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Significance:

This review present collected data from published studies to report what is currently known of the temporal dynamics of DNA methylation in pain conditions. It provides a novel direction for pain research within the field of DNA methylations, suggesting potential approaches for future research and novel DNA methylation modulating treatments in the field of pain.

Time course of DNA methylation in pain conditions: from experimental models to humans

Abstract

Background and Objective: Throughout the last decade, research has uncovered associations between pain and epigenetic alterations caused by environmental factors. Specifically, studies have demonstrated correlations between pain conditions and altered DNA methylation patterns. Thus, DNA methylation has been revealed as a possible modulator or contributor to pain conditions, providing a potential therapeutic target for treatment by DNA methylation modification. To develop such treatments, it is necessary to clarify a wide number of aspects on how DNA methylation affects pain perception; first and foremost, the temporal dynamics. The objective of the present review is to provide an overview of current knowledge on temporal dynamics of DNA methylation in response to pain, and to investigate if a time frame can be established based on the data of currently published studies.

Databases and Data Treatment: PubMed, MEDLINE, Google Scholar and Embase were searched comprehensively for studies of DNA methylation in neuropathic, inflammatory and alternative animal pain models, and in chronic pain patients including Complex Regional Pain Syndrome, chronic postsurgical pain, chronic widespread pain, fibromyalgia and Crohn's disease.

Results: We identified 34 articles highlighting variations in temporal dynamics of DNA methylation across species and between different types of pain. These studies represent a starting point to uncover new insights in the DNA methylation time course in pain.

Conclusions*: N*o time frame can currently be made for the DNA methylation response to pain in any of the reviewed conditions, highlighting an important focus area for future research.

Key words: DNA methylation, temporal dynamics, DNA methylation over time, DNA methyltransferases, Pain, Chronic pain, Epigenetics.

1. Introduction

Chronic pain affects a great proportion of the population worldwide (Massart et al., 2016; Oliveira et al., 2019), posing major health problems affecting the quality of life of the individual (Denk and Mcmahon, 2012; Descalzi et al., 2015; Shao et al., 2017) and leading to significant socioeconomic costs (Denk and Mcmahon, 2012; Descalzi et al., 2015; Shao et al., 2017). The current, revised International Association for the Study of Pain (IASP) definition of pain is: "An

unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage"(Raja et al., 2020). This current definition of pain accentuates that pain is not only a result of tissue damage, but is also an individual and personal experience, influenced by many other potential factors. In recent years, it has become evident that environmental factors may influence the trajectory of pain and the development of acute pain into a disabling chronic condition (Bell et al., 2014). Research on the modulating function of environmental factors on the pain system has recently evolved, and accumulating evidence suggests epigenetic modifications to be one of these modulators (Bell et al., 2014; Crow et al., 2013; Denk and Mcmahon, 2012; Tajerian et al., 2013).

An epigenetic mechanism known to regulate gene expression is DNA methylation (Jaenisch and Bird, 2003; Tochiki et al., 2012). DNA methylation modifications predominantly occur at CpG sites of double-stranded DNA by addition of a methyl group to the fifth carbon of cytosines, generating 5-methylcysotine (5mC) (Bell et al., 2014; Greenberg and Bourc'his, 2019; Michalak et al., 2019). This process is catalyzed by the DNA methyltransferases (DNMTs): DNMT1, DNMT3A and DNMT3b, which establish and maintain the genomic methylation patterns (Michalak et al., 2019). 70-80% of CpGs in the mammalian genome are methylated (Greenberg and Bourc'his, 2019), and DNA methylation is known to be a fundamental mechanism for stable gene repression, genomic stability and processes such as X chromosome inactivation in embryonic development and imprinting (Bell et al., 2014; Greenberg and Bourc'his, 2019; Michalak et al., 2019).

While other epigenetic mechanisms, such as histone methylation and acetylation, mediate fast epigenetic changes, DNA methylation changes are known to be less dynamic, while more stable, constituting long-lasting changes (Kiese et al., 2017; Mews et al., 2014). Interestingly, 5-hydroxymethylcytosine (5hmC), a novel epigenetic mark positively correlated with gene expression and produced by hydroxylation of 5-methylcytosine (Pastor et al., 2013), has recently also been reported present within the somatosensory system, suggesting a potential role in chronic pain development (Chamessian et al., 2017).

These observations make DNA methylation especially interesting to investigate in relation to long-lasting or chronic conditions. DNA methylation alterations are also known to be reversible (Tajerian et al., 2013; Weaver et al., 2004), opening up the possibility of using DNA methylation or demethylation as targets for therapeutic treatment. This is already considered a promising strategy for treatment of different types of diseases, particularly in a variety of cancers (Cheng et al., 2019). Reversing DNA methylation has also been demonstrated to correspond with amelioration of pain, suggesting DNA methylation as a highly promising target for therapeutic treatment of chronic pain (Tajerian et al., 2013).

To develop such a treatment, it is necessary to clarify a substantial number of aspects on how DNA methylation affects pain perception. In other complex multifactorial conditions, studies have investigated DNA methylation rates over time. In cancer, which is characterized by aberrant epigenetic changes, this has been useful in reconstruction of tumor evolution (Kim and Costello, 2017). In the field of pain, DNA methylation studies have explored epigenetics in a range of chronic pain conditions (Bruehl et al., 2019; Ciampi de Andrade et al., 2017; Takenaka et al., 2020), providing accumulating evidence of DNA methylation alterations being functionally relevant to pain mechanisms and nociceptive sensitization (Bell et al., 2014; Gombert et al., 2017). However, these studies often only investigate DNA methylation at one time point, and the temporal dynamics of DNA methylation in pain conditions thereby remain undefined. In order to therapeutically target DNA methylation, it is important to consider the time course; such as when to expect an epigenetic response after induction of acute or long-lasting pain and when the DNA methylation states possibly revert.

The aim of this review was to present collected data from published studies to report what is currently known of the temporal dynamics of DNA methylation in response to pain. We also highlight recent findings linking DNA methylation alterations to pain and discuss potential approaches for novel DNA methylation modulating treatments in the field of pain.

