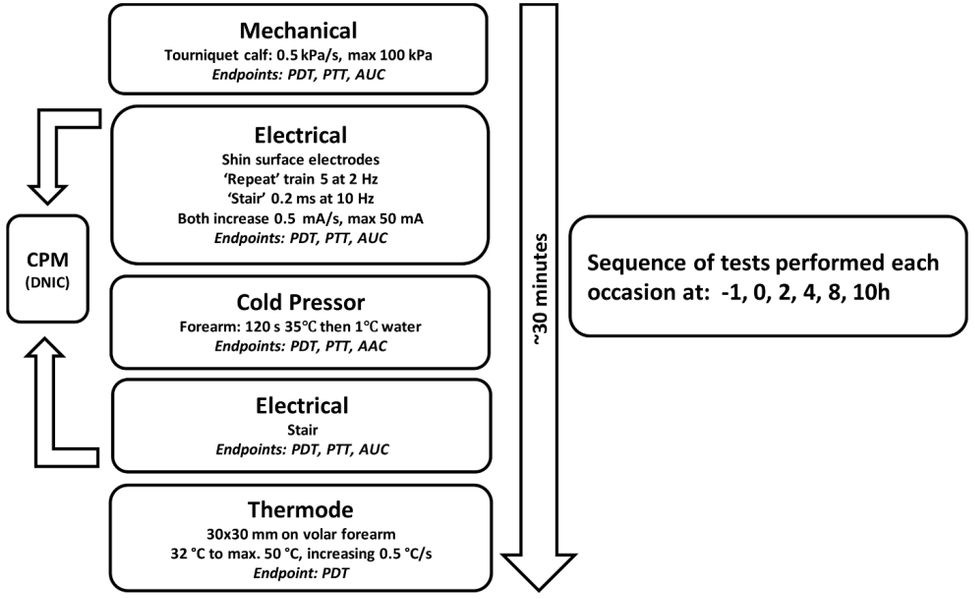
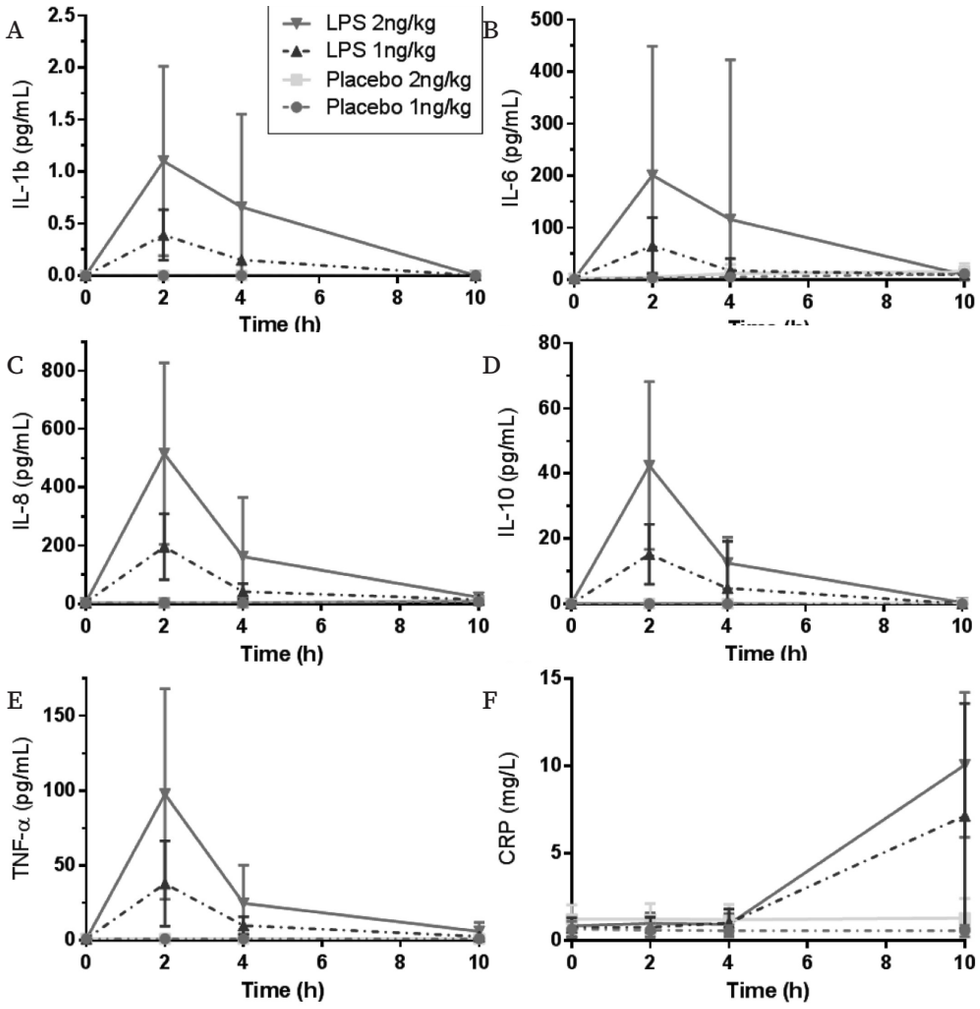
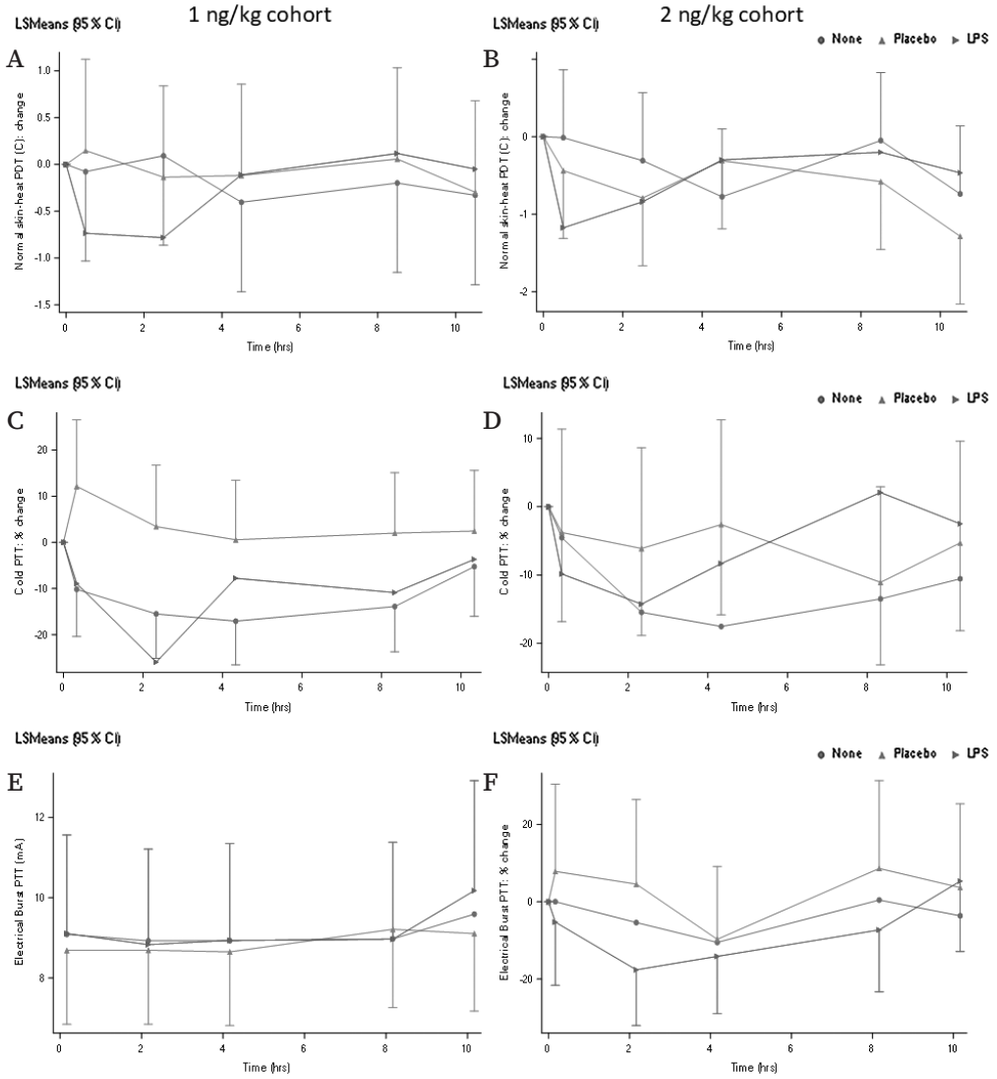


Figure 3 Cytokine concentrations after LPS or placebo administration, measured pre-dose (0h) up until 10h (hours) post-dose.



Vertical lines represent standard deviations. a: IL-1b concentrations (pg/mL), b: IL-6 concentrations (pg/mL), c: IL-8 concentrations (pg/mL), d: IL-10 concentrations (pg/mL), e: TNF-α concentrations (pg/mL), f: CRP concentrations (mg/mL). CRP: C Reactive protein, IL: interleukin, pg/mL: LPS: Lipopolysaccharide, mg or pg/mL: microgram or picogram/milliliter, respectively.

Figure 4 Selection of PainCart test results. Graphs in the left column represent subjects that received 1 ng/kg LPS, right column for subjects that received 2 ng/kg. Data represented as change from baseline in percentages (%), in which baseline has been defined as the pre-dose measurement of that occasion. Values on y-axis represent the least square means change of the 95% confidence interval, time is described in hours on the x-axis. a and b: heat pain PDT; c and d: cold pressor PTT; e and f: electrical bust PTT, g and h: electrical stair PTT; i and j: pressure pain PTT, k and l: CPM PTT.



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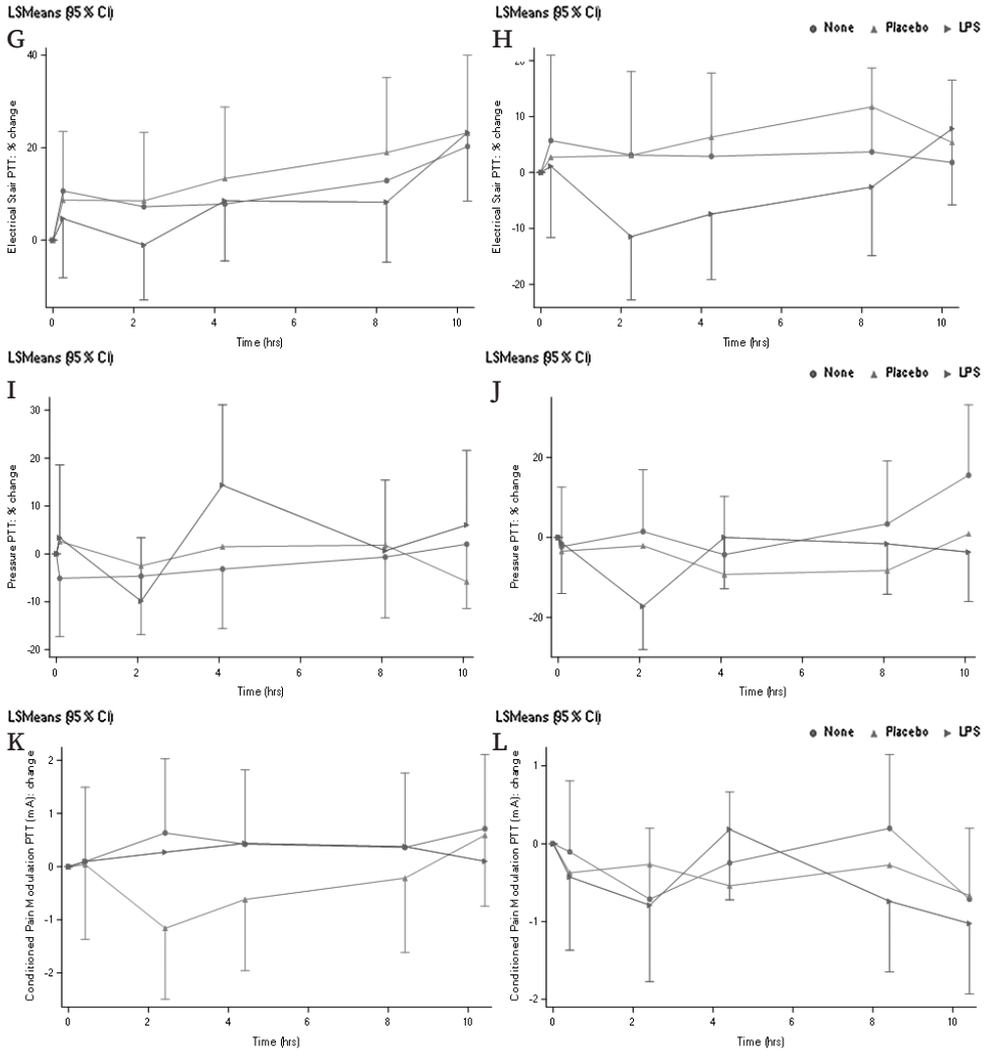
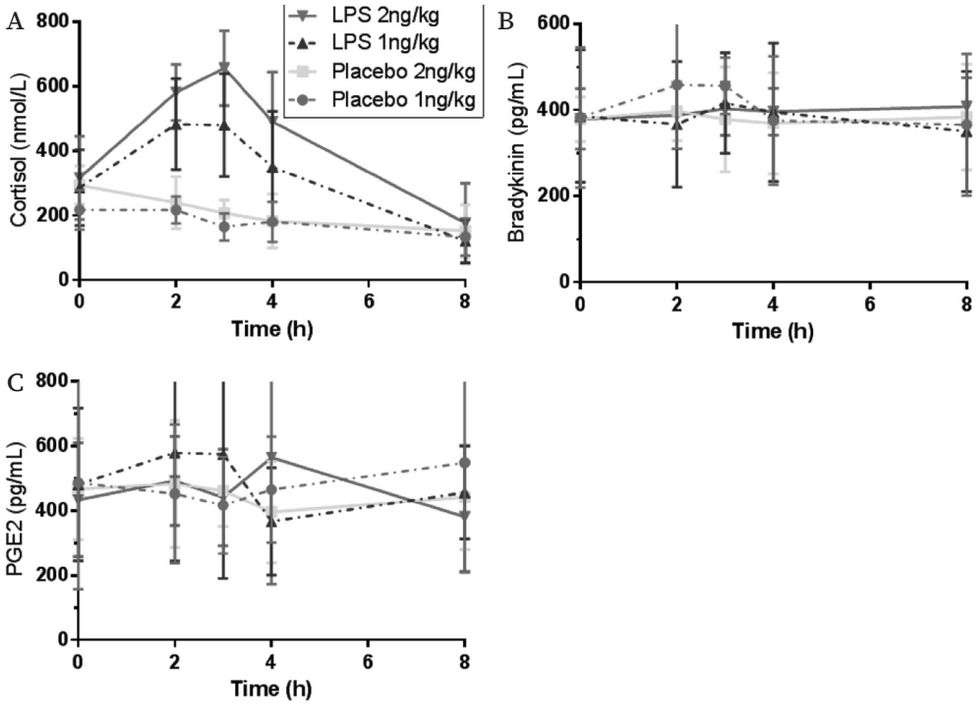


CPM: conditioned pain modulation, PDT: pain detection threshold, PTT: pain tolerance threshold.

Figure 5 Stress hormone concentrations after LPS or placebo administration, measured pre-dose (0h) up until 8h (hours) post-dose. Vertical lines represent standard deviations. a: cortisol concentrations (nnmol/mL). b: bradykinin concentrations (pg/mL). c: PGE_2 concentrations (pg/mL).



LPS: Lipopolysaccharide, PGE: prostaglandin E_2.

[SUPPLEMENTARY MATERIAL AVAILABLE ONLINE AT THE PUBLISHER'S WEBSITE]

CHAPTER 6

A crossover study evaluating the sex-dependent and sensitizing effects of sleep deprivation using a nociceptive test battery in healthy subjects

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ABSTRACT

AIM We assessed whether total sleep deprivation (TSD) in combination with evoked pain tests may be a suitable method to evaluate (novel) central-acting analgesics in healthy subjects.

METHODS This was a two-part randomized crossover study in 24 healthy men and 24 women. Subjects were randomized 1:1 to first complete a day of non-sleep deprived nociceptive threshold testing, followed directly by a TSD-night and a morning of sleep-deprived testing; or first complete the TSD night and morning sleep-deprived testing, to return seven days later for a day of non-sleep deprived testing. A validated pain test battery (heat-, pressure-, electrical burst and stair-, cold pressor pain test, and conditioned pain modulation (CPM) paradigm) and sleep-questionnaires were performed.

RESULTS Subjects were significantly sleepier after TSD as measured using sleepiness questionnaires. Cold pressor pain tolerance (PTT, estimate of difference (ED): -10.8%, 95% CI: -17.5 – -3.6%), CPM PTT (ED: -0.69 mA, 95% CI: -1.36 – -0.03 mA), pressure PTT (ED: -11.2%, 95% CI: -17.5% – -4.3%) and heat pain detection thresholds (PDT, ED: -0.74 °C, 95% CI: -1.34 – -0.14°C) were significantly decreased after TSD compared to the baseline morning assessment in the combined analysis (men + women). Heat hyperalgesia was primarily driven by an effect of TSD in men, whereas cold and pressure hyperalgesia was primarily driven by the effects of TSD observed in women.

CONCLUSIONS TSD induced sex-dependent hyperalgesia on cold-, heat- and pressure pain and CPM response. Results suggests that the TSD model may be suitable to evaluate (novel) analgesics in early-phase drug studies.

INTRODUCTION

Sleep disturbance is a highly prevalent symptom in chronic pain patients – over 50% also report having impaired sleep. [1] Studies in primary insomnia patients or subjects deprived of sleep reported the development of spontaneous pain and increased sensitivity to (experimentally) evoked pain. [2] Therefore, proper sleep is necessary to maintain homeostasis of pain-regulatory processes.

The use of pain models in early-phase pharmacological studies may help to reduce decision-making risks during the translational process from preclinical models to patients with pain, and to determine the biological activity of the studied drug. [3] Most models used to evaluate effects of (novel) analgesics in healthy subjects are evoked pain tasks eliciting nociceptive pain, e.g., pressure or heat application to or on extremities, where there are limited to no disturbances in central pain processing. Such models are less suitable to fully assess the analgesic potential of (novel) neuropathic pain treatments that primarily act in the CNS and target centrally induced lowering of pain thresholds. [4]

Easily adoptable models that do assess effects on central pain processing mostly are based on peripheral input (e.g., secondary hyperalgesia induced in the capsaicin- or ultraviolet-B (UVB) hyperalgesia models rely on peripheral activation), [5] which does not apply to sensitization caused by sleep deprivation. In addition, models that evaluate central effects often have limitations that prevent repetitive use in studies with a cross-over design (which in (pharmacological pain) studies is preferred to limit the required sample size through increased statistical power compared to a parallel design [6]). Reasons precluding repetitive use of other central-acting models may be ethical, or practical. A high subject burden due to unpleasantness of the test procedure is an one example of the former (e.g., intradermal capsaicin injection, or high/low-frequency stimulation), [4,7] and long-term adverse effects another (e.g., the prevalence of post-inflammatory hyperpigmentation with the freeze injury- and ultraviolet-B hyperalgesia models). [8,9] Other models, e.g., secondary hyperalgesia/allodynia induced by topical capsaicin, appear to be only limitedly sensitive to pharmacological interventions, or limitedly reproducible. [5,10]

Sleep deprivation in combination with evoked pain tests may be a suitable alternative to study the effects of centrally acting analgesic drugs. [11] By depriving healthy subjects of sleep, central pain pathways are

affected: disturbed sleep significantly decreased pain tolerance to peripheral mechanical-, heat-, and cold stimuli. [2,12–14]. Sleep deprivation-induced alterations in the endogenous pain inhibition pathway have also been reported (i.e., impaired conditioned pain modulation (CPM) response), a centrally acting mechanism. [2,12–14] Use of sleep deprivation as a tool to evaluate (investigational) analgesics, however, is precluded by discrepant findings. Effects on thermal pain tolerance, for example, differed between those reported by Onen et al. (no significant effect), [15] compared to those by Kundermann et al. (significantly lower heat and cold pain thresholds), [16], and to those by Eichhorn et al. (heat pain solely decreased in women; cold hyperalgesia was induced sex-independently). [13] Given this discrepancy, and to accurately assess a drugs' potential analgesic effect, it is necessary that the sleep deprivation model is first validated without intervention prior to further use in studies with (investigational) analgesics(s).

Here, we investigated the (sex-dependent) effects of sleep deprivation on pain responses in healthy subjects using a comprehensive and validated evoked pain test battery. [17–19]

METHODS

General considerations

This study was performed at the Centre for Human Drug Research, Leiden, The Netherlands in accordance with the Declaration of Helsinki of 1975, as revised in 2013. Approval of the Medical Ethics Committee 'Stichting Beoordeling Ethiek Biomedisch Onderzoek', Assen, the Netherlands, was obtained prior to study start. Results reported here are part of a larger study that also answered distinctly different objectives: to determine the effects of sleep deprivation on driving performance and to determine the validity of an intra-epidermal stimulation method. Those results will be or are reported elsewhere. [20] The trial was registered in the Netherlands Trial Register (NTR number NL7517).

Study subjects and design

Twenty-four men and 24 women were enrolled. All subjects provided consent before any study procedures took place. Interested subjects were

medically screened and enrolled if they were men (part A) or women (part B), 18 – 35 years of age (inclusive), and excluded if they had sleep disturbances, irregular sleeping patterns (e.g., night shifts), or went through a change in time zone(s) seven days prior to the first test day. Regular smokers (> 10 cigarettes or equivalent per day) were excluded from participation, as were those that smoked 24 h before each visit, used (illicit) drugs, consumed alcohol within 24 h prior to each visit, consumed > 8 units of (methyl-)xanthine-holding products per day or consumed this 4 h before each visit. Subjects were acquainted with all tests during a training session (details of tests in section **Study procedures – evoked pain tasks**) that was part of screening procedures. Subjects who did not understand the instructions or who indicated to be intolerable during the pain test training or achieved tolerance at >80% of the maximum input intensity for the cold-, pressure- and/or electrical pain task(s) were excluded. This tolerance threshold of 80% was included to ensure that both an increase and decrease in pain response could be determined during the test visits.

