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The effect of gonadal hormones on the sensation of pain: Quantitative sensory testing in women

Kent Stening



Department of Clinical and Experimental Medicine, Division of Cell Biology, Faculty of Health Sciences, Linköping University, 581 85 Linköping, Sweden

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Patience is passion tamed (L. Abbott)

ORIGINAL PAPERS

The present thesis is based on the following papers referred to in the text by their Roman numerals:

- **I. Stening, K.**, Hammar, M., Berg, G., Blomqvist, A. Pain thresholds and pain tolerance during the ovulatory cycle in healthy women: Quantitative sensory testing. *Manuscript*
- II. Stening, K., Eriksson, O., Wahren, L-K., Berg, G., Hammar, M., Blomqvist, A. (2007) Pain sensations to the cold pressor test in normally menstruating women: comparison with men and relation to menstrual phase and serum sex steroid levels. *American Journal of Physiology, Regulative, Integrated, Comparative Physiology* 293, 1711-1716.
- **III. Stening, K.D.**, Berg, G., Hammar, M., Eriksson, O., Amandusson, Å., Blomqvist, A. Influence of estrogen levels on thermal perception, pain thresholds and pain tolerance: Studies on women undergoing *in vitro* fertilization. *Submitted*
- **IV. Stening, K.D.**, Eriksson, O., Henriksson, K.G., Brynhildsen, J., Lindh-Åstrand, L., Berg, G., Hammar, M., Amandusson, Å., Blomqvist, A. (2011) Hormonal replacement therapy does not affect self-estimated pain or experimental pain responses in postmenopausal women suffering from fibromyalgia: A double-blind, randomized, placebo-controlled trial. *Rheumatology* 50:3, 544-551.

CONTENTS

ABSTRACT	7
SAMMANFATTNING PÅ SVENSKA (Summary in Swedish)	9
INTRODUCTION	13
Definition of pain	13
Distinctions between Sex and Gender and its connection to pain	14
Pain, from receptors to cortex	15
Inhibitory system	18
Pain thresholds and pain tolerance	19
How to measure pain	20
Implications of quantitative sensory tests	21
Psychological implications of experimentally induced pain	23
Sex differentiation, the gonadal hormones and their receptors	24
Hormones throughout the life span	27
The gonadal hormones and pain	29
AIMS	33
METHODS	35
Quantitative sensory tests	35
Subjects	36
Statistics	38

Ethical approval	39
RESULTS AND COMMENTS	41
Paper I	41
Paper II	42
Paper III	43
Paper IV	44
DISCUSSION	47
Hormonal influence on the sensation of pain	47
Methodological remarks	50
The use quantitative sensory tests	50
Test re-test phenomenon due to repeated session design	53
To study hormonal influence	54
About the terms sex and gender	59
Perspective	60
CONCLUSIONS	61
ACKNOWLEDGEMENTS	63
REFERENCES	67

ABSTRACT

Accumulating evidence points to sex differences in pain sensitivity and many chronic pain conditions preferentially affect women. Sex hormones, and in particular estrogens, have been shown to affect pain processing and pain sensitivity in animals, although the findings are divergent. The aim of the research on which this thesis is based was to examine the effect of gonadal hormones on the sensation of pain in women who either presented normal variations in hormonal levels over time or who had been given hormone treatment.

Different quantitative sensory tests (QST) examining temperature thresholds, cold, heat, and pressure pain thresholds, as well as tolerance thresholds for heat and cold, were performed during different hormonal conditions: During hormonal fluctuations throughout the ovulatory cycle (Papers I and II); in women undergoing in vitro fertilization (IVF), a treatment associated with extremely low and high 17β -estradiol levels (Paper III); and before and after hormonal substitution treatment in postmenopausal women suffering from fibromyalgia (Paper IV).

The results showed little changes in pain sensitivity during the ovulatory cycle, with an interaction between 17β -estradiol and progesterone on cold pressor pain as the major finding. No significant changes in pain sensitivity were seen even with the extreme variations in 17β -estradiol levels that occurred during the IVF-treatment. Also, the use of hormonal substitution treatment did not affect pain thresholds or tolerance in postmenopausal women suffering from fibromyalgia.

Session-to-session effects were reported in several studies and seem to be an important factor when using repeated sessions design. Additionally, the present work also emphasizes the use of actual hormonal levels instead of tentative calendar methods when evaluating hormonal effects on the sensation of pain during the menstrual cycle.

The present studies thus indicate that changes in gonadal hormone levels have little effect on experimental pain in women, contrary to what has been reported in animal studies.

SAMMANFATTNING PÅ SVENSKA (Summary in Swedish)

Bakgrund

Smärta är en sensorisk och emotionell upplevelse som de flesta individer på något sätt har erfarenhet av. Könsskillnader gällande smärta är sedan länge beskrivna och påvisade såväl experimentellt som kliniskt, både i djur- och humanstudier. En överrepresentation av kvinnor ses för många kroniska smärttillstånd, såsom fibromyalgi och andra oklara smärttillstånd. En möjlig koppling mellan smärta och kön kan finnas på hormonell nivå. Könshormonerna östradiol, progesteron och testosteron har alla receptorer belägna i centrala nervsystemet. Det är viktigt att påpeka att dessa tre hormoner förekommer hos bägge könen men skiljer sig åt i mängd, relation samt utsöndringsförlopp. Under den fertila kvinnans menstruationscykel varierar halterna av östradiol och progesteron, medan män verkar ha en mer jämn nivå, av framför allt testosteron. I djurförsök har en direkt koppling mellan kroppens smärtsystem och östradiol påvisats i ryggmärgens bakhorn, den del av nervsystemet som mottar den inkommande smärtsignalen. Här finns omkopplingsceller vilka kan aktiveras och via kroppseget morfin, så kallat enkefalin, hämma den inkommande smärtsignalen innan den kommer upp till hjärnan. I djurförsök har det visats att dessa celler även uttrycker östrogenreceptorer och att dessa receptorer, efter att ha aktiverats av östrogen, kan sätta igång produktionen av enkefalin.

Material & metoder

Med hjälp av olika stimuleringsmetoder har smärttrösklar för värme, kyla och tryck, toleranströsklar för värme och kyla, och temperaturdetektionsförmågan undersökts hos fyra olika studiegrupper hos vilka den hormonella miljön kontrollerats via blodprov. Den första delstudien genomfördes på 14 kvinnor i fertil ålder utan hormonbehandling. Mätningarna utfördes under två hormonellt olika faser under tre menstruationscykler, och faserna säkerställdes med blodprov för bestämning av hormonnivåerna. Den andra

delstudien genomfördes på 16 kvinnor i fertil ålder samt på en kontrollgrupp bestående av tio män. Studien var en toleransstudie där smärtstimulering genomfördes via ett s.k. köldpressortest (behållare med isvatten i vilket försökspersonen placerar sin hand så lång tid som möjligt). Fyra smärtstimuleringar utfördes, var sjunde dag under en standardiserad menstruationscykel (28 dagar). Varje stimulering avslutades med blodprovstagning och analys av aktuell östradiol- och progesteronnivå. I den tredje delstudien undersöktes 16 kvinnor som skulle genomgå provrörsbefruktning. Som ett led i denna behandling moduleras kvinnornas hormonnivåer till ytterlighetsvärden. Den första sensoriska testningen ägde rum när kvinnorna inledningsvis var under hormonell nedreglering och uppvisade mycket låga östradiolnivåer, och den andra testningen ägde rum när kvinnorna var under hormonell uppreglering och uppvisade mycket höga östradiolnivåer. Som metodologisk kontroll rekryterades två grupper, en bestående av män (n = 10) och en bestående av kvinnor (n = 9) som stod på så kallade monofasiska p-piller. Den fjärde och sista delstudien genomfördes på 29 postmenopausala kvinnor med fibromyalgidiagnos. Kvinnorna blev randomiserade till behandling med antingen östrogenplåster eller placebo (plåster utan aktiv substans) under åtta veckor. Sensoriska testningar genomfördes före och efter dessa åtta veckors behandling samt vid en avslutande mätning 20 veckor efter utsättandet av behandlingen.

Resultat

Resultatet från delstudie I påvisade en variation i hormonnivåerna under lutealfasen mellan de olika cyklerna vilket belyser vikten av att bestämma menstruationsfasen genom att mäta hormonnivån och inte bara lita till att räkna dagar efter menstruation. Materialet analyserades initialt utifrån kalendermetoden, utan hänsyn till hormonnivåer och därefter gjordes en re-analys efter det att data från hormonellt atypiska menstruationscykler hade uteslutits. En analys baserad på en subgruppering av menstruationsfaserna baserat på uppmätta hormonvärden gjordes också. Resultatmässigt sågs inget samband mellan olika hormonnivåer vare sig på

smärttrösklar eller på smärttolerans. Delstudie II påvisade däremot en signifikant sänkning av smärttröskeln i lutealfasen jämfört med den senfollikulära fasen och vidare påvisades en interaktion mellan östradiol och progesteron, i det att stigande östradiolhalter minskade den ökande smärtkänslighet som var associerad med ökande progesteronnivåer. Via studiens kontrollgrupp kunde även inverkan av inlärning p.g.a. repetitiva mätningar påvisas. I delstudie III påvisades endast förändringar i temperaturkänsligheten samt i köldsmärttrösklar trots extrema skillnader i östradiolnivåerna. Köldsmärttröskeln ändrades även i kontrollgrupperna vilket tyder på att förändringen var en inlärningseffekt. Delstudie IV påvisade som förväntat signifikanta förändringar av den hormonella nivån hos de som behandlades med östrogensubstitution. Det fanns däremot ingen skillnad mellan östrogenbehandling och placebo avseende smärtkänsligheten eller smärtupplevelsen.

Konklusion

Förändringar i könshormonnivåerna resulterade i tre av de fyra delstudierna inte i några mätbara förändringar i smärtkänsligheten. Den enda skillnad som sågs var vid tonisk smärta, där i en delstudie progesteronnivån samvarierade med smärtkänsligheten, ett samband som upphävdes vid stigande östradiolnivåer.

INTRODUCTION

Pain is an important sensation and serves primarily as a protection for the body (Woolf, 2010). However, for some, pain not only serves as a protection, but is experienced day out and day in, without any obvious physical correlates, with severe consequences for health and quality of life. Accumulating evidence points to gender differences in experiencing pain. Thus, differences in pain thresholds, tolerance levels and prevalence of clinical pain conditions are described, with women being more sensitive and more vulnerable to many chronic pain conditions than men (Robinson et al., 1998). One plausible explanation of part of the differences in pain responses between men and women may be the hormonal milieu. The present work focuses on how the gonadal hormones 17β-estradiol and progesterone may influence the sensation of pain.