2. Methods

A comprehensive search of peer-reviewed articles was completed in order to achieve an outline of the temporal dynamics of DNA methylation in response to painful stimuli. The literature search was based on the following search terms: *DNA methylation, methylation, DNA methyltransferase, pain* and *pain model*. The searches were carried out in the following electronic databases: PubMed, MEDLINE, Google Scholar and Embase, considering publications up to February 2020. The search was limited to publications in English language. A preliminary screening of the articles was completed based on their headline and abstract to ensure their relevance. This was followed by a thorough screening based on inclusion criteria and quality assessment. Additionally, references of already retrieved articles were searched to identify potentially relevant studies which did not appear in the traditional search.

The included studies were primary studies on DNA methylation in animal pain models with specified time points of investigation and human studies of DNA methylation in pain conditions. Only few human studies included a time-related aspect of DNA methylation, and for this reason several human studies were included without the time aspect to represent how DNA methylation has been studied in human subjects with different chronic pain conditions.

3. Results

The research process identified 34 peer-reviewed articles, published from year 2011 up until February 2020 to be discussed in the present review. The articles include studies of DNA methylation in neuropathic, inflammatory and alternative pain animal models as well as human studies of subjects with chronic pain including complex regional pain syndrome (CRPS), chronic postsurgical pain (CPSP), chronic widespread pain (CWP), fibromyalgia and Crohn's disease.

3.1. Dynamics of DNA methylation

3.1.1 Dynamics of DNA methylation during a lifetime

Accumulating evidence in research has demonstrated that genome-wide DNA methylation levels change within an individual in the course of the lifetime (Bocklandt et al., 2011; Hannum et al., 2013; Horvath and Raj, 2018). Especially within the first 5 years of life, the methylome has shown to undergo substantial changes of which certain loci continue to manifest persistent early-life DNA methylation changes (Perez et al., 2019). In particular, two main epigenetic reprogramming events remodel DNA methylation in mammalian genomes; first event occurring in the developing germ line and the second event in the pre-implantation embryos. DNA methylation in primordial germ cells (PGCs) is erased during early to mid-gestation in utero and then undergoes re-methylation at different time points in a sex-specific manner (Radford, 2018; Sasaki and Matsui, 2008). Dissimilarly, epigenetic reprogramming during early embryogenesis determines the erasure of most DNA methylation marks inherited from the gametes in order to acquire totipotency and subsequently re-established DNA methylation patterns in lineage-specific regions upon implantation (Radford, 2018; Xu et al., 2020).

Millions of CpG dinucleotides in the human genome have been observed to change with age, also known as *clock CpGs* (Bocklandt et al., 2011; Hannum et al., 2013; Horvath and Raj, 2018). When coupling clock CpGs with specific mathematical algorithms, they can be used to estimate the age of DNA sources. Hence, human DNA methylation data can serve as reliable age estimators, *epigenetic clocks*, and as mortality and lifespan predictors (Hannum et al., 2013; Horvath and Raj, 2018; Michalak et al., 2019). Several models have been developed to measure methylome age, including single and multiple tissue DNA-based age estimators (Bocklandt et al., 2011; Hannum et al., 2013; Horvath and Raj, 2018). These models have established that the rate of methylome aging is influenced by several congenital factors, among others gender and genetic variants, while providing indications of environmental stimuli affecting the methylome (Hannum et al., 2013; Horvath and Raj, 2018). Within the recent years, research has been particularly focused on studying sequences with allele-specific DNA methylation (ASM),

occurring not only when there is an allelic variance, but also on certain loci in which the alleles are identical. A complex interaction between genetic variants and ASM has been revealed, however further studies are required to elucidate how the present crosstalk contribute to human disease susceptibility (Wang et al., 2019a).

3.1.2. Dynamics of DNA methylation in response to environmental stimuli

Monozygotic (MZ) twin studies have demonstrated that even though twins are epigenetically similar at a young age, older MZ twins exhibit substantial genomic differences as well as difference in the overall content of 5mC DNA (Boks et al., 2009; Fraga et al., 2005; Martin, 2005). For instance, studies of MZ twins have demonstrated differences in DNA methylation levels of the *TRPA1* gene promoter, which were strongly associated with differences in heat and pressure pain thresholds (Bell et al., 2014; Gombert et al., 2017). This phenomenon of individual-specific, age-related change in the epigenetic pattern is referred to as *epigenetic drift* (Fraga et al., 2005; Hannum et al., 2013; Jaenisch and Bird, 2003; Martin, 2005), which is suspected to be a result of environmental factors and stochastic changes (Bjornsson et al., 2004; Fraga et al., 2005; Jaenisch and Bird, 2003). The assumption that the environment is partly the cause of epigenetic drift is supported by Fraga and coworkers (2005), who found a more distinct difference in epigenetic markers in older MZ twins who had different lifestyles and had spent less of their lives together (Fraga et al., 2005). Studies have found DNA methylation patterns to be affected by many external factors, such as diet (Cooney et al., 2002; Ingrosso et al., 2003), tobacco (Volkow, 2011), alcohol (Gatta et al., 2019), drug abuse (Gerra et al., 2018), asbestos and metallotoxins (Bjornsson et al., 2004; Christensen et al., 2009). The effect of substance and drug abuse has also been established by thorough investigation (Cadet, 2016; Nestler and Lüscher, 2019); with longitudinal studies revealing an association between prenatal exposure and 5mC changes followed by a subsequent increased risk of adolescent substance abuse (Cecil et al., 2016).

