MECHANISTIC STUDIES OF THE IMPACT OF CHRONIC PAIN ON BRAIN, BEHAVIOUR AND COGNITION IN A RODENT MODEL OF CHEMICALLY INDUCED OSTEOARTHRITIS

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ABSTRACT

Chronic pain has been associated with changes in forebrain regions, including the hippocampus and prefrontal cortex, and impairments in cognitive functions associated with these forebrain regions, including impairments in memory and cognitive flexibility. Osteoarthritis (OA) is a major public health burden, and the main symptom of OA is chronic pain. The impact of OA pain on cognitive function is poorly understood. By combining methods from pain research and behavioural integrative neuroscience, in this PhD work, the impact of OA-like chronic knee pain on selected cognitive functions was investigated in a well-characterised rodent model.

To investigate clinically relevant cognitive deficits associated with chronic pain caused by knee OA, in this study the rat monosodium iodoacetate (MIA) model was combined with translational tests of clinically relevant cognitive tests, including of hippocampus-dependent everyday type memory function, recognition memory and behavioural flexibility. Previous studies using this model of OA pain behaviour have used juvenile albino strains, which show comparatively poor performance in the cognitive tests. Therefore, the first objective was to transfer the MIA model to young adult Lister hooded (LH) rats, a pigmented strain, which is suitable for these cognitive tests (chapter 3). Pain behaviour and joint pathology phenotypes after a standard 1 mg dose of MIA were not robust in young adult LH rats or age and weight matched SD rats. By contrast, injection of 3mg of MIA caused robust pain behaviour, mainly changes in weight-bearing, accompanied by significant cartilage damage and synovitis. MIA-injected rats showed minor changes in locomotor activity with reduced rearing, which may reflect that they put less weight on their hind legs because of knee pain. This dose of MIA was therefore used throughout the thesis project.

To longitudinally assess the impact of OA-related knee pain on hippocampus-dependent place memory, MIA LH rats were tested in the watermaze delayed-matching-to-place (DMP) test, which is highly hippocampus-dependent (chapter 4). There was no evidence of impaired hippocampal memory following induction of the MIA model. No performance parameter on the DMP task was affected by MIA injection up until day 93 after model induction. MIA injected rats showed robust pain behaviour (weight bearing asymmetry), slightly decreased rearing activity and features of knee joint pathology. In this chapter, MIA rats showed some evidence for mildly reduced prepulse inhibition (PPI) at high pulse intensities compared with saline control rats (although this was not replicated in chapter 5 when studied at a later time point following MIA injection).

The impact of OA-like pain on recognition memory and behavioural flexibility was also evaluated in the MIA LH rats (chapter 5). This cohort of MIA injected LH rats was tested in the novel object recognition (NOR) test and in an automated set shifting task. Overall, there was no evidence of impaired recognition memory and behavioural flexibility after induction of chronic MIA up until day 59 after model induction.

Other factors associated with chronic pain in humans may account for why humans experiencing chronic pain have memory impairments, such as the effects of treatment. To test this, the effects of chronic treatment with morphine (3mg/kg twice daily for 7 days) and subsequent withdrawal was evaluated (chapter 6). Pilot work showed that morphine treatment induced an initial antinociceptive effect in LH rats, followed by tolerance and the development of morphine-induced hyperalgesia. Then, to evaluate the potential impact of chronic morphine treatment on both rapid place learning and NOR memory, MIA-injected LH rats were treated with morphine for 10 days (3mg/kg twice daily) or received control injections and were tested on the watermaze DMP task during treatment and at withdrawal. In addition, they were assessed in the NOR test during morphine withdrawal. Morphine had analgesic effects with no evidence of morphine-induced hyperalgesia in the MIA LH rats. In both naïve and MIA LH rats, acute morphine injection promoted hyperactivity. There was no evidence that repeated morphine treatment induced any impairment in rapid place learning performance or recognition memory in MIA-injected rats. However, in this study, MIA-injected LH rats did not show significant object recognition memory 49 days after model induction, which limits the interpretation the lack of morphine effect, but indicates that MIA-induced pain may disrupt such memory at this stage.

Overall, these findings suggest that hippocampus-dependent rapid place learning, NOR memory and behavioural flexibility are not affected by chronic OA-like knee pain in young adult male LH rats. Similarly, sustained treatment with morphine did not affect hippocampal and recognition memory in this model of OA-like knee pain. However, future investigation should be conducted in a wider age range and for longer periods after model induction to exclude the negative impacts of chronic OA pain in cognitive functions.

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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1. CHRONIC PAIN

Pain is defined by the International Association for the Study of Pain (IASP) as "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" (Raja et al., 2020). This definition highlights the fact that the pain experience can be multidimensional, comprising sensation and emotion, and also that pain experience can occur without actual tissue damage. The pain experience is not necessarily proportional to the intensity of a specific pain stimulus and consequentially pain experience can be different between different individuals with similar tissue damage (Tracey and Mantyh, 2007). Pain perception is an experience of nociceptive inputs that can also be influenced by memories, emotional and genetic factors among others (Tracey and Mantyh, 2007).

Pain may also be classified as acute or chronic based upon the length of time it lasts. Acute pain is a survival mechanism essential in our daily life, which signals potentially damaging stimuli and promotes protective behaviours to prevent further injury. On the other hand, pain can become chronic when it lasts for a prolonged period of time, commonly defined as lasting more than 3-6 months in patients (Apkarian et al., 2009), thus losing the biological value in terms of acute survival and becoming a disease. Unfortunately, the mechanisms leading to the transition from acute to chronic pain are still poorly understood.

Chronic pain has become a major health problem in many countries, with around 20% of the population worldwide estimated to be affected by persistent or chronic pain (Hart et al., 2000; Treede et al., 2015) and with no effective medication available to either prevent or treat this disease. Moreover, several studies have reported that chronic pain is also associated with a wide range of comorbidities, such as emotional (depression and anxiety) and cognitive impairments (Roth et al., 2005; Moriarty et al., 2011; Bushnell et al., 2013; McGuire, 2013).

Pain is the major reason chronic pain patients look for medical and health service assistance, however, pain-related psychological distress and possible cognitive impairments are increasingly recognised as having major impact upon people. Previous pain research focused mainly on sensation and the underlying mechanisms, with little focus to the non-sensation components such as cognition and emotion related impacts. Even though this trend has changed in the last decade, the mechanisms underlying the cognitive deficits associated with chronic pain are poorly understood and this area receives relatively little attention. This thesis will be focused on the study of the potential effects of chronic pain on cognitive functions.

1.1.1. MUSCULOSKELETAL PAIN

In an attempt to distinguish the heterogeneous types of pain, an IASP working group had developed a classification system for the different subgroups of chronic pain considering not only the source of pain, but also etiology and affected body location: chronic primary pain, chronic cancer pain, chronic posttraumatic and postsurgical pain, chronic neuropathic pain, chronic headache and orofacial pain, chronic visceral pain, and chronic musculoskeletal pain (Treede et al., 2015, 2019).

Chronic musculoskeletal pain is defined as persistent or recurrent pain, caused by persistent local or systemic inflammation that directly affects bones, joints, tendons or muscles (e.g., rheumatoid arthritis); or caused by disorders of the nervous system that are not linked to musculoskeletal issues themselves but which lead to their development (e.g., multiple sclerosis); or associated with structural changes in the musculoskeletal function (e.g., osteoarthritis) (Treede et al., 2015; Perrot et al., 2019). Osteoarthritis (OA) is one of the most common forms of chronic musculoskeletal pain. This thesis will focus on OA-associated chronic pain.

1.1.2. OSTEOARTHRITIS

OA is the most common form of arthritis and a major public health problem. Between 2005 and 2015 the global prevalence of OA showed an increase of about 32.9% and it was ranked as the 13th highest on the list of contributors to global years lived with disability (Vos et al., 2016). The prevalence of OA varies due to definition/diagnostic criteria used, age categories, countries and study population (Palazzo et al., 2016). The estimated worldwide

population affected is around 250 million people and knee OA is the most common (Dulay et al., 2015; Hunter and Bierma-Zeinstra, 2019). OA is mostly an age-related disease, affecting more frequently aged people (Vos et al., 2016), but other risk factors are also associated with this disease, such as obesity, greater bone density or genetic predisposition (Arden and Nevitt, 2006; Buckwalter and Martin, 2006).

OA is a multifactorial disease, resulting from the progressive degradation of single or multiple joints by an imbalance in the dynamic equilibrium between the breakdown and repair mechanisms of joint tissues (Dieppe and Lohmander, 2005; Egloff et al., 2012; Felson, 2013). The disease manifests first with molecular alterations of joint tissue metabolism, followed by anatomic abnormalities that can lead to illness (Kraus et al., 2015); however the OA chronologic trajectory between molecular and anatomical abnormalities of the joint structure is still not completely well-described.

OA can be divided in two groups regarding its etiology: primary (idiopathic) or secondary (results from trauma/injury or mechanical misalignment). There is also a substantial variability in the course of the disease, structural pathology and response to therapy (Deveza et al., 2017). The huge heterogeneity and variability in this disease have been widely discussed in the pain field (Cruz-Almeida et al., 2013; King et al., 2013), describing the different phenotypes and understanding the mechanisms behind it is necessary to improve the characterization of knee OA and improve target therapies.

OA was previously considered exclusively as a degenerative disease of the cartilage, but is currently known as a multifactorial disease (Piperno et al., 1998; van der Kraan and van den Berg, 2012) whose pathogenesis involves mechanical, inflammatory and metabolic factors which lead to structural derangements as illustrated in Fig.1.1.