Definition of pain

Everyone expresses, in one way or another, the experience of pain. The definition formulated by the International Association for the Study of Pain, "An unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage" (IASP, 1994), points out the subjectivity of pain and that pain is a complex sensation. Thus, pain does not need to be directly associated with tissue damage or specific illness. Clinical pain may be defined according to several factors such as its aetiology, whether or not it is nociceptive or neurogenic, and furthermore to its time duration, whether it is acute or long lasting. Another important distinction with regard to pain, especially in experimental settings, is the distinction between pain and nociception (Aydede and Guzeldere, 2002, Arendt-Nielsen and Yarnitsky, 2009). Pain is the overall experience consisting of both sensory and affective/emotional experiences, while nociception is activation in the nociceptive system, a measurable event that may or may not result in a pain sensation (Loeser and Treede, 2008).

Distinctions between Sex and Gender and its connection to pain

Gonadal hormones may influence pain and result in differences with regard to both clinical and experimental pain between women and men (Fillingim et al., 1999). However, some distinctions are needed. The concepts sex and gender are often used inexactly in the literature (Kim and Nafziger, 2000). Sex is the biological difference between men and women and refers to genetic, hormonal and reproductive differences. while gender refers to characteristics distinguishing women and men. The major aim of the present thesis was to investigate the hormonal influence on pain which in turn may explain the differences between the two sexes. However, this is controversial and explanations of observed differences with regard to pain thresholds and tolerance levels range from purely biological to gender expectations, such as the stereotypical picture that men should be more heroic especially with a woman present in the room, the latter being connected to a presumed in-learned behaviour formed during childhood by the expectations of being a man or women, respectively. However, studies on infants that have not been exposed to gender role expectations have shown differences between the sexes in response to pain, which points to the possibility that innate differences do exist (Guinsburg et al., 2000, Fuller, 2002, Bartocci et al., 2006).

Prevalence studies show women to be more vulnerable to different pain conditions, as mentioned (Mantyselka et al., 2001, Hasselström et al., 2002), and several reviews of the subject support this view (Unruh, 1996, Berkley, 1997, Fillingim et al., 2009, Paller et al., 2009). The list of pain conditions in which women are overrepresented is long and includes fibromyalgia, migraine, tension headache, trigeminal neuralgia, rheumatoid arthritis, carpal tunnel syndrome, and temporomandibular disorders. However, the question why the observed differences exist is not straightforward and full understanding of the condition's aetiology is still missing, although a hormonal influence may be a contributing factor.

This Introduction provides background information on how nociceptive stimuli are transmitted in the body, as well as a discussion of measuring pain as an overall phenomenon. Furthermore, to obtain an understanding of the sex differences, an

overview of the hormonal influence on the differentiation process in men and woman is provided. This is followed by a description of how the hormones act during the reproductive life and finally how hormones may influence the sensation of pain.

Pain, from receptors to cortex

The "normal" pain transmission starts when specific receptors become activated. The receptors for pain, the nociceptors, are described as free nerve endings in the tissue and, when the stimulus excites the receptor, it results in an action potential in the axon that expresses the receptor. The nociceptors are classified into three groups, which respond to their adequate stimuli that may be chemical, mechanical, or thermal. The chemically-responsive receptors are excited by, e.g., bradykinin, serotonin, histamine and acids, whereas the mechanically-responsive receptors react to mechanical stretch. The thermally-responsive receptors, finally, belong to the transient receptor potential (TRP) ion channels and react to warmth and cold, with overlapping thresholds (McKemy, 2005, Reid, 2005). A receptor for cold, TRPM8, is activated at around 30°C down to about 8°C, where it reaches a plateau, hence encompassing both the innocuous and noxious range. At around 17°C, corresponding approximately to the cold pain threshold, a second cold receptor, TRPA1, is activated (McKemy, 2005, Reid, 2005, Foulkes and Wood, 2007, Jones, 2008, Schepers and Ringkamp, 2009). The receptors for warmth, TRPV3 and TRPV4, show a threshold at around 30°C and are activated up to 50°C (Schepers and Ringkamp, 2009). The nociceptors for heat pain, TRPV1 and TRPV2, in turn, have a threshold at around 43°C and 52°C, respectively, with some variation depending on stimulus site (Dyck et al., 1993, Schepers and Ringkamp, 2009).

It seems that thermoreceptors and nociceptors interact to transmit the adequate sensation, because lack of normal thermal sensitivity results in a painful state of pricking pain without thermal quality after stimulation with both noxious heat and cold (Defrin et al., 2002). Moreover, depending on what type of stimulus present, major nociceptors may be activated by more than one stimulus and act polymodally

(Campero et al., 1996, Perl, 2007). The response to the stimuli depends on many factors, such as amplitude and frequency, as in other sensory systems. However, nociceptors differ from other sensory receptors, in that they have high threshold and react first when the stimulus may damage the tissue.

Nociceptive signals are transmitted through two kinds of nerve fibers in the periphery (Dubin and Patapoutian, 2010, Plaghki et al., 2010), Aδ-fibers and C-fibers, to the dorsal horn of the spinal cord. The A δ -fibers are thinly myelinated fibers, with a conduction velocity of 5-30 m/s. Aδ-mediated pain is usually described as sharp, fast or acute pain. The A δ -fibers can further be subdivided in type I and type II due to their activation threshold (Treede et al., 1998). Aδ-fibers have also been shown to transmit the sensation of cold as well as noxious cold (Simone and Kajander, 1997). The Cfibers have a much slower conduction velocity, between 0.5-2 m/s, and elicit the slow or duller type of pain sensation. They are also associated with heat transmission. Also, the C-fibers can to be divided in different groups (Caterina and Julius, 1999), one being neuropeptide producing C-fibers (substance P and calcitonin gene related peptide), and another that is non-peptidergic, and that has been suggested to be involved in pain related to nerve injuries (Stucky and Lewin, 1999, Stucky et al., 2001). However, some C-fibers seem to transmit the sensation of cold in the noxious range (Campero et al., 2009, Campero and Bostock, 2010), and in a bidirectional manner respond both to noxious heat and cold (Cavanaugh et al., 2009).

The pain fibers enter the spinal cord and terminate in several different areas, the so-called Rexed's laminae. Aδ-fibers terminate preferentially in laminae I and V, and C-fibers terminate mainly in laminae I and II (Han et al., 1998, Perl, 1998). In the dorsal horn there are different types of target cells, namely nociceptive-specific cells (NS), thermoreceptive-specific cells (for cold) and polymodal nociceptive cells (HPC) (Craig and Kniffki, 1985, Willis and Westlund, 1997a, D'Mello and Dickenson, 2008). There are also so called WDR (Wide Dynamic Range) neurons that are excited by both noxious and innocuous stimulation, as well as by limb movements (Dubner et al., 1989, Craig, 2004).

The primary afferents make contact through the transmitter substances glutamate and substance P. When the input signal reaches the pre-synaptic side, glutamate and substance P are released in the synaptic cleft and activate receptors on the postsynaptic neuron. Glutamate activates a triad of receptors, both ionotropic and metabotropic types (Zeilhofer, 2005). The AMPA receptor (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) and the NMDA receptor (N-methyl-D-aspartate) are the two major ion-channel receptors and the third major receptor type is metabotropic second messenger linked glutamate receptors. Substance P in turn, activates the NK1 receptor (neurokinin-1) (Mantyh, 2002). The above organization may play a role in pain phenomena, such as central sensitization and allodynia, which are believed to be a change in the sensitivity of neurons in the dorsal horn that is elicited by second messenger systems.

In the dorsal horn, the afferent neurons connect both to local interneurons and ascending pathways (Basbaum et al., 2009). The interneurons may inhibit or attenuate the input signal as described below. There are several ascending pathways of which the most important are the spinothalamic, the spinoreticular and the spinomesencephalic tracts (Willis and Westlund, 1997b, Craig and Dostrovsky, 2001, Willis Jr, 2007). The spinothalamic tract originates in laminae I, V, VII, and VIII and terminates in the thalamus. The spinoreticular tract originates in laminae VII and VIII and terminates in the reticular formation of the brain stem. Finally, the spinomesencephalic tract originates in laminae I and V and terminates in the parabrachial nucleus, including its pontine part, the mesencephalic tectum, and the periaqueductal gray matter (PAG) (Wiberg et al., 1987, Mason, 2005, Brooks and Tracey, 2005).

The ascending pathways, mentioned above, cross the midline directly, transmitting the nociceptive signal contralaterally in the spinal cord, contrary to the ascending pathway for touch, which instead projects ipsilaterally, in the so-called dorsal columns. The thalamus is a major target for the afferent nociceptive input, which involves several different thalamic nuclei. These include the posterior portion of the ventromedial nucleus, the ventrocaudal part of the mediodorsal nucleus, as well as ventral posterior

inferior nucleus, and the ventral posterior nucleus (Craig et al., 1994). These nuclei, in turn, relay the nociceptive information to cortical areas involved in pain processing, giving rise to the integrated sensation of pain (Dostrovsky, 2000, Craig, 2002). Major areas involved in pain processing are the posterior part of the insular cortex, being the primary sensory cortex for pain as well as temperature perception; the anterior cingulate cortex, involved in the affective component of pain; and area 3a of the primary somatosensory cortex (Craig, 2002). Also limbic structures, such as the amygdala, and the prefrontal cortex are activated by nociceptive stimuli, as well as the basal ganglia and other structures belonging to motor systems, such as the cerebellum. As mentioned above, nociceptive stimuli are also transmitted to several other regions than the thalamus. Some terminate in the brain stem (Price, 2002), in which areas for arousal and autonomic regulation are located and a further integration to hypothalamic structures has been shown (Petrovic et al., 2004).

When the sensation reaches central parts of the nervous system, integration takes place and one important function requiring integration is the pain inhibitory system, a descending system from cortex down to the spinal cord (Petrovic et al., 2002a, Petrovic et al., 2002b).

Inhibitory system

The descending pain inhibitory system holds several levels at which the nociceptive transmission can be modulated. When the nociceptive signal passes from the primarily afferents (A- or C-fibers or both) onto neurons in the spinal cord, it may be inhibited or attenuated both by local and central mechanisms (Ossipov et al., 2010). The local mechanisms are mediated by local circuit neurons that release GABA and/or endogenous opioid peptides, such as enkephalins. As will be presented later in this Introduction both GABA and enkephalins have the ability to be regulated by gonadal hormones. The local circuit neurons are connected to descending pathways that originate in central neural structures such as the periaqueductal gray matter (PAG) and the nucleus raphe magnus. The PAG contains receptors for endogenous opioids.

Ligand binding to the receptors activates the descending inhibitory pathways, which terminate in the dorsal horn of the spinal cord. However, non-opioidergic descending pain modulating pathways also exist, in which serotonin and noradrenaline act as transmitters (Zimmermann, 2004). The descending neurons may then inhibit the incoming nociceptive signal by activating the local inhibitory interneurons or by a direct contact to either the primary afferents or their target neurons (Fields et al., 2005, Benarroch, 2008, Ossipov et al., 2010).