Additionally, studies have found social environment and experiences throughout a lifetime to affect DNA methylation patterns, seeing that long-term 5mC alterations have been found in adults and elderly individuals who were subjected to childhood labor and trauma (Marinova et al., 2017; Suderman et al., 2012). Studies in rats have also demonstrated that maternal licking and grooming alter the DNA methylation status of the glucocorticoid receptor gene promoter, compared with offspring with absence of maternal care (Weaver et al., 2004). This correlation between rearing and 5mC alterations persisting to adulthood has also been observed in rhesus macaques in both prefrontal cortex and T cells (Provençal et al., 2012), while early life aggression and prenatal maternal stress have shown to leave stable 5mC signatures in human T

cells after years, particularly in genes involved in immune and inflammatory responses (Cao-Lei et al., 2014; Guillemin et al., 2014).

Growing evidence indicates that early life stressors have implications for the vulnerability to develop painful conditions later in life (Waller et al., 2020). In line with these findings, it has been shown that premature babies exposed to many painful procedures present marked neurodevelopment changes in the nervous system (Williams and Lascelles, 2020). Since anxiety, depression, childhood trauma and adult stressful life events are associated with increased risk of developing chronic pain states (Abdallah and Geha, 2017; Bayram and Erol, 2014), it is plausible that this increased risk is based on 5mC alterations caused by external stressors (Niederberger et al., 2017). However, due to the complexity of chronic pain conditions, more research is necessary to establish how environmental factors affect individual 5mC patterns consequently altering perception of pain and contributing to development of chronic pain conditions.

3.1.3. Epigenetic inheritance

Studies have demonstrated that epigenetic changes acquired in response to environmental exposures, such as early trauma (Gapp et al., 2014), diet behaviors (Dunn and Bale, 2011) and chronic stress (Crews et al., 2012), can be passed along from parents to the next generations. In addition, genetic studies have demonstrated that pain disorders appear to have a heritable component clustering within families. The odds of developing chronic pain states, such as fibromyalgia, are several times higher within families with fibromyalgia patients than in control families (Arnold et al., 2004). While genetic and environmental factors are considered to play a concurrent role in the establishment of ideal ground for chronic pain development, epigenetic inheritance has also been hypothesized to be a contributory factor (Alvarado et al., 2015; Tajerian et al., 2013). In particular, chronic pain has been associated with lasting changes in DNA methylation patterns for which reason it has been hypothesized that DNA methylation is partly responsible for pain chronicity and pain associated co-morbidities by alteration of gene expression (Alvarado et al., 2015).

Based on the current observations, it is very likely that a combination of inherited and acquired epigenetic marks shape the individual 5mC patterns, determining phenotype and consequently affecting the individual perception of pain. Unravelling these processes might provide insight into the mechanisms of chronic pain development and reveal possible therapeutic targets for treatments based on DNA methylation modulation.

3.2. Therapeutic targeting of DNA methylation

Epigenetics-based strategies for treatment of pain diseases are not yet available despite an increased focus on the topic (Niederberger et al., 2017). Research has already demonstrated the possibility of attenuating pain by DNA methylation modulation (Liu et al., 2020; Shao et al., 2017; Sun et al., 2015; Wang et al., 2011). At present, blockade of DNMTs has predominantly been investigated and it has been demonstrated to be the most effective way to prevent DNA hypermethylation (Cheng et al., 2019; Gnyszka et al., 2013). For this purpose, several DNA methylation inhibitors have been developed, including the nucleoside analogs azacitidine, decitabine and zebularine (Cheng et al., 2019; Gnyszka et al., 2013). Among them, azacitidine and decitabine have already been approved for treatment of certain types of cancers as they predominantly affect dividing cells (Cheng et al., 2019). Non-nucleoside analogs have also been developed to prevent aberrant DNA hypermethylation, such as RG108, which has been designed to cause demethylation by blocking DNMT1-activity (Cheng et al., 2019; Shao et al., 2017).

Though these agents show great promise as potential future treatments for pain conditions (Liu et al., 2020; Shao et al., 2017; Sun et al., 2015; Wang et al., 2011), downsides also exist. In particular, common side effects of nucleoside analogs include genomic instability and mutagenic risk. Non-nucleoside analogs do not present similar side effects; however, their efficacy is low compared to azacytidine (Cheng et al., 2019). Another disadvantage of current methylation targeting strategies is the lack of specificity and the risk of causing hypomethylation of the global genome (Cheng et al., 2019).

As proper regulation of DNA methylation is vital, it is necessary to obtain a better understanding of the DNA methylation landscape in chronic pain conditions to therapeutically target the aberrant DNA methylation patterns. Thus, to establish a methylation-targeting therapeutic treatment, the temporal dynamics of DNA methylation in response to pain must be established.

3.3. Temporal dynamics of DNA methylation in response to pain

3.3.1. In vitro studies

To investigate whether hyperexcitation of neurons would lead to upstream epigenetic modifications, an in vitro study by Kiese and coworkers (2017) evaluated histone and DNA methylation changes in cultured rat primary hippocampal neurons following glutamate treatment (Kiese et al., 2017). Increased levels of glutamate are known to play a key role in development of central sensitization, contributing to pain hypersensitivity in inflammatory and

neuropathic pain (Latremoliere and Woolf, 2009). Therefore, this model can be considered to imitate aspects observed in pain conditions. The study by Kiese and coworkers (2017) found histone methylation changes from 3 hours to 2 weeks following glutamate stimulation as well as changes in the DNA methylation pattern 4 weeks after glutamate stimulation (Kiese et al., 2017).

Interestingly, research has shown that histone methylation and DNA methylation are linked and that chromatin states are demonstrated to influence the DNA methylation machinery action to either methylate or fail to methylate a specific domain (Bird, 2002). In addition, histone modifications are not conceived to be stable enough to mediate long-term transcriptional changes, while DNA methylation patterns are considered to be long-lasting but less dynamic than histone modifications (Kiese et al., 2017). This could explain why early histone methylation changes were observed in the mentioned study, while a more long-term reaction was observed for DNA methylation following glutamate treatment.