During the OA process the composition of the cartilage changes. Cartilage is mainly composed of chondrocytes and an extracellular matrix of proteoglycans, collagen fibres and non-collagen proteins (Goldring and Marcu, 2009). Chondrocytes can be considered the housekeepers of this specialized matrix, as in normal situations chondrocytes are relatively inactive and maintain a healthy matrix by a low turnover. In OA, these cells drastically increase their activity to repair the damage leading to an excess of matrix proteins and degradations enzymes. Altered cartilage composition increases susceptibility to disruption by physical forces and initial and small erosions on the surface evolve to deep fissures in the cartilage and later in the calcified areas (Goldring and Marcu, 2009; Hunter and Bierma-Zeinstra, 2019). At the subchondral bone level in OA, bone remodelling and repair increases

and marrow lesions occur, leading to the development of bony outgrowths called osteophytes at joint margins (Hsia et al., 2017) (see Fig. 1.1.).

The production of proinflammatory mediators produced by the hypertrophic chondrocyte activity can also affect the synovium, which consequently releases more proinflammatory mediators, starting a positive feedback loop (Felson, 2013). This pro-inflammatory cascade and its role in OA disease is not yet completely understood, but it is known that chronic inflammation is present in OA and that an inflammatory profile develops with the progression of the disease, with increased infiltration of inflammatory cells into the joint and pain occurrence at this stage (Mora et al., 2018).

The classical symptoms of OA are morning stiffness, swelling, muscle weakness, reduced range of movement, joint instability and pain (Hunter et al., 2008). Pain is the major reason OA patients look for medical and health service assistance. In knee OA, pain is mainly weight-bearing/mechanical pain. In a community cohort of knee OA patients, 97% reported intermittent pain and 35% reported it as “unacceptable” (Liu et al., 2014).

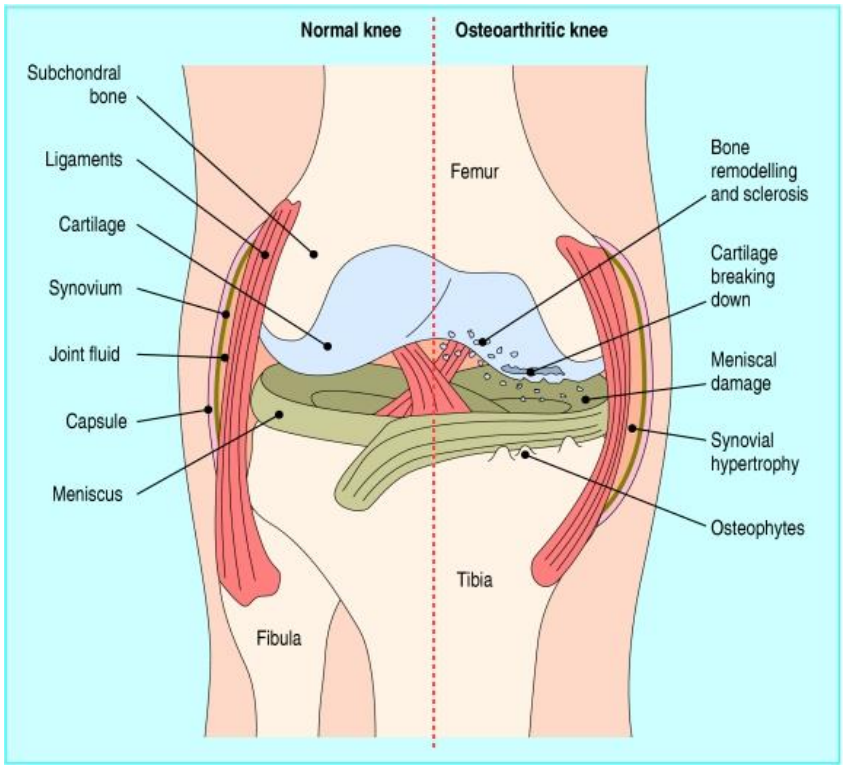

FIGURE 1.1. – OSTEOARTHRITIS PATHOGENIC FEATURES. Healthy vs osteoarthritic knee joint showing the underlying structural changes in the chronic disease. Image from: (Hunter and Felson, 2006).

1.1.2.1. ANIMAL MODELS OF OSTEOARTHRITIS

In order to understand the mechanisms underpinning chronic pain associated with OA, animal models are continuously being improved in order to mimic acute and chronic pain states for a more focused and direct study of the mechanism behind this health problem (Cohen-Solal et al., 2013; Teeple et al., 2013; Pelletier et al., 2015). Animal models provide important information about the mechanisms that lead to the development of a health problem being a crucial tool to study and characterise pathologies and to the development of new therapeutics.

The animal models that better correlate to human OA physiopathology include spontaneous models, such as the Dunkin-Hartley guinea pigs (Wang et al., 2013). However, these models show slow disease progression, which means they are time consuming and expensive and there are no co-aged matched controls. Furthermore, besides suitable time frame to allow reproducible results, there are other factors that should be taken in consideration when selecting the animal model, including the similarity to human pathology and the efficacy to detect a therapeutic effect. Choosing the right animal model of OA can be a challenge, OA is a heterogeneous disease and no single animal model is able to reproduce entirely the human disease. Hence, the most commonly used models in OA studies are the induced models.

OA can be chemically induced with intra-articular injections of substances which can cause damage to the joints, either by damaging the ligaments and tendons (collagenase), or inhibiting chondrocyte metabolism, such as the monosodium iodoacetate (MIA) model (Lampropoulou-Adamidou et al., 2014). As discussed and justified in detail in Chapter 3, the MIA model is the model used in this thesis.

1.2. PAIN PATHWAYS

Pain experience can be established when a peripheral noxious stimulus, whether from mechanical, thermal or chemical source, is applied to the body and transduced into an action potential by specialized primary afferent fibres called nociceptors. Primary afferents are divided in three main types regarding their characteristics: 1) Aβ, large myelinated fibres with higher conduction velocity; 2) Aδ, medium sized myelinated fibres with intermediate conduction velocity and 3) C-fibres , small unmyelinated fibres with slow conduction velocity

(Millan, 1999; Devor, 2009). Primary afferents are distributed throughout the body, including muscles and joints.

When peripheral nociceptors are activated following exposure to noxious stimuli, they transmit information to a proximal nerve terminal in the spinal cord via the primary afferent cell body in the dorsal root ganglion (DRG) (Fig.1.2.). When approaching the spinal cord, bundles of afferents carried in nerve trunks separate and penetrate the dorsal horn grey matter. The nociceptive information is then conducted to the dorsal horn of the spinal cord, where primary afferents synapse with second order neurons. The dorsal horn consists of organized laminae with ten layers called Rexed laminae (Craig, 2003).

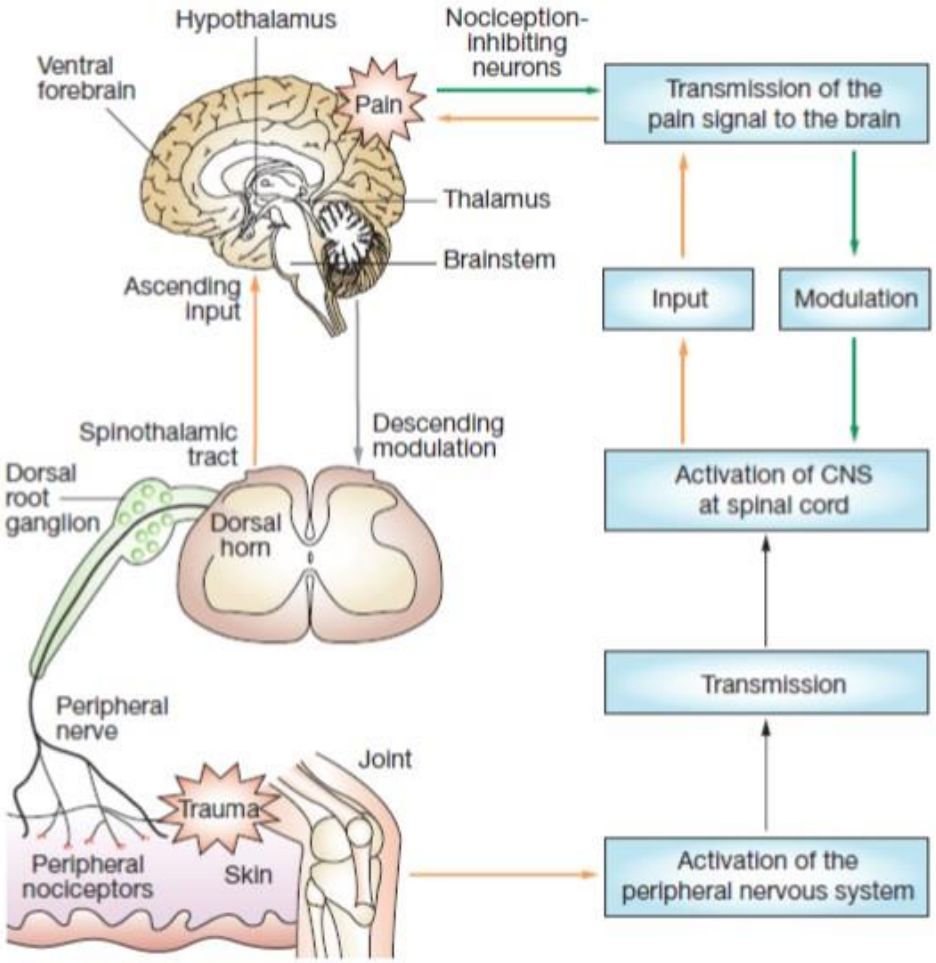

FIGURE 1.2. – PAIN PATHWAY REPRESENTATION. A peripheral activation (thermal, mechanical or chemical) is applied to the body and converted into action potentials by the nociceptor (transduction). This nociceptive information is then conducted to the dorsal horn of the spinal cord via the dorsal root ganglion (transmission). The nociceptive information is then transmitted via ascending pathways to the different supraspinal regions, where it is processed (perception). At this level, descending pathways modulate the nociceptive transmission from supraspinal areas to spinal dorsal horn (descending modulation). Image from (Bingham et al., 2009).