Pain thresholds and pain tolerance

When measuring sensory functions it is important to know whether the threshold or the tolerance level is being examined. O'Driscoll and Jayson (1982) define sensation threshold as "the lowest stimulus at which a sensation is first reported", and pain threshold as "the level of stimulus which will give rise to the first barely perceived pain in an instructed subject under given conditions of noxious stimulation", and finally pain tolerance as "that level of noxious stimulation which can just be tolerated". They also present a couple of important items about the choice of method for pain threshold measurement. In brief, the stimulus must be quantifiable and reproducible, there must be an adequate range between threshold and maximal stimulation, the latter must not produce tissue damage, and finally, the apparatus and used technique should be simple and safe to use. The history of trying to quantify and measure sensory stimuli in "modern time" may start with Magnus Blix and Max von Frey in the late 1800s (Norrsell et al., 1999). By using small hair fibers and needles, von Frey demonstrated the presence of "Schmerzpunkte" in the skin (Pearce, 2005), work which generated the specificity theory built on labelled lines. In contradiction to the labelled line theory, the pattern theory was introduced several years later (Ma, 2010), emphasizing pain as an integrated event (Nathan, 1976). Other pioneers who tried to quantify the sensation of pain were Wolf and Hardy (Hardy and Du Bois, 1940, Hardy et al., 1940), who worked with and developed experimental set-ups, such as the cold pressor test (Wolf and Hardy, 1941, Hardy, 1956), a method used in the present work. The cold pressor test was at the beginning developed to study vasomotor reactions, and can be described in brief as a bucket filled with ice chilled water in which the participant immerses the hand down to the wrist.

Another non-invasive method, used in present work, is the Thermotest, which was developed by Fruhstorfer and Lindblom (Fruhstorfer et al., 1976). It consists of a peltier-based contact thermode that is applied to the skin surface and that generates heat and cold. It is probably, together with pressure algometry, the most often used quantitative sensory test.

How to measure pain

Pain can be measured in different ways. One is by quantification of thresholds and tolerance, as mentioned above. Alternative methods involve the use of different rating scales, such as Visual Analogue Scales (VAS) or numeric scales, or descriptive methods such as pain maps, using describing words and symbols. As for the measurement of thresholds and tolerance, quantitative sensory tests may be performed using different algorithms, such as the *method of limits*, and the *methods of levels* or forced-choice algorithm (Yarnitsky, 1997, Shy et al., 2003). When applying the method of limits, as was used in the present work, the stimuli start on a neutral level and increase until the subject stops it. When using method of levels, the stimulus is presented stepwise and altered according to the response of the subject. For both methods, as well as for all psychophysical methods, repeatability problems seem to exist. The method of limits has shown repeatability problems in several studies (Yarnitsky and Sprecher, 1994). These problems may be related to a learning effect after repeated tests. However, contrary results exist showing the outcome to be stable over time (Wasner and Brock, 2008, Heldestad et al., 2010). An advantage over the method of levels is that the method of limits is easy to perform for the participants and also less time consuming. Claus et al. (1990) showed that forced-choice algorithm required up to six times longer time than the method of limits. For measuring pain thresholds, Dyck et al. (1996) developed an alternative algorithm to the use of method of limits called nonrepeating ascending algorithm, an alternative for overcoming the reaction time bias included in methods of limits. This alternative algorithm is similar in its set-up to the methods of levels and forced-choice algorithm, thereby sharing the same disadvantages.

The use of different rating scales is common in both experimental studies and clinical situations. The 100 mm visual analogue scale marked no pain at one end and worst possible pain at the other end, as well as different verbal and/or numeric scales (Langley and Sheppeard, 1985, Ho et al., 1996, Williamson and Hoggart, 2005), are examples of ratings of pain intensity. In the present study, VAS was used in all papers in different ways. Some problems with VAS are that participants may find it difficult to use a scale with which they need to imagine a feeling they never may have felt, the worst possible pain. Another problem involves the tendency to rate the discomfort instead of the pain intensity. Additionally, it has long been discussed if the scale generates data in ratio or ordinal level (Myles et al., 1999); this aspect is important for the forthcoming choice of statistical method. In this thesis the VAS is regarded as a ratio and therefore parametric analysis was applied. However, the VAS measures just the intensity. A qualitative description of the sensation of pain requires other methods such as pain maps connected to descriptive terms. In the present study a modified pain map was used (Paper IV), describing painful areas of the body (Staud et al., 2004).

Implications of quantitative sensory tests

As mentioned above, the *method of limits* was used in the present work and this algorithm is probably the most commonly used one when measuring pain thresholds (Dotson, 1997, Chong and Cros, 2004, Hansson et al., 2007). When using a thermode in contact with the skin, the C-fiber-mediated warmth and the A δ -fiber-mediated cold sensations are usually experienced at 1-2°C from the normal skin temperature. For the mainly C-fiber-mediated heat induced pain, the threshold is found at around 45°C, while the threshold for the mixed C- and A δ -fiber cold pain threshold is found somewhere around 10-15°C. All psychophysical methods are in one way or another

dependent upon the participating subject's attention, especially the *method of limits* in which the reaction time is essential, with the risk that thresholds are exceeded (Yarnitsky and Ochoa, 1991, Yarnitsky and Sprecher, 1994). By regulating the slope of temperature change to a lower rate ($^{\circ}$ C as standard) this artifact can be minimized (Pertovaara and Kojo, 1985, Hilz et al., 1999, Shy et al., 2003, Palmer and Martin, 2005). However, studies indicate that activation of nociceptors may depend on the stimulus rate, with lower rates mainly activating the C-fibers, whereas higher rates also activate A δ -fibers (Yeomans and Proudfit, 1996).

Other important factors to be considered when using a contact thermode are: (i) the skin temperature; (ii) the choice of stimulation site; and (iii) the contact pressure. A common used baseline temperature is 32°C, to which the skin initially adapts to for a few seconds (Dotson, 1997, Yarnitsky, 1997). The choice of site plays a role (Meh and Denislic, 1994) with less inter-individual variation when the thenar region is stimulated (Hagander et al., 2000). The application of the thermode to the skin surface is then performed with manual pressure, as the manufacturer advises (Somedic), for safety reasons. This may induce a small alteration in adaption pressure to the skin. However, previous studies examined the influence of the adapting pressure and found that it was of little significance (Pavlaković et al., 2008).

Site dependent variations as well as gender differences and intra-individual variations have also been found for other non-invasive methods, such pressure algometry (Buchanan and Midgley, 1987, Fischer, 1987, Rolke et al., 2005). Pressure algometry has, at the same time, been shown to be constant over a long time period in healthy persons (Isselée et al., 2001) and also to present good reproducibility between opposite body areas (Fischer, 1987). Some variations occur with the force rate of the pressure application to the tissue, as reviewed by Jensen et al., 1986), with higher values obtained with increasing application rates. As for thermal painful perception, pressure pain is transmitted by both C- and A δ -fibers (Andrew and Craig, 2002), originating from deeper structures, although skin pressure also plays a role (Kosek et al., 1999). And as for the use of a contact thermode, site differences exist also for pressure pain

and one solution to standardize the performance is to use well defined areas such as the 18 tender points used in the diagnosis of fibromyalgia (Wolfe et al., 1990).

Psychological implications of experimentally induced pain

As the IASP definition of pain implicates, psychological factors are integrated in the sensation of pain (McGrath, 1994), and may act as confounders if the purpose is to study pain as a single dimension, such as the threshold or tolerance level. It is well established in the clinic that the size of an injury does not always mirror the level of experienced pain (Beecher, 1946). Psychological influences on pain have been shown in experimental studies (Jones et al., 2002, Robinson et al., 2010), using similar settings and methods as in present work. The ability to adopt coping strategies, as well as distraction, may influence the result of tolerance tests (Hodes et al., 1990, Petrovic et al., 2000, Frankenstein et al., 2001). Pain thresholds, however, seem to be unaffected (Ahles et al., 1983). Distraction is in fact used as a pain intervention for procedural pain (Kwekkeboom, 2003).

Affective status, such as mood, may also influence the sensation of pain (Bär et al., 2005, Schwier et al., 2010), and increased anxiety and fear of pain have shown to reduce pain thresholds (Buchanan and Midgley, 1987). However, some previous studies (Jensen et al., 2010) showed no influence on experimentally induced pain by negative mood in a group of women suffering from fibromyalgia, hence suggesting distinct mechanisms in the brain for processing experimental pain and negative affect, respectively. Also, earlier experiences of pain have been shown to influence both thresholds and tolerance levels (Dar et al., 1995), which is particularly important when in-between settings and comparisons between subjects are in focus. Another factor that needs consideration when using psychophysical methods is that the sex of the experimenter may influence the outcome (Kállai et al., 2004). Thus, men perform better when a woman is the experimenter (Levine and De Simone, 1991, Kállai et al., 2004). The authors noted a trend towards the opposite phenomenon for women when a man was the experimenter.

Sex differentiation, the gonadal hormones and their receptors

As implied in the beginning of the present thesis, sex and gender influence the sensation of pain. So, how do the sexes differ? The sex steroids, estrogens, androgens, and progesterone are small hydrophobic molecules, which are primarily synthesized in the gonads from cholesterol via several enzymatic steps. Their principal function is to take part in the development, and later, to be one of many regulators of reproductive functions. One step in the sex steroid synthesis is the conversion of estradiol from testosterone by the enzyme aromatase, so both estrogens and androgens exist in, and are important for both sexes. The gonadal hormones are transported in the blood mainly bound to a transport protein called sex hormone-binding globulin and diffuse directly across the cell membrane of their target cells and bind to specific receptor proteins in the cytosol. The steroid-receptor complex then regulates the transcription of specific genes, with the cell and tissue specific characteristics of the transcriptional machinery determining the steroid effect (McDonnell and Norris, 2002).

Estrogens are evolutionary one of the oldest known hormones and biosynthesis of estrogens is present in most species (Lange et al., 2003). It has been suggested that the estrogen receptor may be even older with a principal function as a general transcription factor (Thornton, 2001, Eick and Thornton, 2011), which hence may explain its wide distribution. Two kinds of estrogen receptors are known at present, $ER\alpha$ and $ER\beta$, which activate different genes and have diverse distribution in the body (Tetel and Pfaff, 2010). In fact, most tissues in the body contain receptors for gonadal hormones, including the central nervous system, the reproductive organs and breast tissue, as well as fat tissue, skin, bone and the cardiovascular system. This wide distribution also indicates a variety of functional responses. For example, the ERa activates, among others, genes for opioid receptors and progesterone receptors (Pfaff et al., 2002). Accordingly, estrogens influence and regulate many transmitters and functions in the central nervous system such as dopamine (Dreher et al., 2007), commonly connected to motor and reward functions, serotonin (Robichaud & Debonnel, 2005, Vanderhorst et al., 2005), connected to regulation of mood and wakefulness, and norepinephrine (Curtis et al., 2006), involved in stress response.

With respect to pain processing, the influence of estrogens on the regulation of the endogenous opioids, such as enkephalins, is of major interest, and involves different areas in the brain (Yang et al., 1977, Sar et al., 1978) as well as the spinal cord (Amandusson et al., 1995, 1996). The location of the steroid receptors is intracellular, but recent findings suggest that receptors for estrogens also exist in the plasma membrane (Kelly and Levin, 2001) and activate intracellular G-proteins. This activation gives rise to a rapid effect on the cell by ion-channel activation (Evrard & Balthazart, 2004).