The menstrual cycle may influence (evoked) pain perception. [21–23] To minimize a possible influence on test results, women (part B) were required to use a reliable hormonal contraception method at least 30 days before the first study day until the end of the study. In addition, one of the following was required for participation: A) use of the contraceptive pill continuously (no stop week) throughout the study; B) use of contraceptive pill with a planned stop week, in which the study days are >2 days after re-start of contraceptive pill use; C) in case of other hormonal contraceptives, study days are >2 days after end of withdrawal bleeding.

Subjects in both parts (i.e., A and B) were randomized in a 1:1 ratio (**Figure 1**). Per part, 12 subjects first completed a visit in which two baseline measurement rounds were performed, one in the morning (MORN) and one in the afternoon (AFT). This was followed directly by a night of total sleep deprivation (TSD) and one measurement round post-sleep deprivation (arm 2, **Figure 1**). The other 12 subjects first completed the TSD night and subsequent measurement round, after which they went home to revert to their normal sleep pattern. At least seven days later, these subjects reported back to the clinical unit for a second visit during which the two baseline measurement rounds (MORN and AFT) took place (arm 1, **Figure 1**). This design was identical for both study parts.

Due to the COVID-19 pandemic and related local regulations enforced at time of study conduct of part B, subjects in part B arm 1 were required

to report to clinic at 8.00 instead of 19.00 (see next section) to allow for a day of quarantine and COVID-19 screening; whereas subjects that participated in part B study arm 2 (**Figure 1**) were required to stay an additional full study day at our unit in quarantine including COVID-19 screening. The quarantine day in this arm preceded the regular study visits. Part A was completed before the COVID-19 pandemic thus unaffected.

Study intervention – sleep deprivation

Subjects were deprived from sleep for at least 24 hours at time of the measurements following TSD. The night before TSD, subjects were instructed to go to bed between 22:00-23:00, wake up between 07:00-08:00 the next day and report to the clinic around 19.00 to start the TSD night. To aid subjects in staying awake for the duration of the TSD, a personal activities schedule was created at the start of the visit together with the subject (e.g., playing (video) games, splash cold water in face, or light exercise) to provide structure during the night. Subjects were allowed to deviate from this schedule if, e.g., an activity was found to be effective to promote wakefulness and prevent sleepiness. During the TSD night (19:00 to 07:00), study staff closely monitored the subject and reinforced motivation to ensure compliance and avoided that subjects took naps. Caffeine use was not allowed during TSD.

Study procedures – evoked pain tasks

Pain detection thresholds (PDT) and pain tolerance thresholds (PTT) were evaluated using a multi-modal and fixed-sequence pain test battery at pre-specified timepoints (each test round is indicated with 'T' in **Figure 1**). All subjects thus completed three rounds of pain tests. Details of the procedures have been described extensively elsewhere and therefore only briefly recited here below. [24,25] Measurements were performed in the following sequence: heat pain task, pressure pain task, electrical pain task – repeated stimulus ('burst'), electrical pain task – single stimulus ('stair') (pre-cold pressor, #1), cold pressor pain task, electrical pain task – single stimulus ('stair') (post-cold pressor, #2) and completed with the intra-epidermal stimulation test (results reported elsewhere). [20] Pain intensity for all tests except the heat pain test, was captured using an electronic Visual Analogue Scale (eVAS)-slider. 0 was defined as 'no pain', and 100 as 'worst pain tolerable'. PDT was defined as eVAS > 0; PTT as eVAS = 100.

Heat PDTs were measured with a thermode (contact area: 30mm × 30mm; QSense, Medoc, Israel) placed on the volar forearm that gradually increased from 32 °C with 0.5 °C/s. The test continued until the subject indicated his/her PDT by pushing a button on a hand-held feedback control; or when the safety cut-off of 50 °C was reached. The average of three measurements was used for analysis.

For the pressure pain test, an 11-cm wide tourniquet cuff (VBM Medizintechnik GmbH, Sulz, Germany) was placed over the gastrocnemius muscle. Pressure was computer-controlled with an electro-pneumatic regulator (ITV1030-31F2N3-Q, SMC Corporation, Tokyo, Japan), Power1401mkII analogue–to-digital converter and Spike2 software (CED, Cambridge, UK). Pressure constantly increased with 0.5 kPa/s until PTT or the safety cut-off of 100 kPa was reached.

At start of the cold pressor test, the non-dominant hand was placed in a bath (minimal depth of 200 mm) filled with circulating warm water (35 ± 0.5 °C), for 2 minutes. After 1 min 45 s, a blood pressure cuff was wrapped around the non-dominant upper arm and inflated to 20 mmHg below resting diastolic pressure, limiting warm blood to return to the hand. At 2 min, the hand moved from the warm bath to a similar bath filled with circulating cold water (1.0°C). Using the eVAS slider, PDT and PTT were recorded in seconds. The hand was immediately removed from the cold water when PTT was reported or when the time limit of 120 s was reached, at which point the cuff would also deflate and the hand was removed from the water bath.

Two types of electrical pain paradigms were included (single stimulus ('stair') and repeated stimulus ('burst')), by placing two electrodes (Ag-AgCl) on cleaned skin overlying the left tibial bone. The 'stair' test directly stimulates the nerve and bypasses nociceptors, [26] whereas the 'burst' paradigm serves as proxy for temporal summation/wind-up. [27] With the stair test, single stimuli (10 Hz tetanic pulse; 0.2 ms duration each; intensity increase with 0.5 mA/s) were administered by a constant current stimulator. With the burst paradigm, each single stimulus (train of five, 1 ms square wave pulses repeated at 200 Hz) was repeated five times at the same current intensity, at 2 Hz, and a random interval of 3-8 s between repeats. The intensity increased as with the stair test. Tests stopped automatically when PTT or the safety cut-off of 50 mA was reached.

The electrical stair pain task was performed twice per round: once before and once after the cold pressor test, to evaluate the CPM response. [28] The electrical stair pain test post-cold pressor test (used as test stimulus) was performed as soon as possible after completion of the cold pressor test (used in the CPM paradigm as conditioning stimulus).

Study procedures – questionnaires

Subject-reported sleepiness was collected using the nine-point Karolinska Sleepiness Scale (KSS). [29] Early morning behaviour was characterized using the Leeds Sleep Evaluation Questionnaire (LSEQ) questions 8-10, using a 100 mm eVAS (question 8: how did you feel on waking (ranging from tired to alert), question 9: how do you feel now (ranging from tired to alert), question 10: how would you describe your balance and co-ordination upon awakening (ranging from clumsier than normal to less clumsy than normal)). [30] Both questionnaires were completed at the start of the well-rested measurements, and at the start of the TSD measurements (**Figure 1**).

Statistical considerations and analysis

The CPM response was calculated as the difference between the electrical stair PDT or PTT measured pre-cold pressor test, and the same parameter post-cold pressor test. As the CPM response is generally short-lived, [28] only results of the electrical stair test within 5 minutes after start of the cold pressor test were used for further analysis, to ensure actual CPM effects were evaluated. For CPM PDT and PTT, data of one subject in Part B for well-rested morning and well-rested afternoon state were excluded. For CPM PDT and PTT in Part B, data for two subjects for the well-rested morning state, and for three subjects in the well-rested afternoon state were excluded.

To estimate the differences between groups (sleep deprived/well-rested morning/well-rested afternoon) and sex and the interaction sex and group, data were analysed with a mixed model analysis of variance with fixed factor group, sex and sex by group, and random factor subject. The Kenward-Roger approximation was used to estimate denominator degrees of freedom and model parameters were estimated using the restricted maximum likelihood method. Parameters were initially analysed without transformation. Except those from the heat and CPM-task, all the pain test parameters suggested otherwise, therefore log-transformation was applied. Log-transformed parameters were back-transformed after analysis to allow for interpretation as percentage change.

RESULTS

Subject characteristics

See **Table 1** for subject characteristics. In part A, 28 men were enrolled of which 23 completed all study assessments. In treatment arm 1 (**Figure 1**), one subject had a positive drug test and was replaced; one other withdrew consent. He was not replaced. Data obtained from these two subjects from visit 1 could be used for analysis. In treatment arm 2, one subject had a positive drug test and was replaced; his replacement got sick following minor food poisoning and was also replaced. One other withdrew consent following a headache and was not replaced following protocol regulations. The mean age of subjects for which data was used for final analysis was 26 years (SD ±2.2).

In part B, 24 women were enrolled of which 23 completed all study assessments. In the second treatment arm, one subject withdrew consent during the TSD night following a headache that did not subside. She was not replaced following protocol regulations. The mean age was 25.9 (SD ±3.0).

Results – evoked pain tasks

Significant effects of TSD on the total group (i.e., men and women combined) were observed, as well as in men only (part A) and in women only (Part B) (**Table 2** and **3**, **Figure 2**).

For the cold pressor task, significant effects were noted for the combined- and women group. Cold PTTs were significantly reduced after TSD compared to MORN (combined: Estimate of Difference (ED): -10.8%, 95% Confidence Interval (95% CI): -17.5% – -3.6%, p<.01; women only: ED: -18.5%, 95% CI: -26.9% – -9.1%, p<.001). Men reported a significantly higher cold PDT compared to women (ED: 49.4%, 95% CI: 4.5 -113.6%, p<.05).

CPM response PTT was significantly decreased in the combined group after TSD compared to MORN (ED: -0.69 mA/s, 95% CI: -1.36 mA/s – -0.03

mA/s, p<.05). No significant effects were observed in the men only group or women only group.

Heat PDTs were significantly lower in the combined and men group after TSD compared to the MORN (combined: ED: -0.74 °C, 95% CI: -1.34°C -0.14°C, p = .016; men only: ED: -1.06°C, 95% CI: -1.9°C -0.22°C, p<.05).

Pressure PTTs were significantly decreased for the combined- and women group when comparing the TSD measurement to the MORN (combined: ED: -11.2%, 95% CI: -17.5% – -4.3%, p<.01; women only: ED: -14.7%, 95% CI: -23.1% – -5.3%, p<.01). A significant difference was also noted in the combined group for the AFT versus MORN contrast (ED: -8.1%, 95% CI: -14.6% – -1.2%, p<.05).

No significant effects were noted for any contrast for the electrical burst, or electrical stair pain tests (**Table 2** and 3).

Results – questionnaires

Subjects reported to be significantly sleepier the morning following TSD compared to MORN, as reported on the KSS (ED: 4.3, 95% CI: 3.9 – 4.6, p<.001). Men scored significantly higher than women at both measurements combined (ED: 0.5, 95% CI: 0.2 – 0.9, p<.01). Morning behaviour, as assessed with the LSEQ, was also significantly more tired and unbalanced after TSD compared to behaviour at MORN (ED: -28.23, 95% CI: -32.8 – -23.7, p<.001).

DISCUSSION

We evaluated the effects of TSD on pain perception in healthy men and women. Cold pressor pain, the CPM response, heat pain detection and pressure pain thresholds were significantly lowered; effects were sex-dependent.

The intervention to deprive subjects of sleep was successful, as observed from the KSS (sleepiness) and LSEQ (early morning behavior) questionnaire results. While sleep deprivation significantly reduces attention and vigilance, [31] this likely did not bias results as others also discussed previously. [11] In case of reduced attention, a parallel shift in pain detection and -tolerance is expected – plausibly on each pain modality in approximately the same manner. Both men and women however, could still distinguish pain detection from tolerance as exemplified by significantly

altered tolerance to pressure- and cold pain (PTT), but not detection of pressure and cold pain (PDT, **Table 2**). Our data therefore indicate that subjects were sleep deprived yet sufficiently focused to properly conduct the tests.

Preclinical studies indicate that sleep deprivation-induced hyperalgesia is partly caused by inhibition of the endogenous opioid protein synthesis and lowering the mu- and delta-opioid receptor affinity. [32,33] Other neurotransmitters, such as serotonin (5-HT), appear to play a role in maintaining hyperalgesia. [34,35] This corroborates with clinical evidence showing the importance of opioid- and serotonergic supraspinal mechanisms in the descending pain inhibitory pathway, and the impaired CPM response we and others observed following sleep deprivation. [13,36] The increased sensitivity to the pressure pain test we observed – which aims to activate deep tissue mechanoreceptors – [37] builds on this hypothesis, given that the descending pain pathway, when not impaired, particularly inhibits neural activity residing in deep tissue. [38] The exact pathophysiology underlying the observed sleep deprivation-induced heat and cold hyperalgesia is less clear. Heat-induced hyperalgesia is typically restricted to peripheral sensitization mechanisms. In cold-induced hyperalgesia, both peripheral and central processes are involved but these do not relate to those proposed for sleep deprivation. [11] It is interesting to note that the cold pressor task is commonly employed to study the analgesic effects of opioids in healthy subjects and in patients. [39] Decreased cold pressor thresholds following sleep deprivation may thus support the involvement of a temporarily impaired endogenous opioid system. Further investigation into the underlying mechanisms of sleep deprivation-induced heat and cold hyperalgesia is warranted.

Clinical studies evaluating pain thresholds after sleep deprivation reported inconsistent results due to different readouts, different sleep deprivation protocols, or study designs. [11,13,40] A comprehensive study using quantitative sensory testing was performed to address this and reported results in line with the results discussed here: heat, cold and pressure thresholds were affected by a night of TSD. [11] A recent follow-up study reported sex-dependent effects of TSD on the CPM response and heat hyperalgesia in women and sex-independent effects on cold and mechanical hyperalgesia. [13] Sex-independent effects of TSD on the cold pressor task were also observed in another study that employed a similar design. [40] Our results confirm that TSD induces heat-, pressure- and cold

hyperalgesia. The significantly lowered threshold of heat pain detection in the combined group is largely driven by the effects induced in men, while the significantly lowered threshold of pressure and cold pain tolerance thresholds are largely driven by the effects induced in women (**Table 3**, **Figure 2**). As the pathophysiology of heat and cold hyperalgesia caused by TSD is yet to be elucidated, it is difficult to dissect why effects on the cold pressor test were seemingly more sex-dependent in our study. The distinct pressure pain protocols used may have played a role in a few of the other discrepancies observed. Where Schuh-Hofer et al. and Eichhorn et al. evaluated more superficial and local pain using an algometer and used this for evaluation of the CPM response, [11,13] we used a pressure cuff around the leg that also target deep tissue nociceptors – which are primarily affected by the descending pain pathway as stated above – and used the electrical stair task as test stimulus for CPM.

Currently available neuropathic pain treatments are only efficacious to a limited extent, resulting in an unmet clinical need. [41] There is great interest in developing drugs for neuropathic pain in general, and in drugs that can treat central sensitization specifically. Here, we showed that TSD lowered pain thresholds and impaired the CPM response, suggesting that TSD alters descending input from the CNS. [13,15,16] In this study we also observed altered neural activity by increased detection probability of a double-pulse electric intra-epidermal stimulus, that further suggested increased facilitation or decreased inhibition (results published elsewhere [20]). While not strictly related to central sensitization, an imbalance between descending inhibition and ascending facilitation of pain signals, is one of the changes found in patients with chronic pain states. [42,43] Sleep disruption also alters other central pain processes, in a sex-dependent manner: an impaired CPM response and increase in temporal summation (a phenomenon often reported in chronic pain disorders) was observed in females, [13,44] whereas secondary hyperalgesia (a model for neuropathic pain) was induced in males. [44,45] We discussed in the introduction that, while surrogate models for central sensitization in humans do exist, [4] they mostly require peripheral input and are of limited use in experimental pain studies with a cross-over design. TSD in combination with nociceptive testing may therefore offer an alternative method to evaluate central mechanisms that play a role in chronic pain [11] and may be suitable to demonstrate and quantify effects of analgesics aimed to treat central sensitization and neuropathic pain. We aim to confirm this

assumption in a next study that will include the same pain test battery and TSD model in which we will administer drugs used to treat neuropathic pain to both males and females. In case a sex-dependent drug response is observed in that next study, results may also aid clinicians in making drug prescription decisions for pain patients suffering from sleep disorders. No opioids will be tested in that next study, as sleep restriction – a similar yet distinct model to TSD – has been shown to attenuate morphine analgesia. [46] To the best of the authors knowledge, that is the only published study which employed the TSD (or similar) model in context of analgesic drug testing. It thus remains to be seen which nociceptive test following TSD will be most sensitive to drug effects.

Results presented here are to be read with the following considerations. First, only short-term effects of TSD over one night were assessed, while pain including that due to sleep disturbance is mostly a chronic process. Study results can therefore be used for method development, but are only of limited use for understanding the pathophysiology of sleep deprivation-induced/conditioned pain. [34] Additionally, the age of enrolled subjects was relatively young, limiting our conclusions. A young age range was included as this study was part of a larger study in which we also assessed the effects of TSD on driving performance. [31] Recruiting older subjects for that test was considered unsafe. We did not correct for multiple statistical testing, which may have led to false positives in our results. This was deemed acceptable as the study was exploratory of nature, and commonly adopted approach in early-phase exploratory drug studies.

In conclusion, TSD induced sex-dependent hyperalgesia on cold-, heat- and pressure pain and impaired the CPM response. This confirmed TSD as a method to alter central pain processes. Our data suggest that the model may be used to evaluate (novel) analgesics in experimental pain studies that (partially) target central processes. Investigators should be aware of a sex-dependent response when using the model.

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Table 3 Subject characteristics.

Demographic category	Total (n=50)	Men (n=26)	Women (n=24)
AGE (YEARS)			
Mean (SD)	26.1 (2.6)	26 (2.2)	25.9 (3)
WEIGHT (KG)			
Mean (SD)	70.8 (11.6)	77.5 (11.2)	64.1 (7.5)
Height (cm)			
Mean (SD)	176 (10.1)	183.1 (8.5)	168.6 (4.9)
BMI (KG/M^2)			
Mean (SD)	22.8 (2.4)	23 (2.1)	22.7 (2.7)
FITZPATRICK SKIN TYPE, N (%)			
Type I	3 (6%)	0	3 (12%)
Type II	28 (56%)	13 (50%)	15 (63%)
Type III	17 (34%)	13 (50%)	4 (17%)
Type IV	2 (4%)	0	2 (8%)

Table references subjects that completed the study or were replacement subjects for those that could not complete the study and were replaced (see methods section) BMI: Body Mass Index, cm: centimetres, kg: kilograms. m2: square meters, n: number of subjects, SD: standard deviation.

Table 2 Results of primary evoked pain task endpoints, study parts combined.

Test	Combined group (all measurements n=47)			Men vs Women
	SD vs MORN	SD vs AFT	AFT vs MORN	
COLD PRESSOR				
PDT	-11.5%, p=.32 (-30.6 – 12.9%)	-18.8%. p=.09 (-36.2 – 3.4%)	9%, p=.47 (-14.1 – 38.3%)	**49.4%, p=.03 (4.5 – 113.6%)**
PTT	**-10,8%, p=.004 (-17.5 – -3.6%)**	**-8.2%, p=.03 (-15 – -0.7%)**	-2.9%, p=.45 (-10 – 4.8%)	22.9%, p=.24 (-13 – 73.7%)
ELECTRICAL BURST				
PDT	4.4%, p=.63 (-12.7 – 24.9%)	-7.2%, p=.41 (-22.4 – 11%)	12.5%, p=.19 (-5.7 – 34.1%)	-10.2%, p=.47 (-33.1 – 20.7%)
PTT	-3.2, p=.54 (-12.8 – 7.5%)	-7.6%, p=.14 (-16.7 – 2.7%)	4.7%, p=.37 (-5.5 – 16%)	-1.2%, p=.92 (-23.5 – 27.6%)
ELECTRICAL STAIR				
PDT	13.5%, p=.12 (-3.3 – 33.3%)	2.7%, p=.74 (-12.5 – 20.6%)	10.5%, p=.21 (-5.7 – 29.4%)	-8.3%, p=.53 (-30.4 – 20.9%)
PTT	.9%, p=.84 (-7.6 – 10.2%)	-4.6%, p=.27 (-12.7 – 4.2%)	5.8%, p=.2 (-2.9 – 15.4%)	1.6%, p=.88 (-17.4 – 24.8%)
CPM				
PDT	0.06mA, p=.87 (-0.7 – 0.82 mA)	-0.46mA, p=.24 (-1.22 – 0.31 mA)	0.52mA, p=.18 (-0.25 – 1.28 mA)	-0.29mA, p=.42 (-0.99 – 0.42 mA)
PTT	**-0.69mA, p=.04 (-1.36 --0.03 mA)**	-0.49mA, p=.15 (-1.16 – 0.18 mA)	-0.2mA, p=.55 (-0.87 – 0.47 mA)	0.37mA, p=.2 (-0.2 – 0.95 mA)
HEAT				
PDT	**-0.74°C, p=.02 (-1.34 – -0.14 °C)**	-0.41°C, p=.18 (-1.01 – 0.19 °C)	-0.33°C, p=.27 (-0.92 – 0.26°C)	1.21°C, p=.1 (-0.26 – 2.68 °C)
PRESSURE				
PDT	-6.1%, p=.35 (-17.8 – 7.2%)	-4.2%, p=.53 (-16.2 – 9.6%)	-2%, p=.76 (-14.2 – 11.8%)	32.3%, p=.16 (-10.5 – 95.6%)
PTT	**-11.2%, p=.002 (-17.5 – 4.3%)**	-3.3%, p=.37 (-10.1 – 4.1%)	**-8.1%, p=.02 (-14.6 – -1.2%)**	21.1%, p=.07 (-1.9 – 49.6%)

Statistical analysis of evoked pain task endpoints for both study parts (A and B) combined. EDs, p-values and 95% CIs (between brackets) are referenced for indicated contrasts. Values are presented either in the unit in which they were measured, or in % for tests for which the data were log-transformed. Data in bold and italic denote significant effects ($p < 0.05$). EDs <0 are in favour of first mentioned condition of the contrast (e.g., SD in SD vs MORN), >0 in favour of second mentioned condition. °C: degrees Celsius; AFT: well-rested afternoon condition, 95% CI: 95% confidence interval, CPM: conditioned pain modulation paradigm, ED: estimate of difference, El stair/burst: Electrical stair (single stimulus) and electrical burst (repeated stimulus) pain tests, mA: milliampere, MORN: well-rested morning condition, n: number of subjects, PDT/PTT: pain detection/tolerance threshold, SD: sleep deprived morning condition.

Table 3 Results of primary evoked pain task endpoints per study part.