Projections from some second-order neurons ascend along the anterior and posterior tracts transmitting nociceptive information to the brain via the ascending pathways, including the spinothalamic, spinoreticular and spinomesencephalic tracts (Almeida et al., 2004). The ascending projections in the spinothalamic tract mainly originate in layers I and V of the dorsal horn and this tract is organised in two main pathways, the ventral and the dorsal (Todd, 2010). Although they ascend separately, the two tracts merge at the medulla level. This tract mainly projects to thalamus, which is subsequently distributed to several cortical structures. Neurons in the spinoreticular tract project mainly into the lateral and dorsal reticular nucleus and the neurons in the spinomesencephalic tract end in the periaqueductal gray (PAG) (Todd, 2010). Directly or indirectly, altogether, these ascending pathways distribute the information to supraspinal nuclei (Tracey and Mantyh, 2007).

The supraspinal pathways involved in the processing of pain-related information comprise a large matrix of regions, including cortical and subcortical regions. Brain imaging studies have been a crucial tool to allow a better understanding of this complex network and to unveil the regions involved (Apkarian et al., 2005). Areas such as somatosensory cortices, anterior cingulate cortex, insular cortex, thalamus and PFC are some of the most commonly reported to be involved in pain processing. However with the improvement of the imaging techniques the understanding of this complex network has been increasing and other areas have been found to be involved in pain processing [see for review (Brooks and Tracey, 2005; Ong et al., 2019)].

After supra-spinal processing, the brain modulates spinal cord activity through descending pathways to either facilitate (pronociception) or inhibit (antinociception) pain perception (Millan, 1999, 2002). Some of the key brain areas involved in pain processing are the PAG, rostral ventromedial medulla (RVM), somatosensory cortex, amygdala, insular anterior cingulate and prefrontal cortices, hippocampus and thalamus (Wager, 2004; Apkarian et al., 2005; Ochsner et al., 2006).

1.3. PERIPHERAL AND CENTRAL SENSITIZATION

After repeated and intense noxious stimuli, the nociceptive system can suffer sensitization and the threshold for its activation reduces and leads to an amplification of the subsequent inputs (Latremoliere and Woolf, 2009). Pain sensitization is often present in patients suffering from chronic pain, including OA patients (Soni et al., 2019), a normally non-noxious stimulus such as walking, standing or climbing stairs can be perceived as painful

(Dimitroulas et al., 2014). The reasons for this sensitization are likely multi-factorial, representing both inflammatory and neuropathic components (Thakur et al., 2014).

Tissue damage and the concurrent inflammation causes cellular disruption, sympathetic neuron discharge and activation/infiltration of immune cells (Pattison et al., 2021), which promote the release of a large variety of inflammatory mediators (Raoof et al., 2018). In OA, inflammation promotes the local release of factors such as tumor necrosis factor α (TNFα), interleukin 1β (IL-1β), IL-6, and IL-17, H^+, prostaglandin E2, ATP and growth factors [see for review (Pattison et al., 2021)] (Fig.1.3.). The resulting effects of some or all these mediators released at the level of the damaged joint (Schaible, 2012) contribute to an "increased responsiveness and reduced threshold of nociceptors to stimulation of their receptive field" - peripheral sensitization (Loeser and Treede, 2008). In acute and normal situations, nociceptors are able to recover from this sensitization after the tissue heals or the inflammation resolves but unfortunately the acute plasticity changes do not always resolve and lead to chronic pain development (Loeser and Treede, 2008). The causes for these two possible outcomes are not yet known. Peripheral sensitization can induce an increased responsiveness, where a noxious stimulus can cause a stronger pain experience than under normal conditions (hyperalgesia) (Woolf and Salter, 2000) and can also lead to a reduced threshold of the nociceptors and the perception of an innocuous stimulus becomes painful (allodynia) (Merksey and Bogduk, 1994).

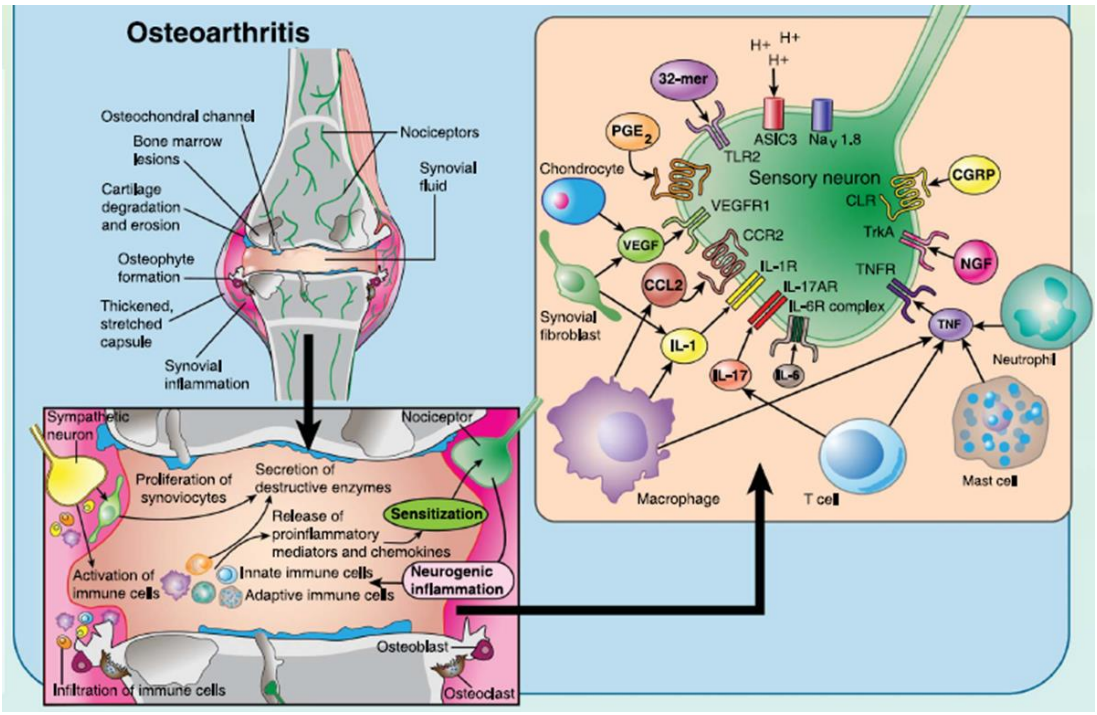

FIGURE 1.3. – CELL-CELL INTERACTIONS IN OSTEOARTHRITIS. Image from (Pattison et al., 2021).

In addition, pathological neuronal inputs from the damaged tissue can also cause complex changes in the central nervous system leading to central sensitization. At the spinal level, receptive fields of neurones expand and neurones have reduced thresholds (Latremoliere and Woolf, 2009). The sensitized neurones can also release pro-inflammatory mediators further sensitizing the spinal neurones and activating microglia or astrocytes (Woolf and Salter, 2000). Many mechanisms can contribute to central sensitization, driven by different effector (such as PKA, PKC, CaMKII, and ERK1/2), changes in the threshold and activation of N-methyl-D-aspartate (NMDA) receptor and, the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, alterations in ions channels and in the release of GABA and glycine release occurs. Consequently, nociceptive neurons can display increased spontaneous activity, reduction in threshold for activation and/or enlargement of receptive field by conversion of nociceptive-specific neurons to wide dynamic neurons (Latremoliere and Woolf, 2009).

Central sensitization is present in many chronic pain conditions and can manifest differently from pain conditions (Arendt-Nielsen et al., 2018). Central sensitization may explain why patients with OA show different features and why patients complain of more pain than was expected with the structural changes observed (Arendt-Nielsen et al., 2015). Patients at advanced stages of OA exhibit pain features consistent with central sensitization, including hyperalgesia at both the site of damage and remote areas from the affected joints, and changes in conditioned pain modulation, indicative of altered descending inhibitory pain mechanisms (Lluch et al., 2014).

The peripheral and centralized pain mechanisms in OA and their developmental time frame still is not completely understood. A major issue associated with the lack of knowledge about central sensitization in OA is the impact upon the development of new therapeutic approaches, as traditional treatments are mainly focused on peripheral nociceptive mechanisms and reducing joint pain (Murphy et al., 2012). Two approaches are used to clinically assess pain sensitization in OA patients, questionnaires and experimental assessments, such as pressure and thermal pain thresholds (Arendt-Nielsen, 2017). Importantly, translational human OA features of sensitization have been reported, such as local and wide-spread hyperalgesia, central integration of repeated nociceptive inputs (temporal summation or central hyperexcitability) and descending modulation (Arendt-Nielsen, 2017).

Central sensitization has not only spinal components, but also supraspinal components (Suzuki and Dickenson, 2005), for example, patients with OA exhibit altered activity in two important supraspinal brain areas linked with pain processing, the anterior cingulate cortex and rostral ventromedial medulla (Soni et al., 2019).

1.4. SUPRA-SPINAL CHANGES IN CHRONIC PAIN

Brain imaging in humans helped highlight brain areas potentially involved in pain and also in cognitive processing, and it is now clear that circuits responsible for the processing of nociceptive information are also involved in cognitive processes. This may help to understand the impact of chronic pain in cognitive functions. The persistent nociceptive inputs associated with chronic pain conditions may disrupt or compete with cognitive inputs leading to cognitive impairments. Imaging studies indicate that the anterior midcingulate cortex is involved in both cognitive and pain control (Shackman et al., 2011), an example of overlapping between the pain and cognition networks.

Structural changes have been reported in patients with chronic pain in different brain regions, including reductions (Apkarian et al., 2004; Kuchinad et al., 2007; Valfrè et al., 2007) and increases in volume (Rocca et al., 2006) in specific brain regions. Self-reported pain assessed using sensitivity questionnaire was positively correlated with larger grey matter volume in the parahippocampal gyrus, extending to the hippocampus (Ruscheweyh et al., 2018). Furthermore, volumetric reduction changes in the PFC, have been observed in patients with various types of chronic pain, including chronic lower back pain and fibromyalgia (Kelley and Domesick, 1982; Kuchinad et al., 2007; Moriarty et al., 2011). Importantly, ongoing spontaneous knee OA pain was reported to engage medial prefrontal-limbic cortical areas (Parks et al., 2012).