The receptors for progesterone exist in two isoforms, PR-A and PR-B and are often co-expressed in the cells (Conneely et al., 2002). In addition to effects on its own receptors, as well as on the estrogen receptors, progesterone acts on other systems. Its metabolite, allopregnanolone, is well known as a GABA-A receptor agonist (Pluchino et al., 2006), and GABA, in turn, is the major inhibitory neurotransmitter in the CNS, involved in sedative, anxiolytic, and anticonvulsive effects (Bitran et al., 1991, Belelli and Lambert, 2005). It also seems that progesterone, as well as estrogens, has membrane bound receptors that may act through G-proteins in a rapid manner (Karteris et al., 2006, Mourot et al., 2006).

While progesterone, estrogens and androgens are important for both men and women, they are at the same time important factors that differentiate women from men. The hormonal influence begins in the development of the foetus, in the sex differentiation process. Our phenotype is predicted by the genes, males having one X and one Y chromosome and females having two X chromosomes. The Y chromosome contains a gene sequence *Sex Determining Region Y* for *testis determining factor* (TDF), which controls the differentiation of the gonads to become testes (Vilain and McCabe, 1998, Sim et al., 2008), a development process that occurs around week seven. If TDF is not present, the gonads by default differentiate into ovaries.

When the gonads differentiate to testes, they start to produce *Mullerian duct inhibiting hormone* and testosterone, which act in the further development process to the male sex. In the female development, hormones seem to have a less prominent role, or in

other words, when TDF is not present, the foetus develops to a female. The role of sex hormones in the development of the central nervous system is complicated, and paradoxically, as shown in rats, estrogens are the major hormones that govern the masculinization process. This process seems to be dose dependent, with low doses of estradiol being more potent than testosterone on the central nervous system. Testosterone diffuses into the nerve cells and is converted to estradiol by aromatase, an intracellular enzyme. So the effect of testosterone on the central nervous system is in part an intracellular estrogenic event. New-born female rats have shown to produce a liver protein, alpha-fetoprotein, that binds estrogens and that inhibits hormonal influences on the brain (Bakker et al., 2006). However, alpha-fetoprotein does not have the same effect in humans. In primates, it has been shown that androgens play a role in the masculinization process. In humans, behavioural studies on girls with congenital adrenal hyperplasia, a condition associated with elevated prenatal testosterone levels, have shown more male behaviour in childhood (Nordenstrom et al., 2002, Pasterski et al., 2011).

So, while the male central nervous system is influenced by sex hormones in one way or another, the female brain is more or less protected against gonadal hormonal influence, or exposed to very low hormonal levels during the foetal development. The different hormonal milieu causes structural sex differences (Bowers et al., 2010). They are widespread in the central nervous system in areas such as cerebral cortex, hippocampus, amygdala, hypothalamus and corpus callosum. Fitch and Denenberg (1998) point out the following mechanisms to explain the dimorphism that exists between the sexes: (i) different estrogen levels, higher in the male because of the aromatization process; (ii) the time point for high estrogen levels occurs in developmentally critical periods; and (iii) different distribution and expression of target receptors, which in turn may be connected directly to a genetic mechanism exerted by the sex chromosomes in the differentiation process (Carruth et al., 2002, Arnold et al., 2004). Alternative views exist (De Vries, 2004), implying that sex differences in a structural manner do exist but that they exist entirely to compensate functional differences due to physiological events such as hormonal fluctuations.

Hormones throughout the life span

As mentioned above, sex hormones influence the growing foetus, and they do so in distinct ways in males and females. The secretion of estrogens increases in women when puberty starts somewhere between the ages of 11 and 16 years and has a cyclic variation until the end of reproductive life. The two major female sex hormones, 17β-estradiol and progesterone, act under the influence of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary, which in turn are under influence of gonadotropin releasing hormone (GnRH) from the hypothalamus. FSH and LH are then together with estradiol and progesterone involved in a cyclic variation via both a positive and negative feedback system during the ovulatory or menstrual cycle (Buffet et al., 1998).

The normal ovulatory cycle is on average 28 days, and can be divided into the follicular phase, the ovulation and the luteal phase. However, variations from the normal profile seem to be common (Alliende, 2002), and constitute an important factor when hormonal influence on e.g. pain sensitivity is to be studied. When the follicular phase begins, the plasma levels of FSH and LH are high thus stimulating the follicle growth leading to increased production of estradiol. A peak in estradiol levels occurs just before the ovulation and suppresses FSH secretion while at the same time triggering a short but large secretion of LH, the so called mid-cycle LH surge. This LH surge causes ovulation from the mature follicle and converts the follicle into a corpus luteum which mainly produces progesterone. The level of progesterone thus increases rapidly during the luteal phase, whereas estradiol levels decrease just after the ovulation, but then display a second peak under the influence of the corpus luteum (Fig.1).

If an implantation occurs, the cyclic fluctuation stops and a continued secretion of estradiol and progesterone takes place. Now the placenta acts as the major producer of regulatory hormones (Lacroix et al., 2002), producing chorionic gonadotropin (CG), which takes over and increases the stimulation of the corpus luteum. Otherwise, if the

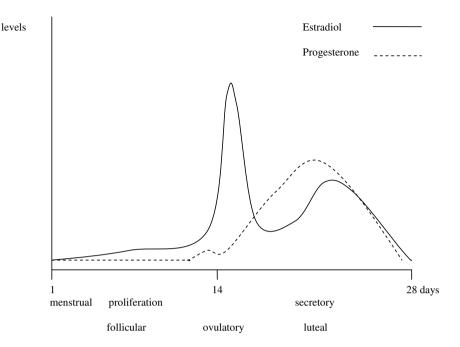


Figure 1. A schematic presentation of the human female ovulatory cycle, standardized to 28 days. Estradiol (solid line) has two peaks in the human cycle, one just before the ovulation and one, together with progesterone (dashed line), during the luteal phase. The follicular phase includes the menstrual phase and the following proliferation phase, which reflect the event in uterus before ovulation. Note that the concentration scales for estradiol and progesterone are not identical. Modified from Buffet et al. (1998).

ovum is not implanted, the luteal production of estradiol and progesterone ceases and the endometrium becomes ischemic, which results in menses. In addition to their effects on the reproductive functions, the gonadal hormones also influence the nervous system during the menstrual cycle. Structural plastic changes have been shown in several brain areas (Protopopescu et al., 2008, Pletzer et al., 2010), some of them involved in affective functions.

When reproductive life ends, at an average age of 51 years (Pollycove et al., 2011), the production of estrogens – and progesterone – from the ovaries decreases. After a few years it has almost ceased, but women have some other source of production of estrogens after menopause, such as fat tissue that synthesizes small levels of estrogens

mainly from androgens produced in the adrenals and the ovarian stroma via the aromatization process mentioned above.

After menopause a decrease in endogenous opioid production occurs, and this decrease has been suggested to play an important role in the symptomatology of post-menopausal problems (Berg et al., 1988, Genazzani et al., 1988), with hot flushes and other symptoms (Odell, 2001). These symptoms can be reduced with hormone replacement therapy, which increases endogenous opioid peptide levels (Nappi et al., 1990, Spencer et al., 1990). A connection between menopause and the chronic pain syndrome fibromyalgia has been suggested because of the earlier onset of menopause in women suffering from fibromyalgia (Waxman and Zatzkis, 1986, Pamuk et al., 2009). Of additional interest are the discussions on non-scientific blogs and web pages where women describe their experience of problems connected to increased or worsening pain during the menstrual cycle or in connection to the menopausal transition.

Men have the same regulating hormones as women, but not the cyclic variation. Instead, men have more or less constant circulating levels of testosterone, which is the most important sex steroid for men. However, males also secrete estrogens, produced by the testes, and synthesized from androgens by aromatisation (Brodie et al., 2001). The role of estrogens in men seems to be widespread in many tissues and organs, as it is in women, and estrogen receptor expressing cells have been found in several brain regions, adipose and muscle tissues, and the immune and circulatory system (Lombardi et al., 2001).

The gonadal hormones and pain

Hormonal regulation of pain has been examined in animals as well as in humans, both in experimental and clinical studies, but with partly contradictory results. Estrogens have been reported to increase as well as decrease pain sensitivity, which in turn may be due to different activation of the two known estrogen receptors (Coulombe et al.,

2011). In animal studies a rapid increase in enkephalin mRNA in the spinal cord was shown after a bolus injection of estradiol (Amandusson et al., 1999), suggesting a connection between nociceptive control and gonadal hormones. Furthermore, the absence of endogenously produced estrogens in aromatase knock-out mice has been shown to increase nociceptive behaviour (Multon et al., 2005), an effect that was reduced by subsequent estradiol administration. Gear et al. (1996) showed that estrogens enhanced the antinociceptive effect of kappa opioid agonists, results consistent with those of studies simulating the hormonal profile that occurs during pregnancy (Dawson-Basoa and Gintzler, 1996). Other authors (Fan et al., 2007) have shown that estrogens influence the pain system in early development. Some (Sternberg et al., 2004) suggest that the pain inhibitory system differs between the sexes, as has been demonstrated in animals (Mogil et al., 1993). Thus, in a stress-induced analgesia paradigm male rats displayed an endogenous opioid dependent analgesia, whereas female rats showed an estrogen dependent non-opioidergic analgesia that was attenuated by gonadectomy and restored by estrogen substitution. In other test paradigms the opioid-dependent antinociception in females, but not males, was shown to require the concomitant activation of spinal μ- and κ-opioid receptors (Chakrabarti et al., 2010).

During pregnancy as well as during the ovulatory cycle, also progesterone may play a role in the regulation of pain sensitivity, but its role is much less examined than that of estrogens. However, both central (Kavaliers and Wiebe, 1987) and spinal mechanisms (LaCroix-Fralish et al., 2008) have been suggested for progesterone. The findings are diverse, with some studies reporting an antinociceptive effect of progesterone (Coronel et al., 2011), while others show pronociceptive effects (Waxman et al., 2010).

In experimental studies in humans, contradictory results exist regarding the sex differences as well as the hormonal influence on the sensation of pain. This may be due to small samples, different techniques and test setups, as reviewed by Sherman and LeResche (2006), and Greenspan et al. (2007). Studies based on large samples often show differences between the sexes in experimental settings, as for example in studies of pressure pain (Woodrow et al., 1972) and cold pain tolerance (Walsh et al.,

1989). It has been discussed if these differences are due to physiological or psychological factors or to a combination. However, results from studies investigating autonomic reactions such as pupil reflexes (Ellermeier and Westphal, 1995) and cardiac response (Maixner and Humphrey, 1993) during painful stimulation also show differences between the sexes. A common way to examine the hormonal influence on pain sensitivity is to follow menstrual cycle fluctuations, but again the results are contradictory, as reviewed by Reily et al. (1999), Sherman and LeResche (2006), and Martin (2009). In healthy subjects, increased pain sensitivity has been reported during the follicular phase (Hapidou and Rollman, 1998, Hellstrom and Lundberg, 2000, Bajaj et al., 2001), but others show increased pain sensitivity during the luteal phase (Hapidou and De Catanzaro, 1988, Fillingim et al., 1997, Tassorelli et al., 2002), and some find no differences at all (Kowalczyk et al., 2006, Soderberg et al., 2006).