3.3.2. In vivo studies

In search of potential biomarkers and novel treatment options for chronic pain diseases, clinical (Chidambaran et al., 2017, 2019) and animal studies (Pan et al., 2014; Wang et al., 2016) have been conducted investigating epigenetic changes in relation to pain. In animal models, this has mainly been investigated in mice or rats by induction of inflammatory pain through subcutaneous injection of Complete Freund's Adjuvant (CFA) or by induction of neuropathic pain through surgical injury such as chronic constriction injury (CCI), spinal nerve ligation (SNL) and spared nerve injury (SNI), followed by analyses of epigenetic changes (Descalzi et al., 2015; Garriga et al., 2018; Massart et al., 2016; Pan et al., 2014; Wang et al., 2016). In the present animal pain models, behavioral changes have been observed in response to tactile and thermal stimuli (Garriga et al., 2018; Massart et al., 2016; Oliveira et al., 2019), which can be compared with the change observed in chronic pain patients. Differentially methylated regions have been found in neuropathic and inflammatory pain models at global level and in specific gene promoters. Consistently, differential expression of the DNA methylation enzymes, DNMT3a, DNMT3b, Methyl CpG binding protein 2 (MeCP2) and DNMT1, in specific range of time and depending on the pain induction model, were evidenced, however with contrasting results. Interestingly, the observed epigenetic regulation involves mainly genes related to the transduction signal and glutamate, inflammatory and pain pathways. Additional studies on the identified gene networks might lead to a better understanding of the association between these specific patterns of co-expression and their regulation in regard to the pain processes timing, thereby providing insight into the pathology of chronic pain.

An overview of animal studies investigating DNA methylation changes following pain induction is illustrated in *Table 1* and described in the following paragraphs.

3.3.2.1. Neuropathic pain models

Based on the current studies, the general cohesiveness between findings from neuropathic pain models appears to be very limited while a general difference appears in time course patterns between rodent species.

Studies of DNA methylation changes in CCI neuropathic pain models have reported increased global DNA methylation and increased methylation of specific promoters; Glutamate decarboxylase 1 (GAD1) promotor and Mu opioid receptor gene (MOR) gene promotor, in rodent lumbar spinal cords 14 days following CCI surgery (Shao et al., 2017; Wang et al., 2011, 2016). In a study by Shao and coworkers (2017), an even higher increase in methylation was detected in the MOR promoter from mouse lumbar spinal cords at day 7 post CCI while no methylation changes were observed at day 1 (Shao et al., 2017). Coherent with the methylation patterns following CCI, studies show significant upregulations of DNMT3a and MeCP2 gene expression levels at day 14 following CCI, while downregulation of MBD2 levels (Wang et al., 2011, 2016; Zhao et al., 2017). Further, studies show upregulations of DNMT3a levels at day 3 to 14 after CCI surgery (Shao et al., 2017; Zhao et al., 2017), though these regulations could not be detected at day 1 or 2 (Shao et al., 2017; Zhao et al., 2017). Based on these findings, it is possible that methylation changes could be detectable as early as 3 days post CCI. However, it is still unclear if changes in methylation could be detected later than 14 days after CCI surgery.

Different from the CCI findings, studies on SNL neuropathic pain models have shown global DNA methylation changes in rat dorsal root ganglia (DRG) as soon as 24 hours and up to 3 weeks post SNL (Garriga et al., 2018; Gölzenleuchter et al., 2015), and specific methylation changes have also been found within this time range, including demethylation of the CXC motif chemokine receptor 3 (CXCR3) and G protein-coupled receptor 151 (GPR151) gene promotors, and increased methylation of the Potassium Voltage-Gated Channel Subfamily A Member 2 (KCNA2) gene promotor (Jiang et al., 2017, 2018; Zhao et al., 2017). This demonstrates a longer time span of DNA methylation changes following SNL as well as an earlier onset. Significant upregulations of DNMT3a in rat DRG have been observed at day 3 to 7 post SNL. However, this was followed by a normalization of the DNMT3a concentration at day 14 (Zhao et al., 2017). The same study found no change in DNMT3b expression within 14 days post SNL (Zhao et al., 2017), which is in contrast with findings in mouse lumbar spinal cords illustrating a decrease in DNMT3b expression from day 1 to 21 following SNL surgery (Jiang et al., 2017, 2018). Additionally, no changes in DNMT1 and DNMT3a expression were found in mouse lumbar

spinal cords at day 10 post SNL (Jiang et al., 2017) implying a difference in the epigenetic response between the two species.

Fitting into the timeframe of CCI and SNL induced pain methylation patterns, findings from an SNI neuropathic pain model have demonstrated specific methylation of the glutamate ionotropic receptor NMDA Type Subunit 2B (GRIN2B) gene promotor in mouse spinal dorsal horn at day 7 following SNI surgery (Wang et al., 2019b). Studies have also investigated global DNA methylation patterns in rodent prefrontal cortex (PFC), T-cells and amygdala, respectively, and found the methylation patterns to be regulated at 6 months and 9 months following SNI surgery (Massart et al., 2016; Tajerian et al., 2013), revealing a long methylation time-span in these tissues and cells. In contrast, mouse visual cortex and thalamus were investigated 6 months after SNI, but no changes in global DNA methylation patterns were observed (Tajerian et al., 2013).

Similar to SNL studies, findings on regulation of DNMT3b following SNI appear contradictory. A study by Pollema-Mays and coworkers (2014) found increased expression of DNMT3b in rat DRG from day 7 to 28 following SNI surgery (Pollema-Mays et al., 2014), while another study by Tochiki and coworkers (2012) found no alterations of DNMT3b in rat superficial dorsal horn (Tochiki et al., 2012). Furthermore, the DNMT1 expression has been found to be downregulated at day 7 by Tochiki and coworkers (2012), while Pollema-Mays and coworkers (2014) found no regulation at day 7, while an upregulation of DNMT1 at day 14 following SNI (Pollema-Mays et al., 2014; Tochiki et al., 2012). The study by Pollema-Mays and coworkers (2014) also found upregulation of DNMT3a at day 14 following SNI (Pollema-Mays et al., 2014), while Tochiki and coworkers (2012) found no alterations at day 7 (Tochiki et al., 2012). However, these findings are not necessarily contradictory if the onset of alterations were later than day 7 following SNI surgery. Finally, significant downregulations of MeCP2 expression have been observed on day 7 (Tochiki et al., 2012), possibly supporting the findings of DNA methylation changes in mouse spinal dorsal horn 7 days following SNI.