Clinical studies have showed altered functional network in some pain conditions, such as fibromyalgia (Ichesco et al., 2014), chronic widespread pain (van Ettinger-Veenstra et al., 2019) and ankylosing spondylitis (Hemington et al., 2016). Several studies provide evidence that OA is associated with brain anatomical and functional alterations (Gwilym et al., 2010; Cottam et al., 2018; Barroso et al., 2020). Baliki and colleagues have showed that different chronic pain types have different “brain signatures” (Baliki et al., 2011). In knee OA patients, grey matter density was reported to be reduced in the insular and mid anterior cingulate cortex, paracentral lobule, hippocampus and regions of the inferior cortex (Baliki et al., 2011). Mao and colleagues have also reported a small reduction of grey matter in the hippocampus

of knee OA patients compared with controls (Mao et al., 2016). A recent study conducted in patient with chronic knee OA also reported disruption in the functional connectivity (Cottam et al., 2018). Brain network alteration were also shown in rats after induction of MIA model (Abaei et al., 2016).

Although imaging techniques in human patients play a fundamental role in identifying associations between changes in brain function and chronic pain, they are limited in advancing understanding of the underlying mechanisms. Here, animal models have been a valuable tool to dissect these changes at a cellular and network level. For example, a study conducted in an induced neuropathic pain model in rats reported increased length and branching of dendrites and spine density of pyramidal neurons in acute slices of the medial PFC contralateral compared with control rats (Metz et al., 2009).

Apart from the morphological changes, several studies have reported neurochemical changes. There is a huge number of neurotrophic factors, cytokines, enzymes and neurotransmitters commonly involved in both pain and cognitive processing, and disruption of the normal activity of these components can lead to cognitive impairments. Some examples are the involvement of the brain-derived neurotrophic factors (BDNF), the glial cells and neurotransmitter GABA. All these components have been implicated in both pain and cognitive functions (check review for more information (Moriarty et al., 2011)).

1.5. COGNITIVE CHANGES IN CHRONIC PAIN

Cognition is defined as acquisition, processing, storage and retrieval of information by the brain (Spence, 1996). Cognitive function is crucial for an independent life and is also vital for the engagement with and success of a therapy. Pain itself has a cognitive-evaluative component (requires learning, recall of past experiences and active decision making). In this way, pain and cognition can overlap.

A review of the literature around cognitive deficits in chronic pain reveal there are two main models used to explain cognitive impairments: those that suggest cognitive impairments are an indirect consequence of chronic pain, and those that suggest these deficits are a direct consequence of chronic pain (Moriarty et al., 2011). Indirect models mainly attribute cognitive impairments in chronic pain patients to depression, anxiety, insomnia or medication (Landrø et al., 2013). Contrasting with these ideas, there is the emerging idea that chronic pain processing may disrupt directly brain regions with crucial

roles in cognitive functions and, consequently, causes cognitive deficits (Moriarty et al., 2011).

It has been suggested that the association of cognitive deficits and chronic pain reflects competition for the same neural network (Legrain et al., 2009b, 2009a; Moriarty et al., 2011). Processing of the nociceptive inputs in chronic pain patients is very persistent and can compete with other sensory inputs for processing resources, resulting in impaired cognitive functions. Cognitive neuroscience studies have highlighted the important roles of areas such as hippocampus and PFC in memory and attention. These areas as mention above, are also strongly associated with chronic pain conditions. It is important to note that these brain areas also have collateral reciprocal connections to each other and, generally, nociceptive information is not processed by a single and isolated brain region.

Clinical studies have been providing evidence that cognition may be impaired in some chronic pain conditions. Some examples of cognitive dysfunction that have been associated with chronic pain patients are memory, including working, spatial, recognition and long-term memory (Hart et al., 2000; Dick and Rashiq, 2007; Yong Liu et al., 2017); attention (Eccleston et al., 1997; Dick et al., 2002; Dick and Rashiq, 2007; Oosterman et al., 2011); decision making (Apkarian et al., 2004; Berryman et al., 2013); and mental flexibility (Karp et al., 2006; Moriarty et al., 2017).

Cognition can be greatly impacted by factors such as age and emotional states such as depression and anxiety, as well as by chronic pain, but the current literature is not always consensual on the impact of each of them or their comorbid effects. One of the major limitations of the clinical studies in this field is the lack of baseline measures and consequently the establishment of appropriate control groups. Preclinical studies on the other hand, allow to study and explore the mechanisms of cognition-pain interactions with the use of well-established animal models of chronic pain and translational neurocognitive tasks.

Preclinical studies have also contributed with evidence that chronic pain has negative impacts on cognition in rodents (for review see (Moriarty et al., 2011)). Attention deficits in rodents with inflammatory pain showed lower accuracy, higher perseverative and premature responses comparing with control animals (Pais-Vieira et al., 2009). Also, behavioural flexibility was impaired in rodents with chronic pain conditions (Brown and Tait, 2014; Murray et al., 2015; Moriarty et al., 2016a; Cowen et al., 2018). Additionally, long-term potentiation (LTP), the main form of measuring the activity-dependent synaptic plasticity,

was impaired in the hippocampus of nerve-injured mice, suggesting pain-related reduction in LTP might be an important driver of cognitive impairments in chronic pain (Ji et al., 2010).

Decision-making impairments in rodents were observed in studies using two different models of inflammatory chronic pain (kaolin and carrageenan model and complete Freund's Adjuvant (CFA) model), where pain was associated with a preference for a ‘‘high-risk’’ lever associated with larger but more infrequent rewards than the alternative lever (Pais-Vieira et al., 2009). Similar results were also observed in a model of visceral pain (Cao et al., 2016). Increased neuronal excitability and synaptic transmission in the basolateral amygdala was associated with decreased activity in the mPFC and consequently impaired decision-making performance in an arthritis pain rodent model. The pharmacological deactivation of the basolateral amygdala was able to increase activity in mPFC neurons and restore normal task performance in the gambling task (Ji et al., 2010).

Cardoso-Cruz and colleagues have reported that the induction of neuropathic pain in a rat model impaired performance on a working memory task by disrupting PFC-hippocampus connectivity in a frequency-dependent memory (Cardoso-Cruz et al., 2019). They show that typically healthy rats exhibit higher theta frequency connectivity levels between these areas compared with neuropathic rats and that these levels were a good predictor of good performances. The interaction between the hippocampus and PFC seems also crucial for social memory behaviour (Sun et al., 2020). Once more, these studies provide evidence that pain may induce brain plasticity alterations that may be associated with cognitive impairments in chronic pain patients.

Although cognitive deficits are widely acknowledged in relation to chronic pain, the distinct cognitive processes affected in distinct chronic pain conditions remain to be clarified. Furthermore, little is known about the specific effects of musculoskeletal pain on cognitive function, but even less is known regarding the relationship between OA pain and cognitive deficits. Some clinical studies conducted in groups of chronic patients with different conditions, including musculoskeletal pain patients have found impairments in working memory and verbal episodic memory performance (Oosterman et al., 2011), mental flexibility (Karp et al., 2006) and attention (Dick et al., 2002). However, as previously mentioned different chronic pain conditions can have different brain signatures (Baliki et al., 2011) showing the importance to further address cognitive deficits in particular pain conditions.

1.6. THESIS OUTLINE

In this thesis, we used a chemical-induced model of OA knee pain in a rodent model to test the hypothesis that persistent nociceptive inputs associated with chronic OA pain disrupts or competes with cognitive inputs leading to cognitive impairments, such as hippocampus-dependent memory, recognition memory and cognitive flexibility. Additionally, we investigated how sustained opioid treatment affected memory function in this OA rodent model. In sum, the main objectives were:

1) To transfer/validate the MIA model to adult Lister hooded rats.

2) To investigate hippocampus-dependent memory in MIA-injected adult male LH rats.

3) To study recognition memory and behavioural flexibility in MIA-injected adult male LH rats.

4) To explore the possible effects of long-term morphine treatment on memory in MIA-treated LH rats.

CHAPTER 2

GENERAL METHODS

General methodologies and techniques are described in this chapter as are sources of animals, collected tissues, consumables and equipment. Techniques and procedures specific for any study are described in the respective chapter.

2.1. ANIMALS

All animal care and experimental procedures were conducted in accordance with the requirements of the UK Home Office Animals Scientific Procedures) Act (1986) and the International Association for the Study of Pain. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010).

Young adult male Lister Hooded (LH) and Sprague Dawley (SD) rats from Charles River UK were used in this project. Animal numbers can be found in each chapter. LH rats weighed between 250-275g and SD rats weighed between 275-300g (rats of both strains were approximately 2 months of age) at the start of the experiments. The age was an important consideration when planning this project, previous studies have been describing the behavioural phenotype changes and key brain developmental processes across comparable ages in humans and in rats (Semple et al., 2013), for example evidence suggests that GABAergic and dopaminergic systems are only fully mature in rats at 2-3 months of age (i.e., postnatal day 60-90). Furthermore, an old radiographic skeletal study suggests that the rat bone is only completely mature around 60-80 postnatal day (Hughes and Tanner, 1970).

Rats were housed 4 per individually ventilated cages (IVC) cages under temperature-controlled conditions and under a 12 h light-dark cycle with lights-on between 7:00am and 7:00pm. Animals had food and water available ad libitum. First, rats had a period of acclimatisation following their arrival, they were habituated to handling by the experimenter for a few days before any experimental procedure or apparatus habituation. All behavioural testing was carried out during the light phase.