Across the menstrual phase, different activation patterns in the brain have been shown, such as in affective responses, which increase in phases connected with low estrogen levels (de Leeuw et al., 2006). However, others, also using brain imaging techniques (Choi et al., 2006), found a different activation pattern, with higher pain ratings and unpleasantness during the luteal phase than during the follicular phase. In contrast, patients suffering from pain reported less pain in phases connected with high estrogen levels (Hellstrom and Anderberg, 2003). However, in women suffering from fibromyalgia (Okifuji and Turk, 2006), no difference was found throughout the menstrual cycle on experimentally induced ischemic pain.

The diagnosis fibromyalgia is especially interesting, because the majority suffering from this syndrome are women and the condition is most prevalent in childbearing age (Weir et al., 2006) or during the menopausal transitions (Waxman and Zatzkis, 1986), periods characterized by changes in the hormonal milieu. Thus, the influence of sex hormones adds to a large number of hypotheses on the aetiology of fibromyalgia that include a disturbance in the hypothalamic-pituitary-adrenal axis, disturbance in the autonomic system, immunologic involvement, as well as genetic or hereditary factors (Bradley, 2009, Clauw, 2009, Nielsen and Henriksson, 2007).

AIMS

The aim of the research on which this thesis is based was to examine the influence of the gonadal hormones 17β -estradiol and progesterone on the sensation of pain. The thesis is based on four studies, with their respective specific aims:

- To study the influence of the hormonal fluctuations during the ovulatory cycle on pain threshold and tolerance for cold, heat and pressure in healthy women of fertile age
- II) To study the influence of hormonal fluctuations during the ovulatory cycle on pain tolerance to noxious cold in healthy women of fertile age.
- III) To study the influence of extreme variations in estradiol levels on pain threshold and tolerance in women undergoing *in vitro* fertilization.
- IV) To study the effects of hormone replacement therapy on pain thresholds and pain tolerance in postmenopausal women suffering from fibromyalgia.

METHODS

Quantitative sensory tests

In the present work, the quantification of pain thresholds and pain tolerance during different hormonal levels was the primary focus. Some modifications of the methods used were carried out between the studies in order to improve study design and facilitate analysis. Further details are described in the method sections of Papers I-IV.

A set of different quantitative sensory tests (QST) was used. Temperature thresholds were determined by a Thermotest (Somedic, Hörby Sweden) instrument, which uses a peltier based thermode that generates warm, cold, noxious heat, and noxious cold. The surface area of the thermode was 2.5 x 5 cm and the thermode was applied to the participant's thenar region. All measurements started at 32°C, which is considered as neutral to the skin temperature and commonly used in QST settings. The temperature was then increased or decreased with a rate of 1°C/second until the participant reached the detection threshold, pain threshold or her/his tolerance level according to the methods of limits. The participant pressed a turn-off button at the level reached and the temperature was then set again at 32°C. For safety precautions, the apparatus had a cut-off limit at 2°C and 52°C, respectively. Repeated recordings were done with an interstimulus interval of 4-6 seconds.

Pressure pain thresholds were detected with a pressure algometer (Somedic, Hörby Sweden). A 1 cm probe was attached with a force rate of 100 kPa/second to four specific tender points bilaterally, corresponding to defined tender points used in the diagnosis of fibromyalgia (Wolfe et al., 1990): the midpoint of the upper border of the trapezius muscle, a point distal to the lateral epicondyle of the elbow, the upper outer quadrant of the gluteal region, and the medial fat pad proximal to the knee joint line.

A modified Cold Pressor test was used to determine cold pain tolerance (Walsh et al., 1989, Hirsch and Liebert, 1998, Mitchell et al., 2004). The participant immersed one hand down to the wrist in a water tub (2.8 l), filled with ice-chilled water (1.5 \pm 0.5°C).

The temperature was monitored with a steel probe digital thermometer (VEE GEE Scientific, Kirkland, WA, USA). In the studies reported in papers I, II and IV the circulation in the tub was done manually. In the study reported in Paper III the set-up was improved with an air-driven electric pump to induce circulation of the water.

Subjects

In the study reported in Paper I, 14 healthy women of fertile age who did not use hormone based contraceptives were recruited. Before inclusion, they were told to follow and record one menstrual cycle. If the recorded cycle was considered to be normal in length, the women were included in the study. They were then followed over three menstrual cycles. QST for temperature detection thresholds, cold and heat pain thresholds, pressure pain thresholds, and pain tolerance thresholds for heat and cold were performed twice in each cycle: in the early follicular phase and during the midluteal phase (Fig. 2). Menstrual cycle phases were verified by venous blood samples with measurements of $s-17\beta$ -estradiol and s-progesterone.

Paper II, again based on research performed on healthy women of fertile age (n = 16) without hormone based contraceptive treatment, was a tolerance study. A modified cold pressor test was used as stimulus and the participants were tested once a week during a four week period (Fig. 2). A computerized VAS-rating was used during the sessions, which made the set-up less sensitive to the fact that participants sometimes reached the cut-off limits. After each session a blood sample was drawn to verify hormonal status. The pain ratings during the cold pressor test were correlated to the serum hormonal levels. A control group of men (n = 10) was used to evaluate possible session-to-session effects defined as changed tolerance in the same direction between sessions.

In the study for Paper III, 16 women undergoing *in vitro* fertilization were tested for temperature detection thresholds, cold and heat pain thresholds, pressure pain thresholds, and pain tolerance thresholds for heat and cold during (i) the initial hormonal down-regulation phase, induced by a GnRH-analogue (Suprecur[®], Sanofi-

Aventis, Paris, France or Synarela[®], Pfizer Inc., New York, USA), and (ii) the subsequent up-regulation phase induced by FSH stimulation (Gonal-f[®], Merck Serano S.A., Geneva, Switzerland or Puregon[®], Schering-Plough AB, Stockholm, Sweden or Menopur[®], Ferring S.A. Holding, Lausanne, Switzerland) (Fig. 2). Considering the within group design, two control groups of 10 age-matched men and 9 women, respectively, were used for evaluation of eventual session-to session effects.

In the study for Paper IV, the participants were postmenopausal women, who had had their last menstrual bleeding at least six month ago, and who suffered from fibromyalgia (n = 29). They were recruited from the Pain Clinic at Linköping University Hospital. The women were randomized to treatment either with transdermal estrogen patches (Evorel[®], 50ug 17-β estradiol/daily, Janssen-Cilag, Sollentuna, Sweden) or with a placebo patch for a period of eight weeks, according to a double blinded protocol administrated by the pharmacy at Linköping University Hospital. QST for temperature detection thresholds, cold and heat pain thresholds, pressure pain thresholds, and pain tolerance thresholds for heat and cold were performed before treatment start and after eight weeks of treatment, and with a final session twenty weeks after termination of treatment (Fig. 2). At each session the participant was told to perform a self-estimation of perceived pain using a modified pain map.

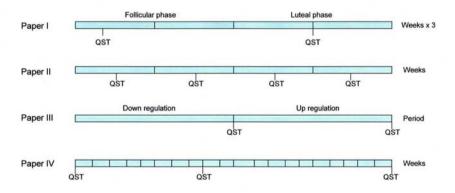


Figure 2. A schematic overview of respective timeline for the studies in Papers I-IV and when the QST sessions were performed. In Papers I, II and IV, each segment represents one week. In Paper III the segments represent the different treatment stages. In Paper I, QST was performed twice during three consecutive cycles.

Statistics

Due to differences in design between Papers I-IV, including both within and inbetween measurements as well as a different number of sessions, several statistical analyses were performed; the details are presented in the respective papers. In Papers I, III and IV, the sample size was calculated for a significance level at 0.05 with 85% power based on found differences in a previous pilot study (Stening, 1999) and in paper II by estimation from normative data on the cold pressor test (Walsh et al., 1989). The pilot study was performed on 14 healthy postmenopausal women undergoing transdermal estrogen treatment and with a similar set-up as in paper IV. In the pilot study, QST was performed twice, before and after eight weeks of treatment, and showed a 4°C reduction in cold pain threshold. Considering eventual test-retest phenomena as part of the explanation for the obtained reduction in threshold, we based our power calculation on half of that difference.

In the study for Paper I, repeated measures were performed and the material was analysed with a General Linear Model (GLM) in different settings. The material was then split into different subgroups, analysed with a three-way analysis of variance (ANOVA) with a mixed model design followed by Tukey's *post hoc* test.

In Paper II, the GLM was also used to show interaction effects and to analyse the effect of eventual session-to-session effects.

In Paper III, an ANOVA was used as main analysis. Student's paired *t*-test was used for within-group comparisons. For between groups comparisons Student's independent *t*-test was used. A linear regression was used to compare how the treatment group changed between sessions with how the average of the two control groups changed between sessions.

In Paper IV the sample size was based on a power calculation as described above, in turn based on the pilot study, but with the addition of 10% to secure adequate power in case some participants would choose to leave the study. The statistical analysis was

then performed with an ANOVA according to repeated measures and use of control group.

Ethical approval

Pain induction in voluntary individuals gives rise to several ethical considerations, as expressed by IASP's Ethical guidelines for pain research in humans (Charlton, 1995). All the studies were approved by the local ethics committee in Linköping, Sweden, and adhere to the principles of the Declaration of Helsinki. All subjects gave their written as well as oral consent to participate. They were informed that they could discontinue the study whenever they wanted and without giving any reason for their decision. The use of estrogen treatment is a regime connected to adverse effects and risks, and a major health investigation of the participants was done before eventual inclusion. All the participating women in Paper IV were counseled and treated by a gynecologist and a specialized nurse concerning their treatment during the studies. All adverse effects were registered and reported and the women were followed-up at the last session, twenty weeks after termination of the treatment. The study was performed according to Good Clinical Practice and approved by the Medical Products Agency, Uppsala, Sweden (151:662/01) and reported to ClinicalTrials.gov Registration; http://www.clinicaltrials.gov; NCT01087593.