Ultimately, at this stage limited information is available on the actual onset and permanence of DNA methylation in neuropathic pain models with respect to the development and onset of the neuropathic nociceptive behaviors.

3.3.2.2. Inflammatory pain models

In CFA-induced inflammatory pain models, DNA methylation changes have been detected within specific promoters in rodent DRG on day 3 and day 6 following CFA injection, including demethylation of the CXC motif chemokine receptor 4 (CXCR4) and Cystathionine Beta-Synthase (CBS) gene promotors, and increased methylation of the miRNA-2019 promotor (Li et al., 2018;

Pan et al., 2014; Qi et al., 2012), proving an early onset of epigenetic changes in response to pain similar to what was found in neuropathic SNL models. Moreover, a study by Tochiki and coworkers (2012) observed upregulations of DNMT3b, DNMT3a2, DNMT1 and MeCP2 levels in rat superficial dorsal horn at day 7 following CFA injection (Tochiki et al., 2012), which could indicate a methylation process lasting for longer than the detected 6 days.

Though upregulation of DNMT3b levels were found at day 7, studies have shown contradictory findings on day 3 following CFA injection in rats; Feng Li and coworkers (2018) found a significant decrease in DRG on day 3 (Li et al., 2018) and Qi and coworkers (2012) found no altered expression of DNMT3b on day 3 following CFA injection (Qi et al., 2012). However, the studies agree that no alterations of DNMT3a expression exist at day 3 following CFA injection (Li et al., 2018; Qi et al., 2012). Another study by Oliveira and coworkers (2019) found a significantly increased expression of DNMT3a2 in mouse DRG 6 hours after CFA injection with no detectable increase at day 1 or day 2 following the injection (Oliveira et al., 2019).

Similar to the findings from the neuropathic pain models, limited information is available at this stage on the actual onset and permanence of DNA methylation in inflammatory pain models with respect to the development and onset of the inflammatory nociceptive behaviors.

3.3.2.3. Alternative pain models

Some studies have investigated DNA methylation through alternative pain models. Sun and coworkers (2015) investigated DNA methylation patterns in incisional pain by making incisions into hind paws of mice (Sun et al., 2015). Through this model they found increased global DNA methylation in the skin surrounding the incision at day 1 and 3 after incision, while DNMT3b expression was increased 6 hours after incision (Sun et al., 2015). However, no change in DNMT3b expression was found at 24 hours and 72 hours after incision, neither in the spinal cord nor in the DRG (Sun et al., 2015). Moreover, no changes in DNMT1 or DNMT3a expression were detected at any of the investigated time points in either skin, spinal cord or DRG (Sun et al., 2015).

A study by Mao and coworkers (2019) also investigated DNA methylation regulators in a paclitaxel-induced neuropathic pain model, in which they found a significantly increased DNMT3a expression in rat DRG at day 7 and 14 following the first injection of paclitaxel. No increase was found on day 21 (Mao et al., 2019). Further, this study did not find significant changes in DNMT1 and DNMT3b at any of the three time points (Mao et al., 2019).

Overall, the presented findings from animal pain models include information on DNA methylation changes in response to pain providing indications of DNA methylation patterns

within different types of pain. The findings also show possible variances in DNA methylation patterns between types of pain and between species. However, no conclusions can be made with regard to the time frame for any of the pain induction animal models.

3.3.2.4. Reverting DNA methylation modifications in animal studies

Although animals are known to recover from traumatic injuries in nature, studies of recovery in experimental chronic settings are difficult to execute. However, models of reverted nociceptive injury are available for study (Dableh et al., 2011), and hence the dynamics of DNA methylation changes and their possible reversal can be analyzed. Studying the possible reversal of certain epigenetic modifications in more detail could be a focal point for future research.

3.3.2.5. Studies in healthy human subjects

Several MZ twin studies have investigated the associations between variations in DNA methylation and pain sensitivity. For instance, a study by Bell and coworkers (2014) observed a strong association between DNA methylation levels and pain sensitivity scores finding differential methylation of the pain gene *TRPA1* and other potential pain gene candidates in individuals with low heat pain thresholds (Bell et al., 2014). These findings were supported by Gombert and coworkers (2017), who found hypermethylation of *TRPA1* CpG sites in subjects with low threshold for pressure pain (Gombert et al., 2017). Collectively, these findings show correlations between pain sensitivity and altered methylation levels even in healthy subjects.

3.3.2.6. Clinical studies

The correlation between DNA methylation changes and pain in human subjects has been studied in several chronic pain conditions such as CRPS, chronic postsurgical pain (CPSP), chronic widespread pain (CWP), fibromyalgia and Crohn's disease. Notably, in the majority of these studies, DNA methylation levels were only evaluated at one time-point. A brief overview of these clinical studies is illustrated in *Table 2*.

In some studies, DNA methylation levels were assessed prior to pain induction with the aim of finding epigenetic indicators of disease. In particular, Chidambaran and coworkers (2017;2019) investigated DNA methylation levels in peripheral blood samples acquired prior to spinal surgery with the aim of finding epigenetic predictors of CPSP (Chidambaran et al., 2017, 2019). These studies found DNA methylation of *OPRM1* CpG sites to be associated with CPSP

along with correlations between DNA methylation patterns in CPSP and child anxiety sensitivity (Chidambaran et al., 2017, 2019).