2.2. INTRA-ARTICULAR INJECTIONS

Osteoarthritis was chemically induced with a single intra-articular injection of monosodium iodoacetate (MIA). For induction of MIA-induced arthritis, each rat was anesthetised with isoflurane (isoflurane 2.5 – 3% in 1L/min O2). Each rat was placed in a chamber with this volatile mixture until areflexic (confirmed by pinching of hind paws). The rat was then placed in a supine position and the skin around the knee joint of both legs shaved, the diameter of the knee joints were measured using a digital electronic calliper and, then, the skin of left joint was swabbed with chlorhexidine (Animal care Ltd, Dunning ton, York). Each rat received a single intra-articular injection of 50 µL of either MIA (1 mg/50 µL saline or 3 mg/50 µL saline; Sigma) or the same volume of sterile 0.9% normal saline (control animals) through the infra-patellar ligament of the left knee using a 30-gauge 8-mm needle (Guingamp et al., 1997; Bove et al., 2003; Sagar et al., 2010). The knee joint was held in around 90˚ flexion, then the needle was inserted through the suprapatellar ligament with the joint to administer the compound.

Animals were allocated to treatment groups before the model induction based on the behaviour baseline measurements; treatment allocations were adjusted to match groups for their baseline measurements. The experimenter conducting the knee injections was blind to treatment. Right (un-injected) and the saline injected knee joint were used as controls.

2.3. PAIN BEHAVIOUR MEASURMENTS

Baseline measures were obtained prior to the intra-articular injections of MIA-induced arthritis and then repeated postoperatively at specific time points (described in each study). The effects of intra-articular injection of MIA/saline were assessed using two behavioural tests: i) weight distribution through the injured and contralateral limb using an incapacitance tester (Bove et al., 2003) and ii) mechanical sensitivity measuring the hindpaw withdrawal thresholds by application of von Frey monofilaments in the plantar surface of the paw (Chaplan et al., 1994; Combe et al., 2004; Sagar et al., 2011).

2.3.1. WEIGHT-BEARING TEST

The change in distribution of weight across the hind limbs associated with osteoarthritic knee pain was assessed by measuring changes in the weight distribution between ipsilateral (osteoarthritic) and contralateral paw (control). Rats were first habituated in the Perspex box

prior to start of the study for at least 3 days in order to get used to their surroundings and to reduce the stress levels during the data collection. Animals were placed in an incapacitance (Linton Instrumentation Diss, Norfolk, UK) tester in a way that each hind paw rested on a separate scaler plate to that force exerted by each limb can be measured (Fig.2.1.). The average of the force exerted in 3 seconds was measured in grams and the collected data corresponds to the mean of three consecutive readings. Changes in hind paw weight distribution were calculated by the following equation:

$$(Ipsilateral/Contralateral + Ipsilateral)\ x\ 100\ .$$

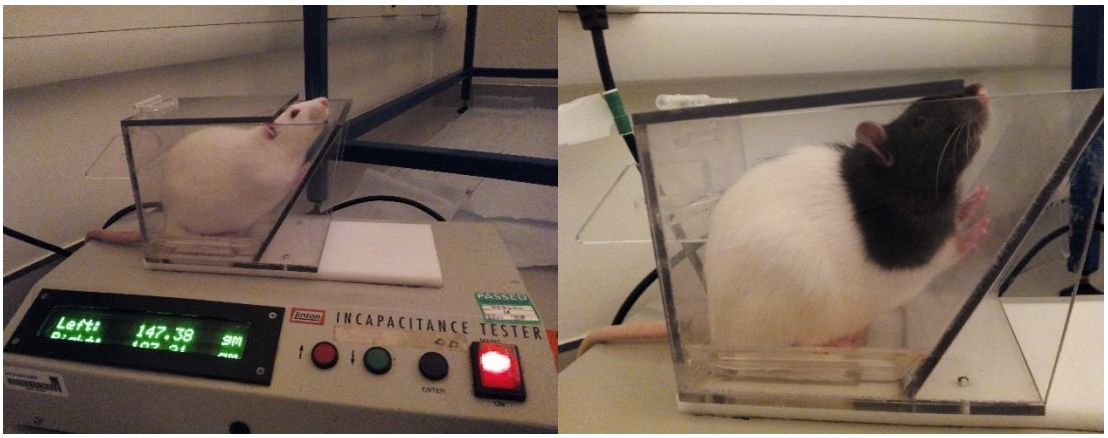

FIGURE 2.1. – INCAPACITANCE METER USED FOR WEIGHT BEARING ASYMMETRY ASSESSMENTS. The incapacitance meter consists in a Perspex box with a lid to hold the animal, two transducer pads in the bottom allow to record weight distributed to either hind limb by the rat. The average of the force exerted in 3 seconds was measured in grams and the collected data corresponds to the mean of three consecutive readings.

2.3.2. PAW WITHDRAWAL THRESHOLD TEST

The development of mechanical allodynia was assessed using Von Frey monofilaments test (Fig.2.2.). Rats were first habituated to the apparatus at least 3 days before testing. On the first two days, animals were only placed in the chambers for approximately 30 min with no stimuli, on the third day of habituation, after the 30 min habituation 6g was applied to the plantar surface of the animals in order to habituate the animals at the presence of the

FIGURE 2.2. – VON-FREY CAGES USED FOR MEASURING MECHANICAL ALLODYNIA. Rats were placed in Plexiglas wire bottom test cages and hindpaw withdrawal threshold was measured by applying Von-Frey monofilaments of different forces with the up-down method.

stimuli. In each day of testing rats were placed singly into the cages 15-20 min prior to the assessment. Hind paw withdrawal threshold (PWT) was measured using a modified up-down method (Chaplan et al., 1994; Sagar et al., 2011). Von Frey monofilaments (Semmes-Weinstein monofilaments of bending forces 0.6, 1, 1.4, 2, 4, 6, 8, 10 and 15; the force values are presented in grams) were applied perpendicular to the plantar surface of both paws of the rats for 3s period in ascending order, starting with 6g hair. If fewer than two applications elicited a withdrawal response, the hair with the next highest force was applied. Once a positive response was established, the paw was retested with the next descending Von Frey monofilament until no response occurred. A positive response was considered if the paw was sharply withdrawn or flinching or licking occurred. The lowest weight of monofilament which elicited a reflex was noted as a PWT.

2.4. SENSORIMOTOR ACTIVITY MEASURMENTS

The effects of intra-articular injection of MIA/saline were assessed using two behavioural tests: i) open-field locomotor activity test, to assess potential impairments in the locomotor activity and ii) startle/prepulse inhibition test to evaluate brain function. Sensorimotor activity measures were also obtained prior to the intra-articular injections of MIA-induced arthritis, and postoperatively at specific time points (described in each study). Sensorimotor activity measurements were conducted one day after pain behaviour measurements.

2.4.1. LOCOMOTOR ACTIVITY TEST

To assess locomotor activity rats were placed individually in the centre of Perspex chambers (39.5cm long x 23.5cm wide x 24.5cm deep) with metal grid lids for 30 minutes (Fig.2.3.). Locomotor activity was measured similar to previous studies (Pezze et al., 2014). 2 levels of a 4x8 photobeam configuration allowed measuring the locomotor activity, the break of two consecutive photobeams generates a locomotor count (Photobeam Activity System; San Diego Instruments). The total locomotor activity was calculated for each 10 min block of the overall time of the test.

FIGURE 2.3. – OPEN-FIELD CHAMBER USED TO MEASURE LOCOMOTOR ACTIVITY. Rats were placed in Plexiglas chambers for 30 minutes and locomotor activity was measured by break of photobeams.

2.4.2. STARTLE-PREPULSE INHIBITION TESTS

Startle is a reflex evoked by sudden stimuli, which can be tactile, visual or acoustic. While, prepulse Inhibition (PPI) is a phenomenon in which a suppression of a startle response occurs when a weak stimulus (prepulse) precedes a subsequent stronger startle stimulus. Startle/PPI test has been used in both humans and rodents as a measure of sensorimotor gating. Brain areas such hippocampus, prefrontal cortex, nucleus accumbens and amygdala seem to play an important role in Startle/PPI (Koch, 1999).

The measurements were conducted similar to previous studies (Pezze et al., 2014) using four well-lit (15W) and ventilated sound-attenuated chambers (39x38x58cm3), each one with a clear Perspex cylinder (8.8cm diameter, 19.5cm long) inside (Fig.2.4.). The cylinders

were linked to a solid Perspex base also linked to an accelerometer to measure the individual whole-body startle response by a Reflex Testing software (San Diego Instruments, US). Centrally above the cylinders was located speakers that presented the background and acoustic stimuli produced by a noise generator controlled by the SR-Lab system (San Diego Instruments, US).

Startle/ PPI test was divided in four main parts, the acclimatization and 3 test blocks. In the acclimatization rat is exposed to a 62-dB (A) background noise for a period of 5 minutes, the background noise continues through all the session (23 minutes). In the first block, 10 startle pulses of 120-dB (A) of 40ms each were presented alone. In the second block, to measure the PPI, 5 different type of pulse (120-, 84-, 80-, 76-, 72-dB (A)) were presented 10 times each, in pseudorandom order and with a variable interval of 10 to 20s duration. The percentage of PPI (%PPI) induced for each pulse intensity was calculated using the follow formula: %PPI = [(mean amplitude on pulse-alone trials – mean startle amplitude on prepulse-plus-pulse trials) / (mean startle amplitude on pulse-alone trial)] X 100. Finally, in the third block 5 startle pulses of 120-dB (A) completed the session.