RESULTS AND COMMENTS

Pain thresholds and pain tolerance did not change during the ovulatory cycle in healthy women (Paper I)

A set of QSTs was performed on 14 healthy women on two different occasions during their menstrual cycle, during the early follicular phase and the mid-luteal phase. This is a common design for evaluating the influence of gonadal hormones on pain sensitivity. However, despite the fact that a normal menstrual cycle preceded the first cycle during which QST was performed, the hormonal levels recorded were not always consistent with the assumed cycle phase. Some values obtained during the presumed mid-luteal phase were more representative for the ovulatory phase and some for the late luteal phase. The data obtained were therefore analysed in several different ways, first by the calendar method, followed by a re-analysis after hormonally atypical cycles had been omitted. As a third strategy, the data were subdivided into three different groups, based on the hormonal profiles of the cycles. The main finding was a slightly increased heat pain threshold on one of two thenar regions and increased heat pain tolerance during the luteal phase compared with follicular phase when atypical cycles had been omitted. We then re-analysed the material with session as covariate in the statistical model. This analysis showed a strong session-to-session effect (P < 0.001 - 0.01), and no phase effect (P = 0.33 and P = 0.78 for heat pain threshold and P = 0.45 and P = 0.25 for heat pain tolerance), suggesting that the observed changes of these two variables were all due to study design. Thus, there was no clear evidence that pain sensitivity changed during the menstrual cycle.

Comments: At the time this study was carried out (2004) most previous studies used indirect methods to assess the cycle phase, hence deducting – and not measuring hormonal levels (Sherman and LeResche, 2006). We found anovulatory cycles without high progesterone levels during the presumed midluteal phase, as well as atypical hormonal levels, despite the fact that normal menstrual cycles were reported by the participants. This emphasises the need for measuring actual hormone levels in studies

examining pain sensitivity across the menstrual cycle. The observed variations in hormonal levels also influenced the ability to analyse the material according to the initial power calculation, which was based on an assumed similarity between consecutive cycles and a within subject design that in general requires fewer subjects than an in-between design. This in turn resulted in the decision to analyse the material in several steps, starting with the calendar method followed by analysis after omission of hormonally atypical cycles, and finally the stratification of the cycles according to hormone profile. The finding that cycle phase did not influence pain sensitivity goes in line with observations in several other studies (Soderberg et al., 2006, Kowalczyk et al., 2006, Klatzkin et al., 2010, Teepker et al., 2010), although contradictory observations have been reported (Ring et al., 2009).

Pain sensations to the cold pressor test in normally menstruating women vary with serum sex steroid levels (Paper II)

Sixteen healthy women without hormonal contraceptive treatment were examined with a cold pressor test once a week over a standardized cycle period of 28 days. As a methodological control for session-to-session effects a group of 10 men was included. The result showed a lower pain threshold, represented by shortened time period before the stimulus was considered painful, during the late luteal phase compared with the late follicular phase (P = 0.04). In-depth analysis of the pain sensitivity at different hormonal levels demonstrated decreasing pain threshold and higher perceived pain ratings with increasing progesterone concentrations. In addition, there was an interaction between estradiol and progesterone in that the increased pain intensity that was associated with high levels of progesterone in the luteal phase was reduced with increasing estradiol concentrations ($F_{1,38} = 5.79$, P = 0.02).

Comments: In this second study, we only used one stimulus, the cold pressor test and developed a computerized VAS. The use of the computerized VAS permitted more sensitive analyses than in our previous studies and partly overcame the problem that some participants reached the cut-off time limit in the cold pressure test, which in

previous analyses may have blunted the result. In contrast to the procedure for Paper I, we also used a control group to evaluate session-to-session effects. Our data contradict those reported in a previous study using the cold pressor test (Kowalczyk et al., 2006) that did not find any relationship between pain sensititivy and hormonal levels, but are consistent with the results from animal studies (Kuba et al., 2006). The results, however, raise further questions concerning whether it is the actual, static hormonal levels or the dynamic shift, in this case the drop in progesterone from high to low levels in the late luteal phase, that may trigger the change in pain sensitivity.

Dichotomous levels of 17β -estradiol seem to have a minor influence on pain threshold and tolerance in women undergoing in vitro fertilization (Paper III)

Sixteen women undergoing in vitro fertilization were examined with a set of quantitative sensory tests during hormonal down-regulation and up-regulation, respectively. As methodical control, a group of men and a group of women using monophasic contraceptives were recruited. The hormonal treatment in association with the IVF regimen yielded as expected very large differences in serum 17β-estradiol levels, with a close to 80-fold increase between the down-regulation phase and upregulation phase. The QSTs revealed significant changes in thermal perception thresholds (P = 0.003) with reduced discrimination capacity during the up-regulation phase. There was also a significant difference for cold pain threshold, which changed from 11.5° C to 14.5° C (P = 0.04) between the two sessions, suggesting increased pain sensitivity during the up-regulation phase. However, a similar but not significant change in cold pain thresholds was also found in both control groups, implying that session-to-session effects may account for the observed observation. Heat pain thresholds, heat tolerance, pressure pain thresholds and the cold pressor test showed no significant differences between the sessions. Thus, the data suggest that even very large changes in estradiol levels do not result in changes in pain sensitivity.

Comments: The very limited changes in pain sensitivity seen in studies I and II may be explained by the fact that the normal fluctuation of gonadal hormones during the menstrual cycle are small. Therefore, analysis of pain sensitivity in women undergoing in-vitro fertilization is an attractive alternative. This study is to our knowledge the third (Tsen et al., 2001, Nisenblat et al., 2010) that has used the IVF-set up as a model to study hormonal effects. The result goes largely in the same direction as in the previously published studies. Despite the extreme variation in hormonal levels most of the variables did not change between the test sessions, with the exception of thermal perception thresholds and cold pain thresholds, which not were examined in previous studies. However, as described above the change in cold pain threshold could probably be explained by a session-to-session effect.

Pain sensitivity in postmenopausal women suffering from fibromyalgia is not influenced by hormonal replacement therapy (paper IV)

Twenty-nine postmenopausal women suffering from fibromyalgia were included and randomized to either treatment with 17β-estradiol or placebo for a period of eight weeks. QST was performed before and after treatment, with a final session twenty weeks after termination of treatment. A significant increase in hormonal levels was shown as expected in the estradiol treated group, while no change was noted in the placebo treated group. However, one woman in the placebo group showed signs of endogenous hormonal activity and she was therefore excluded from the study. No differences between the estradiol and placebo treated groups regarding thresholds for pain and temperature, or for pain tolerance were found. A similar result was obtained for the overall pain experience as measured by the modified pain map. Session-to-session effects, with increasing thresholds over time irrespective of treatment, were observed for several variables in one or the other treatment groups, with increasing thresholds for temperature perception, and cold and heat pain tolerance, and reduced thresholds for pressure pain (over one of the four tender points) and the cold pressor test, as well as increased VAS scoring of the latter.

Comments: This is to our knowledge the first study to evaluate transdermal estradiol treatment in an experimental setting involving experimentally induced pain. Because of the lack of any effect of the treatment, the study was terminated when half of the planned number of participants had been recruited; this decision was also influenced by new recommendations from the National Medical Products Agency about hormonal substitution treatment. The findings show that experimentally produced pain, as well as self-reported on-going pain in postmenopausal women suffering from fibromyalgia did not respond to hormonal substitution treatment. While there are numerous anecdotal reports on e.g. web fora on the beneficial effects of estrogen treatment for fibromyalgia, it is possible that these effects relate to alleviation of other symptoms than pain, such as fatigue, sleep disturbances, and depressed mood.

DISCUSSION

The overall aim of the research on which the present thesis is based was to study the effects of the gonadal hormones 17β -estradiol and progesterone on the sensation of experimentally induced pain in different groups of women that showed variations in their hormonal milieu, such as during different hormonal phases throughout the menstruation cycle, before and after estrogen substitution treatment, and during dichotomous hormonal levels due to in vitro fertilisation treatment. The overall results are discussed in the following paragraphs together with certain methodological remarks.

Hormonal influence on the sensation of pain

Accumulating evidence in the literature points to sex and gender differences in the sensation of pain (Fillingim et al., 2009). However, while hormonal influence on pain sensitivity is readily observed in experimental animals (Dawson-Basoa and Gintzler, 1996, Craft et al., 2004, Sanoja and Cervero, 2008), it has largely been elusive in studies on humans. Here, with the exception of the changes seen in the pain ratings in the cold pressor test (Paper II), there was little or no effect on pain sensitivity as a result of changing hormone levels. Not even extreme variations in estradiol levels as in study III, resulted in any significant changes in pain threshold or tolerance, and hormonal substitution treatment, used in study IV, had no other effect on the observed variables than did the placebo.

However, it is important to remember that, similar to the present work, the majority of studies in humans focus on threshold and tolerance measurements and that this may not reflect all aspects of pain sensitivity. An alternative hypothesis was presented by Tousignant-Laflamme and Marchand (2009), suggesting a hormonal influence on diffuse noxious inhibitory control (DNIC) instead of actual thresholds. This idea agrees with the suggested differences in the pain inhibitory system between the sexes (Sternberg et al., 2004, Loyd and Murphy, 2009), that will be discussed further below.

As pointed out in the Introduction, estradiol and progesterone may influence many systems and this hormonal involvement may be mediated by both organizational and activational effects. The strength of this influence may be both event specific as during pregnancy, or restricted to level dependent interaction, as suggested by the result in Paper II. Accordingly, the hormonal changes occurring during the menstrual cycle may be too small to induce any but subtle changes in pain sensitivity and thresholds. Notably, other studies using similar design as in Papers I and II, i.e. pain threshold measurements throughout the menstrual cycle, did not find any relationship between hormonal levels and thresholds either (Teepker et al., 2010, Kowalczyk et al., 2010). However, as referred to above, sex specific differences have been shown for DNIC (Quiton and Greenspan, 2007, Popescu et al., 2010), and the endogenous pain control has also been reported to be influenced by hormonal contraceptive treatment (Rezaii and Ernberg, 2010), hence suggesting hormonal control. Furthermore, as pointed out by Soderberg et al. (2006), site specific effects may also exist, such as variation in cold pain perception on the mammilla, but not on other body regions across the menstrual cycle. Further methodological considerations will be discussed below. A sympathetic involvement, due to the ability of gonadal hormones to influence the vascular tone in response to cold may also account for observed sex differences. Thus, Vierck et al. (2008) showed that female rats was more aversive to cold than male rats and, contrary, heat stimulation was more aversive in male rats.

In line with observations from animal experiments, while not the focus of the present work, clear sex differences in cold pain tolerance were seen between men and women (Papers II and III). However, in the study of IVF-treated women (Paper III), we found that these women showed less tolerance, independent of the treatment phase, not only in comparison with men, but also in comparison with the women in the control group, suggesting that stress may also be an important factor. Infertility may be associated with elevated stress, anxiety and depression (Csemiczky et al., 2000, Kee et al., 2000), factors that may influence the outcomes of experimental pain tests (Jones et al., 2002, Robinson et al., 2010), especially tolerance tests (Manning and Fillingim, 2002).