Test	Men			Women		
	SD (n=24) vs MORN (n=23)	SD (n=24) vs AFT (n=23)	AFT (n=23) vs MORN (n=23)	SD (n=23) vs MORN (n=24)	SD (n=23) vs AFT (n=24)	AFT (n=24) vs MORN(n=24)
COLD PRESSOR						
PDT	4.6%, p=.8 (-26 – 47.8%)	-14.2%, p=.38 (-39 – 20.7%)	21.8%, p=.24 (-12.9–70.4%)	-25.1%, p=.1 (-46.8 – 5.5%)	-23.2%, p=.13 (-45.5 – 8.2%)	-2.5%, p=.88 (-30.4–36.6%)
PTT	-2.4%, p=.66 (-12.6 – 9%)	-0.6%, p=.91 (-11 – 11%)	-1.8%, p=.73 (-11.8 – 9.3%)	**-18.5%, p=.0003 (-26.9– -9.1%)**	**-15.1%, p=.004 (-23.9– -5.4%)**	-3.9%, p=.46 (-13.7 – 7%)
ELECTRICAL BURST						
PDT	22.3%, p=.12 (-4.9 – 57.1%)	4%, p=.75 (-19 – 33.7%)	17.5%, p=.2 (-8.2 – 50.3%)	-10.8%, p=.38 (-30.9–15.1%)	-17.2%, p=.15 (-35.8 – 6.9%)	7.7%, p=.56 (-16.3–38.5%)
PTT	2%, p=.79 (-11.9–18.2%)	-2.4%, p=.75 (-15.7–13.1%)	4.5%, p=.54 (-9.5 – 20.7%)	-8.2%, p=.26 (-20.9 – 6.6%)	-12.5%, p=.08 (-24.6 – 1.6%)	4.9%, p=.52 (-9.4 – 21.5%)
ELECTRICAL STAIR						
PDT	19.7%, p=.12 (-4.5–49.9%)	6.4%, p=.58 (-15–33.3%)	12.4%, p=.3 (-9.9–40.2%)	7.6%, p=.52 (-14.4–35.3%)	-0.8%, p=.94 (-21.1–24.7%)	8.5%, p=.47 (-13.4 – 36%
PTT	9.3%, p=.16 (-3.5 – 23.6%)	-0.6%, p=.93 (-12.1–12.5%)	9.9%, p=.12 (-2.6 – 24%)	-6.8%, p=.27 (-17.7 – 5.7%)	-8.5%, p=.16 (-19.3 – 3.7%)	1.9%, p=.76 (-9.9 – 15.3%)
CPM						
PDT	-0.27mA, p=.61 (-1.32 – 0.78 mA)	-0.41mA, p=.43 (-1.46 – 0.63 mA)	0.14mA, p=.78 (-0.9 – 1.19 mA)	0.39mA, p=.48 (-0.71 – 1.5 mA)	-0.5mA, p=.38 (-1.61 – 0.62 mA)	0.89mA, p=.12 (-0.24 – 2.02 mA)
PTT	-0.59mA, p=.2 (-1.51 – 0.32 mA)	-0.68mA, p=.14 (-1.59 – 0.24 mA)	0.08mA, p=.86 (-0.83 – 1 mA)	-0.79mA, p=.11 (-1.76 – 0.17 mA)	-0.31mA, p=.54 (-1.28 – 0.67 mA)	-0.49mA, p=.33 (-1.47 – 0.5 mA)
HEAT						
PDT	**-1.06°C, p=.01 (-1.9 – -0.22 °C)**	-0.61°C, p=.15 (-1.45 – 0.23 °C)	-0.45°C, p=.28 (-1.27 – 0.37°C)	-0.42°C, p=.34 (-1.27 – 0.44 °C)	-0.21°C, p=.63 (-1.06 – 0.64 °C)	-0.21°C, p=.62 (-1.05 – 0.63°C)

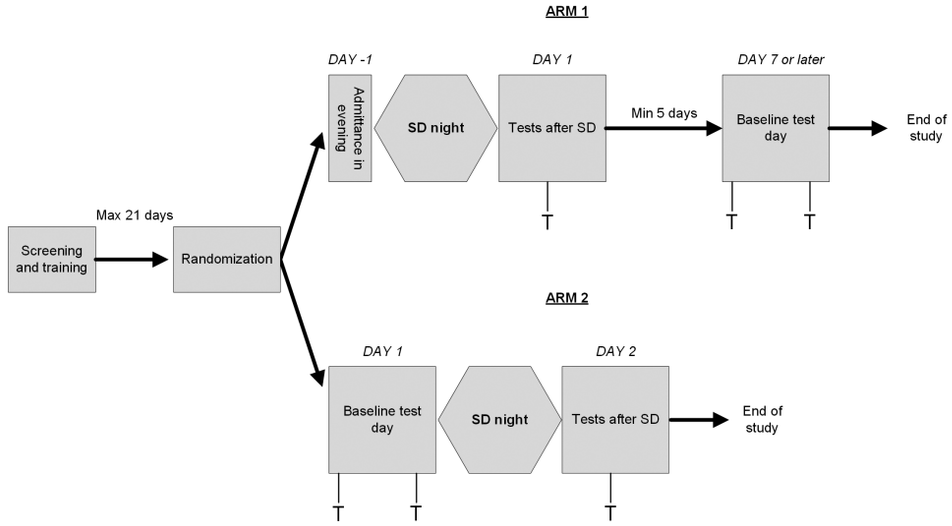
(Table continues on next page)

(Continuation Table 3)

Test	Men			Women		
	SD (n=24) vs MORN (n=23)	SD (n=24) vs AFT (n=23)	AFT (n=23) vs MORN (n=23)	SD (n=23) vs MORN (n=24)	SD (n=23) vs AFT (n=24)	AFT (n=24) vs MORN(n=24)
PRESSURE						
PDT	-6.6%, p=.48 (-22.7 – 13.0%)	-12.2%, p=.18 (-27.6 – 6.4%)	6.4%, p=.52 (-12 – 28.7%)	-5.7%, p=.54 (-21.7 – 13.7%)	4.6%, p=.63 (-13.2 – 26.1%)	-9.8%, p=.26 (-25 – 8.3%)
PTT	-7.5%, p=.14 (-16.8 – 2.8%)	0.9%, p=.86 (-9 – 11.9%)	-8.4%, p=.1 (-17.4 – 1.7%)	**-14.7%, p=.003 (-23.1 – -5.3%)**	-7.4%, p=.15 (-16.5 – 2.8%)	-7.9%, p=.12 (-16.9 – 2.1%)

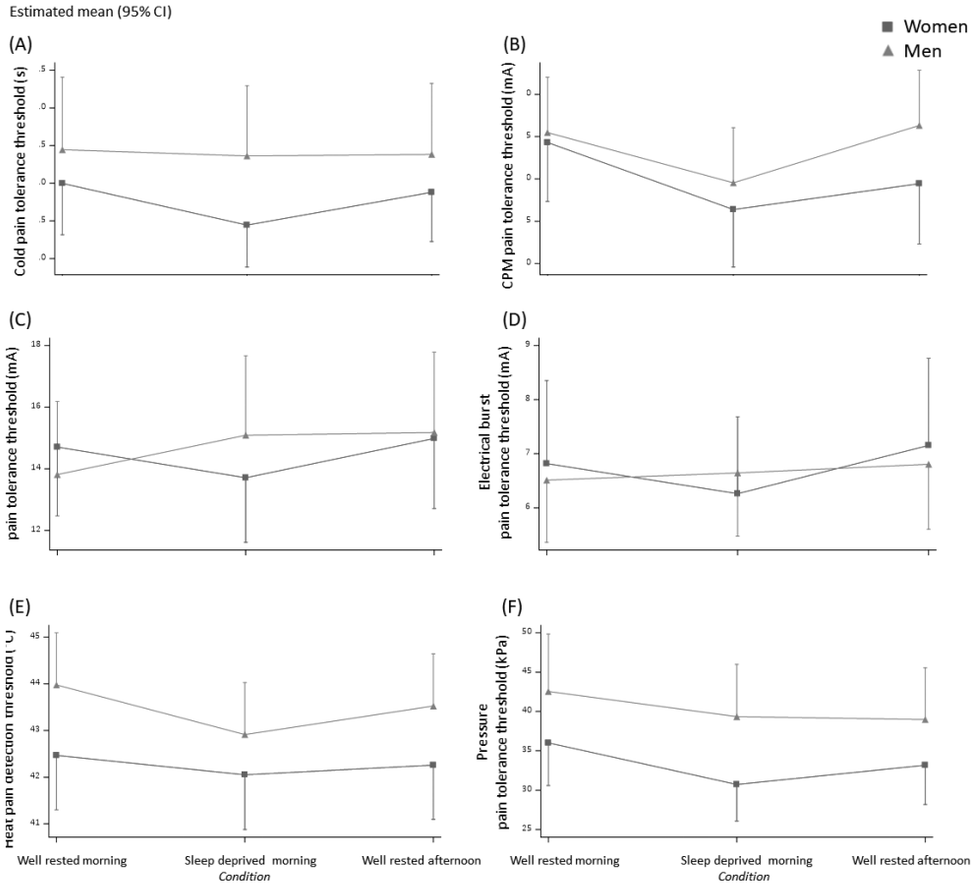
Statistical analysis of evoked pain task endpoints per study part (part A: men, part B: women). EDs, p-values and 95% CIs (between brackets) are referenced for indicated contrasts. Values are presented either in the unit in which they were measured, or in % for tests for which the data were log-transformed. Data in bold and italic denote significant effects ($p < 0.05$). EDs <0 are in favour of first mentioned condition of the contrast (e.g., SD in SD vs MORN), >0 in favour of second mentioned condition. °C: degrees Celsius; AFT: well-rested afternoon condition, 95% CI: 95% confidence interval, CPM: conditioned pain modulation paradigm, ED: estimate of difference, El stair/burst: Electrical stair (single stimulus) and electrical burst (repeated stimulus) pain tests, mA: milliampere, MORN: well-rested morning condition, n: number of subjects, PDT/PTT: pain detection/tolerance threshold, SD: sleep deprived morning condition.

Figure 1 Study design. Schematic study design of both part A and B. Due to the COVID-19 pandemic, subjects in part B arm 1 were required to report to clinic at 8.00 instead of 19.00 to allow for a day of quarantine and COVID-19 screening; whereas subjects that participated in part B study arm 2 were required to stay an additional full study day at our unit in quarantine including COVID-19 screening (see methods section). The quarantine day in this arm preceded the regular study visits. Part A was completed before the COVID-19 pandemic thus unaffected.



T: moments that test rounds were performed.

Figure 2 Selection of evoked pain test parameter results. Graphical presentation of estimated means and 95% CIs of primary evoked pain test results per study part (part A: men, part B: women) and per condition tested (well-rested morning, sleep deprived morning and well-rested afternoon).



a) cold pressor PTT b) CPM PTT, c) electrical stair PTT, d) electrical burst PTT, e) heat PDT, f) pressure PTT. °C: degrees Celsius; 95% CI: 95% confidence interval, CPM: conditioned pain modulation paradigm, Electrical stair/burst: Electrical stair (single stimulus) and electrical burst (repeated stimulus) pain tests, kPa: kilopascal, mA: milliampere, PDT/PTT: pain detection/tolerance threshold, s: seconds.

CHAPTER 7

Simultaneous measurement of intra-epidermal electric detection thresholds and evoked potentials for observation of nociceptive processing following sleep deprivation

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ABSTRACT

Sleep deprivation has been shown to increase pain intensity and decrease pain thresholds in healthy subjects. In chronic pain patients, sleep impairment often worsens the perceived pain intensity. This increased pain perception is the result of altered nociceptive processing. We recently developed a method to quantify and monitor altered nociceptive processing by simultaneous tracking of psychophysical detection thresholds and recording of evoked cortical potentials during intra-epidermal electric stimulation.

In this study, we assessed the sensitivity of nociceptive detection thresholds and evoked potentials to altered nociceptive processing after sleep deprivation in an exploratory study with 24 healthy male and 24 healthy female subjects. In each subject, we tracked nociceptive detection thresholds and recorded central evoked potentials in response to 180 single- and 180 double-pulse intra-epidermal electric stimuli. Results showed that the detection thresholds for single- and double-pulse stimuli and the average central evoked potential for single-pulse stimuli were significantly decreased after sleep deprivation. When analyzed separated by sex, these effects were only significant in the male population. Multivariate analysis showed that the decrease of central evoked potential was associated with a decrease of task-related evoked activity. Measurement repetition led to a decrease of the detection threshold to double-pulse stimuli in the mixed and the female population, but did not significantly affect any other outcome measures.

These results suggest that simultaneous tracking of psychophysical detection thresholds and evoked potentials is a useful method to observe altered nociceptive processing after sleep deprivation, but is also sensitive to sex differences and measurement repetition.

INTRODUCTION

Despite ample research efforts, there are only few biomarkers that can be used for objective monitoring and stratification of chronic pain patients. Patients with chronic pain often experience sensations of pain in response to a non-nociceptive input (i.e., allodynia), or an increased sensation of pain in response to a nociceptive input (i.e., hyperalgesia). A current challenge is to find biomarkers that can identify alterations in nociceptive processing leading to or involved in chronic pain on an individual level. The identification of such biomarkers could allow for patient stratification into functionally distinct groups, and may enable prediction of treatment efficacy per individual. [1] Furthermore, the development of such mechanism-based biomarkers can make it possible to accurately quantify the effects of analgesic drugs on nociceptive processing, which may provide an important proof-of-concept tool in early phase clinical pharmacology studies.

Key aspects in many types of chronic pain, including fibromyalgia, headache, and complex regional pain syndrome, are central sensitization and reduced endogenous modulation of nociceptive input. [2,3] Therefore, recent studies have focused on measuring the effect of central sensitization or reduced inhibition induced by experimental pain models, e.g., capsaicin-induced secondary hyperalgesia. [4,5] One method to centrally alter pain perception is by depriving healthy individuals of sleep. [6] In this model, both central sensitization and reduced endogenous inhibition are thought to increase pain perception. [7] Various studies have demonstrated a close relation between sleep impairments and an increased sensitivity to pain stimuli. In healthy subjects, sleep deprivation has been shown to cause hyperalgesic responses and an altered evoked cortical response, i.e., a decreased amplitude and increased habituation of the P2 in laser evoked potentials. [6,8] Another recent study demonstrated impaired conditioned pain modulation and facilitation of temporal pain summation following 24 h of total sleep deprivation in healthy subjects. [9] Impaired pain inhibition on one hand, and enhanced pain facilitation on the other, have both been related to various chronic pain conditions such as musculoskeletal, visceral, and neuropathic pain. [7] These observations suggest that sleep deficiency leads to altered central nociceptive processing, and an associated increase in pain perception. The sleep deprivation model may therefore be ideal to generate

biomarkers that aim to quantify altered central nociceptive processing in healthy volunteer and chronic pain patient populations.

Recently, we developed a method for the characterization of both peripheral and central nociceptive processing by measuring the effect of nociceptive stimulus properties on detection probability and cortical evoked potentials (EPS). Nociceptive nerve fibers in the skin are activated using low-intensity intra-epidermal electric stimulation with cathodic square-wave pulses. [10] Inhibition and facilitation of repeated nociceptive input are explored by varying the number of pulses and the interpulse interval, [11–13] based on the concept that central [e.g., temporal summation, short-term synaptic plasticity] [14] or peripheral [e.g., subthreshold or suprathreshold super-excitability] [15] neural mechanisms can attenuate or amplify neural activation by a second pulse dependent on its time with respect to the first pulse.

During a single measurement session, single- and double-pulse stimuli are applied according to an adaptive method of limits to track corresponding nociceptive detection thresholds while recording the electroencephalogram (EEG) to measure associated EPS. [11,16] This combination of outcome measures potentially provides a unique insight into nociceptive processing. Nociceptive detection thresholds can be used to observe altered sensitivity. [17–19] In addition, the reliability of detecting the corresponding stimulus level (i.e., the minimum needed for a subject to detect nociception) is quantified by the detection probability slope. [20] EPS can be used as biomarker for altered nociception, such as in the case of central sensitization, [21] attentional modulation, [22] and placebo analgesia. [23] We believe that both outcomes (i.e., EPS and nociceptive detection thresholds) measure different aspects of nociceptive processing and should be combined in a single experiment. After an initial demonstration that both techniques could be efficiently combined, [16] we showed how the combined method may be used for studying the effect of intra-epidermal stimulus properties on nociceptive detection thresholds and EPS in a healthy population. [24]

This combined method was developed with the goal of identifying combinations of psychophysical and neurophysiological features that could aid diagnosis and stratification of chronic pain patients, and as a proof-of-concept tool to characterize the effects of (investigational) analgesics in early phase clinical studies. Here, we examined if we could

register altered nociceptive processing following sleep deprivation using this method in an exploratory study with 24 healthy male and 24 healthy female subjects. We study the feasibility of using the combination of nociceptive detection thresholds and EPS to observe altered nociceptive processing following sleep deprivation in both sexes.

METHODS

The work presented here was part of a study at the Centre for Human Drug Research (Leiden, The Netherlands) in which also other nociceptive pain tasks were performed. During the first part of this study, 24 male subjects were included. During the second part, 24 female subjects were included. In each part subjects participated in a measurement session (described below) after a night of sleep deprivation (sleep deprived occasion) and after a normal night of sleep (control occasion) (**Figure 1**). On the sleep deprived occasion, subjects were deprived of their sleep by remaining awake a full night under supervision of a research assistant, after which the subjects participated in one measurement session in the morning. To ensure wakefulness of the subjects, they were closely monitored the entire night. To minimize the chance of creating a bias in study results, the interactions between subject and research assistant were kept to a minimum at night. In addition, the morning measurements were performed by a different assistant than the assistant that monitored the subject(s) during the sleep deprivation night. On the control occasion, subjects participated to two measurement sessions following a normal night of sleep, one in the morning and one in the afternoon. Participants were asked to go to sleep between 22:00 and 23:00, and to wake up between 7:00 and 8:00, on the night preceding the control occasion. The order of both occasions was randomized. If the sleep deprived occasion preceded the control occasion, a minimum resting period of at least 5 days was required. In practice, this resting period was either 7 or 8 days on all occasions.

The study received approval from a Medical Review and Ethics Committee (Foundation BEBO, Assen, The Netherlands) before study start, and was performed in accordance with the Declaration of Helsinki. All subjects provided written informed consent prior to any study assessments taking place. The study has prospectively been registered in the Dutch Trial Register (NTR) as NTR7517.

Participants

A total of 24 healthy male (age 26.2±2.1) and 24 healthy female (age 25.9±3.0) participants were enrolled. Participants were recruited via media advertisement or from the subjects' database of the Centre for Human Drug Research, Leiden, The Netherlands. Inclusion criteria were an age between 23 and 35 years, to reduce the potential influence of age on outcome measures, and a body mass index between 18 and 32 kg/m^2, to exclude underweight or extremely overweight individuals. Exclusion criteria were a history or symptoms of any significant disease, history or presence of sleep disorders, a change in time zones 7 days prior to the study period, average usage of tobacco products equivalent to or more than 10 cigarettes per day, average usage of (methyl)-xanthines of more than 8 units per day, and inability to refrain from usage during the study occasions. No usage of (illicit) drugs was permitted from 3 days prior to each study period until discharge. Consumption of alcohol or tobacco- and nicotine-containing products was not permitted from 24 h prior to each scheduled visit until discharge. Participants underwent a urine drug screening and alcohol breath test on each arrival at the clinical research unit, i.e., before the start of each occasion. In addition, participants were not allowed to consume excessive amounts of caffeine, defined as more than 800 mg per day, from 2 days prior to each visit. Participants fully abstained from using caffeine-containing products from 4 h prior to each visit until discharge. No prescription medications and over-the-counter medications, except for contraceptive pill usage, were permitted within 14 days prior to the first occasion, or less than 5 half-lives, and during the course of the study. In addition, no vitamin, mineral, herbal, and dietary supplements were permitted within 7 days prior to the first occasion, or less than 5 half-lives, and during the course of the study.

To minimize a possible influence of the menstrual cycle on pain perception, females were required to use a reliable method of hormonal contraception at least 30 days before the first study day until the end of the study. Females were required to use their own hormonal anticonception (prescribed by their general practitioner of gynecologist) continuously during study participation or were only allowed to participate if the study days were more than 2 days after re-start of contraceptive pill use or after bleeding withdrawal. This to prevent possible variations caused by the menstrual cycle. No side effects of hormonal contraception were reported.

Stimuli

Participants received intra-epidermal electric pulses applied by a constant current stimulator (NociTRACK AmbuStim, University of Twente, Enschede, The Netherlands). Intra-epidermal electric stimulation at intensities of less than twice the detection threshold preferentially activates Aδ-fibers in the skin. [10,25,26] Stimuli were applied via an electrode attached to the volar lower arm at the side of the dominant hand (**Figure 2**). The electrode consisted of an array of 5 interconnected microneedles embedded in silicone, each needle protruding 0.5 mm from the electrode surface. Previous studies using this electrode showed that stimulation resulted in a sharp pricking sensation, [27] and similar latencies of response times and evoked N1, N2 and P2 peaks in comparison with earlier studies using intra-epidermal and laser stimulation. [24] In addition to single-pulse stimuli, double-pulse stimuli were used to observe potential effects of inhibition or facilitation of repeated nociceptive input. [11–13] As such, two stimulus types were used in this study:

- A single 210 μs pulse
- A double 210 μs pulse with an inter-pulse interval (IPI) of 10 ms.

Procedure

While seated in a comfortable chair, participants were instructed to focus their attention on the stimulation electrode, to reduce the potential influence of (variations in) spatial attention. First, a rough estimate of the detection threshold was obtained using a normal staircase procedure with a stepsize of 0.025 mA. The participant was instructed to hold a button, and to release the button as soon as a stimulus was perceived. Second, an accurate estimate of the detection threshold was obtained using an adaptive and randomized psychophysical method of limits, also referred to as 'threshold tracking', designed to estimate detection thresholds with a potential drift. [28] Participants were instructed to hold a button, and to briefly release the button when a stimulus was perceived. A vector of 5 stimulus amplitudes was initialized with a stepsize of 0.025 mA around the initial estimate of the detection threshold. For each stimulus, a value was randomly chosen from this vector. When the stimulus was detected, the vector was decreased by 0.025 mA. When the stimulus was not detected, the vector was increased by 0.025 mA. This process was repeated for a

total of 180 single- and 180 double-pulse stimuli, during a time period of approximately 20 min. The interval between two consecutive stimuli was randomized with a uniform distribution of 2.5–3.5 s.

Electroencephalography recording

During the entire detection threshold tracking procedure, the scalp EEG was recorded at 32 Ag/AgCl electrodes located on the scalp according to the international 10/20 system. Electrode impedance was kept below 5 kΩ. To reduce eye blink and movement artefacts, participants were asked to fix their gaze at one spot on the wall and blink as few times as possible while pressing the response button and focusing their attention on the received stimuli.