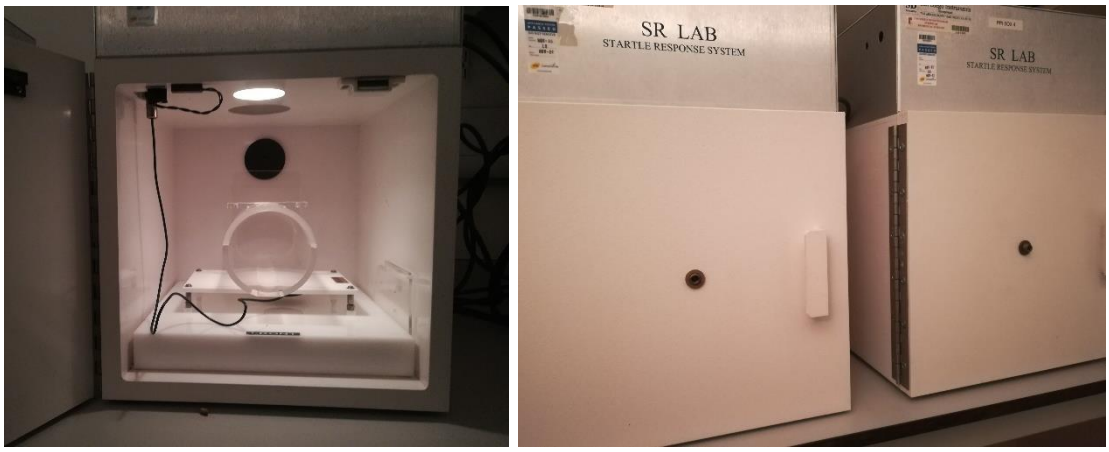

FIGURE 2.4. – STARTLE/PREPULSE INHIBITION CHAMBER USED TO MEASURE SENSORIMOTOR GATING. Rats were placed in a Plexiglas cylinder inside a sound-attenuated chamber. The cylinder was linked to an accelerometer to measure the startle response to the different pulse intensities presented during the test.

2.5. TISSUE COLLECTION AND TISSUE PROCESSING

At the end of each study, animals were anesthetized with a lethal dose of sodium pentobarbitone (1-1.5 mL Euthatal, intraperitoneal), and then transcardially perfused with

0.9% saline followed by 4% paraformaldehyde (PFA) (around 250-300 ml of each solution was used to perfuse each animal).

Blood was collected during the perfusion with saline. Brains, spinal cords and DRGs were carefully excised and kept in the same fixative (PFA 4%) overnight and then stored in 30% sucrose azide solution at 4^{o}C for cryprotection.

Both ipsilateral and contralateral knees were also collected and stored in the fixative for 72h. Knee joints were then transferred to an ethylenediaminetetraacetic acid (EDTA) + 7.5% polyvinylpyroolidene (PVP) solution for decalcification (5 to 6 weeks, agitated at room temperature). Solution was changed once a week. This process allows to soften the heavily mineralised tissue and consequently to obtain an adequate and satisfactory thin section for histological purposes.

After decalcification, knee joints were split in a frontal plane so medial and lateral orientation was maintained. Knees were kept stretched with help of forceps and then, with a sharp razor split along the lateral ligament (medial collateral ligament) (Kraus et al., 2010). Both halves, anterior and posterior parts, were placed in separated cassettes. Then, the trimmed joints in the cassettes were processed by standard histological techniques and mounted in paraffin wax at King's Mill Hospital by an experienced laboratory technician (Roger Hill). Twenty-four 5µm sections were cut in total from each paraffin block (from both anterior and posterior halves of the knee). 8 serial 5µm sections were obtained from 3 different levels (post, medial and frontal part) and 1 section from each level were stained and used for scoring. Paraffin sections were cut using the microtome at City Hospital by Sara or by an experienced histology technician (Mohsen Seyed).

2.6. HISTOLOGICAL STAINING

Histological techniques were used to detect chemical components of cells and tissues to assess OA progression in the knee joints. Haematoxylin and Eosin (H&E) and Safranin-O-Fast Green are the most commonly used histological stain techniques to detect chemical components of cells and tissues to assess OA progression in the knee joints.

H&E staining is used to assess basic structures/tissue morphology (Schmitz et al., 2010). Haematoxylin is basic dye with high affinity for acidic structures such as nucleus, staining them blue/purple, while eosin is an acidic dye that stains basic structures such as cytoplasm, staining them pinkish (Fig.2.5.). Areas with high proteoglycan content are stained bluish.

Paraffin wax sections were first dewaxed in xylene (x2) for 5 min, then rehydrated in graded ethanol (100% and 70%, both x2 for 5 min), and then washed in distilled water for 5 min. Sections were then immersed in the nuclear staining (Mayer's haematoxylin) for 15 min, rinsing in running tap water until the water was discoloured and sections turned blue. Sections were then dipped for 30 seconds to 1 min in the second dye (eosin) to stain all other tissue structures and rinsing in tap water again to differentiate the staining. Sections were then dehydrated through graded ethanol (70% and 100%, both x2 for 5 min) and dipped into xylene (x2, 5 min). Finally, slides were mounted in DPX and covered using 64 mm cover glass and allowed to dry in the fume hood.

Safranin-O-Fast green is highly used to study cartilage and bone structures(Schmitz et al., 2010). Safranin-O is a cationic dye that stains proteoglycan in normal cartilage. Proteoglycans are stained dark pink-red and the red colour intensity is a measure of cartilage damage. Fast green stains the subchondral bone in blue/green (Fig.2.6.). Paraffin wax embedded sections were fist dewaxed in xylene (x2) for 5 min, then rehydrated in graded ethanol (100% and 70%, both x2 for 5 min), and then washed in distilled water for 5 min. Sections were then immersed in the Weigert's haematoxylin dye for 2 min, rinsing in running tap water for 1 min and then in acid alcohol solution for 20 sec and subsequently in tap water again for 3 min.

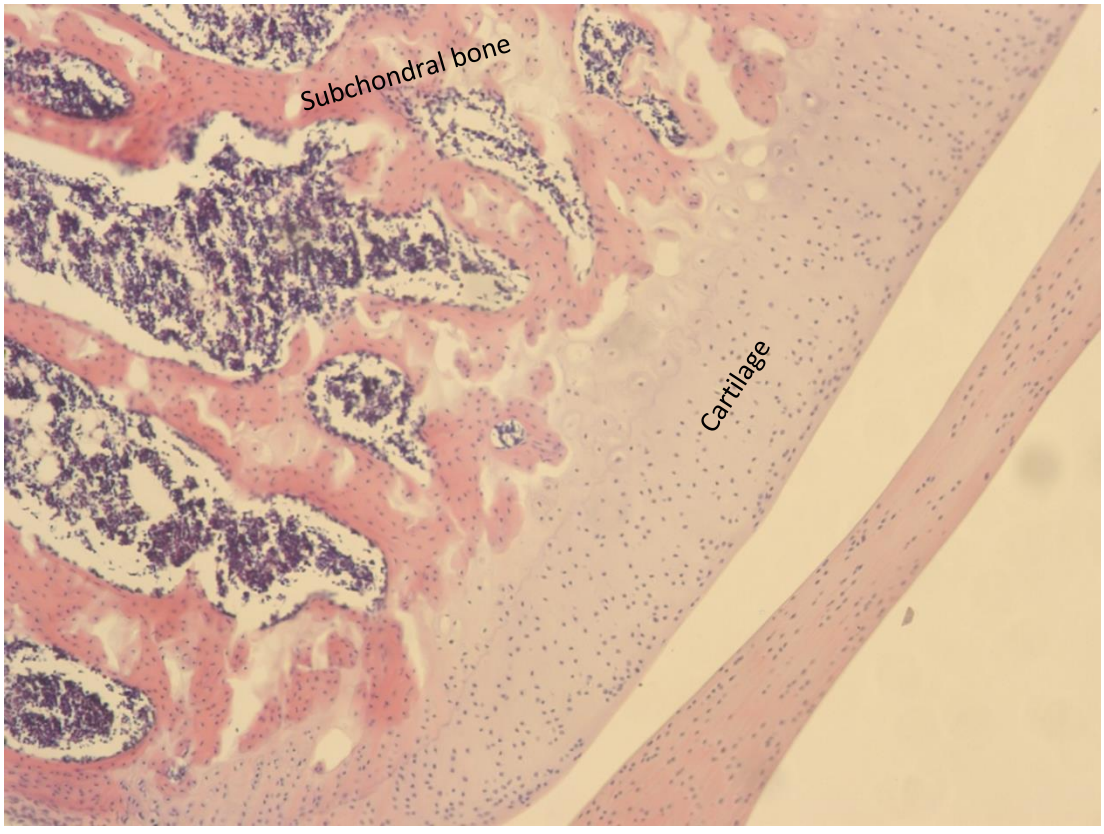

FIGURE 2.5. – HAEMATOXYLIN AND EOSIN STAINED SECTION OF RAT TIBIA. Cartilage matrix – pink (bluish), bone, fibrotic tissue – pink to red, nuclei – blue, cytoplasm – pink to red.

Sections were then immersed for 5 min in the fast green, briefly dipped into acetic acid for 1 sec and immersed in Safranin-O for 5 min. To differentiate staining, sections were submersed in running tap water for 5 min and then dehydrated in 100% ethanol (x2 for 5 min) and dipped into xylene (x2, 5 min). Finally, slides were mounted in DPX and covered using 64 mm cover glass and allowed to dry in the fume hood.