The only significant change in examined sensory parameters that occurred during the IVF-treatment, and that could not be explained by the repeated measurement design, was decreased thermal perception thresholds when the estradiol levels were high, pointing to effects on non-noxious sensory functions. This is in contrast to the findings in Papers I and IV, in which the thermal perception threshold did not change significantly, and points to the possibility that supraphysiological levels of estrogens may play a role. In rats, estrogens have been shown to regulate the size of the sensory receptive fields (Kow and Pfaff, 1973, Tashiro et al., 2007) as well as central sensory processing (Reed et al., 2009), but they are also known to influence the skin, involving increased content of collagen and moistening factors (Shah and Maibach, 2001, Hall and Phillips, 2005). Furthermore, the changed thermal discrimination may also be due to cognitive components such as elevated reaction time. The reaction time is essential when evaluating the thermal detection threshold because of the short time duration between stimulus start and detection level. Estrogens have been shown to interact with the dopaminergic system (Colzato et al., 2010), involved in cognitive control and motor functions, factors that influence the reaction time. An impaired response time has been reported during the late follicular phase compared with the luteal phase in different psychological experiments (Gasbarri et al., 2008), suggesting that high levels of estradiol could impair the sensory detection threshold.

Hormonal substitution treatment (Paper IV) showed no effect on pain threshold or pain tolerance, nor on self-estimated pain in women suffering from fibromyalgia. Neither was there any effect on spontaneously reported pain, as determined by the pain map used by the participants. Positive effects on pain of hormonal replacement therapy have been reported. For example, the Women's Health Initiative study (Hays et al., 2003), involving over 16,000 women, showed an attenuation of bodily pain after estradiol substitution treatment. However, it is likely that this large sample was not representative for fibromyalgia patients, but rather represented other musculoskeletal conditions. Estrogen treatment has been shown to influence both the repair of muscle tissue (Tiidus, 2005) as well as having osteoprotective functions (Nakamura et al., 2007), effects that may explain the observed self-estimated improvement. In contrast, while the aetiology and pathophysiology of fibromyalgia remain obscure, there is

evidence that central, rather than peripheral, mechanisms are involved (Nielsen and Henriksson, 2007, Clauw, 2009, Petersel et al., 2011), such as disturbed endogenous pain inhibition (Kosek et al., 1996a, b, Julien et al., 2005, Jensen et al., 2009, Kosek and Hansson, 1997, Kosek et al., 1997) and reduced central μ -opioid receptor availability (Harris et al., 2007). Another complicating fact is that women suffering from fibromyalgia most likely do not constitute a homogeneous group with respect to the symptomatology, suggesting different aetiology (Hurtig et al., 2001, Giesecke et al., 2003), which in turn implies that they respond differently to different interventions. Our sample size was too small to determine the effect on eventual subgroups.

Finally, it should also be noted that the patients involved in the present study had suffered from chronic on-going pain for several years, which may have resulted in plastic changes in the nervous system (Woolf and Salter, 2000, Reichling and Levine, 2009). Therefore, it remains possible that studies on women with shorter illness history could have yielded different results.

Methodical remarks

The use of quantitative sensory tests

The use of quantitative sensory tests is associated with advantages as well as limitations depending on the choice of the algorithm used. The tests can be standardized and are easily reproducible; however, they basically measure thresholds rather than perceived pain intensity. As mentioned in the Introduction, the *methods of limits* are reaction time sensitive, which may result in higher thresholds. However, at the same time this algorithm involves short and understandable instructions and the test procedures are thereby easy to perform, as well as being timesaving in comparison to other algorithms. Studies presenting normative data (Magerl et al., 2010, Nezeri et al., 2011a) and standardized procedures (Rolke et al., 2006) may serve as guidelines; a direct comparison between studies requires the same procedure, algorithm, age, sex, etc. The selection of methods is therefore an area of consideration. In the present thesis, the

choice fell upon the use of thermal and mechanical stimulations, which are non-invasive and generate sensations that are likely to be familiar to the participant. We also considered the ability to use both brief and tonic stimulations, as well as stimulation from both the skin and deeper structures in our battery of tests as important, since different stimuli may be differently affected by the hormone status (Fillingim and Ness, 2000, Fillingim et al., 2009). Type of method as well as site and depth of stimulation (Giamberardino et al., 1997b) have been shown to influence the outcome. Moreover, Neziri et al. (2011b) have suggested that different types of stimuli represent different dimensions of pain, probably due to their activation of different kinds of afferents and pathways. They suggest the use of multimodal assessment instead of examining a single variable. One disadvantage inherent in contact methods such as thermotest and pressure algometry relates to the concomitant activation of other fibers than nociceptive fibers, which provide an inbuilt artifact in the method. This may be crucial when evaluating the effect of analgesics.

In some of the initial studies, several methodological problems were encountered. For example, many participants reached the cut-off levels in pain tolerance tests, which created difficulties in the statistical analysis. Therefore, some methodological improvements were made in designing the studies for Papers II and III. In Paper II, a computerized VAS was used in the cold pressor test, making it more independent of the cut-off limit. This set-up was also used in Paper III. The thermotest set-up was also changed. The operating range for the used version of the Thermotest equipment can be set between 0-52°C. However, while the apparatus displays a linear temperature fall of 1°C/s down to +10°C, it produces less accurate temperature changes below that point. Considering that, the cut-off for cold pain was in Papers I and IV set to 5°C, thus not using the full temperature range of the apparatus. However, because some participants reached the cut-off temperature, it was in Paper III changed to 2°C. Furthermore, when measuring heat pain thresholds, 10 stimuli were used in the study reported in Paper III instead of 4, in an attempt to detect any habituation or sensitization effects. These were evaluated by examining threshold changes throughout the session, however, no significant change was found. An increase in the temperature increase rate up to 3°C/s was also used in Paper III for the heat tolerance test to stimulate both the C and Aδfibers (Yeomans and Proudfit, 1996), of course with the risk that the measured thresholds were too high since the participant may not have responded fast enough, as pointed out by others (Yarnitsky and Ochoa, 1991, Yarnitsky and Sprecher, 1994). As mentioned in the Introduction, the use of pressure algometry may also generate overestimations because of the high speed with which the pressure is applied. Unfortunately, few studies presenting normative values exist (Jensen et al., 1986, Fischer, 1987, Nussbaum and Downes, 1998, Rolke et al., 2005) and the method is lacking standardization. In the present work the force rate was set to 100kPa/sec. A lower force rate might have yielded different result, especially in the subjects examined in Paper IV.

To induce a tonic pain sensation the choice fell on the cold pressor test, which rapidly produces a quick, severe, aching pain, generally tolerable for just a few minutes. The pain sensation is probably induced by a combination of stimulation of cold pain receptors in the skin and vascular reactions (Kreh et al., 1984). The response to the CPT has been further shown to divide participants into high and low responders, respectively (Chen et al., 1989). This observation, together with the demonstrated test re-test variations, as pointed out in Paper II, has raised some criticism towards the test. However, alternative methods to induce tonic pain with the same time duration are rare, the closest alternative probably being the ischemic tourniquet test (Smith et al., 1966). This test has been shown to be useful when evaluating the effect of gonadal hormones on pain (Fillingim et al., 1997), but, nevertheless, in our set of tests it would have been too time consuming to use, with a described tolerance time up to 20 minutes. The longer sessions may increase the risk for poorer performance of the participant due to loss of attention. As presented in the Introduction, several psychological factors may influence the outcome from the QST, such as attention and alertness as well as distraction.

Test re-test phenomenon due to repeated session design

All studies included in the present thesis were in one way or another based on a repeated sessions design, in which the participant is examined several times with the same standardized procedure (Vickers, 2003). With this design, based on within subject differences, some problems should be discussed. As mentioned in the Introduction, psychophysical measurements have been reported to generate test re-test bias. This is particularly important to take into consideration when there is no control group, as in Paper I. The test re-test phenomenon, defined as gradual change of the outcome in the same direction throughout the sessions is dependent on several factors that may influence the results and limit the validity of the study. An important factor is the learning or training effect connected to the situation and the method used, with the actual performance being influenced by the previous session. Another factor is the time span between the sessions.

Session-to-session effects were obvious in Paper II. It was therefore included as one factor in the statistical analysis, the GLM-model (Cnaan et al., 1997), which provided tools to handle the between and within subject data together with nested data as well as covariates. A trend towards session-to session effects was also seen in Papers I and III. In paper I we expanded the analysis and included session as a covariate to account for this phenomenon, and in paper III, we used two groups of controls that displayed stabile hormonal levels over time to evaluate interaction between group and session in the ANOVA, to determine session-to-session effects.

Tendencies to session-to-session effects are also seen in studies by others, such as Teepker et al. (2010), using similar set-up for different stimuli, e.g. pressure, electric and heat pain thresholds. Studies evaluating the test re-test phenomenon according to the *methods of limits* show in general a small variation between sessions and report that cold pain thresholds vary more than heat pain thresholds, but within acceptable limits for clinical comparisons (Wasner et al., 2008, Sand et al., 2010, Moloney et al., 2011, Wylde et al., 2011).

To study hormonal influence

The body of evidence showing hormonal interaction on the pain system is enormous but for the most part built on studies in animals. Work in this laboratory by Amandusson et al. (Amandusson et al., 1995) described estrogen receptors in the superficial spinal dorsal horn, and showed that these receptors were expressed by enkephalinergic neurons (Amandusson et al., 1996), and that administration of estradiol increased the expression of enkephalin mRNA (Amandusson et al., 1999), hence suggesting that estrogen could influence pain processing in the spinal cord. Others (Evrard, 2006) found a local aromatase production in the spinal dorsal horn and suggested a regulatory function by locally produced estrogens on sensory information. Estrogen receptors have also been found in the dorsal root ganglion cells (Chaban et al., 2003, Ma et al., 2005, Papka et al., 2001), with the function to regulate the activity of ATP on the P2X receptor and thereby modulate pain transmission. Estrogens also affect the dorsal horn ganglion cells (Sarajari and Oblinger, 2010), by modulating the production of substance P, thereby being antinociceptive. However, contradictory data also exist. Thus, estrogens have been shown to alter NMDA receptor activity and to be pro-nociceptive in the spinal cord, which may be one of the mechanisms behind reported sex differences for visceral pain (Tang et al., 2008, Ji et al., 2011, Mayer et al., 1999). Moreover, estrogens have been shown to act also on supraspinal levels associated with nociceptive processing, such as the PAG (Laflamme et al., 1998, Vanderhorst et al., 2002, McEwen, 2002, Smith et al., 2006). The use of knock-out animals (Multon et al., 2005) has shown that estrogen deficiency increases pain in some models, and gonadectomy increases pain sensitivity (Pajot et al., 2003), whereas estrogen substitution to gonadectomized rats reduces induced pain (Mannino et al., 2007).

The possibility to study the hormonal influence on pain is more limited in humans than in animals. One way is to use the hormonal fluctuations throughout the menstrual cycle, a method also used in animal studies, in which hormonal fluctuations throughout the so called estrous cycle have shown to influence the reaction to painful stimuli (Martínez-Gómez et al., 1994, Giamberardino et al., 1997a, Vincler et al., 2001). However a direct translation of the results from animals to humans is not possible: The estrous cycle of

rodents is much shorter and consists of the phases metestrus-diestrus-proestrus-estrus (Fillingim and Ness, 2000), including one single peak of estradiol in the proestrus phase, directly followed by a peak for progesterone, which thus differs from the hormonal pattern during the human ovulatory cycle.