Analysis

EFFECT OF STIMULUS PROPERTIES AND SLEEP DEPRIVATION ON DETECTION PROBABILITY The effect of stimulus properties and sleep deprivation on the detection probability was analyzed for the male group, female group, and the combination of both groups using a generalized linear mixed model in R, estimated using the lme4 and MASS toolboxes. [29,30] We used the statistical model in (1), where the log-odds of stimulus detection (ln(Pd1–Pd)) is modulated by the effects and interaction of stimulus type (TYP), i.e., single- or double-pulse, stimulus amplitude (AMP) and condition (C) and by the effects and interaction of trial number (TRL) and condition (C). We also added terms for measurement number (M) and occasion (O) to account for potential confounding. Condition, measurement, and occasion were modeled as categorical. All within-subject fixed effects were also included as random effects grouped by subject

$$\ln\left(\frac{P_d}{1-P_d}\right) \sim 1 + AMP * TYP * C + TRL * C + M + O + (1 + AMP * TYP * C + TRL * C + M + O|S) \quad (1)$$

Before GLMR analysis of the dataset, outliers were excluded, defined as measurements in which the detection threshold was estimated smaller than 0 or larger than 1.6 mA, or where the slope of the psychometric curve was estimated smaller than 0 or larger than 100 mA^{-1}. Effect significance was tested using a two-tailed type-III test using Wald–Chi-square statistics.

Detection thresholds and slopes were computed using the estimated model coefficients. Differences of detection thresholds and slopes between the sleep deprived measurement and the first control measurement and between both control measurements were tested by generating a posterior distribution of each model coefficient with 20,000 samples using the ARM package in R. [32] Subsequently, these posterior distributions were used to compute the distribution, confidence intervals, and significance of the (difference between) detection thresholds.

PREPROCESSING OF EEG DATA The scalp EEG data was pre-processed using Fieldtrip. [33] Epochs were extracted from the EEG from 0.5 s before to 1.0 s after the stimulus. Eye blink and movement artefacts were identified and removed using independent component analysis, [34] resulting in removal of 2 independent components on average. Epochs with excessive EMG activity were excluded from analysis based on visual inspection. Subsequently, epochs were bandpass-filtered from 0.1 to 40 Hz and baseline-corrected using the interval ranging from − 0.5 s to 0.0 s relative to stimulus onset.

GRAND AVERAGE EVOKED POTENTIAL The Cz-M1M2 derivation was used for analysis of the central EP, as previous studies showed that these channels (Cz, M1, and M2) have the largest SNR for intra-epidermal electric EPS in healthy participants, when using a 32-channel electrode configuration. [24] Grand average waveforms at the identified latency at the Cz-M1M2 derivation were computed by averaging all trials seperated by measurement number (1 or 2), stimulus type (single- or double-pulse), and condition (with or without sleep deprivation), resulting in 180 trials per average. A positive peak (P2) was defined as the most positive peak between 300 and 500 ms at Cz-M1M2 and selected for further analysis. The differences of average EP at Cz-M1M2 between the sleep deprived measurement and the first control measurement and between both control measurements were tested at the identified P2 latency (390 ms) using a two-tailed paired-sample *t* test.

EFFECT OF STIMULUS PROPERTIES ON EVOKED POTENTIAL The effect of stimulus properties and sleep deprivation on the EP at P2 latency was analyzed for the male group, female group, and the combination of both groups using a linear mixed model in Matlab (version 2017b,

MathWorks, Inc.). We used the statistical model in (2), similar to the model for analysis of detection probability in (1), but including a term for additional cortical activity evoked by stimulus detection (D) which could decrease with respect to the trial number (TRL), and also vary with respect to condition (C). Condition, stimulus detection, measurement, and occasion were modeled as categorical

$$U_{EEG} \sim 1 + AMP * TYP * C + TRL * D * C + M + O + (1 + AMP * TYP * C + TRL * D * C + M + O|S) \quad (2)$$

Significance of the effect coefficients was assessed using a two-tailed *t* test using Satterthwaite's method for estimation of the degrees of freedom.

RESULTS

Exclusion of outliers

In the first part of the study (males), 7 out of 72 measurements were excluded due to an incomplete measurement, as a result of technical problems with the measurement setup. For the analysis of EEG, 3 out of the remaining 65 measurements were excluded due to extreme noise caused by a faulty electrode. For the analysis of detection probability, 16 out of the remaining 65 measurements were excluded due to poor task performance leading to unreliable detection thresholds or slopes as defined in the section **Analysis – Effect of stimulus properties and sleep deprivation on detection probability.**

In the second part of the study (females), 4 out of 72 measurements were excluded due to an incomplete measurement, as a result of technical problems with the measurement setup. For the analysis of EEG, 3 out of the remaining 68 measurements were excluded due to extreme noise caused by a faulty electrode. For the analysis of detection probability, 2 out of the remaining 68 measurements were excluded due to poor task performance leading to unreliable detection thresholds or slopes as defined in the section **Analysis – Effect of stimulus properties and sleep deprivation on detection probability.**

Effect of stimulus properties and sleep deprivation on detection probability

The effect of stimulus properties and sleep deprivation on detection probability is shown in **Table 1**. The random-effects covariance matrices associated with each generalized linear mixed model fit are available in **Appendix I**. In all groups, significant effects on the detection probability were observed for the intercept, amplitude, type, trial number, and the interaction between amplitude and type. The detection probability increases with respect to the amplitude and decreases over the number of trials. The positive coefficients for type and the interaction between amplitude and type shows that addition of a second pulse to the stimulus increases detection probability. An additional significant effect of stimulus type is observed in the combined group, as well as male group only. The combination of both groups and the female group show an additional significant effect of measurement, and of the interaction between amplitude, type, and condition.

Detection thresholds derived from the coefficient estimates are shown in Table 2. For the combined group and the male group, the estimate of the detection threshold is significantly lower for both single-pulse and double-pulse stimuli after sleep deprivation. The female group shows a similar non-significant trend after sleep deprivation. For the combination of both groups and the female group, the estimate of the detection threshold is significantly lower for both single-pulse and double-pulse stimuli during the second control measurement. The male group shows a similar non-significant trend during the second control measurement.

Detection probability slopes derived from the coefficient estimates are shown in **Table 3**. The slope appears to increase in all groups after sleep deprivation. However, this increase was only significant in the female group for double-pulse stimuli.

GRAND AVERAGE EVOKED POTENTIAL The difference between sleep deprived and control measurements for each group is shown in the time domain at the Cz-M1M2 derivation in **Figure 3**. For the combination of both groups and the male group, there was a significant decrease in maximum EP amplitude in response to detected single- and double-pulse stimuli after sleep deprivation. For the female group, there was no

significant difference in maximum EP amplitude between sleep deprived on control measurements. For all groups, there was no significant difference in EP between both control measurements.

EFFECT OF STIMULUS PROPERTIES AND SLEEP DEPRIVATION ON EVOKED POTENTIAL The effects of stimulus properties and sleep deprivation on the EP at 390 ms latency on the Cz-M1M2 derivation were quantified by linear mixed regression based on Equation 2 and a *t* test of each computed effect coefficient. Results for each group are shown in **Table 4**. The random-effects covariance matrices associated with each linear mixed model fit are available in **Appendix A**. For each group, significant effects of stimulus properties on the EP were found for stimulus detection, trial number, amplitude, and the interaction between amplitude and type. For the combination of both groups and for the male group, a significant interaction between sleep deprivation and stimulus detection was found. For this interaction between sleep deprivation and stimulus detection, effect coefficients of − 1.28 and − 2.21 were found for the combination of both groups and for the male group, respectively, which means that the EP in response to detected stimuli decreased by − 1.28 and − 2.21 μV after sleep deprivation.

DISCUSSION

In search of a composite biomarker for altered nociceptive processing, we combined techniques to simultaneously measure detection thresholds and EPS in response to nociceptive intra-epidermal electric stimulation. We explored if this combination of techniques could be used to observe changes in nociceptive processing following sleep deprivation in a male and female population. We found that intra-epidermal electric detection thresholds and EPS both decreased after 24 h of sleep deprivation in a combined group of healthy male and female subjects.

The effects of intra-epidermal electric stimulus properties on the detection probability were similar to the effects observed in the previous studies. [11,24] supporting the validity of our results. Similar to these earlier observations on unchallenged healthy subjects, we observed a general positive effect of stimulus amplitude and the interaction between amplitude and type on detection probability (**Table 1**). Both effects indicate that the detection probability increased when the stimulus amplitude of

single- or double-pulse stimuli increased, which is associated with an increased recruitment of peripheral nerve fibers at increased currents. The detection probability also increased following addition of a second pulse as a result of the temporal summation of neural activity elicited by both pulses, which was signified by the positive effect of stimulus type and the positive interaction between stimulus amplitude and stimulus type in generalized linear mixed regression (**Table 1**). The detection probability decreased over the number of trials, plausibly due to a decreased attention or physiological habituation to the stimulus. In addition, there was a significant interaction between stimulus amplitude, type, and sleep deprivation for the mixed population, suggesting that the effect of adding a second pulse on the detection probability is increased after sleep deprivation. This interaction suggests an increased facilitation or decreased inhibition of neural activity evoked by the second pulse following sleep deprivation. A potential explanation for increased facilitation of the second pulse is increased temporal summation, as originally defined by Price et al., [35] which has also been shown to be increased following sleep deprivation using modern temporal summation paradigms. [36,37]

Nociceptive detection thresholds for intra-epidermal electric stimulation were decreased following sleep deprivation. These detection thresholds were computed from generalized linear mixed regression coefficients, [38] and statistically tested through Monte Carlo simulation of detection threshold distributions. As a result, we found that in a mixed population (i.e., male and female groups combined) detection thresholds for both types of stimuli decreased after sleep deprivation. Earlier studies have examined the effects of sleep deprivation using mechanical and thermal pain (detection) thresholds. Some of these studies support that pain thresholds are decreased following sleep deprivation, having observed a significant decrease in mechanical and heat pain thresholds due to sleep deprivation. [39–42] However, not all studies found a significant correlation between pain thresholds and sleep deprivation. [43,44] We demonstrated here that the nociceptive intra-epidermal electric detection thresholds to single-pulse and double-pulse stimuli were decreased in a mixed population, while noting that both detection thresholds were also significantly decreased during the second control measurement. As such, any repeated measures designs involving nociceptive detection thresholds should account for this effect by randomization of the measurement order.

Intra-epidermal stimulation evoked a cortical response with a maximum at 390 ms, which was decreased following sleep deprivation. The latency of this evoked response was similar to the P2 potential measured in response to nociceptive stimuli in previous studies. [12,24,45] We used the Cz-M1M2 derivation to study the influence of sleep deprivation and stimulus properties on evoked cortical activity at this latency. We found a significant decrease of the P2 amplitude in response to detected single- and double-pulse stimuli after sleep deprivation, while the waveform remained similar during both control measurements. Regression analysis showed a significant interaction between sleep deprivation and stimulus detection, suggesting that sleep deprivation mainly resulted in a reduction of task-related cortical activity.

A decrease of P2 amplitude at Cz-M1M2 has also been related to reduced stimulus intensity and reduced stimulus salience in earlier studies, [46,47] which appears contradictory to the notion that sleep deprivation causes hyperalgesia. [48] A decreased P2 amplitude might reflect a decreased attention, [49] as a result of sleep deprivation. However, decreased attention appears contradictory to our observation that sleep deprivation results in a higher nociceptive detection thresholds, which suggests that participants are more sensitive to nociceptive input following sleep deprivation. This simultaneous increase of sensitivity and decrease of measured cortical activity was also found in three recent studies assessing pain sensitivity. [6,50,51] Hypotheses for this phenomenon in these studies include loss of attention or a reduction in cortical cognitive or perceptual mechanisms. However, a recent FMRI study suggests the reduction of cortical activity following sleep deprivation is associated with a reduction of stimulus evoked activity in the insula and the anterior cingulate cortex, which are both involved in the endogenous modulation of pain. [52] Although the origin of this phenomenon is reason of debate, it shows that detection thresholds and EPS are measuring distinct aspects of nociceptive processing and are useful to combine to study effects of sleep deprivation on nociception. Further experimental and modelling studies are necessary to better explain why an increased nociceptive sensitivity and a decreased EP are both observed following sleep deprivation in this and other studies.

To the best of our knowledge, this is the first study to examine the effect of sleep deprivation on nociceptive detection thresholds and EPS in both a male and a female population. In fact, a few studies have been done to

identify sex differences in nociceptive processing before and after sleep deprivation. [37,53] To start with, there was a large difference in detection task performance between males and females, as a total of 16 measurements had to be removed due to unreliable detection thresholds in the male group in comparison to only 2 measurements in the female group. This difference was also observed in the detection slopes [quantifying detection (un)certainty], which were lower for male subjects on all occasions. Furthermore, this difference between both groups was larger on the control occasion than on the sleep deprived occasion. The observed difference in task performance might be attributed to a greater sensitivity to noxious stimuli in females. [54] However, other sex-related differences in sensitivity, cognitive performance, and attention cannot be excluded based on the current results.

Separate analysis of the results for a male and a female population suggests that outcomes are dependent on sex. While average detection thresholds decreased for both stimulus types in both groups, this decrease was only significant in the male population when analyzed in separate groups. On the other hand, only the female population showed an increased effect of double-pulse stimuli on detection probability following sleep deprivation, potentially associated with increased temporal summation of pulses. The grand average EP amplitude was significantly decreased after sleep deprivation in the male population and regression analysis showed a significant decrease in task-related activity following sleep deprivation in the male population only. Divergent sex-dependent effects of sleep deprivation on nociceptive processing and pain have been noted previously. Smith et al. observed that a significant increase of capsaicin-induced secondary hyperalgesia following sleep deprivation only occurred in males, while a significant increase of nociceptive temporal summation following sleep deprivation mostly occurred in females. [37] Furthermore, Eichhorn et al. observed that the decrease in endogenous inhibitory control associated with sleep deprivation only occurred in females. [53] From those results as well as ours, it is clear that there are not only significant differences in nociception and pain between the sexes, [55] but also that the effect of sleep deprivation on nociceptive processing and pain might depend on sex.

Limitations

There are several limitations that should be addressed before adopting this method in further clinical or pharmacological studies. This was an exploratory study, as this was the first study to examine intra-epidermal electric detection thresholds and EPS following sleep deprivation, and no prior data were available to formulate hypotheses and perform a sample size calculation. Although this study included a larger group of participants than earlier studies showing significant effects of sleep deprivation on nociceptive detection thresholds [ranging from 6, to 20 participants] [39,42] or EPS [ranging from 12 (Schuh-Hofer et al. 2015) to 33 participants], [51] this study might still lack sufficient power to observe some of the sex-dependent effects of sleep deprivation.

Several other choices in our current study design might have impacted study results, and are important to address in potential follow-up studies. In the current study, the male and female population were recruited in two time periods with an interval of 1.5 years. As such, potential confounding by the time period in which the experiments were performed (e.g., COVID-19 risk mitigation measures, seasonal effects, and potentially other unknown factors) on the sex-dependent effects observed in this study, cannot be excluded. Follow-up studies should therefore recruit and test participants in the same time period. Females were required to use their own hormonal contraception continuously during study participation to prevent an influence of potential hormonal variations caused by the menstrual cycle on pain perception. [56] Nevertheless, this might limit generalizability of our current observations to females who do not take hormonal contraception. The effect of hormones on nociceptive processing following sleep deprivation remains undocumented, and further studies are needed to provide more insight in the potential influence of hormones on sleep and nociception. Another potential bias in outcomes might have been introduced by the time gap between occasions. As in half of the subjects, the second occasion was preceded by a resting period of at least 5 days, while in the other half, the second occasion was preceded by the first (separated by one night), this could have led to a bias in outcomes due to potential familiarization effects in the second half. Future experiments might avoid such a bias by including an equal resting period between each occasion. Experiments with male and female participants were performed by a mixed population of research assistants of both

sexes. As the gender of the experimenter can influence reported pain measures, [57–59] this could have led to additional variance of outcomes between subjects.

Conclusion

Observation of altered nociceptive detection thresholds and EPS following sleep deprivation in male and female populations shows that it is feasible to evaluate impaired nociceptive processing following sleep deprivation in a human population based on intra-epidermal detection thresholds and EPS. Some effects were only observed in either a male or a female population, such as a decrease of the intra-epidermal electric detection threshold or a decrease of the EP, and might be sex-dependent. The current results suggest that intra-epidermal electric detection thresholds and EPS could be helpful in exploring the link between sleep impairment and chronic pain in future studies. Nevertheless, it remains important to note that, like any method relying on participant report (e.g., questionnaires, quantitative sensory testing), nociceptive detection thresholds and EPS might be influenced by attention and learning processes. Developing nociception biomarkers that are unbiased by psychological states remains a current challenge for pain science. The possibilities of combining the sleep deprivation model with more objective measures of nociception and pain are exciting, as they allow to translate results from earlier pharmacological animal studies using sleep deprivation, e.g., [60–62] to humans with potential applications in the identification of analgesic and sedative compounds.

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Table 1 Effect of stimulus properties on the detection probability for the male group (M), the female group (F) and the combination of both (All), computed using GLMR.

Stimulus Property	Coeff. (All)	Coeff. (M)	Coeff. (F)	χ^2 (All)	χ^2 (M)	χ^2 (F)	*p* (All)	*p* (M)	*p* (F)
(Intercept)	**-3.50**	**-3.19**	**-3.44**	**172.51**	**80.24**	**69.25**	**<.001**	**<.001**	**<.001**
Amplitude (AMP)	**6.10**	**4.45**	**7.52**	**148.42**	**85.06**	**98.05**	**<.001**	**<.001**	**<.001**
Type (TYP)				**6.01**	**11.83**	0.66	**<.05**	**<.001**	.42
Type 2	**-0.39**	**-0.85**	-0.19						
Trial number (TRL)	**-0.52**	**-0.41**	**-0.62**	**108.89**	**26.88**	**104.33**	**<.001**	**<.001**	**<.001**
Measurement (M)				**5.90**	0.82	**4.33**	**<.05**	.37	**<.05**
Measurement 2	**0.64**	0.31	**0.77**						
Occasion (O)				2.22	0.97	2.10	.14	.32	.14
Occasion 2	0.30	-0.48	-0.32						
Condition (C)				0.11	1.64	0.01	.74	.20	.90
Sleep Dep.	0.14	0.85	0.08						
Amplitude × Type				**52.81**	**20.69**	**38.09**	**<.001**	**<.001**	**<.001**
Amplitude × Type 2	**6.69**	**5.74**	**7.81**						
Amplitude × Condition				1.09	0.82	1.07	.30	.36	.30
Amplitude × Sleep Dep.	1.23	1.14	1.88						
Type × Condition				0.10	1.40	0.70	.75	.23	.40
Type 2 × Sleep Dep.	-0.13	0.54	-0.60						
Trial number × Condition				0.06	0.06	0.16	.80	.81	.69
Trial number × Sleep Dep.	-0.02	-0.03	-0.04						
Amplitude × Type × Condition				**3.74**	0.52	**3.90**	**.05**	.47	**<.05**
Amplitude × Type 2 × Sleep Dep.	**3.18**	1.36	**5.19**						

Significance was assessed using type-III Wald Chi-square statistics with one degree of freedom. All effect coefficients are expressed in log-odds per unit with the units mA^{-1} for amplitude and $(100\ trials)^{-1}$ for trial number. The numbers of measurement and occasion refer to the moments at which the procedure was conducted as described in Fig. 1. Significant values (p<.05) are shown in bold.

Table 2 Detection thresholds for the male group (M), the female group (F) and the combination of both (All) per stimulus type (in).

Stimulus Type	Thresh. (All)	Thresh. (M)	Thresh. (F)	95% CI (All)	95% CI (M)	95% CI (F)
Single-pulse, Control 1	0.57	0.72	0.46	[0.48 0.69]	[0.55 0.94]	[0.37 0.55]
Single-pulse, Control 2	**0.47***	0.65	**0.35***	[0.38 0.57]	[0.43 0.92]	**[0.31 0.41]**
Single-pulse, Sleep Dep.	**0.46***	**0.42****	0.36	**[0.38 0.58]**	**[0.28 0.62]**	[0.29 0.48]
Double-pulse, Control 1	0.30	0.40	0.24	[0.25 0.38]	[0.29 0.59]	[0.19 0.29]
Double-pulse, Control 2	**0.25***	0.37	**0.19***	[0.21 0.32]	[0.24 0.58]	**[0.16 0.22]**
Double-pulse, Sleep Dep.	**0.23***	**0.21*****	0.18	**[0.18 0.29]**	**[0.14 0.31]**	[0.14 0.24]

Control 1 and Control 2 refer to the first and second control measurement in Fig. 1 respectively. Each significant difference of the sleep deprived measurement or the second control measurement with respect to the first control measurement is denoted with * (p<0.05), ** (p<0.01) and *** (p<0.001). Detection thresholds with a significant difference with respect to the first control occasion (p<.05) and associated confidence intervals are shown in bold.

Table 3 Detection probability slopes for the male group (M), the female group (F) and the combination of both (All) per stimulus type (in).

Stimulus Type	Slope (All)	Slope (M)	Slope (F)	95% CI (All)	95% CI (M)	95% CI (F)
Single-pulse, Control 1 & 2	6.11	4.45	7.52	[5.14 7.07]	[3.56 5.35]	[6.04 8.99]
Single-pulse, Sleep Dep.	7.32	5.59	9.42	[5.23 9.49]	[3.26 7.95]	[6.34 12.44]
Double-pulse, Control 1 & 2	12.79	10.18	15.33	[10.46 15.13]	[7.06 13.30]	[12.34 18.33]
Double-pulse, Sleep Dep.	17.18	12.66	**22.40***	[12.43 22.00]	[7.75 17.68]	**[15.80 28.84]**

Control 1 and Control 2 refer to the first and second control measurement in Fig. 1 respectively. Each significant difference of the sleep deprived measurement with respect to the control measurements is denoted with * (p<0.05), ** (p<0.01) and *** (p<0.001). Slopes with a significant difference with respect to both control occasions (p<.05) and associated confidence intervals are shown in bold.

Table 4 The coefficient estimates, t-values and corresponding p-values for the effect of stimulus properties on the EP at 390ms (Cz-M1M2) in the male group (M), the female group (F) and the combination of both (All).