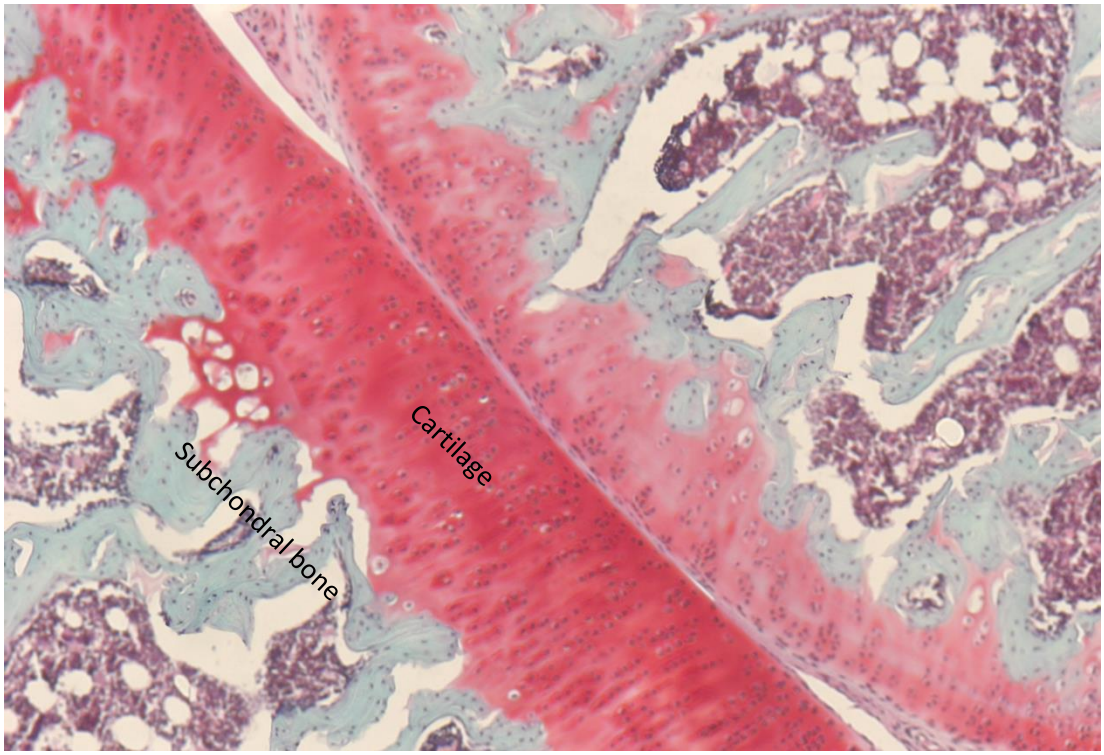

FIGURE 2.6. – SAFRANIN-O FAST GREEN STAINED SECTION OF RAT TIBIA. Cartilage matrix – pink to red, underlying bone – blue/green, nuclei – dark blue/black, cytoplasm – grey green/blue.

2.7. HISTOPATHOLOGICAL SCORING

After staining, images were collected using a Zeiss Axioscop50 microscope (Carl Zeiss Ltd, Welwyn Garden City, UK), captured using a video camera (AxioCam MRmZeiss) and analysed using Axiovision real 4.8 software. Experimenters were blinded to experimental group. Once again, scoring were carried out by Sara or by an experienced histology technician (Mohsen Seyed). In the first study, a reliability analysis between Mohesen's and Sara's scorings was carried out (intraclass correlation (ICC)>0.90, $p<0.001$ for all the scoring parameters).

Microscopic scoring for OA cartilage pathology was carried out as described in Table I.

TABLE I – PARAMETERS USED FOR HISTOPATHOLOGICAL SCORING OF MIA MODEL.

	Score:
Cartilage damage (Janusz et al., 2002)	0= normal 1= minimal superficial zone only 2= mild extends into the upper middle zone 3= moderate well into the middle zone 4= marked into the deep zone but not to tidemark 5= severe full thickness degeneration to tidemark **Cartilage integrity** = Cartilage damage x cartilage damage involvement (1, 2 or 3). Involvement is the extent of damage in the tibial plateau – this area is divided in 3 equal parts along the surface)
Synovial inflammation (Mapp et al., 2008)	0= lining cell layer 1-2 cells thick 1= lining cell layer 3-5 cells thick 2= lining cell layer 6-8 cells thick and/or mild increase in cellularity 3= lining cell layer >9 cells thick and/or severe increase in cellularity
Osteophytes(Janusz et al., 2002)	0= no changes 1= mild (<40 μm) 2= moderate (40–160 μm) 3= severe (>160 μM) (size is measured using an ocular micrometer)
Chondrocytes (Pritzker et al., 2006)	0= present in the cartilage 1= not present in the cartilage
Proteoglycans (Pritzker et al., 2006)	0= normal 1= mild loss 2= moderate loss 3= severe loss 4= complete loss

CHAPTER 3

MONOSODIUM IODOACETATE-INDUCED OSTEOARTHRITIS-LIKE KNEE PAIN IN LISTER HOODED RATS

3.1. INTRODUCTION

Chronic pain is a major public health problem worldwide with no effective treatment. Pain is the principal reason why chronic pain patients seek medical assistance and pain research is mainly focused on the nociceptive / sensation components. Nevertheless there is evidence that this disease may also be associated with other comorbidities, including cognitive impairments (Moriarty et al., 2011). However, pain research has only recently started to focus on the non-sensation components of the disease and understanding of the relationship between chronic pain and cognition and underlying mechanisms is limited. Overall, understanding of the impact of chronic pain on cognition is variable depending upon the pain state, in the case of osteoarthritis (OA) it is rather limited.

Animal models can provide important information and highlight relevant mechanisms that lead to the development of a health problem, and for that they are a crucial tool to study and characterise pathologies and for the development of new therapeutics. The selection of the most appropriate animal model and strain to address the study question is crucial to obtain the most clinically relevant information and maximise the benefits of the research.

3.1.1. MONOSODIUM-IODOACETATE MODEL

As mentioned in the general introduction, there are several animal models that replicate aspects of human OA physiopathology, including spontaneous, surgically- and chemically-induced models. No single model is able to reproduce entirely the human disease, however the spontaneous models are widely accepted because they mimic the natural occurrence of OA. However, the models most commonly used are induced models that in contrast with the spontaneous models allow the use of appropriate age-matched controls and exhibit less heterogeneity, and consequently require lower numbers of animals per group.

Intra-articular injection of monosodium iodoacetate MIA has been widely used to model the development of OA and associated pain responses, and to test potential new drug therapies aimed at resolving the painful symptoms (Lampropoulou-Adamidou et al., 2014). This model has been widely used due to the rapid timecourse in the development of joint pathology, the high reproducibility (Guzman et al., 2003; Marker and Pomonis, 2012; Pitcher et al., 2016) and it does not require any additional surgery and therefore avoids postoperative pain. MIA is an inhibitor of the enzyme glyceraldehyde-3-phosphate dehydrogenase, and therefore an inhibitor of glycolysis (Jiang et al., 2013). Intra-articular injection of MIA induces chondrocytic death that leads to destruction of cartilage in several different animals species, including rodents, rabbits and guinea pigs (Kim et al., 2018).

3.1.2. FEATURES OF THE MIA MODEL

Although the MIA model does not mimic the natural occurrence of OA in humans it is associated with similar features of knee pathology (Guingamp et al., 1997). OA is a multifactorial disease and besides cartilage degradation, synovitis (infiltration of inflammatory cells in the synovium space) (Dulay et al., 2015) and fibrillation is also present (Piperno et al., 1998). The intra-articular injection of MIA results in chrondrocyte death in the tibial plateaux and femoral condyles which leads to loss of cartilage integrity, osteophyte formation and progressive proteoglycan loss (Janusz et al., 2001, 2002; Pritzker et al., 2006). At later stages, cartilage fibrillation, resorption and subchondral bone degradation also occur (Janusz et al., 2002; Mapp et al., 2013).

As in people with unilateral knee OA (Christiansen and Stevens-Lapsley, 2010), rodents with unilateral joint injury also exhibit weight bearing asymmetry (Bove et al., 2003). Weight bearing asymmetry is an indicator of standing pain, patients and rodents with OA tend to lean less or put less weight on the injured joint, being considered an indirect measurement of knee pain. The MIA model is associated with pain related behaviours, such as asymmetry of hind paw weight distribution and lowered hindpaw withdrawal thresholds (mechanical allodynia) (Bove et al., 2003; Sagar et al., 2010, 2011). Measuring this pain behaviour provides indirect surrogate measures of alterations in the peripheral and central processing underlying OA-related pain, as changes in weight bearing represent changes in both peripheral and central processes, while lowered paw withdrawal thresholds is mainly centrally driven (Graven-Nielsen and Arendt-Nielsen, 2002).

As described in the general introduction, there are multiple lines of evidence from preclinical and clinical studies for central sensitization in pain processing in OA (Arendt-Nielsen et al., 2015). Evidence for central sensitization includes the development of hyperalgesia, i.e., increased responses to a painful stimulus, allodynia, painful responses to an otherwise innocuous stimulus, and changes in spinal neuronal thresholds (Lluch et al., 2014; Havelin et al., 2016; Soni et al., 2019). In the MIA model in rats, enhanced spinal responses to mechanical stimulation of the hindpaw skin were demonstrated (Sagar et al., 2010). Changes in hindpaw mechanical thresholds may indicate referred (distal) pain (Arendt-Nielsen et al., 2018), and can be assed in rodents by measuring changes in withdrawal thresholds at remote sites to the primary injury (Nwosu et al., 2016a).

Overall, there is strong evidence that a single intra-articular injection of MIA leads to histological, biochemical and pain behaviour changes. Both the extent of knee pathology and pain behaviour are dose and time dependent, with higher concentration of MIA leading to greater severity at earlier time points – Table I (Bove et al., 2003; Sagar et al., 2010; Ferreira-Gomes et al., 2012; Mapp et al., 2013; Ogbonna et al., 2013; Aso et al., 2016; Nwosu et al., 2016b).

3.1.3. RAT STRAINS USED TO TEST COGNITIVE FUNCTION AND NOCICEPTIVE RESPONDING

The main aim of this thesis was to study the potential impact of chronic OA pain on cognitive function and to address this several cognitive tasks were conducted. Besides selecting an appropriate pain model, an appropriate rat strain is also an important factor to consider. As mentioned before, the MIA model has been widely used to measure pain behaviour, including in our lab; however, previous studies have been mainly conducted in young albino rat strains as showed in Table I. Unfortunately, albino rats show comparatively poorer performances in translational tests of clinically relevant cognitive functions. A study conducted in several pigmented strains, such as Lister hooded (LH) rats and Long Evans rats, albino strains, such as SD and Wistar rats, and also wild rats has showed that pigmented and wild animals have significant higher visual acuity when compared to albino strains (Prusky et al., 2002). The poor visual acuity of the albino strains can lead to worse performances in specific behaviour tasks, making the strain selection a very important factor to take into account when vision-mediated behavioural task, such as the Morris watermaze task or other behavioural maze tasks are involved. Other studies showed that non-albino strain performed

better in visual learning and memory tasks (Yau et al., 1994; Higgins et al., 2007; Kumar et al., 2015).