Numerous other studies have investigated DNA methylation patterns during pain conditions such as a study by Takenaka and coworkers (2020), who investigated DNA methylation levels from blood samples of chronic pain patients and preoperative patients comprising a diverse group of subjects with neuropathic pain, non-neuropathic pain, chronic low back pain and no pain, respectively (Takenaka et al., 2020). When dividing these subjects into groups based on DN4 (Douleur Neuropathique 4 questionnaire) pain sensitivity scores, Takenaka and coworkers (2020) found a likely association between DNA methylation rates of the promoter region of the *TRPA1* gene and DN4 pain scores (Takenaka et al., 2020). Studies by Sukenaga and coworkers (2016) and Gombert and coworkers (2020) reported similar findings (Gombert et al., 2020; Sukenaga et al., 2016). These studies found *TRPA1* methylation levels in whole blood samples of chronic pain patients and patients suffering from Crohn's disease to correlate with pain symptoms and low pressure and mechanical pain thresholds, respectively (Gombert et al., 2020; Sukenaga et al., 2016). Based on previous *TRPA1* methylation studies, Achenbach and coworkers (2019) also investigated the methylation levels of the *TRPA1* promoter region of patients with painful multi-somatoform disorder (MSD) and healthy volunteers in relation to childhood trauma. An association was found between DNA methylation patterns and mechanical pain sensitivity in healthy subjects exposed to childhood trauma. Further, significantly higher methylation rates were found in MSD patients with severe childhood trauma when compared with MSD patients with no trauma (Achenbach et al., 2019).

DNA methylation patterns during pain conditions were also investigated by Ciampi de Andrade and coworkers (2017), Menzies and coworkers (2013) and Gerra and coworkers (manuscript under revision, 2020) revealing alterations of DNA methylation patterns in blood samples from fibromyalgia patients (Ciampi de Andrade et al., 2017; Menzies et al., 2013). Similarly, significant differentially methylated regions in several genes, such as *RE1-silencing transcription factor* (*REST*), *Monoamine oxidase B* (*MAOB*) and *collagen type I, alpha 2 chain* (*COL1A2*) were identified analyzing blood samples from CWP patients (Burri et al., 2016). A characteristic DNA methylation landscape of differentially methylated genes was also found in Crohn's Disease patients by Li Yim and coworkers (2016)(Li Yim et al., 2016). Several of these differentially methylated genes are known to be implicated in immune response processes and inflammatory pathways (Li Yim et al., 2016).

While some studies evaluated DNA methylation patterns prior to surgery or at a random time point during chronic pain conditions, a study by Bruehl and coworkers (2019) investigated DNA methylation profiles in patients who had experienced military trauma and subsequently undergone post-traumatic amputation surgery (Bruehl et al., 2019). From whole blood

extracted within a period of 3-18 months post traumatic injury, differentially methylated CpG sites were found between subjects who developed CRPS and non-CRPS neuropathic pain patients (Bruehl et al., 2019). Unfortunately, only few human studies have investigated methylation changes at specific time points as well as over time in response to different pain input. However, a study by Livshits and coworkers (2017) investigated the association between DNA methylation levels and CWP affection status in a longitudinal study of MZ twin pairs with and without CWP as well as in non-related individuals (Livshits et al., 2017). Through repeated DNA methylation measurements conducted ≥3 years apart, changes were identified in methylation levels in CWP-affected subjects compared with healthy subjects, with methylation changes closely associated with genes such as *IL17A*, *ADIPOR2*, and *TNFRSF13B* (Livshits et al., 2017).

3.3.2.7. Reverting DNA methylation modifications in human studies

Until now, no longitudinal studies have investigated the possible reversion of DNA methylation modifications in neither human experimental pain nor clinical studies. In case short-lasting experimental painful provocations can trigger DNA methylation modifications, this would be an interesting model to use for studying the possible reverting dynamics of the modifications. Another possible model could be to follow patients with long lasting pain conditions who become pain free, for instance after replacement of an arthritic joint. Nonetheless, this calls for further research.

4. Summary and future perspectives

The field of epigenetics has rapidly evolved within the last decade and it has become evident that epigenetic modulators, including DNA methylation, play a role in pain conditions and general pain sensitivity. While an association between DNA methylation changes and pain is clear, no studies have focused on establishing the temporal dynamics of DNA methylation in response to pain; in particular the onset of DNA methylation changes and the potential normalization of DNA methylation levels.

Animal models of neuropathic and inflammatory pain have provided the possibility to investigate DNA methylation levels at several time points following induction of pain presenting an ideal way to assess a DNA methylation timeline and the effect in different types of pain. Another advantage of animal models is that DNA methylation levels can be investigated in different tissues and organs. However, only few studies have been conducted, and with many

inconsistencies and shortcomings the current studies are insufficient to establish a cohesive DNA methylation time frame in response to pain.

As the DNA methylation timeline in animals would most likely differ from the human, human studies of DNA methylation after induction of experimental pain provocation would be an ideal model. However, it is a challenge to set up human studies investigating DNA methylation levels prior to and continuously following pain induction under safe and ethical conditions. Regardless, several human studies of DNA methylation in pain conditions have been conducted and epigenetic alterations have been demonstrated to represent a mechanism by which an acute pain experience can evolve into pathological processes as central sensitization, causing chronic pain syndromes. However, current studies are mostly limited to observations of only one time point during painful conditions, due to the difficulty in objectifying pain in humans and exploring these modifications long-term, for which reasons the dynamics of DNA methylation in pain conditions remain poorly understood. These issues might be overcome by pairing molecular analyses with Quantitative Sensory Testing techniques, a psychophysical approach allowing better quantification of sensory alterations in both healthy individuals and patients (Georgopoulos et al., 2019). In addition, longitudinal experimental designs with repeated measures and appropriate power could clarify whether epigenetic mechanisms represent the cause or the effect of pain-associated diseases.

Another obstacle in human studies is that the majority of human studies explore DNA methylation patterns in peripheral blood mononuclear cells which may not reflect changes in the central pain mechanisms, possibly contributing to the difficulties in establishing any causal relationship among the differences highlighted. Alternatively, animal studies analyze mainly root ganglia, spinal cord and brain tissues, allowing a better understanding of the pathophysiological mechanisms of pain. Nonetheless, it should be noted that the periphery in some cases reflect changes in signaling pathways at a central level and therefore peripheral cells represent a source of biomarkers for pain states (Kwok et al., 2012).