Stimulus Property	Coeff. (All)	Coeff. (M)	Coeff. (F)	*t* (All)	*df*	*t* (M)	*df*	*t* (F)	*df*	p (All)	p (M)	p (F)
(Intercept)	0.89	1.61	0.06	1.54	*31.2*	1.49	*15.6*	0.12	*50.8*	.13	0.15	.91
DETECTION (D)												
Detected	**7.02**	**7.71**	**6.43**	**11.10**	***44.6***	**7.45**	***22.0***	**8.45**	***21.7***	**<.001**	**<.001**	**<.001**
Amplitude	**2.40**	**2.54**	**2.58**	**4.11**	***35.1***	**2.60**	***13.5***	**3.04**	***34.0***	**<.001**	**<.05**	**<.01**
TYPE												
Type 2	-0.37	-0.59	-0.50	-1.04	*108.3*	-1.05	*48.9*	-0.85	*29.8*	.30	.30	.40
Trial number (TRL)	**-0.57**	**-0.54**	**-0.65**	**-3.34**	***53.3***	**-2.30**	***27.1***	**-2.62**	***25.8***	**<.01**	**<.05**	**<.05**
MEASUREMENT (M)												
Measure-ment 2	-0.17	-0.76	0.18	-0.42	*23.4*	-1.22	*14.8*	0.37	*18.5*	.68	.24	.72

Stimulus Property	Coeff. (All)	Coeff. (M)	Coeff. (F)	*t* (All)	*df*	*t* (M)	*df*	*t* (F)	*df*	p (All)	p (M)	p (F)
OCCASION (O)												
Occasion 2	-0.44	-0.53	0.14	-1.38	*21.0*	-1.17	*13.5*	0.30	*13.5*	.18	.26	.77
CONDITION (C)												
Sleep dep.	-0.42	-1.57	0.03	-0.46	*29.8*	-1.05	*16.1*	0.02	*19.1*	.65	.31	.98
AMPLITUDE × TYPE												
Amplitude × Type 2	**3.60**	**3.60**	**4.65**	**4.08**	***11.3***	**4.22**	***12.1***	**2.37**	***11.7***	**<.01**	**<.01**	**<.05**
TRIAL NUMBER × DETECTION												
Trial number × Detected	-0.55	-0.34	-0.72	-1.96	*43.7*	-0.84	*21.1*	-1.85	*23.4*	.06	.41	.08
DETECTION × CONDITION												
Detected × Sleep dep.	**-1.28**	**-2.21**	-0.99	**-2.39**	***45.3***	**-3.13**	***22.7***	-1.28	*23.3*	**<.05**	**<.01**	.21
AMPLITUDE × CONDITION												
Amplitude × Sleep dep.	0.56	1.61	0.61	0.58	*25.4*	1.00	*14.0*	0.37	*11.7*	.57	.34	.72
TYPE × CONDITION												
Type 2 × Sleep dep.	0.36	0.44	-1.07	0.49	*44.0*	0.43	*31.1*	-0.98	*23.3*	.63	.67	.34
TRIAL NUMBER × CONDITION												
Trial number × Sleep dep.	-0.09	-0.65	0.20	-0.30	*44.0*	-1.52	*22.5*	0.45	*23.2*	.76	.14	.65
AMP. × TYPE × CONDITION												
Amp. × Type 2 × Sleep dep.	-1.29	-0.33	5.24	-0.97	*16.8*	0.25	*13.5*	1.54	*16.4*	.35	.81	.14
TRIAL NUM. × DET. × COND.												
Trial num. × Det. × Sleep dep.	-0.45	-0.14	-0.69	-0.93	*45.9*	-0.21	*26.9*	-0.98	*21.8*	.36	.83	.34

All effect coefficients are expressed in μV per unit with the units mA^{-1} for amplitude and $(100\ trials)^{-1}$ for trial number. The numbers of measurement and occasion refer to the moments at which the procedure was conducted as described in Fig. 1. Significant values (p<.05) are shown in bold.

Figure 1 Participants were measured on two occasions: after a night of sleep deprivation (1 measurement) and after a normal night of sleep (2 measurements). If the sleep deprived occasion preceded the control occasion, a resting period of at least 5 days was used between both occasions.

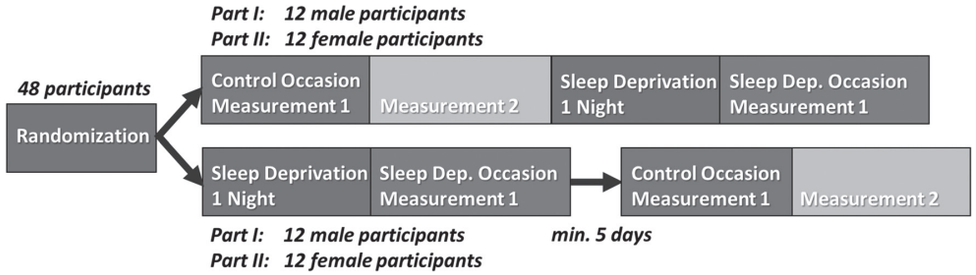


Figure 2 Electrode placement on the volar forearm on the side of the dominant hand (top), and electrode dimensions (bottom).

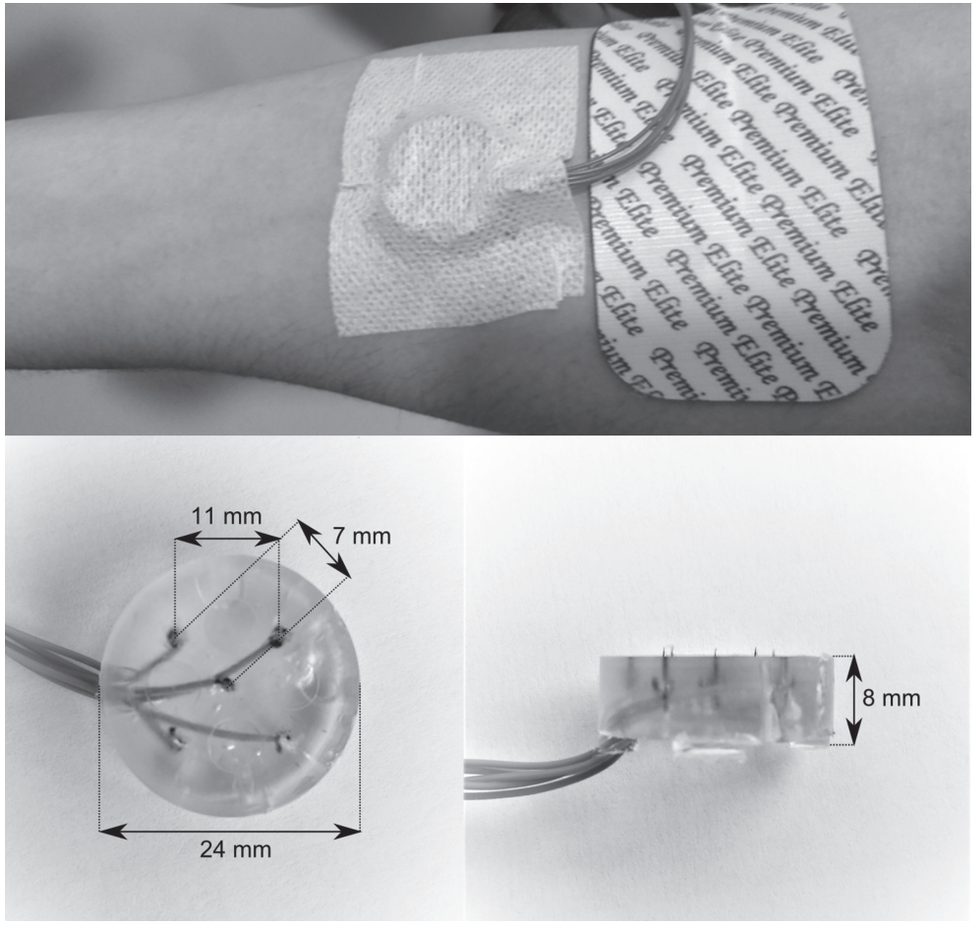
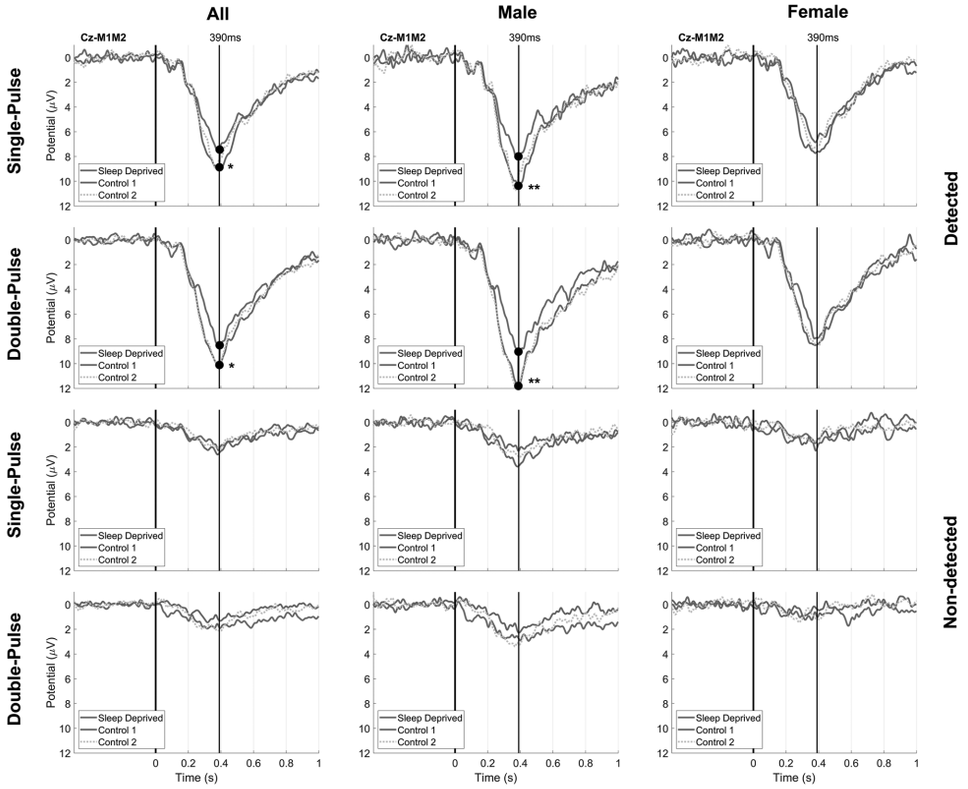


Figure 3 Grand average EP in each group in response to single-pulse and double pulse intra-epidermal stimuli at Cz-M1M2 for participants with normal sleep during a first and a second measurement (Control M1 and Control M2 respectively) and after 24 hours of sleep deprivation. There was a significant difference in maximum EP amplitude at Cz-M1M2 between the sleep deprived and the first control measurement for detected single- and double-pulse stimuli in the male group and the combination of both groups.



Significance is indicated with * (p<.05) and ** (p<.01).

[SUPPLEMENTARY MATERIAL AVAILABLE ONLINE AT THE PUBLISHER'S WEBSITE]

CHAPTER 8

Investigation of the sensitizing properties of a topical ethanolic 1% capsaicin formulation, and its applicability in a nociceptive test battery

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In preparation

ABSTRACT

OBJECTIVE In experimental context, capsaicin is used as a model to temporarily induce cutaneous sensitization to heat and mechanical stimuli, and alter skin properties. These effects, however, vary between capsaicin formulations. We investigated whether an ethanolic 1% capsaicin formulation could induce sensitization, and whether it interfered with other pain tests, to determine if it could be included in a multi-modal test battery and used in early-phase analgesic drug studies.

METHODS This was a two-period open-label study in ten healthy male volunteers. Sensitization induced by ethanolic 1% capsaicin was evaluated by determining pain thresholds using a heat pain test, Von Frey test, laser evoked potentials, and in combination with an validated evoked pain test battery (pressure-, cold pressor, electrical burst and electrical stair pain tests). Skin redness, erythema and blood perfusion were evaluated using multispectral- and laser speckle contrast imaging. Data were analyzed with a repeated-measures ANCOVA.

RESULTS Ethanolic 1% capsaicin induced a mean peak pain of 4.4 (on an 11-point scale, t = 0.5 h), significantly induced primary heat sensitization (Estimate of Difference (ED) primary vs untreated area: -9.8%, p<.0001) and secondary mechanical allodynia (response in time different from '0': p<.05). The secondary allodynic response was more evident in Period 1 compared to Period 2, but did not significantly differ (p=.211). Capsaicin also increased skin redness (ED: 0.11 Arbitrary Units (AU), p<.0001) and blood perfusion (ED: 44 AU, p<.0001) compared to untreated skin, and did not clearly interfere with other pain models.

CONCLUSIONS The ethanolic 1% capsaicin solution induced stable primary sensitization, skin redness, was well-tolerated and did not evidently interfere with other tests. Secondary allodynia was induced significantly, yet variable. The ethanolic 1% capsaicin solution is suitable as model for use in early-phase drug studies in the context of a multi-modal nociceptive test battery, but leaves room for further improvement.

INTRODUCTION

Capsaicin, the active component in chili peppers, is a chemical irritant often used in clinical setting. Capsaicin induces burning and painful sensations through highly selective interaction with transient receptor potential cation channel subfamily V member 1 (TRPV1), present on C-fibers and a subset of Aδ-fibers. [1–3] Conversely, prolonged exposure to high concentrations (e.g., 8%) leads to analgesia lasting for months by reducing TRPV1-expressing nociceptive nerve endings. [4] TRPV1 can be activated by noxious heat (≥ 43 °C) and physical abrasion, allowing capsaicin in lower concentrations (≤ 3%) to be used as a challenge agent to induce primary sensitization to heat and mechanical stimuli by modulating peripheral afferent nerves at the treated site. [5,6] Capsaicin also exerts effects in the central nervous system (CNS) by sensitizing nociceptive neurons to their normal or subthreshold afferent input (i.e., central sensitization), which is hypothesized to be due to transiently increased neuronal excitability in the dorsal horn. [7,8] Capsaicin-induced central sensitization may, for example, be evaluated by quantifying the mechanical allodynic response in the area surrounding the site where the capsaicin was administered (i.e., secondary allodynia).

In experimental settings, capsaicin is mostly either topically administered as a cream or ethanolic solution, or injected intradermally. [6,7,9–14] While scientific publications about the effects of capsaicin on primary sensitization and neurogenic inflammation (e.g., erythema and vasodilation) mostly agree, reports on capsaicin-induced secondary allodynia do not: only half of the studies demonstrate secondary effects of topical capsaicin formulations. [15] In a previous human experimental pain study, we could only demonstrate primary, but not secondary effects of a 1% capsaicin cream formulation. [16]

For a topical drug to be efficacious, sufficient skin penetration of the active ingredient and skin permeation are key. However, skin penetration is significantly influenced by a drug's physicochemical properties – and therefore challenging to optimize. [17] The cream formulation we previously tested may have inadequately penetrated the skin, thereby limiting induction of secondary allodynia. Chemical penetration enhancers including ethanol can increase (topical) drug flux and skin permeation, which may lead to higher efficacy of the active ingredient. [18–20] An ethanolic capsaicin solution formulation may therefore be superior in

inducing secondary allodynia, as suggested by other studies with such a formulation. [6,9,13,21,22]

For an experimental pain model to be of use for evaluating (novel) analgesics in the context of a multimodal pain test battery, it may not influence other measurements and the results should be reproducible. [16] A proof-of-concept trial which validates the use of a new model in context of existing methods is therefore warranted. In our clinical unit, we use a validated multi-modal test battery with distinct tests that do not interfere with one another, and allow for profiling and benchmarking of drugs against each other. [23]

Here, we evaluated whether a topical ethanolic 1% capsaicin solution could induce primary and secondary sensitization without influencing other tests, to serve as an extension to our nociceptive test battery.

METHODS

General considerations

The study was conducted at the Centre for Human Drug Research (CHDR, Leiden, The Netherlands), in accordance with the Declaration of Helsinki of 1975, its amendments and the Guideline for Good Clinical Practice. This study was registered in the Netherlands Trial Register under No. 7704, ToetsingOnline No. NL68698.056. and approved by the Medical Ethics Committee Stichting Beoordeling Ethiek Biomedisch Onderzoek (Stichting BEBO, Assen, The Netherlands) before any assessments took place.

Study design

This was a two-period, open-label, proof-of-concept study in 10 healthy males. Enrolled subjects attended the clinic on two identical visits lasting one full day each, with a wash-out of at least 7 days in-between. A telephonic follow-up 5-9 days after the last capsaicin administration (i.e., last dose) concluded study participation.

Screening was planned up to 42 days before the first study day. Written informed consent was voluntarily provided by all subjects prior to any assessments taking place. Male volunteers, aged 18-45 (inclusive), with a BMI between 18-30 kg/m2 (inclusive) and that were overtly healthy as

confirmed by a comprehensive medical evaluation including vital signs, medical history review and previous or chronic pain symptoms, were eligible to participate. Subjects that reported to have significant allergic reactions (urticaria or anaphylaxis) to capsaicin, or with a dark skin type (Fitzpatrick V and VI), widespread acne, tattoos or scarring on the volar forearms were excluded.

The 1% capsaicin solution (see next section) was applied at screening to make subjects familiar with the sensation and exclude those allergic to the solution or reporting to have intolerable pain after administration. In addition, a training session for all pain tests except the von Frey assessment was part of the screening assessments to familiarize subjects. Those indicating to be intolerable or too tolerant were excluded. Subjects were found to be too tolerant when achieving tolerance at >80% of maximum input intensity for the pressure, electrical or cold pain test The training also included determination of the individualized laser stimulus threshold for the LEP assessment (test procedures details in section **Study procedures**).

Study drug

A 60% ethanolic, topical 1% capsaicin solution was used and produced under Good Manufacturing Practice (GMP) conditions at Tiofarma BV, Oud-Beijerland, The Netherlands. The solution contained capsicum oleoresin US Pharmacopeia (USP), ethanol 96% pure and purified water.

At screening and in the morning of both study days, 50uL (= 0.5 mg) of the 1% capsaicin ethanolic solution was applied topically on a predefined 3×3 cm area on the dominant volar forearm (i.e., primary area), after which it was occluded for 30 minutes. Household film was used for occlusion for the first two subjects on the first study period, but pressed the capsaicin outside of the intended area of application and therefore was replaced by Tegaderm film of 6x7 cm (3M, USA) for all subsequent study days. A 3×3 cm silicone mall placed prevented the solution from spreading outside the intended area of application. Remaining solution was carefully wiped off the skin towards the middle of the 3×3 cm area after 30 minutes.

Adverse events (AEs) were recorded to confirm safeness of using the ethanolic 1% capsaicin formulation.

Study procedures

EVOKED PAIN TEST BATTERY At screening and during each visit, a validated battery of pain tests was performed (**Figure 1**). All pain tests were equipped with a maximum safety cut-off to minimize the risk of harming subjects. [24]

The pain test battery was performed as previously described. [25,26] In summary, subjects were assigned to a separate room that did not have any form of distraction and seated comfortably in a chair. For each test but the heat pain test, subjects were asked to hold a hand-held electronic visual analogue scale (eVAS), with which they could indicate their currently perceived pain intensity. The eVAS ranged from 0 up to 100. 0 was defined as 'no pain', sliding > 0 defined the Pain Detection Threshold (PDT), and 100 defined the Pain Tolerance Threshold (PTT; 'worst pain tolerable').

For the heat pain tests on capsaicin-treated and untreated skin, a 3×3cm thermode (QSense, Medoc, Israel) was placed first on the area where capsaicin was applied (i.e., primary/capsaicin-treated area). The thermode gradually increased with 0.5°C/s starting from 32°C. Subjects were given a hand-held feedback control and asked to click the button on the control when the heat stimulus was first perceived as painful (PDT). As a safety precaution, no heat PTT was measured and 50°C was used as cut-off temperature. This procedure was repeated next on the non-dominant arm at an area contralateral to that of where capsaicin was (planned to be) applied (i.e., control/untreated area). Per timepoint and per area, the average of triplicate measurements was used for further analysis.

The short-form McGill Pain Questionnaire (SF-MPQ, Dutch version) was used to evaluate the affective and sensory components of the pain perceived following the heat-, pressure-, electrical burst-, electrical stair- and cold pressor pain task. [27,28]

LASER EVOKED POTENTIAL (LEP) ASSESSMENT To assess changes in cortical brain response, LEPs were recorded following laser stimulation (LS) in a quiet room with minimal illumination using adequate safety precautions. A laser (Nd:Yap, Stimul 1340, Electronic Engineering) generated stimuli with a 5 mm diameter, 5 msec duration, random inter-stimulus intervals of 6-8 s and individual stimulus strength as defined at screening (see next paragraph). 20 stimuli were administered per location, during study days first on the secondary area (dominant arm, **Figure 2**), then control area (non-dominant arm) and ending with the

primary area (dominant arm). The stimulation site was moved slightly within the defined area after each stimulus to avoid skin damage and nociceptor sensitization/habituation. [21,29,30] After each set of 20 stimulations, the subject reported peak pain using an 11-point NRS for each specific area. Cortical responses to LS were recorded using an EEG system (REFA32, Twente Medical Systems international (TMSi), Oldenzaal, the Netherlands) and collected with a 10-20 cap system. To minimize recording artefacts, subjects were instructed to keep their eyes open, focus and stay relaxed. Subjects were asked to push a hand-held reaction button when a stimulus was felt. The amplitude (μV) and latency (msec) of the maximum negative peak between 150 and 300 msec (N2), the maximum positive peak between 250 and 360 msec (P2) and N2P2 peaks that were observed in each EEG were used for analysis.

As part of the screening procedures, the individual threshold of each subject was determined using a validated script, following related literature. [22] Briefly, the laser stimulus was pointed at non-treated skin on the non-dominant volar forearm. The stimulus increased from 0 to a maximum of 2.0 J, with increments of 0.25 J/step. Subjects reported if the stimulation was perceived as a sharp pinprick by answering a concise yes/no question following each step. If the stimulus was felt as a sharp pinprick or 2.0 J was reached, the ramp was repeated for a total of three times. The average result was multiplied by 1.5 and, if needed, rounded off to a lower value for safety purposes. That threshold was used for that specific subject throughout the remainder of the study.

SECONDARY MECHANICAL ALLODYNIA (VON FREY TEST) At the start of each study visit, eight spokes that divided a circle equally were drawn on the volar forearm to quantify the area of secondary allodynia (**Figure 2**). Pre-capsaicin application, individualized perception to mechanical pain was determined using Von Frey filaments (OptiHair, MRC systems GmbH, Heidelberg, Germany) with strengths of 128, 256, 362 and 512 mN. The strength preceding the one the subject reported as being just painful at first was used to determine allodynia for that subject.