As presented in Papers I and II, it was found that the hormonal levels throughout the menstrual cycle fluctuated as expected, but there were large differences between cycles both within and between subjects. Nevertheless, to follow menstrual cycle fluctuations is the most common way to study hormonal influence on the sensation of pain in humans. However, the use of the calendar to deduce cycle phase involves considerable uncertainty. Fehring et al. (2006) reported a within subject cycle variability of more than 7 days in 42 % of a sample of 165 women and concluded that a variation in cycle length between 7-14 days is not unusual. These observations may help explain the differences in results on pain sensitivity during different menstrual phases that do exist in the literature (e.g. Rao et al., 1987, Amodei and Nelson-Gray, 1989, Fillingim et al., 1997, Cimino et al., 2000, Kowalczyk et al., 2006, Ring et al., 2009, Klatzkin et al., 2010, Teepker et al., 2010).

Studying changes in pain sensitivity during the menstrual cycle, we obtained different results in Papers I and II. The difference may be explained by different study design as well as the method of analysis. Our data point to the need for further studies using other tonic stimuli in addition to the cold pressor test, such as tonic heat (Naert et al., 2008) or an ischemic pain (Fillingim et al. (1997).

Another model for studying hormonal effects on pain is to study women undergoing in vitro fertilization, a treatment regime connected with large variation in estradiol levels, which in turn may mimic studies performed in animals using supra physiological doses of estradiol. To our knowledge, in addition to the present work (Paper III), only two previous studies have examined pain sensitivity in women treated with IVF (Tsen et al., 2001, Nisenblat et al. 2010). However, they differ in the tests that were used. The study by Tsen et al. (2001) measured pressure pain thresholds and used a modified cold pressor test with parameters similar to the activation time recorded by us in papers II

and III. The study by Nisenblat et al. (2010) only used the thermotest method for heat pain. The overall result from the two previous studies points to a pronociceptive effect of estrogens, rather than an antinociceptive effect. While we observed a similar pronociceptive effect, with reduced threshold for cold pain when estrogen levels were high, our data suggests that this effect was a session-to-session effect rather than an effect of the changing levels of estrogens. Neither Tsen et al. (2001) nor Nisenblat et al. (2010) measured this effect.

It is important to point out that in Paper III only the estradiol level, and not the progesterone level changed between test sessions. The role of progesterone on the sensation of pain is far less well investigated than that of estrogens. During pregnancy the phenomenon pregnancy induced analgesia (PIA) occurs, with an elevation of pain thresholds and tolerance preceding the forthcoming labour (Gintzler and Bohan, 1990, Carvalho et al., 2006). The PIA, which is supposed to be elicited by the elevated estradiol and progesterone levels, has been shown to be naloxone sensitive (Gintzler and Bohan, 1990), hence involving the endogenous opioidergic system. Interestingly, neither estradiol nor progesterone alone was sufficient to produce PIA, but increased levels of both hormones were needed (Dawson-Basoa and Gintzler, 1993). While progesterone may affect GABA-A receptors through its metabolite allopregnanolone, it also influences progesterone receptors that have been found in similar areas as estrogen receptors (Kastrup et al., 1999, Labombarda et al., 2010). A direct effect of progesterone on pain processing by the modulation of GABAergic neurons in the PAG has been demonstrated (Lovick and Devall, 2009), suggesting that progesterone is a component that sets the tune in the descending pain inhibitory system. Moreover, progesterone may influence nociceptive transmission by dorsal root ganglion cells by modulating the P2X3 receptor (Fan et al., 2011). Also at other sites, progesterone may influence GABAergic neurons, and GABA may act together with opioids in controlling neural excitability, including that important for nociceptive transmission (Melcangic and Bowery, 1996, Zinder and Dar, 1999).

As described above, the use of menstrual cycle fluctuations, as well as the use of the huge variations in estradiol levels during IVF-treatment, are two ways to study

hormonal influence on the sensation of pain in humans. A common method in animal research is hormonal substitution treatment following castration. However, substitution treatment to study the effect of pain sensitivity in human is not common and is subject to several legal restrictions. To the best of our knowledge, the study reported in Paper IV is the first one that used an experimental method to examine the effect of hormonal substitution treatment on the sensation of pain in women with fibromyalgia. In previous studies in which the effect of substitution treatment has been examined, as in the studies by Wise (2000), and Fillingim and Edwards (2001), participating women were on treatment when the studied started, and the data on the variables that were studied were compared with those obtained from a non-treated control group of women or from men.

Hormonal replacement therapy has long been used for the alleviation of menopausal symptoms such as hot flushes, and has become common during the last decades of the 20^{th} century (Nelson et al., 2002). These symptoms have been connected to a neuroendocrine event, whereby estrogens influence β -endorphin (Nappi et al., 1990, Spencer et al., 1990). For a considerable time, the beneficial role of estrogens was far more often discussed than were the potential risks involved, as evidenced by the citation below (Maddison, 1973):

"If the slide shows poor cornification and the patient has symptoms and signs of oestrogen deficiency she needs replacement therapy. I use natural conjugated oestrogens, since there are practically no side-effects with this preparation. The cycle can be either 4 weeks or (for older women) 5 weeks. For the first 14 days in the 4-week cycle, or 21 days in the 5-week cycle, the patient takes one conjugated oestrogen tablet, either 0-625 mg. or 1-25 mg. or 2-5 mg.; for the next 7 days she takes the same plus a progesterone tablet-e.g., norethisterone acetate 2-5 mg. ('Norlutin A'); and then for the next 7 days either nothing or a 0-625 mg. tablet of conjugated oestrogen, during which time she will have a withdrawal bleeding. With this regimen women past the menopause maintain a satisfactory level of oestrogen and progesterone and obtain great benefit from the substitution therapy. In many cases therapy has been continuous over 10 years. More and more women today will be coming forward to their doctors for this treatment"

However, a growing mass of evidence has challenged the described benefits connected to the treatment, and two studies in particular seem to have influenced the view of hormone replacement therapy more than others, the WHI (Women's Health Initiative Study) (Rossouw et al., 2002) and HERS (Heart and Estrogen/progestin Replacement Study) (Hulley et al., 1998), respectively. These have led to modifications in prescriptive guidelines and furthermore to changed attitudes toward treatment in the general population (Hoffmann et al., 2005, Lindh-Åstrand et al., 2007).

While we in the present study found no effect of estrogen substitution either on experimental pain or spontaneous pain among the participants, estrogen substitution has shown various effects in animals. Thus, both pro-nociceptive (Tang et al., 2008, Ji et al., 2011) and anti-nociceptive effects (Liuzzi et al., 1999, Claiborne et al., 2006, Thompson et al., 2008) have been reported, and these differences seem to be related to treatment length, with anti-nociceptive effects seen after longer treatment time (Liuzzi et al., 1999). However, contrasting data also exist. Thus, it has been suggested that the anti-nociceptive effect of estrogen substitution is exerted through the serotoninergic system, but that because of down-regulation of estrogen receptors, this effect declines over time (Ito et al., 2004).

Estrogen substitution treatment in humans, in turn, has been associated both with increased risks for low back pain problems (Brynhildsen et al., 1998, Wijnhoven et al., 2006), as well as the alleviation of similar conditions (Kyllönen et al., 1999). Aloisi et al. (2007) examined a group of transsexual women and men who received hormonal treatment before sex change operation, and reported that the occurrence of pain changed in a high percentage of the subjects. Moreover, case studies in which aromatase inhibitors have been used, a treatment regime associated with estrogen depletion, report elevated pain (Nemitz et al., 2008, Bertolini et al., 2011). Furthermore, treatment with GnRH-agonists (as used in Paper III) for endometriosis was reported to elicit fibromyalgia-like symptoms (Toussirot and Wendling, 2001). Analogous effects are described in women who terminate their substitution treatment. They have been reported to display increased musculoskeletal stiffness and bodily pain (Ockene et al., 2005).

About the terms sex and gender

The terms sex and gender, respectively, have generally been used in the present thesis as synonyms that reflect the biological sex in different ways, which in turn is one of several ways to handle the terminology (Pryzgoda and Chrisler, 2000, Diamond, 2002). As pointed out by Torgrimson and Minson (2005), the use of the word gender has increased tremendously in the literature during the past years. To study a biological event such as hormonal influence may refer more to sex rather than gender if one wants to differentiate the meanings of the words, with the latter referring more to behavior and expectations or to an identity, a discussion outside the scope of the present work. However, to evaluate sex and gender differences was not the aim of the present thesis despite the use of men as control group in Papers II and III. The studies were not designed for that purpose. Yet, the results may need a comment. In a comparison between men and women, it was above all the tolerance threshold with the subsequent subjective rating that differed, while pain thresholds were quite similar. However, the fact that a gaunt woman tolerated more than a former military attack diver, as was seen in the present work, points to the possibility that individual differences with regard to pain are substantial, and cross over the sex and gender limits.

However, it is important to have the context in mind. The results were obtained in an experimental setting and may therefore not be representative for the clinical situation, in which women are found to be more vulnerable to chronic painful conditions than men. This in turn is one part of the health paradox (Hammarstrom and Hovelius, 1994) between the sexes. Despite the higher morbidity in women they have a longer life span than men. During the past few years, several explanations trying to solve the paradox have been presented, including the view on the influence of estrogens, which has gone from being universally good for everything to a more balanced view today.

Perspectives

The foci of the present thesis were the involvement and influence of gonadal hormones on the sensation of pain. Estradiol and progesterone influence several neurotransmitters, and thereby functions, and their role for endogenous opioids and GABAergic mechanisms may open for new interventions. However, to label one or another hormone as either pro- or anti-nociceptive seems to be a simplification. The results from studies on gonadal hormones, and sex and gender differences, are often met with criticism because of misconceptions favoring one sex over another as being e.g. less pain sensitive or more heroic. Yet, the major point is not to make comparisons, but rather to focus on creating a deeper understanding about differences and thereby hopefully improving individualized treatment regimes in the future.

CONCLUSIONS

Both estrogens and progesterone are involved in the nervous system functions, including areas and pathways for pain transmission, and a regulatory function on pain sensitivity has been demonstrated in animals. In the present thesis, I examined the role of gonadal hormones on the sensation of pain in women. The results showed that most measures of pain sensitivity varied little across the menstrual cycle, but that in a tonic pain test there were decreased pain threshold and higher pain ratings with increasing progesterone concentrations, an effect that was attenuated with increasing levels of 17β-estradiol. Changes of estrogen levels alone, and even with the extreme variations that are associated with IVF-treatment, had little effect on pain sensitivity. Also, there was no effect of transdermal estrogen treatment on pain thresholds and tolerance in postmenopausal women suffering from fibromyalgia. Session-to-session effects were observed in several studies and seem to be an important factor in studies using a repeated sessions design. In addition, the present work also emphasizes the need to control actual hormonal values when evaluating variation of pain sensitivity across the menstrual cycle.

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