Future studies should focus on the temporal dynamics including onset and normalization of DNA methylation patterns in experimental and clinical pain conditions, preferably through longitudinal studies with repeated measures and appropriate power. The DNA methylation landscape should also be investigated in several types of chronic pain, in different tissues and in conditions in which pain can be reverted, such as joint replacement. This could provide a better understanding of DNA methylation processes in pain, clarifying why certain experiences or substance-induced DNA methylation signatures remain stable whereas others change over time. Moreover, this could provide a basis for identification of epigenetic biomarkers, ultimately leading to better treatment options for pain conditions.

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Author Contributions

L. Arendt-Nielsen conceptualized the review and critically revised the manuscript.
L. M. Johansen and M. C. Gerra acquisitioned and reviewed the literature, wrote and critically revised the manuscript. All authors discussed the results, commented on the manuscript and approved final version to be published.

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DNA methylation in animal pain models

STUDY	**SPECIES**	**TISSUE**	**PAIN MODEL**	**REGULATORY ELEMENTS** Time point(s) of detected alterations of DNA methylation regulatory elements post pain induction	**TIME POINTS** of detected DNA methylation alterations post pain induction	**DNA METHYLATION DETECTION ASSAY**
Wang et al (2016)	Rat	Lumbar spinal cord	CCI	14 days (DNMT3a, DNMT3b, MeCP2 and MBD2 mRNA)	14 days (*GAD 1* promoter)	Pyromark CpG Assay on bisulfite-modified DNA
Wang et al (2011)	Rat	Lumbar spinal cord	CCI	14 days (MeCP2 protein)	14 days	ELISA with Methylamp™ Global DNA Methylation Quantification kit
Shao et al (2017)	Mouse	Lumbar spinal cord	CCI	3 days – 14 days (DNMT3a protein) No significant regulations were detected at day 1 and 2, and time points after 14 days were not investigated	7 days – 14 days (*MOR* gene promoter) No significant remodeling was detected at day 1, and a decline was observed at day 14	Methylation specific PCR on bisulfite-modified DNA
Zhao et al (2017)	Rat	L5 DRG	CCI SNL	7 days (DNMT3a mRNA and protein) 3 days – 7 days (DNMT3a mRNA and protein) No significant regulation was detected at day 14	- 7 days (*Kcna2* gene promoter)	Bisulfite sequencing and bisulfite pyro-sequencing assay
Garriga et al (2018)	Rat	DRG	SNL	-	3 days – 3 weeks	Digital Restriction Enzyme Analysis of Methylation (DREAM) and Reduced representation bisulfite sequencing

						(RRBS)
Gölzenleuchter et al (2015)	Rat	DRG	SNL	-	24 hours	Reduced representation bisulfite sequencing (RRBS)
Jiang et al (2017)	Mouse	Lumbar spinal cord, dorsal horn	SNL	1, 3 and 10 days (DNMT3b mRNA) No significant regulation was detected at day 21 10 days (DNMT3b protein)	10 days (*Cxcr3* promoter)	Bisulfite sequencing PCR (BSP) and Methylation specific PCR (MSP)
Jiang et al. (2018)	Mouse	Lumbar spinal cord, dorsal horn	SNL	1, 3, 10 and 21 days (DNMT3b protein)	10 days (*GPR151* gene promoter)	Bisulfite sequencing PCR (BSP) and Methylation specific PCR (MSP)
Massart et al. (2016)	Rat	T-cells and PFC	SNI	-	9 months	Methylated DNA immunoprecipitation (MeDIP) microarrays and Gene-specific real-time PCR
Pollema-Mays et al (2014)	Rat	DRG	SNI	1 week – 4 weeks (DNMT3b mRNA) 2 weeks (DNMT1 and DNMT3a mRNA) No significant regulation was detected at week 1 and 4	-	-
Tajerian et al (2013)	Mouse	PFC and amygdala	SNI	-	6 months	Luminometric Methylation Assay (LUMA)

Wang et al (2019)	Mouse	Spinal dorsal horn	SNI	-	7 days (CpG sites of *GRIN2B* gene 5'-regulatory area, including promoter)	Bisulfite sequencing and pyrosequencing
Tochiki et al (2012)	Rat	Spinal cord, superficial dorsal horn	SNI CFA (into ankle joint)	7 days (MeCP2 and DNMT1 mRNA) 7 days (MeCP2, DNMT1, DNMT3a2 and DNMT3b mRNA)	-	-
Qi et al (2012)	Rat	DRG	CFA (into hindpaw)	-	3 days (*cbs* gene promoter)	Bisulfite sequencing PCR (BSP) and Methylation specific PCR (MSP)
Feng Li et al (2018)	Rat	DRG	CFA (Intraplantar injection)	3 days (DNMT3b mRNA) No significant regulation was detected for DNMT3a	3 days (CpG region of *CXCR4* gene promoter)	Bisulfite sequencing PCR (BSP) and Methylation specific PCR (MSP)
Oliveira et al (2019)	Mouse	DRG	CFA (into hind paw)	6 hours (DNMT3a2 mRNA) No significant regulation was detected at 1, 3, 24 and 48 hours	-	-
Pan et al (2014)	Mouse	Lumbar spinal cord	CFA (into hind paw)	-	6 days (miR-219 promoter)	Bisulfite sequencing

Sun et al (2015)	Mouse	Skin surrounding incision Spinal cord DRG	Incision (into hind paw)	6 hours (DNMT3b mRNA) No significant regulation was detected at 24 or 72 hours No significant regulation was detected for DNMT1 or DNMT3a) No significant regulation was detected in spinal cord or DRG	1 – 3 days	Fluorometric assay (MethylFlash Methylated DNA Quantification)
Mao et al (2019)	Rat	DRG	Paclitaxel-induced neuropathic pain	14 days (DNMT3a protein)	-	-

Tabel 1: Overview of in vivo animal studies investigating DNA methylation patterns following pain induction. References, species, tissues investigated, pain models and DNA methylation detection assays used are reported for each study. Time points in which DNA methylation and DNA methylation regulatory elements have been measured post pain induction are indicated. Time points of significant alterations and time points showing no alterations are included.