After capsaicin administration, the assessment started on the north spoke at the most outer point from the center of the primary area and moved to the middle with steps of 5 mm. Once sensation changed from nearly painful to painful, that point was determined to be the border of the allodynic area. This assessment was repeated for all spokes in a clockwise fashion. The allodynic area was quantified in mm^2 using individual

values for each spoke. A 5 mm border zone for each spoke surrounding the primary area (i.e., the 3×3cm capsaicin application area) was used to minimize the risk of reporting false positive effects.

SKIN ANALYSIS – ERYTHEMA AND BLOOD FLOW ASSESSMENTS
The assessments were performed in a temperature-controlled area (approximately 22°C) where subjects were accommodated to the temperature for at least 15 min. Procedures employed to assess effects of capsaicin on the skin using multispectral and laser speckle contrast imaging, have been described extensively described elsewhere. [31–33]

In brief, capsaicin-induced erythema (defined here as the CIELAB colour space a* value) and redness (defined as the haemoglobin average level [34]) were measured using a multispectral imaging device (Antera 3D, Miravex, Dublin, Ireland). The CIELAB a* value is a colorimetric score of redness based on the harmonized CIELAB color space, whereas the haemoglobin score is based on an algorithm of the Antera 3D device that assesses skin redness. The regions of interest for both assessments were size matched at the site of application, and at the untreated site used as control (i.e., same area of skin on the dominant and non-dominant arm, respectively). Skin blood perfusion (i.e., basal blood flow) was quantified using Laser Speckle Contrast imaging (LSCI; PeriCam PSI System, Perimed AB, Järfälla, Sweden). [31]

Statistical considerations and analysis

Analysis was performed using SAS for Windows version 9.4 (SAS Institute Inc., Cary, NC, USA). No adjustments for multiple comparisons were employed as the study was explorative.

For the Von Frey test, secondary mechanical allodynia was defined as a response > 0mm^2 outside the primary and border area. A responder was defined as reporting to have an area of secondary mechanical allodynia > 0mm^2 in both study periods. Intra-subject variability was visualized by calculating the mean difference of the allodynic response per subject (i.e. Period 1-Period 2). Von Frey test results for the first study period of the first two subjects were excluded from analysis as the solution accidently spread outside the primary area, and therefore was deemed to preclude proper assessment of effects on the secondary area (also see **Figure 2**.)
Repeatedly measured pharmacodynamic data were analyzed with a

mixed model analysis of covariance with group, area (if applicable: control, primary or secondary area), time, visit, and interaction effects as fixed factors and subject, subject by area and subject by time as random factors and the (average) baseline measurement as covariate. Contrasts for primary versus (vs) control, and if applicable secondary vs control, primary vs secondary and secondary mechanical allodynia vs '0' were calculated within the model.

For each endpoint, estimates of the difference (ED) were generated for specified contrasts, and back transformed EDs in percentage for log transformed parameters. In addition, 95% confidence intervals (95% CI; in % for log-transformed parameters) and Least Square Means (LSMean) (the geometric means for log transformed parameters), and the p-value for each applicable contrast was reported.

RESULTS

Demographics and safety

A summary of subject characteristics is included in **Table 1**. 10 male subjects were enrolled as planned and completed the study.

Seven subjects reported a total of 16 AEs, of which eight were related to LS study procedures and four were reported as a mild burning sensation on the capsaicin application site. Other AEs were unrelated to study conduct (e.g., nasopharyngitis). One AE was moderate in severity (second degree burn after laser stimulation), all others were mild.

Pain test results

EVOKED PAIN TEST BATTERY Results are summarized in **Table 2**. Capsaicin significantly lowered heat PDTs on treated skin compared to heat PDTs on untreated skin (ED: -9.8%; 95% CI: -10.8 – -8.6%; p<0.001), which peaked around 2 h post-administration and lasted until the last measured timepoint (10 h post-administration) (**Figure 3**). Effects were similar in both periods. The affective and sensory perception of heat pain were both significantly increased as noted on the SF-MPQ (affective, ED: 0.07; 95% CI: 0.01 – 0.13; p <.05; sensory, ED: .16, 95% CI: 0.11 – 0.20; p <.001).

No significant differences over-time, or period effects (period 2 vs period 1) were noted for PDT or PTT for the other evoked pain models included

in the test battery (i.e., cold pressor-, electrical burst-, electrical stair-, pressure pain test and CPM paradigm) (**Table 2** and **3**). No effects were observed on the SF-MPQ endpoints for the cold pressor-, electrical burst-, electrical stair- and pressure pain test.

SUBJECTIVE PAIN RATING AND VON FREY TEST Subjective capsaicin pain ratings using a NRS were transiently and significantly increased after application, peaking shortly after administration (t = 0.5 h) to an estimated mean of 4.4 (on 11-point scale; effect over time p <.001). Pain ratings were comparable in both periods (estimated mean Period 1: 1.6, Period 2: 1.5; p=0.368) (**Figure 3**; **Table 2**).

Capsaicin significantly induced secondary mechanical allodynia (response for n=10 subjects over time versus '0' (i.e., no secondary allodynic response): estimated mean period 1: 728.7 mm^2, estimated mean period 2: 497.8 mm^2; p<.05). (**Figure 3**; **Table 2**). Only a subset of subjects (n=6, of n=8 that could be included in the analysis (see section **Statistical considerations and analysis**) reported to have allodynia in both periods.

Although no significant difference between periods was observed (period 2 versus period 1: ED: -230.8 mm^2; 95% CI: -717.6 – 255.9 mm^2; p>.2), effects were consistently more pronounced in period 1 than period 2, with the exception of the 30 min time point (**Figure 4**; **Table 2**).

LEP TEST Pain response to LS as measured with a NRS significantly differed between the primary and control area (ED: 0.76, 95% CI: 0.46 – 1.06, p<.0001), and between the secondary and control area (ED: 0.57, 95% CI: 0.27 – 0.87, p<.001). (**Table 2**).

No significant effects of capsaicin were noted for the response time to LS, or peak-to-peak amplitude for the N2-P2 peaks (**Table 2**).

Skin analysis

Compared to the untreated area, capsaicin significantly increased skin blood perfusion on the treated area (ED: 44.02 arbitrary units (AU); 95% CI: 39.20 – 48.84; p<.001) and significantly induced skin redness (haemoglobin levels for treated vs untreated skin: ED: 0.11 AU; 95% CI: 0.07 – 0.15 AU, p <.001). No significant erythemic effect was observed (CIELAB a* score treated vs untreated skin, ED: .37 AU, 95% CI: -0.08 – 0.82; p>.1) (**Figure 3**, **Table 2**).

DISCUSSION

We evaluated the sensitizing effects of an 1% capsaicin ethanolic solution, when incorporated as model in a validated evoked pain test battery. Results indicate that this formulation induced significant and tolerable primary heat sensitization without evidently influencing other pain tests. Secondary mechanical allodynia was also significantly induced, yet variable and only observed in a subset of subjects.

Various capsaicin formulations and administration routes are used in experimental context to induce sensitization, each with its own strengths and weaknesses. Intradermal (ID) injection, for example, can elicit reproducible and long-lasting secondary allodynia without requiring other possibly influencing factors as a heat sensitization procedure (i.e., kindling with a heat thermode), a tactic commonly employed for topical formulations to stabilize and increase duration of capsaicin effects. [35] While 80-100% of subjects receiving an ID capsaicin injection report to have secondary allodynia, [35] we deliberately decided not to test an ID formulation. The procedure is more difficult and invasive compared to topical application, but also induces a nearly maximal pain sensation (NRS of ~9 out of 10), [36] which likely will result in significant subject drop-out rates in early-phase drug studies with a multi-period cross-over design that require (highly painful) injections in every study period. We develop the capsaicin model for such designs specifically, therefore believe an ID injection is not suitable. The limited increase in NRS (a maximum LSM of 4.4) confirms that the ethanolic formulation is tolerable and applicable for its intended use (**Figure 3**).

Rather, hoping to increase the secondary allodynic response, we opted to change our formulation to one with enhanced skin penetrability by switching from cream to an ethanolic solution at a concentration (i.e., 60% ethanol) found stable by the manufacturer. Primary sensitization to heat was evidently more induced by the ethanolic- compared to the cream formulation (ED of Heat PDT vs control ethanolic formulation: -9.8%; ED of Heat PDT vs control cream formulation -3.85%), and secondary allodynia was repeatably induced by the ethanolic formulation whereas the cream formulation could not produce any notable effects. [16] These results suggest that between the formulations we tested, the ethanolic option indeed is superior in inducing secondary sensitization.

Nonetheless, also when using the ethanolic solution formulation, only a subset (n = 6) of subjects reported to have secondary mechanical allodynia in both study periods and the allodynic area was limited (**Figure 3**). It is not likely that a higher concentration would have yielded more pronounced effects, as a recent review found no correlation between topical capsaicin concentrations and allodynic area. [35] Conversely, a correlation was found between heat-kindling of the treated area, and enhancement of the allodynic response. [35] We did not employ that procedure here, as we did not observe any differentiating effects of kindling in the previous capsaicin cream study. [16] While publicly available literature only sparsely discusses the duration of the allodynic response, the kindling interval in our previous study with capsaicin cream (every ~2 hours) was not in line with other studies that successfully employed the procedure (every 45 min). [10,16,37,38] It is suggested to re-evaluate whether kindling may be used to further optimize the capsaicin model.

We found no significant difference in the secondary allodynic response between both periods (ED: -230.8 mm2; p>.2), although it should be noted that effects in period 1 were evidently more pronounced at all timepoints except for 30 min (**Figure 4**). Decreased sensitization of TRPV1 is known from e.g. high-dose (8%) topical capsaicin administrations, [4] but in experimental study-context a decreased response after repeated capsaicin administration is only described once elsewhere. [39] Cavallone et al. reported decreased secondary hyperalgesia to Von Frey testing – but not for allodynia using brush strokes – after second capsaicin administration. They determined this was in contrast to others as well. [39] Further studies with our ethanolic 1% capsaicin formulation are warranted to confirm whether the decreased response was a chance finding due to small sample size, or rather due to habituation effects.

As an alternative to the use of capsaicin to induce secondary sensitization, other models may be considered, such as high-frequency electrical stimulation (HFS). This relatively novel method selectively induces secondary allodynia at an amplitude similar to ID capsaicin and lasts for several hours. [40,41] Unpleasantness of the procedure is reported to be meagerly lower than ID capsaicin injection, so it has to be evaluated if that will preclude its use in multi-period crossover drug studies. [42,43]

We found no significant influence of the 1% capsaicin ethanolic formulation on any of the other nociceptive tests (**Figure 3**, **Table 2**). The (LSMean) pain thresholds over the day were comparable to the previous

capsaicin study, when taking into consideration that the data of the other study is of a larger sample (n=18) and placebo treatment arm of a cross-over study, where this was an open-label proof-of-concept study in n=10 subjects without treatment (**Table 3**). In this study, we did note an unexpected slight decrease over time of heat PDTs on untreated skin that was comparable in both periods (**Figure 3**). There was no such trend on untreated skin in the proof-of-concept capsaicin cream formulation study. [16] We could not find a clear reason as to why in this study heat PDTs on untreated skin were decreased. We did change the assessment order (i.e., for heat PDT first evaluate the treated area before the untreated area, where it was performed vice versa in the capsaicin cream study), and switched to an air-cooled thermode (QSense 3×3 cm thermode, Medoc) from a water-cooled one (TSAII 3×3 cm thermode, Medoc, Israel). We do not believe that the change in order or change of capsaicin formulation is causative, as the heat tests were performed on two distinct extremities (i.e., dominant and non-dominant arm). We cannot rule out that the change of equipment is causative, as heat PDTs on untreated skin were also slightly decreased in two other studies with the same capsaicin cream that evaluated heat PDTs using the air-cooled QSense rather than water-cooled TSAII device. [26,44] We do believe it is unlikely as we followed the same test procedures and used a thermode of equal size (3×3 cm – studies suggest that mainly the contact area may affect the allodynic response). [39,45] Nevertheless, these observations do not limit the use of the ethanolic formulation in early-phase drug studies, as long as designed properly. In a placebo-controlled cross-over design, subjects are balanced per calculated contrast. This ensures that such variability, if any, is present evenly in every period and thus effectively is cancelled-out when calculating analgesic drug effects compared to placebo response. [46]

One other key characteristic of capsaicin is that it transiently increases skin redness and induces erythema. [47,48] Haemoglobin average levels, a surrogate for skin redness, and blood perfusion were significantly increased by capsaicin as expected (both p<.0001; **Table 2**). While evidently increased shortly after capsaicin administration, we found no significant effect on the erythemic response (evaluated with CIELAB a* score) (**Table 2**). Sparse evaluation of this endpoint due to the many other tests performed on a single day, in combination with black marker drawings on the skin (used for quantification of the allodynic response (**Figure 2**) may have hindered proper evaluation of CIELAB a* values. This confounding

effect was not noted on the other skin analysis endpoints as the haemoglobin score is based on an algorithm rather than colorimetric scoring with the CIELAB a* results, [34] and because skin blood low was measured using laser speckle imaging rather than colour spectrum-based imaging.

The results presented here are to be read with the following considerations. Pain perception and the contribution of TRPV1 to nociceptor excitability – through interaction with gonadal hormones –significantly differs between men and women. [49,50] To optimize our chance to find significant effects in this exploratory study, we only included men – which limits the conclusions drawn to that population. The temperature ramp setting used for determining heat PDTs was accidently set too low (0.1 °C/s instead of 0.5 °C/s) during data collection of both periods for one subject, and of one period for two subjects. As a conservative approach, the data presented here are without those results, while noting that this error did not affect the effects noted (ED heat PDT capsaicin- vs untreated skin, without incorrect measurements: -9.8%, same ED with all measurements included: -10%). Lastly, the instructions given to subjects for reporting of secondary mechanical allodynia may have been suboptimal (i.e., when a change from ''nearly painful to painful'' was perceived). While a Von Frey strength was determined pre-dose for which the subjects confirmed to not feel pain (see section **Secondary mechanical allodynia (von Frey test)**), we cannot rule out that 1) this perception changed over-time, and 2) that the allodynia was perceived similarly across the whole area where it was quantified (**Figure 2**). For future studies, it may be less biased and more in line with procedures of other investigators to 1) perform a baseline measurement (i.e. pre-capsaicin) identical to how the test is performed at subsequent timepoints, and 2) ask subjects for the distinct detection of an increase in perception to the mechanical stimulus (i.e. *'distinct change in sensation as increased burning, tenderness, more intense pricking, or an unpleasant sensation'*). [38,39]

In conclusion, the 1% capsaicin ethanolic formulation induced primary heat sensitization, secondary mechanical allodynia, and increased skin blood flow and erythema. The pain induced by the capsaicin application was tolerable, without evidently interfering with other measurements. The model therefore is suitable for use in early-phase drug studies as extension of a multi-modal nociceptive test battery, but leaves room for further improvement.

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Table 1 Summary of Demographic characteristics.

Demographic category	Number (N = 10)
SEX, N (%)	
Male	10 (100%)
AGE (YEARS)	
Mean (SD)	23 (3.7)
WEIGHT (KG)	
Mean (SD)	77.3 (7.8)
HEIGHT (CM)	
Mean (SD)	181.7 (5.3)
BMI (KG/M^2)	
Mean (SD)	23.4 (1.5)
FITZPATRICK SKIN TYPE	
Type II	4 (40%)
Type III	4 (40%)
Type IV	2 (20%)

m: centimeters, kilograms, m^2: square meters, SD: standard deviation.

Table 2 Summary of statistical analysis.

Endpoint	Contrast	1st LSM[1]	2nd LSM[1]	ED	95% CI Lower	95% CI Upper	p-value
NRS CAPSAICIN PAIN							
	Time (response-'0')						**<.0001**
	Period 2-Period 1	1.5	1.6	-0.2	-0.6	0.2	.37
	Von Frey Time (response-'0')						**<.05**
	Period 2-Period 1	299.4 mm^2	492.3 mm^2	-193 mm^2	-521.5 mm^2	135.5 mm^2	.21
HEAT PDT							
	Primary-control	36.1 °C	40 °C	-9.8%	-10.8%	-8.6%	**<.0001**
	Period	-	-		-	-	0.63
	MPQ: affective (primary-control)	.38	.31	.07	.01	.13	**.02**
	MPQ: sensory (primary-control)	.70	.55	.16	.11	.2	**<.0001**
LS							
Reaction time	Primary-control	724.3 ms	720.8 ms	3.5 ms	-29 ms	35.9 ms	.83
	Secondary-control	715.5 ms	720.8 ms	-5.3 ms	-38.5 ms	27.9 ms	.75
Amplitude N2-P2 *peaks*	Primary-control	23.9 uV	24.1 uV	-0.2 uV	-2 uV	1.5 uV	.79
	Secondary-control	23.1 uV	24.1 uV	-1 uV	-3 uV	0.9 uV	.3
NRS *pain*	Primary-control	6.1	5.3	0.8	0.5	1.1	**<.0001**
	Secondary-control	5.9	5.3	0.6	0.3	0.9	**.0002**
SKIN ANALYSIS – BLOOD PERFUSION							
Basal flow	Primary-control	84.4 AU	40.4 AU	44 AU	39.2 AU	48.8 AU	**<.0001**
SKIN ANALYSIS – ERYTHEMA							
Haemoglobin average level	Primary-control	0.9 AU	0.8 AU	0.1 AU	0.1 AU	0.1 AU	**<.0001**
*Skin colour CIELab a**	Primary-control	11.3 AU	10.9 AU	0.37 AU	-0.1 AU	0.8 AU	.1

(Table continues on next page)

(Continuation Table 2)

Endpoint	Contrast	1st LSM[1]	2nd LSM[1]	ED	95% CI Lower	95% CI Upper	p-value
ELECTRICAL STAIR (SINGLE STIMULUS)							
	PDT: time						.89
	PDT: Period 2-Period 1	6.7 mA	6.1 mA	10.1%	-17.4%	46.6%	.47
	PTT: time						.49
	PTT: Period 2-Period 1	16.2 mA	16.7 mA	-2.7%	-15.3%	11.7%	.66
ELECTRICAL BURST (REPEATED STIMULUS)							
	PDT: time						.76
	PDT: Period 2-Period 1	1.5 mA	1.6 mA	-5.9%	-39%	45.4%	.76
	PTT: time						.4
	PTT: Period 2-Period 1	7.3 mA	7.8 mA	-6.4%	-21.6%	11.8%	.42
PRESSURE							
	PDT: time						.74
	PDT: Period 2-Period 1	15.3 kPa	15.4 kPa	-0.8%	-13.8%	14.2%	.91
	PTT: time						.11
	PTT: Period 2-Period 1	40.5 kPa	36.5 kPa	10.9%	-8.4%	34.2%	.25
COLD PRESSOR							
	PDT: time						.63
	PDT: Period 2-Period 1	5.7 s	5.2 s	9%	-22.5%	53.3%	.61
	PTT: time						.13
	PTT: Period 2-Period 1	23.2 s	18.8 s	23%	-0.3%	51.8%	.05
CPM							
	PDT: time						.66
	PDT: Period 2-Period 1	0.8 mA	0.2 mA	0.6 mA	-1.4 mA	2.6 mA	.52
	PTT: time						.33
	PTT: Period 2-Period 1	0.4 mA	0.8 mA	-0.4 mA	-2.6 mA	1.8 mA	.7

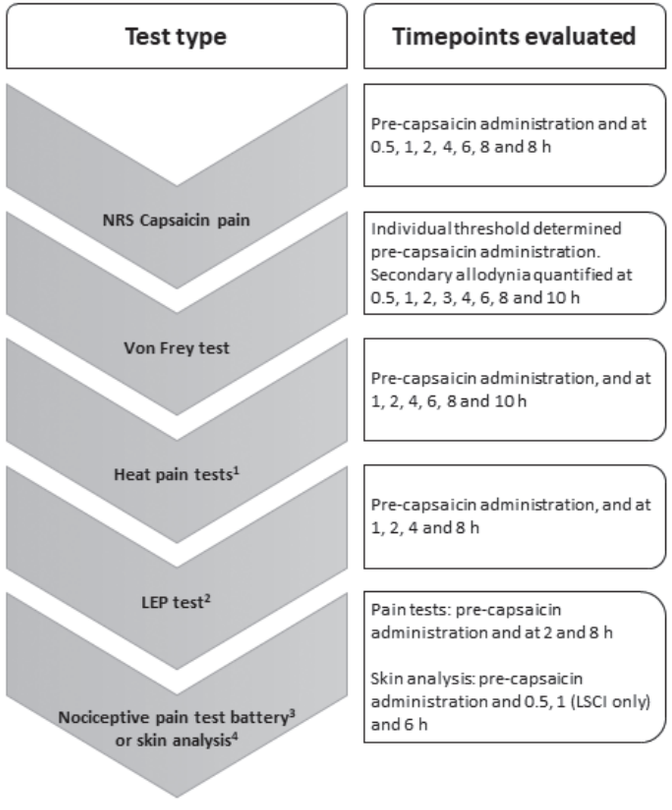
Selection of test results. 1: 'first' and 'second' LSM refers to the LSM of the first/second mentioned condition in the contrast (e.g., first LSM of primary – control refers to the LSM of the primary area). Positive ED values favor the first mentioned in the contrast (e.g. primary area, in the primary area – control area contrast) and vice versa. 'primary' is the area of skin treated with capsaicin, 'secondary' the area surrounding the treated (i.e., primary) area (both on the dominant arm); 'control' is the area on untreated skin (on the non-dominant arm). The contrast 'time' describes whether LSMs for specified test differed significantly over-time. 95% CI: 95% confidence Interval, CPM: conditioned pain modulation, ED: Estimate Of Difference, LSM: least square means, mm^2: square millimeters, MPQ: short-form McGill pain questionnaire, N2: maximum negative peak between 50 and 200 msec; P2: maximum positive peak between 150 and 500 msec; uV: microvolt. PDT/PTT: pain detection/tolerance threshold.

Table 3 Comparison of nociceptive test results between two CHDR studies using a capsaicin model.

Time (h) or contrast	1% capsaicin ethanolic solution (i.e., current) study N=10		Capsaicin cream formulation study [16] N=18	
	PDT (95% CI)	PTT (95% CI)	PDT	PTT
PRESSURE PAIN (kPa)				
1	-	-	11.4	41.8
2	-	-	11.0	42.3
3	15.17 (13.4 – 17.1)	37.6 (34 – 41.5)	12.2	44.5
4	-	-	12.6	42.4
6	-	-	11.5	43
9	15.5 (13.7 – 17.5)	39.3 (35.6 – 43.5)	-	-
10	-	-	12.6	41.4
COLD PRESSOR PAIN (S)				
1	-	-	4.0	23.0
2	-	-	3.9	23.5
3	5.6 (3.8 – 8.3)	22 (18.7 – 25.8)	3.7	22.9
4	-	-	3.8	22.2
6	-	-	3.1	23.7
9	5.2 (3.5 – 7.7)	19.9 (16.9 – 23.4)	-	-
10	-	-	3.4	21.9
ELECTRICAL STAIR PAIN (SINGLE STIMULUS, mA)				
1	-	-	6	22.3
2	-	-	5.1	21.8
3	6.4 (5.3 – 7.7)	16.3 (14.9 – 18.7)	6.5	21.7
4	-	-	6.3	21.8
6	-	-	5.6	22.2
9	6.3 (5.2 – 7.7)	16.7 (14.9 – 18.2)	-	-
10	-	-	5.3	22

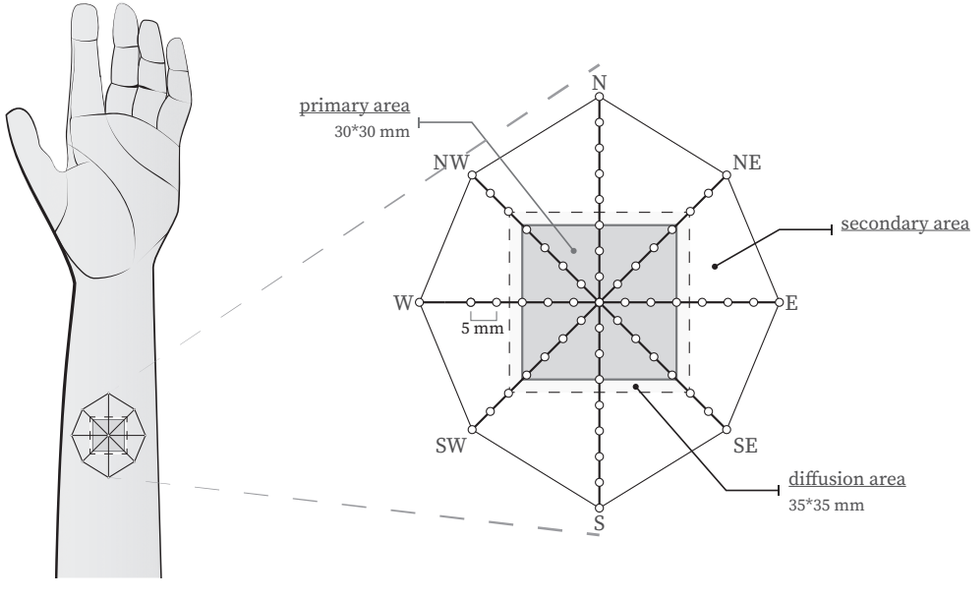
Comparison of PDT and PTTs per time point between current study that evaluated the 1% capsaicin ethanolic formulation, and the previous study that evaluated the 1% capsaicin cream formulation (Siebenga et al., 2020). [16] Data are presented as LSM's, including 95% CI's between parentheses for the current study. Statistical analysis of potential period effects (i.e., difference between LSM's in period 2 versus period 1) are included for the current study as well, presented in . For the previous study), data of the period in which subjects received placebo are included (reference [16]). '-': pain test not performed at respective timepoint for that study, 95% CI: 95% Confidence Interval, h: hour, kPa: kilopascal, LSM: least square means, mA: milliamperes, n : number of subjects, NA: not applicable, PDT/PTT: pain detection/tolerance threshold, s: seconds.

Figure 1 Order of assessments. Refer to the methods section for details of test procedures. Timepoints are protocol time (in hours) post-capsaicin administration, unless stated otherwise. 1: First heat pain test on capsaicin-treated skin followed by heat pain test on untreated skin. 2: First on treated area (i.e., primary area on dominant arm), followed by the untreated area (non-dominant arm) and the secondary area (i.e., surrounding primary area on dominant arm). 3: order: pressure pain test, electrical burst pain test, electrical stair pain test, cold pressor pain test, electrical stair pain test (repeat for evaluation of CPM). 4: First on capsaicin-treated area, then on control area (contralateral area on non-dominant arm). At coinciding timepoints first LSCI followed by MSCI.



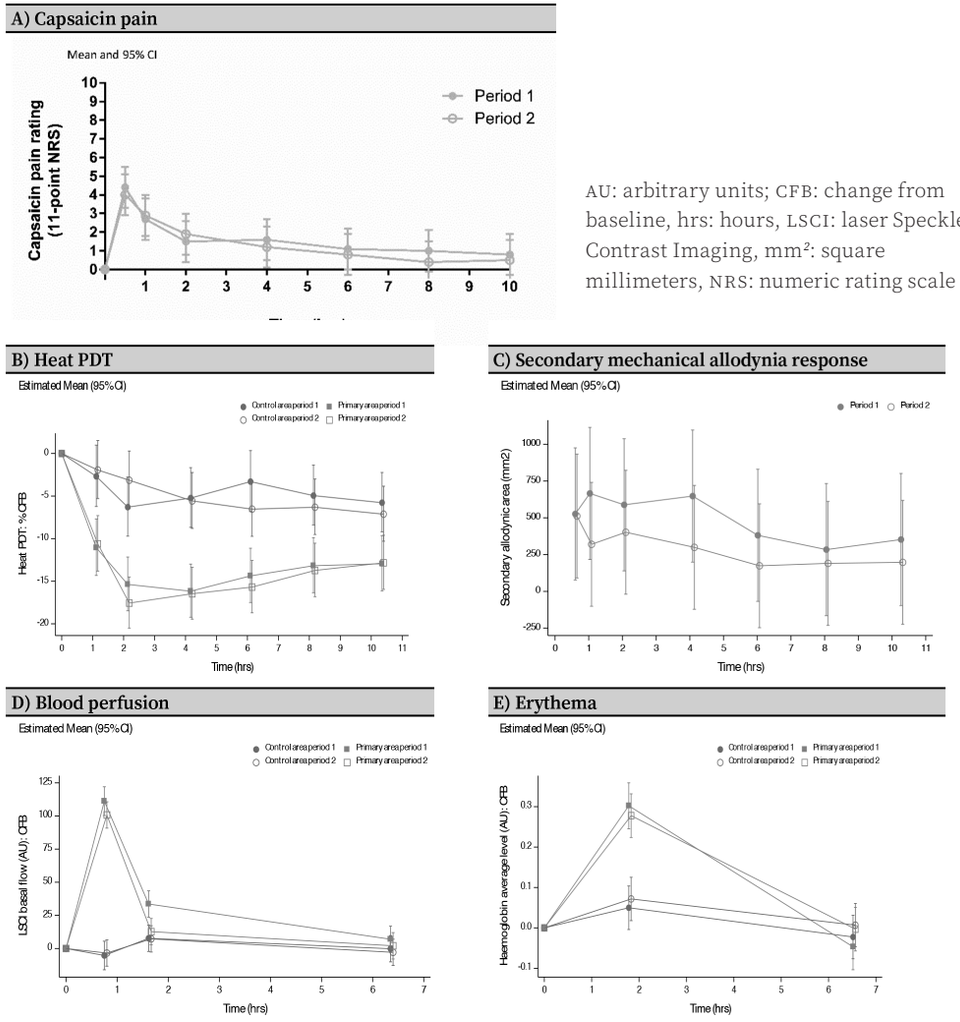
CPM = Conditioned Pain Modulation, h: hour, LSCI: Laser Speckle Contrast Imaging, MSCI: Multispectral Imaging, NRS: 11-point numeric rating scale.

Figure 2 Illustration of secondary mechanical allodynia assessment. Eight spokes divided a circle equally were drawn on the volar forearm to quantify the area of secondary allodynia. Assessment started on the north spoke at the most outer point from the center of the primary area (#1) and moved to the middle with steps of 5 mm. Once sensation changed from nearly painful to painful, that point was determined to be the border of the allodynic area. This assessment was repeated for all spokes in clockwise fashion. The allodynic area was quantified in mm2 using individual values for each spoke. A 5 mm border zone for each spoke surrounding the primary area (i.e. the 3×3cm capsaicin application area) was employed to minimize the risk of reporting false positive effects.



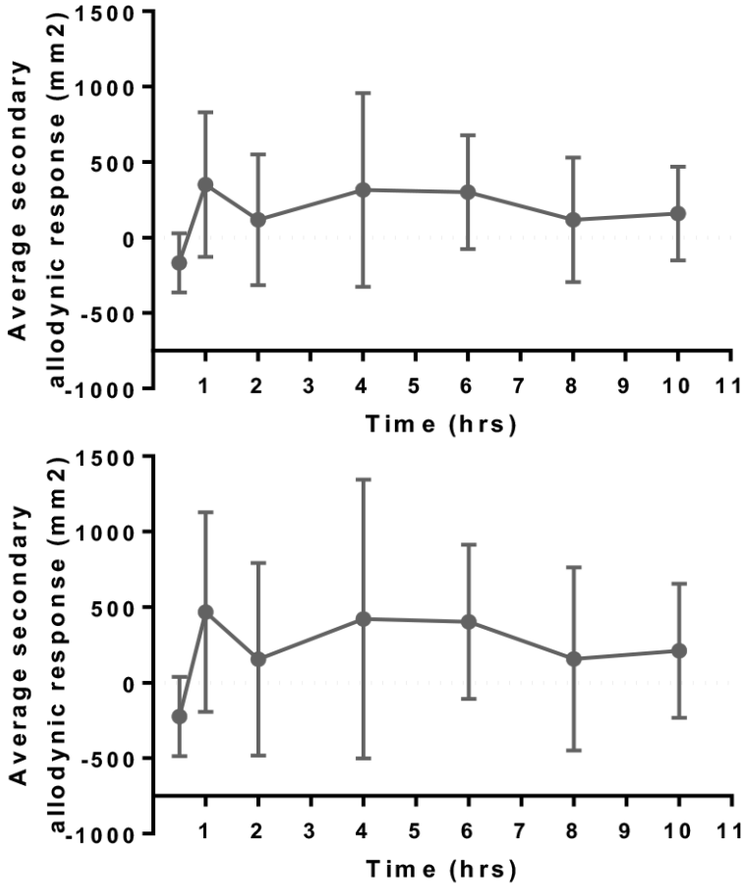
N: North, NE: Northeast, E: East, SE: Southeast, S: South, SW: Southwest, W: West, NW: Northwest, mm: millimeters. *The authors wish to thank Folkert van Meurs for illustrating this Figure.*

Figure 3 Overview of change from baseline time profiles. Effect-time profiles over the day for A) Subjective capsaicin pain rating using NRS, B) heat PDTs, C) secondary mechanical allodynia response, D) blood perfusion (i.e., basal blood flow), E) erythema (i.e., average haemoglobin level). Data are presented as estimated means with 95% confidence intervals. 'primary area' describe effects on the capsaicin treated area (i.e., on the 3×3cm area on the dominant arm), 'control area' describe the effect on untreated skin (i.e., on the contralateral area of where capsaicin was applied, on the non-dominant arm) 'period 1/2' describe the study period in which the observations were made.



AU: arbitrary units; CFB: change from baseline, hrs: hours, LSCI: laser Speckle Contrast Imaging, mm²: square millimeters, NRS: numeric rating scale

Figure 4 Intra-subject (within-subject) variability of the secondary allodynic response per measured time point. Data represented as mean difference in response between Period 1 – (minus) Period 2, with 95% confidence intervals. A) response for all subjects included in analysis (n=8); B) response for subjects defined as responder (n=6). Positive values indicate response was greater in Period 1; negative values indicate the response was greater in Period 2.



hrs: hours, mm²: square millimeters

CHAPTER 9

General discussion and conclusion

Scientific progress, and in particular development of drugs, has been accelerating with unprecedented speed. Where discovery of medicines initially was based on herbal knowledge (e.g., aspirin has been formulated from the willow bark), drugs are now discovered by high-throughput screening of libraries containing candidate molecules for their biological activity (i.e., combinatorial chemistry) or screening of molecules for their interaction with a biomolecule proposed to yield therapeutic benefit (i.e., rational drug design). Pharmaceutical and biotechnological companies utilize these approaches and leverage improved knowledge of biological targets to discover and develop novel, often highly selective (analgesic) drugs that are expected to yield improved clinical utility over classical medicines with fewer dose-limiting adverse effects.

By redefining drug discovery, drug development strategies should be revised as well. Biological processes are known to vary widely between species, which is also true for pain signalling. An example described in this thesis is the clear difference in availability of voltage-gated sodium channel (Na_V)1.8 and Na_V1.9-positive sensory neurons between humans and mice. [1] A wide range of preclinical models have been developed to mimic human pain disease phenotypes, but their predictive value is questionable. [2] Although animal models remain a vital tool in drug testing, they commonly are not equipped to accurately predict the full nature of a drug's therapeutic effects. [3] As a further complication, costs associated with human trials are ever increasing, [4] warranting careful decision making on a drug's potential early in the clinical development process.

By including biomarkers that allow for measuring pain signalling in early-phase drug studies, important data on (dose-dependent) effects can be generated, which can save costs in later-phase trials. It must be noted that, while of importance, biomarkers mostly are models for clinically relevant endpoints at best, e.g., in healthy volunteers they can only mimic a specific part of a certain (pain) pathology. Another challenge is that many novel drugs are increasingly target-selective, and may have effects on (pain) pathways that often have yet to be clinically proven relevant. Previously validated methods should therefore be scrutinized for their validity to establish Proof-of-Mechanism or Proof-of-Concept (POM, POC; **Chapter 1**) of new drug classes. In parallel, improved selectivity necessitates further refinement of human experimental models to more accurately represent aspects of clinical disease or symptoms targeted. Based on these advances, the studies described in this thesis were conducted: a

quest for finding suitable biomarkers, by developing and testing models for usability to evaluate Na_V inhibitors, the third-to-most studied analgesic drug class in early-phase drug development (**Chapter 1**).

In **Chapter 1**, we defined that a proper (analgesic) biomarker should be able to demonstrate a clear, consistent drug response across different studies, and should demonstrate it consistently for drugs of the same class. [5] By using PainCart – the fixed-sequence nociceptive test battery employed in the studies described in this thesis –, in combination with either the topical 1% capsaicin cream model or ultraviolet (UV)B model that were developed previously, [6] we assessed in **Chapters 2-4** which currently available methods are suitable to consistently demonstrate effects of Na_V inhibitors on nociceptive thresholds. Altered cold pressor pain thresholds proved to be the most reproducible biomarker, by responding to three Na_V inhibitors (VX-128, VX-150 and mexiletine) and aligning with our hypothesis described in **Chapter 1**. In that Chapter, we also suggested to include the capsaicin model in studies evaluating Na_V inhibitors, but observed that neither of the selective Na_V1.8 inhibitors tested with that model (i.e., VX-150 nor VX-128; **Chapter 2** and **3**, respectively) affected capsaicin-induced heat pain thresholds. This may be explained as Na_V1.7 rather than Na_V1.8 is linked to inherited erythromelalgia ('man on fire' syndrome), supported by the finding that selective Na_V1.7 inhibitors PF-05089771 reduced burning-like symptoms in a phase II trial. [7,8] It may, however, also be concluded that the topical 1% capsaicin cream model is suboptimal for studying analgesics, as the same model failed to show effects of tramadol or duloxetine. [6] This led us to performing the study described in **Chapter 8**.

A pharmacological biomarker should also clearly (and when applicable, dose-dependently) respond to therapeutic dose levels of drugs. [5] The tests included in the PainCart battery have previously been profiled using a variety of registered analgesics, including the Na_V-blocking antiepiLEPtic phenytoin that significantly affected nociceptive thresholds in the electrical stair pain paradigm. [9] It was, however, concluded that insufficient plasma concentrations of phenytoin were reached, preventing use of that data for evaluation of this biomarker criterion. In **Chapter 4** we noted that the cold pressor test significantly responded to therapeutic doses of Na_V inhibitor mexiletine, but not lacosamide. Based on the differential characteristics of mexiletine and lacosamide – preferential modulation of Na_V1.8 versus Na_V1.7, respectively – we discussed that

biomarker selection should depend on which Na_V-subtype is primarily targeted. Corroborated by evidence from the VX-128 and VX-150 studies (**Chapter 2 and 3**), this thesis supports the use of the cold pressor test as biomarker for $Na_V1.8$-induced analgesia. The effects (or lack thereof) of lacosamide on nociceptive thresholds using PainCart provide further evidence that evoked pain models may not be suitable for evaluating $Na_V1.7$-preferential analgesics: others could also not demonstrate analgesics effects of lacosamide on experimental pain models, and no effects could be demonstrated of selective $Na_V1.7$ inhibitor PF-05089771. [6,10] While at time of discovery, in 2006, the role of $Na_V1.7$ in pain signalling was considered a major breakthrough, $Na_V1.7$ inhibitors have withhold their pain potential as none has been registered as of yet. [11] Without evidently efficacious $Na_V1.7$ inhibitors available to test, it remains difficult to draw unambiguous conclusions on the validity of currently available methods for that subtype. It may be that other methods such as the nerve excitability threshold tracking test are more suitable, as it demonstrated POM of lacosamide on, e.g., motor nerve excitability and sodium channel conductance. [12]

Having established that there is still ample room for improvement in the development of suitable biomarkers for profiling of (selective) Na_V inhibitors in healthy volunteers, hyperalgesia-inducing methods were considered. As stated in **Chapter 1**, a suitable biomarker should have a plausible relationship with the pharmacology of the tested drug class, and with the disease pathophysiology. A key aspect in many types of chronic pain such as fibromyalgia and neuropathic pain syndromes, is central sensitization – defined by the International Association for the Study of Pain (IASP) as *'an increased responsiveness of nociceptors in the central nervous system to either normal or sub-threshold afferent input'*. [13,14] Central sensitization may manifest as symptoms such as hyperalgesia and allodynia. [13] These relate to conditions caused by nociceptor hyperexcitability, a mechanism targeted by Na_V-inhibiting drugs – and by selective $Na_V1.8$ inhibitors in particular. [15] Inducing hyperalgesia in healthy volunteers therefore was determined as potentially leading to suitable pharmacodynamic biomarkers for Na_V inhibitors – and/or other analgesic drug classes.

Wishing to further expand our knowledge on hyperalgesia testing and expand our range of models in healthy volunteers, we examined whether two distinct models – total sleep deprivation (TSD) and topical application of 1% capsaicin ethanolic solution – could be used in experimental

clinical trial-context to induce hyperalgesia and/or allodynia. We noted that TSD induced sex-dependent hyperalgesia on cold-, heat- and pressure pain, and altered the conditioned pain modulation response (**Chapter 6**), as well as nociceptive processing (**Chapter 7**). The 1% capsaicin ethanolic solution model was found to increase sensitization to heat and induce secondary allodynia (**Chapter 8**). Those results confirmed suitability of these methods in healthy volunteer drug studies, but follow-up studies with pharmacological interventions are warranted to adequately test if they are sensitive to drug effects as well.

The role of Na_V-1.7 and Na_V-1.8 in inflammatory pain is through modulation by kinases such as PKA (protein kinase) and P38 MAPK (mitogen-activated protein kinase). [16] Following injury or inflammation, various inflammatory cells and mediators (e.g., macrophages, neutrophils, mast cells) are recruited to the affected tissue that subsequently increase the level of a set of kinases, including P38 MAPK. Na_V-1.7 and Na_V-1.8 – channels that are upregulated in nociceptors that innervate the affected tissue – become phosphorylated and modulated, resulting in increased ectopic action potential generation and ultimately to hyperalgesia and allodynia. [16] The human endotoxemia model (i.e., systemically administering lipopolysaccharide (LPS)) can be used to induce systemic inflammation and P38 MAPK signalling. [17] We tested in **Chapter 5** whether this could translate into a systemic inflammatory hyperalgesia model. However, LPS was not able to evoke clear, consistent and dose-dependent, inflammatory hyperalgesia, failing as challenge model to be part of a suitable biomarker for profiling effects of analgesics.

In the present thesis, we have attempted to address certain issues that analgesic drug developers are facing in the early-phases of clinical drug development, by reviewing applicable tools for the top-10 most-developed analgesics in early-phase clinical development (**Chapter 1**), by using those methods to profile investigational and registered analgesic compounds (**Chapters 2-4**), and finally by exploring other tools that may further improve predictability of a drugs' anti-hyperalgesic effects in healthy volunteers (**Chapters 5-8**). While two methods are suitable for further testing, we need to note that – except for the sleep deprivation model – most studies were only performed in male volunteers, and only mimicked one or few aspect(s) of the complex and multifactorial symptom that is pain. As such, psychological and psychosocial factors that play a role in pain chronification were left out-of-scope. Mostly as they are (yet) unfeasible

and/or unethical to test in study context (e.g., exposing healthy subjects to irreversible or prolonged pain), but also because of the exploratory nature of this research. Novel technologies including augmented/virtual reality (VR) may serve a purpose here, as they may aid in further refining methods and assessing aspects of pain that have been infeasible to test without putting the safety of volunteers at risk. While VR in pain research till date primarily has been used to temporarily inhibit the pain perception by introducing immersive images (e.g., an interactive snowy canyon environment during the treatment of burn wounds), [18] VR simulation possibly may also be used as biomarker to enhance the pain experience and assess the affective component of pain perception. Preliminary results from a study using such a method at the Centre for Human Drug Research (CHDR) seem promising and suggest for a follow-up study to evaluate drug effects targeting affective pain mechanisms. [19]

In an industry with exhaustive lead times such as the pharmaceutical sector, improving methods is key in reducing the time needed to bring medical products onto the market. Recently, the European Medicines Agency released a guidance to help developers navigate through the most important regulatory requirements in the clinical development trajectory of advanced therapy medical products (ATMPs), stipulating to answer important questions about the drug's therapeutic potential in a timely manner. [20] Although most analgesics are not identified as ATMPs, the same approach should apply. Drug developers and clinical researchers that aid in this process, are advised to design early-phase studies in such a way that allow to demonstrate POM and/or POC early-on in healthy volunteer studies, or in well-chosen patient (sub-)populations – but not to test neither and leave questions unanswered till late. The results described here offer an opportunity to aid in this process and refine pain research, in an effort to bring therapies with improved clinical efficacy to the pain patients in need.