Abbreviations: Cbs, Cystathionine Beta-Synthase; CCI, chronic constriction injury; CFA, complete Freund's adjuvant; Cxcr, C-X-C motif chemokine receptor; DNMT, DNA methyltransferase; DRG, dorsal root ganglia; GAD1, glutamate decarboxylase 1; GPR151, G protein-coupled receptor 151; GRIN2B, glutamate ionotropic receptor NMDA Type Subunit 2B; Kcna2, potassium voltage-gated channel subfamily A member 2; MBD2, methyl-CpG binding domain protein 2; MeCP2, methyl CpG binding protein 2; miR-219, microRNA 219; MOR, mu opioid receptor; PFC, prefrontal cortex; SNI, spared nerve injury; SNL, spinal nerve ligation.

DNA methylation in clinical studies

STUDY	SAMPLE	SUBJECTS	TIMEPOINT(S) OF SAMPLING	SIGNIFICANT FINDINGS	DNA METHYLATION DETECTION ASSAY
Chidambaran et al. (2017)	Peripheral blood	Patient undergoing spinal surgery (n=133)	Prior to surgery	Correlation between DNA methylation of *OPRM1* CpG sites and CPSP	Pyrosequencing, CpGs at *OPRM1* promoter
Chidambaran et al. (2019)	Peripheral blood	Subjects with (n = 16) and without chronic postsurgical pain (CPSP) (n = 40)	Prior to surgery	Correlation between DNA methylation patterns in CPSP and child anxiety sensitivity	Human Infinium MethylationEPIC BeadChip microarrays
Takenaka et al. (2020)	Blood	Patients with chronic pain (n=24) (neuropathic pain, non-neuropathic pain, chronic low back pain), preoperative patients (n=24)	One timepoint during neuropathic pain follow-up in neuropathic pain patients. One timepoint prior to surgery in preoperative patients	Association between DNA methylation at *TRPA1* promoter and DN4 pain scores	Illumina HumanMethylation450 BeadChip, genome-wide methylation assay
Sukenaga et al. (2016)	Whole blood	Chronic pain patients (n=12)	One timepoint during chronic pain follow-up	Correlation between *TRPA1* methylation levels and neuropathic pain symptoms.	Illumina HumanMethylation450 BeadChip, genome-wide methylation assay
Gombert et al. (2020)	Whole blood	Patients suffering from Crohn's disease (n=55) and healthy participants (n=75)	One timepoint during Crohn's disease progression	Correlation between *TRPA1* methylation levels and pain symptoms, low pressure thresholds and mechanical pain thresholds	Targeted bisulfite Sequencing PCRs
Achenbach et al. (2019)	Whole blood	Patients with MSD (n= 151) and healthy subjects (n= 149). Relative subgroups with childhood trauma	One timepoint for each group of subjects	Associations between DNA methylation patterns and mechanical pain sensitivity in healthy subjects exposed to childhood trauma. Further, significantly higher methylation rates were found in MSD patients with severe childhood trauma when compared with MSD patients with no trauma	Targeted bisulfite sequencing

Ciampi de Andrade et al. (2017)	Peripheral blood	Fibromyalgia patients (n = 24) and healthy subjects (n = 24)	One timepoint during fibromyalgia progression, compared to controls	Changes in DNA methylation patterns in fibromyalgia patients when compared to healthy controls	Illumina-HumanMethylation450 BeadChips.
Menzies et al. (2013)	Whole blood	Fibromyalgia patients (n=8) and healthy subjects	One timepoint during fibromyalgia progression, compared to controls	Different DNA methylation patterns in fibromyalgia patients when compared to healthy controls	450 K HumanMethylation Chip, genome wide methylation assay
Burri et al. (2016)	Peripheral blood	Unrelated CWP patients (n=281) and discordant CWP monozygotic twin pairs (n=33)	One timepoint during CWP progression	Differentially methylated regions in genes such as *REST*, *MAOB* and *COL1A2* in CWP patients	Illumina HumanMethylation 450k BeadChip, genome wide methylation assay
Li Yim et al. (2016)	Peripheral blood	Crohn's disease patients (n=18), healthy matched controls (n=25)	One timepoint during Crohn's disease progression	Altered methylation in genes related to immune response and inflammatory pathways in Crohn's disease patients	Illumina HumanMethylation 450k BeadChip, genome wide methylation assay
Bruehl et al. (2019)	Whole blood	Individuals developing CRPS (n = 9) and non-CRPS neuropathic pain (n = 38) following amputation surgery	Within 3-18 months following traumatic injury	Differentially methylated CpG sites were associated with CRPS in neuropathic pain patients	Illumina HumanMethylation 450k BeadChip, genome wide methylation assay
Livshits (2017)	Whole blood	Monozygotic twin pairs discordant for CWP (n = 50), and an independent dataset (n = 1608 twins)	Repeated DNA methylation measurements conducted ≥3 years apart	Methylation changes in *IL17A*, *ADIPOR2*, and *TNFRSF13B* genes in CWP-affected subjects	MeDIP-sequencing

Table 2: Overview of clinical studies investigating DNA methylation patterns in relation to pain conditions. References, sample- and subject type, timepoint of sampling and significant findings are reported for each study.

Abbreviations: *ADIPOR2*, Adiponectin receptor 2; *COL1A2*, collagen type I, alpha 2 chain; CPSP, central post-stroke pain; CRPS, Complex regional pain syndrome; CWP, Chronic widespread pain; DN4, Douleur Neuropathique 4; IL17A, Interleukin-17A; *MAOB*, Monoamine oxidase B; MSD, painful multi-somatoform disorder; MZ, monozygotic; *OPRM1*, Opioid Receptor Mu 1; *REST*, RE1-silencing transcription factor; *TNFRSF13B*, TNF Receptor Superfamily Member 13B; *TRPA1*, Transient Receptor Potential Cation Channel Subfamily A Member 1.