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APPENDICES

NEDERLANDSE SAMENVATTING

De ontwikkeling van innovatieve geneesmiddelen is in recente jaren sterk geëvolueerd. [1] Aanvankelijk was de ontdekking van medicijnen gebaseerd op basale kennis van kruiden; zo is aspirine bijvoorbeeld geformuleerd uit wilgenbast. Vandaag de dag combineren farmaceutische en biotechnologische bedrijven echter technologisch geavanceerde methodologieën met toegenomen kennis van biologische doelwitten ('targets') om nieuwe geneesmiddelen te ontwikkelen die vaak zeer selectief op een of enkele doelwit(ten) aangrijpen. Het zijn medicijnen die behandelingen naar een nieuw niveau tillen. In dit proefschrift zijn ze daarom aangeduid als 'next-generation': een nieuwe generatie van – in geval van dit proefschrift – pijnstillers; analgetica. Het is de verwachting dat deze nieuwe middelen, wanneer ze gebruikt zullen worden, meer therapeutisch effect en minder (dosisbeperkende) bijwerkingen zullen hebben dan de analgetica die momenteel voorgeschreven worden.

Door de verhoogde selectiviteit en nieuwe doelwitten waar deze nieuwe analgetica op aangrijpen, moeten klinische ontwikkelingsstrategieën ook worden herzien. Biologische processen variëren sterk tussen (dier) soorten; dit is niet anders in het geval van pijn, en pijnsignalering. Een voorbeeld dat direct relateert aan dit proefschrift is het verschil in aanwezigheid van spanningsafhankelijke natriumkanalen (Na_V -kanalen) tussen diersoorten. Na_V-kanalen zijn een soort poriën die, onder andere, op sensorische neuronen ('gevoelszenuwen') aanwezig zijn. Door open en dicht te gaan onder invloed van bepaalde factoren, spelen Na_V -kanalen een belangrijke rol in het doorgeven van pijnsignalen naar de hersenen. Analgetica die ingrijpen op het blokkeren van deze kanalen worden Na_V -blokkers genoemd. Subtypes van Na_V -kanalen, zoals $Na_V1.8$ en $Na_V1.9$, zijn bij mensen op sensibele zenuwen meer aanwezig dan bij muizen. [2] Ondanks dat diermodellen een essentieel hulpmiddel blijven bij het testen en veilig ontwikkelen van geneesmiddelen, impliceert dit verschil in aanwezigheid van Na_V-kanalen dat diermodellen dikwijls niet in staat zijn om de volledige aard van de therapeutische effecten van een (nieuw) geneesmiddel nauwkeurig te voorspellen. [3] Bovendien nemen de kosten van klinische onderzoeken steeds verder toe. [4]

Door in vroeg-klinisch onderzoek (fase I/II-A) met nieuw te testen analgetica gebruik te maken van biomarkers die (veranderingen in) pijnsignalering kunnen aantonen, is het mogelijk om belangrijke resultaten over (dosisafhankelijke) effecten te generen. Biomarkers kunnen de eerste signalen van effectiviteit van een onderzoeksmiddel detecteren, en kostenbesparend werken door al aspecten van het onderzoeksmiddel te testen die anders in duurder, laat-fase onderzoek (fase II-B/III) nog moeten worden getest. Het werken met biomarkers kent echter ook uitdagingen. Net zoals bij preklinische modellen, staan ook biomarkers in mensen slechts model voor klinisch relevante eindpunten. Daarnaast hebben biomarkers vaak slechts betrekking op een specifiek aspect van een bepaalde (pijn) pathologie. Een andere uitdaging is dat veel nieuwe geneesmiddelen in toenemende mate selectief zijn en op nieuwe doelwitten aangrijpen. De daaruit voortvloeiende effecten in de mens, en/of klinische relevantie van die effecten, zijn dikwijls nog niet duidelijk aangetoond. Eerder gevalideerde methodologie moet daarom opnieuw worden doorgelicht op hun validiteit om Proof-of-Mechanism of Proof-of-Concept (POM, POC) van zulke nieuwe geneesmiddelklassen vast te kunnen stellen. In dit proefschrift definieerden we POM als bewijs dat het analgeticum op de beoogde locatie in het lichaam zijn beoogde farmacologische effect heeft. POC werd als term gebruikt voor aangetoonde analgetische effecten in patiënten, of op experimentele modellen in gezonde vrijwilligers (**Hoofstuk 1**).

Naast het opnieuw beoordelen van de validiteit van huidige modellen, vereist de verhoogde selectiviteit van geneesmiddelen in sommige gevallen verdere verfijning van (humane) experimentele modellen. Dit is nodig om adequaat het aspect van de ziekte of het symptoom na te kunnen bootsen waar het geneesmiddel selectief op zou moeten aangrijpen. Op basis van deze punten werden de studies die in dit proefschrift staan beschreven, uitgevoerd. Deze thesis is daarom tweeledig. Enerzijds beschrijft het een zoektocht naar adequate biomarkers voor het aantonen van de effectiviteit van (selectieve) Na_V-blokkers, – een van de meest geteste analgeticum klassen in vroege fase geneesmiddelonderzoek (**Hoofdstuk 1**). Anderzijds beschrijft het verfijning en verder ontwikkeling van experimentele modellen als biomarkers.

In **Hoofdstuk 1** definieerden we dat een goede (analgetische) biomarker in staat moet zijn om in meerdere onderzoeken een duidelijke, consistente respons van (genees)middelen aan te kunnen tonen. Deze respons moet daarnaast vergelijkbaar zijn voor verschillende (genees)middelen binnen dezelfde klasse. [5] We maakten daarbij gebruik van de PainCart, een gevalideerde nociceptieve testbatterij van het Centre For Human Drug Research (CHDR). [6] Nociceptie is het vermogen om weefselbeschadiging

of dreigende weefselbeschadiging waar te nemen; nociceptoren zijn zenuwuiteinden die signalen van beschadigd weefsel detecteren. De testen die onderdeel zijn van de PainCart induceren elk selectief druk-, elektrische-, hitte of koude pijn. Collectief zijn ze in staat om specifieke (veranderingen in) nociceptie aan te tonen. De PainCart testbatterij werd uitgevoerd in combinatie met het eerder ontwikkelde topicale 1% capsaïcine crème-model (**Hoofdstuk 2,3**) of eerder ontwikkelde ultraviolet (UV) B-model (**Hoofdstuk 4**). Het capsaïcine crème-model maakt gebruik van de irriterende effecten van het actieve ingrediënt van hete pepers: capsaïcine. Met het UVB model werd op een stukje huid op de rug een soort zonnebrand nagebootst en lokale ontsteking geïnduceerd. Veranderingen op pijndrempels bij de koude pijntest (cold pressor test) bleek de meest reproduceerbare biomarker te zijn, door gevoelig te zijn voor analgetische effecten van drie Na_V -blokkers (VX-128, VX-150 en mexiletine). In **Hoofdstuk 1** suggereerden we dat, naast veranderingen op de cold pressor test, beïnvloeding van het capsaïcine-model mogelijk ook een adequate biomarker voor het aantonen van Na_V -blokker effecten zou kunnen zijn. Het capsaïcine crème-model bleek echter niet gevoelig voor de geteste selectieve $Na_V1.8$-blokkers (VX-128 en VX-150; respectievelijk **Hoofdstuk 2**, **3**). Mogelijk is het capsaïcine model beter in staat om effecten van Na_V-blokkers die op andere doelwitten aangrijpen, zoals $Na_V1.7$, aan te tonen. Er kan echter ook worden geconcludeerd dat dit topicale 1% capsaïcine crème-model suboptimaal is voor het bestuderen van analgetica, omdat het ook geen effecten van tramadol of duloxetine kon aantonen. [7] Dit gaf ons reden om het onderzoek uit te voeren wat in **Hoofdstuk 8** beschreven staat.

Een farmacologische biomarker moet behalve consistent ook duidelijk (en indien van toepassing, dosisafhankelijk) reageren op therapeutische dosisniveaus van (genees)middelen. [5] In **Hoofdstuk 4** merkten we op dat Na_V -blokker mexiletine, maar niet lacosamide, pijndrempels bij de cold pressor test significant beïnvloedde. Op basis van de differentiërende kenmerken van mexiletine en lacosamide – respectievelijk preferentiële modulatie van $Na_V1.8$ versus $Na_V1.7$ – concludeerden we dat het bij het selecteren van de juiste biomarker belangrijk is op welk Na_V -subtype het te testen analgeticum preferentieel aangrijpt. Op basis van de resultaten uit **Hoofdstukken 2, 3** en **4** ondersteunt dit proefschrift het gebruik van pijdrempels gemeten met de cold pressor test als biomarker voor $Na_V1.8$-geïnduceerde analgesie. De effecten (of het ontbreken daarvan) van lacosamide op nociceptieve drempels leveren bewijs dat de gebruikte modellen

mogelijk niet optimaal zijn voor het testen van analgetica die preferentieel op $Na_V1.7$ aangrijpen, een conclusie die in lijn is met eerder onderzoek. [7,8] De rol van $Na_V1.7$ in pijnsignalering werd ten tijde van ontdekking in 2006 als een grote doorbraak beschouwd. De potentie van deze doorbraak hebben $Na_V1.7$ -blokkers echter nog niet waargemaakt; ondanks meerdere pogingen zijn er (nog) geen $Na_V1.7$ -selectieve blokkers die geregistreerd hebben kunnen worden. [9] Totdat aangetoond effectieve $Na_V1.7$-blokkers beschikbaar komen om biomarkers mee te valideren, blijft het moeilijk om ondubbelzinnige conclusies te trekken over de toepasbaarheid van momenteel beschikbare biomarkers voor middelen die $Na_V1.7$ als hoofddoelwit hebben. Het zou kunnen zijn dat andere methoden, zoals de 'nerve excitability threshold trackingtest', geschikter zijn, omdat dat model wel POM van lacosamide kon aantonen. [10]

Met de resultaten uit **Hoofstukken 2, 3** en **4** kon ook worden vastgesteld dat er nog voldoende ruimte is voor het verfijnen van biomarkers voor het profileren van (selectieve) Na_V-blokkers in vroege-fase geneesmiddelonderzoek. Zoals in **Hoofdstuk 1** vermeld, moet een geschikte biomarker een op z'n minst plausibele relatie hebben met de farmacologie van de geteste geneesmiddelklasse, en met de pathofysiologie van de ziekte. Een belangrijk aspect bij veel soorten chronische pijn, zoals fibromyalgie en neuropathische pijnsyndromen is centrale sensitisatie; wat gedefinieerd is door de International Association for the Study of Pain (IASP) als *'een verhoogde respons van nociceptoren in het centrale zenuwstelsel op normale afferente input of afferente input die zich net onder de prikkeldrempel bevindt*'. [11,12] Centrale sensitisatie kan zich manifesteren als symptomen zoals hyperalgesie en allodynie. [11] Deze symptomen hebben betrekking op aandoeningen die worden veroorzaakt door overprikkelbaarheid van nociceptoren. Dat is een mechanisme waarop Na_V-blokkerende (genees) middelen – en in het bijzonder selectieve $Na_V1.8$-blokkers – zich richten. [13] Het induceren en moduleren van hyperalgesie bij gezonde vrijwilligers werd zodoende als een mogelijke biomarker voor effectiviteit van Na_V-blokkers geschat, en verder onderzocht in de overige hoofdstukken van deze thesis.

In de context van experimentele klinische studies onderzochten we in **Hoofdstuk 5-8** geschiktheid van enkele methodes om hyperalgesie en/of allodynie te induceren. Een ervan was totale slaapdeprivatie (TSD; **Hoofdstuk 6, 7**). Hierbij werden proefpersonen minimaal 24 uur wakker gehouden en het effect van TSD op nociceptie bepaald. Een ander model

wat onderzocht werd, was topicale toediening van 1% capsaïcine op alcoholbasis (ethanolische oplossing aangebracht op de onderarm). Dit model was een aanpassing van de formulering van het capsaïcine crème-model gebruikt in **Hoofdstukken 2** en **3**. Met de aangepaste formulering beoogden we een uitgesprokener effect te induceren. TSD induceerde geslachtsafhankelijke hyperalgesie op koude-, hitte- en drukpijn (**Hoofdstuk 6**). Ook bracht TSD veranderingen teweeg in de geconditioneerde pijnmodulatierespons (**Hoofdstuk 6**) en nociceptieve verwerking (**Hoofdstuk 7**). Het topicale 1% capsaïcine in ethanolische oplossing-model verhoogde bij gezonde mannen de gevoeligheid voor hitte, en induceerde allodynie in het aangrenzende gebied waar de capsaicine was aangebracht ('secundaire allodynie', **Hoofdstuk 8**). De resultaten bevestigden dat beide modellen van nut kunnen zijn in vroeg-fase geneesmiddelonderzoek met gezonde proefpersonen. Wel zijn vervolgonderzoeken met farmacologische interventies nodig om te kunnen beoordelen of beide modellen ook duidelijke (dosisafhankelijke) effecten van (genees)middelen kunnen aantonen, en voor welke klasse zij een adequate biomarker kunnen zijn.

$Na_V1.7$ en $Na_V1.8$ spelen een rol bij ontstekingspijn door modulatie van kinasen zoals PKA (protein kinase) en P38 MAPK (mitogen-activated protein kinase). [14] Verschillende ontstekingscellen en andersoortige mediatoren (bijvoorbeeld macrofagen, neutrofielen en mestcellen) gaan na een verwonding of ontsteking naar het aangetaste weefsel. Dit verhoogt vervolgens de aanwezigheid van kinases zoals P38 MAPK. $Na_V1.77$ en $Na_V1.8$, die toenemen in nociceptoren wanneer het geïnnerveerde weefsel wordt aangetast, worden gefosforyleerd en gemoduleerd, wat uiteindelijk tot hyperalgesie en allodynie kan leiden. [14] Het humane endotoxemiemodel (dat wil zeggen, systemische toediening van lipopolysaccharide (LPS)) kan in klinische studies worden gebruikt om een systemische ontsteking na te bootsen en P38 MAPK-signalering te induceren. [15] LPS zijn moleculen die voorkomen in het buitenmembraan van gramnegatieve bacteriën. In **Hoofdstuk 5** onderzochten we of systemische toediening van LPS zich zou kunnen vertalen in een bruikbaar inflammatoir hyperalgesiemodel. Met de doses die wij testten was LPS niet in staat om duidelijke, consistente en dosisafhankelijke effecten op te wekken. We concludeerden daarom dat het model niet bruikbaar is om effecten van analgetica, in de context van vroeg-fase geneesmiddelonderzoek, mee aan te tonen.

In dit proefschrift hebben we getracht enkele problemen uit te lichten en aan te pakken, waarmee farmaceutische en biotechnologische bedrijven in vroeg-fase geneesmiddelen onderzoek dikwijls worden geconfronteerd wanneer zij analgetica ontwikkelen. Zo hebben we gekeken naar de toepasbaarheid van beschikbare modellen voor de top-10 meest geteste analgetica in vroeg-fase ontwikkeling (**Hoofdstuk 1**), hebben we enkele van de voorgestelde modellen uit **Hoofdstuk 1** gebruikt om geregistreerde analgetica en analgetica in ontwikkeling te profileren (**Hoofdstuk 2-4**); en hebben we onderzocht of andere methodologieën mogelijk ook bruikbaar zijn voor het aantonen van anti-hyperalgetische effecten van analgetica in gezonde vrijwilligers (**Hoofdstuk 5-8**). Alhoewel enkele van de geteste modellen bruikbaar lijken, moet worden vermeld dat, met uitzondering van de studie met het TSD model, alleen mannen in de studies hebben meegedaan. Daarnaast bootsten de modellen slechts één of enkele aspecten van pijn na, terwijl pijn juist een complex en multifactorieel symptoom is. Zo werden psychologische en psychosociale factoren die bijdragen aan de chronificatie van pijn buiten beschouwing gelaten. Meestal omdat het (nog) niet mogelijk en/of onethisch is om zulke aspecten in onderzoeks-context te testen (denk bijvoorbeeld aan gezonde proefpersonen bloot te moeten stellen aan onomkeerbare of langdurige pijn), maar ook vanwege de exploratieve insteek van deze onderzoeken. Nieuwe technologieën, waaronder augmented/virtual reality (AR/VR), kunnen hier echter een rol gaan spelen. Zo kan VR-simulatie bijvoorbeeld een affectieve component aan de pijnprikkel toe te voegen waardoor de pijnbeleving intenser wordt.

In een industrie met lange ontwikkeltijden zoals de farmaceutische en biotechnologische sector, is het verbeteren van methodologie essentieel bij het minimaliseren van de tijd die nodig is om een product op de markt te brengen. Onlangs heeft het Europees Geneesmiddelenbureau (de EMA) een richtsnoer gepubliceerd om ontwikkelaars te helpen navigeren door de belangrijkste wettelijke vereisten behorend bij het klinische ontwikkelingstraject van medische producten voor geavanceerde therapieën (advanced therapy medical products; ATMPs). Het richtsnoer stelt dat belangrijke openstaande vragen over het therapeutisch potentieel van het te testen geneesmiddel vroegtijdig in het ontwikkeltraject moeten worden beantwoord. [16] Hoewel de meeste analgetica niet als ATMPs classificeren, zouden dezelfde richtlijnen moeten gelden.

Geneesmiddelontwikkelaars en klinische onderzoekers die in het vroegfase deel van het ontwikkeltraject betrokken zijn, wordt geadviseerd om klinische studies zo te ontwerpen dat POM en/of POC al kan worden bestudeerd in gezonde vrijwilligers en/of goedgekozen patiënt(sub)populaties. Er kan een selectie in deze opties worden gemaakt, maar het heeft nooit de voorkeur om belangrijke vragen over het therapeutisch potentieel tot laat in het traject onbeantwoord te laten. De resultaten beschreven in deze thesis bieden de mogelijkheid om het ontwikkelproces van analgetica te ondersteunen en zo pijngeneesmiddelonderzoek te verbeteren, zodat therapieën met verbeterde klinische werkzaamheid versneld bij de pijnpatiënten in nood kunnen komen.

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CURRICULUM VITAE

Hemme Jacob Hijma was born in Leiden, The Netherlands on January 27th, 1992. After completing secondary school at the *Stedelijk Gymnasium* in Leiden in 2010, he subsequently moved to Amsterdam and enrolled in the bachelor's (BSc) program in Biomedical Sciences at the *Vrije Universiteit* (VU), with a focus on biomedical interventions in the latter part of that study. Next, he enrolled in the master's program (MSc) Science and Business Management at *Utrecht University*, which comprises science-focussed topics in the first year and business management-related ones in the second. During his master's, Hemme completed internships investigating genetics at the *Regenerative Medicine Center Utrecht*; and opportunities for innovation in clinical trials at *Janssen, pharmaceutical companies of Johnson & Johnson*. He obtained his MSc degree in 2017 and further pursued his interest in drug development by starting working as a Clinical Scientist in the *Neurology and Pain* department at CHDR. Under the supervision of Prof. dr. G.J. Groeneveld and Prof. dr. J. Burggraaf, he also performed the research described in this thesis during that period. Since 2021, Hemme works as an Experienced Clinical Scientist at CHDR supervising Clinical Scientists. He has completed his training to become a board-certified clinical pharmacologist in 2022. Hemme lives together with his partner France in Leiden.

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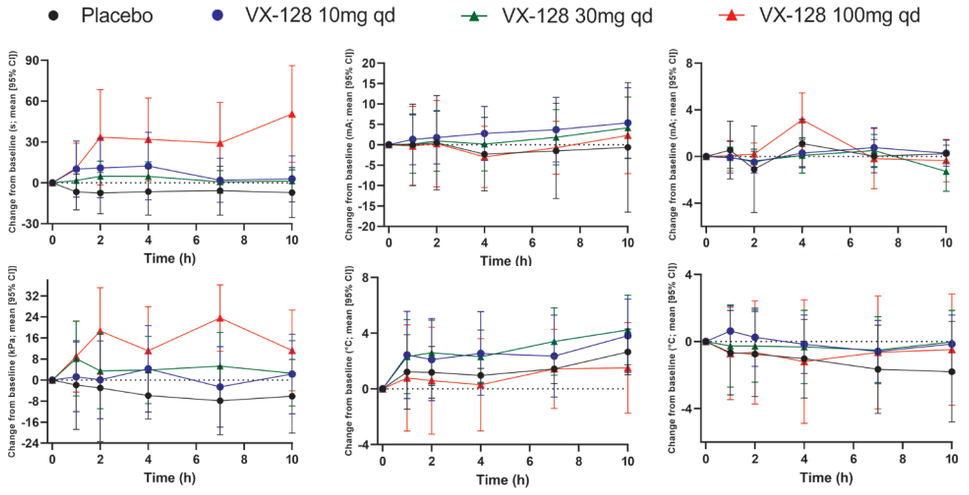
Selection of evoked pain test results – change from baseline. A. Mean (95% CI) Cold Pressor Pain Test results: Pain Tolerance Threshold on Day 1; B. Mean (95% CI) Electrical Stimulation pain test: Pain Tolerance Threshold on Day 1; C. Mean (95% CI) Conditioned pain modulation: Pain Tolerance Threshold on Day 1; D. Mean (95% CI) Pressure Pain test: Pain Tolerance Threshold on Day 1; E. Mean (95% CI) Capsaicin-induced pain test: Pain Detection Threshold on Day 1; F. Mean (95% CI) Thermal pain test (on control/ untreated skin): Pain Detection Threshold on Day 1 Effects of placebo (n=5), VX-128 10 mg QD (n=10), VX-128 30 mg QD (n=10) and VX-128 100 mg QD (n=10) on selected evoked pain test endpoints determined on Day 1 of study part B. Descriptive statistical analysis was performed; data are represented as means with 95% CI. Effects of VX-128 were noted for cold pressor PTT at the highest tested dose (100 mg QD) and suggestive dose-dependent effects of VX-128 for pressure pain PTT.



A. Cold Pressor PTT; B. Electrical Stimulation PTT; C. Conditioned Pain Modulation PTT; D. Pressure PTT; E. Capsaicin-induced PDT; F: Thermal PDT (on control/untreated skin). Abbreviations: °C: degrees Celsius, CI: confidence interval, h: hour(s), kPa: kilopascal, mA: milliamperes, n= sample size, PDT: pain detection threshold, PTT: pain tolerance threshold, s: seconds, SD: standard deviation.