3.1.3.1. CHRONIC PAIN MODELS IN LISTER HOODED RATS

For decades pigmented strains have been the first choice for assessment of navigation and memory in rodents (Clemens et al., 2014), as these animals are usually more accurate and faster at learning the tasks, allowing detection of impairments in specific conditions more precisely. In addition, LH rats show increased locomotor activity and novelty-induced behaviour (Clemens et al., 2014). For these reasons, LH rats were selected to be used in this thesis.

Previous studies of pain conditions have been performed in LH rats, various models have been studied including intra-plantar injection of formalin to induce inflammation (Butler et al., 2011), spinal nerve ligation (Moriarty et al., 2016a), inguinal hernia repair (Bree et al., 2016) and complete Freund's Adjuvant (Pais-Vieira et al., 2009). However, to our knowledge, models of OA pain have yet to be studied in LH rats.

3.1.4. CHAPTER AIMS

MIA model has been widely used to investigate pain mechanisms, associated behaviour and possible treatments. The intra-articular injection of this glycolysis inhibitor promotes characteristic pain behaviour, degradation of cartilage, as well as other OA features in several laboratory animals, such as rodent, rabbits and guineas pigs. Albino rat strains have been the most commonly used in pain laboratories, however these strains are less suitable for cognitive behaviour studies, due to poor performances in comparative translational tests of clinically relevant cognitive functions. In this chapter, in order to overcome this drawback when testing our hypothesis that chronic OA pain may affect cognitive function, chronic OA-like knee pain induced by MIA model was studied in LH rats with the objective of identifying a suitable dose to induce a robust pain phenotype and knee pathology in this strain of rats.

TABLE I- SUMMARY OF SOME MIA DOSES IN PAIN BEHAVIOUR AND KNEE HISTOLOGY. WB: WEIGHT BEARING; VF: VON-FREY TEST.

Paper	Strain	Age	MIA	Time point	Pain behaviour	Knee histology
(Nwosu et al. 2016)	**Sprague Dawley rat**	250-300 g	0.1 mg or 1 mg (50 µL)	Until day 20 (1mg) and day 42 (0.1mg)	**WB**: 1 mg resulted in WB asymmetry while 0.1 mg did not sig. alter the WB. **PWT:** Both doses reduced PWT.	At **D20**, both cartilage damage and abnormal chondrocyte morphology were comparable for the two doses of MIA. However, proteoglycan loss only reached significant difference for 1 mg group, compared to the control.
(Sagar et al. 2010)	**Sprague-Dawley rat**	160-190 g	0.3 mg, 1 mg, or 3mg (50 µl)	Until day 28	0.3 mg MIA did not produced change in pain behaviour on day 14 **WB**: 1 mg and 3 mg produced changes in weight bearing (slightly higher asymmetry with higher dose) **PWT**: All doses reduced PWT. 0.3 and 1 mg showed similar results. 3 mg produced a more robust decrease in PWT.	1 mg produced comparable changes in cartilage and subchondral bone on days 14 and 28, mild synovitis only on day 28. 3 mg MIA produced severe changes in cartilage, subchondral bone, and synovitis, increasing from day 14 to day 28. No results from 0.3 mg Decreases in hind paw withdrawal thresholds were significantly correlated with changes in cartilage subchondral bone and synovium histology on day 28 but not day 14 following injection of MIA.
(Bove et al. 2003)	**Wistar rats**	175-200 g	0.1 mg, 0.3 mg, 1mg or 3 mg (50 µl)	Days 1, 3, 7 and 14	**WB**: concentration-dependent increase in the WB asymmetry	Only assessed in rats that had received 1 mg of MIA at days 7 and 14. Cartilage damage, chondrocyte loss and loss of proteoglycan, increasing severity from day 14 to day 28.
(Aso et al. 2016)	**Sprague Dawley rats**	250-300 g	3 mg/ 25 µl	Week 2 and 6	**WB**: reduction from week 1 with peak at week 6 **PWT:** continuous reduction from week 1 to week 3 and then a slightly reverse was observed from week 4 to week 6.	At **2weeks**, severe damage of the cartilage and subchondral bone changes. At **6 weeks**, the subchondral bone damage was further aggravated and fibrillation is present.
(Ferreira-Gomes et al. 2012)	**Wistar rats**	230 ±20 g	0.3 mg, 1 mg, or 2mg (25 µl)	Until day 31	**Knee-bend** and **CATwalk**: Dose-dependent increase	Dose- and time-dependent. Higher dose leads to higher severity in early time points.
(Udo et al. 2016)	**Wistar rats**	270-285 g	0.1 mg, 0.2 mg, 0.5 mg or 1mg (50 µl)	Week 2, 4, 6, 8 and 12	no behaviour	Dose- and time-dependent.
(Mapp et al. 2013)	**Sprague Dawley rat**	~180 g	1mg/ 50 µl	Until Day49	MIA- injected rats did not display **WB** asymmetry. **PWT** were also reduced. Saline animals also showed a small decrease in PWT.	Synovitis, osteophytes, chondropathy and vascular channels reduction were observed since D14. The severity of the histology pathology increased with time. By day 49: extensive loss of proteoglycan and absence of chondrocytes.
(Ogbonna, Clark, and Malcangio 2015)	**C57Bl/6 mice**	3, 15 or 22 months old (20–40 g)	1mg/10uL	Until day 28	**WB**: All ages showed significant changes in WB (15-month-old showed the greatest asymmetry in the 15-month-old and attenuated in the 22-month-old mice). **PWT**: No changes in PWT was observed in both 15- and 22- month-old mice. Reduced PWT in the 3-month-old mice	3- to 22-month-old mice showed cartilage damage and loss of proteoglycan.

3.2. METHODS

Refer to Chapter 2 for general methodology.

3.2.1. ANIMALS

A total of 48 adult male Lister hooded (LH) rats weighing between 253-283g and 24 Sprague-Dawley (SD) weighing between 275-300g (rats of both strains were approximately 2-3 months of age; Charles Rivers, UK) were used in this study. The age was an important consideration when planning this project to ensure complete brain and bone maturation, as discussed in chapter 2.

3.2.2. PAIN BEHAVIOUR AND SENSORIMOTOR ACTIVITY

The model of OA pain was induced with a single intra-articular injection of MIA (as described in 2.2.). Each rat was injected with 50 µL of MIA (1 mg/50 µL or 3 mg/50 µL saline) or the same volume of sterile 0.9% saline solution (control animals) through the infra-patellar ligament. Rats were initially allocated to the treatment groups before model induction based on the pain and sensorimotor activity baseline measurements to match the prospective treatment groups for their baseline measurements as closely as possible. In each cage, half of the animals received treatment and the other half saline. The experimenter (S.G.) was blinded to the treatment allocations throughout the data collection and analysis.

Baseline measures of pain behaviour and sensorimotor activity were obtained prior to MIA-induced arthritis (Day 0) and then measures were taken at specific postoperative time points as described in Fig.3.1. Pain behaviour were assessed using two behavioural tests: i) weight distribution through the injured and contralateral limb using an incapacitance tester, and ii) mechanical sensitivity measurement of hindpaw withdrawal thresholds by application of von Frey monofilaments to the plantar surface of the paw (as described in 2.3.). Potential effects of the model on the sensorimotor activity were assessed using two behavioural tests: i) open-field locomotor activity test, to assess potential impairments in the locomotor activity, and ii) startle/prepulse inhibition test to evaluate brain function (as described in 2.4).

3.2.4. EXPERIMENTAL DESIGN

Three different experiments were conducted:

Experiment 1 – LH rats injected with 1 mg MIA: a total of 24 LH rats were used in two separate replicates. Rats received a single intra-articular injection of either MIA (1 mg/50 µL; n=12) or the same volume of sterile saline solution (controls; n=12). After MIA injection one rat presented breathing difficulties and was humanely killed and excluded from the study. One extra rat was excluded from weight-bearing test due to intense stress when placed into the chamber. Pain behaviour and sensorimotor activity measurements were collected at baseline, and then from day 3 to day 42, twice a week until the endpoint Day 49. (Fig3.1.A).

Experiment 2 – 20 LH and 20 SD rats were used in this experiment. Half of the rats of each strain were injected with 50 µL of MIA (1 mg/50 µL), the other half were injected with the same volume of sterile saline solution (controls). Pain behaviour measurements were collected at baseline, days 14 and 28 after model induction to prevent habituation to the tests due to repeated testing. Sensorimotor activity was assessed at baseline, days 15 and 29. Endpoint: Day 35. (Fig.3.1.B).

Experiment 3 – this experiment was conducted as a pilot study and all the 4 LH and the 4 SD rats were injected with 3 mg/50 µL of MIA. Pain behaviour was assessed at baseline days 7, 14, 21 and 28. Endpoint: Day 35. (Fig.3.1.C).

At the end of each experiment, rats were anesthetized with a lethal dose of sodium pentobarbitone and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA). Brains, spinal cords, DRGs and knees were carefully excised, preserved and stored. Knee joint sections were stained with haematoxylin and eosin- or safranin-O/fast green and then scored for overall joint morphology and proteoglycan loss. Total cartilage joint damage, osteophyte, proteoglycan loss, synovial inflammation and chondrocyte presence were scored to evaluate the severity of the knee joint pathology as described in 2.7.

3.2.5. STATISTICAL ANALYSIS

GraphPad Prism 8 and IBM SPSS Statistics 24 were used to generate the graphs and perform the statistical analysis. Results from the pain behaviour studies were analysed using

an analysis of variance (ANOVA), with group for between-subjects and time as repeated measures/within-subjects variable of testing day. Results from the sensorimotor behaviour studies were analysed using a mixed design factorial ANOVA test with testing day and task blocks (or pulse intensity in the PPI test) as within-subject factors and group as between-subjects factor. Bonferroni multiple comparison was used as *post-hoc* testing. Knee pathology was analysed with Kruskal-wallis test since normality was violated.

$p<0.05$ was considered to represent a significant difference and all results were expressed as mean ± standard error (SEM).

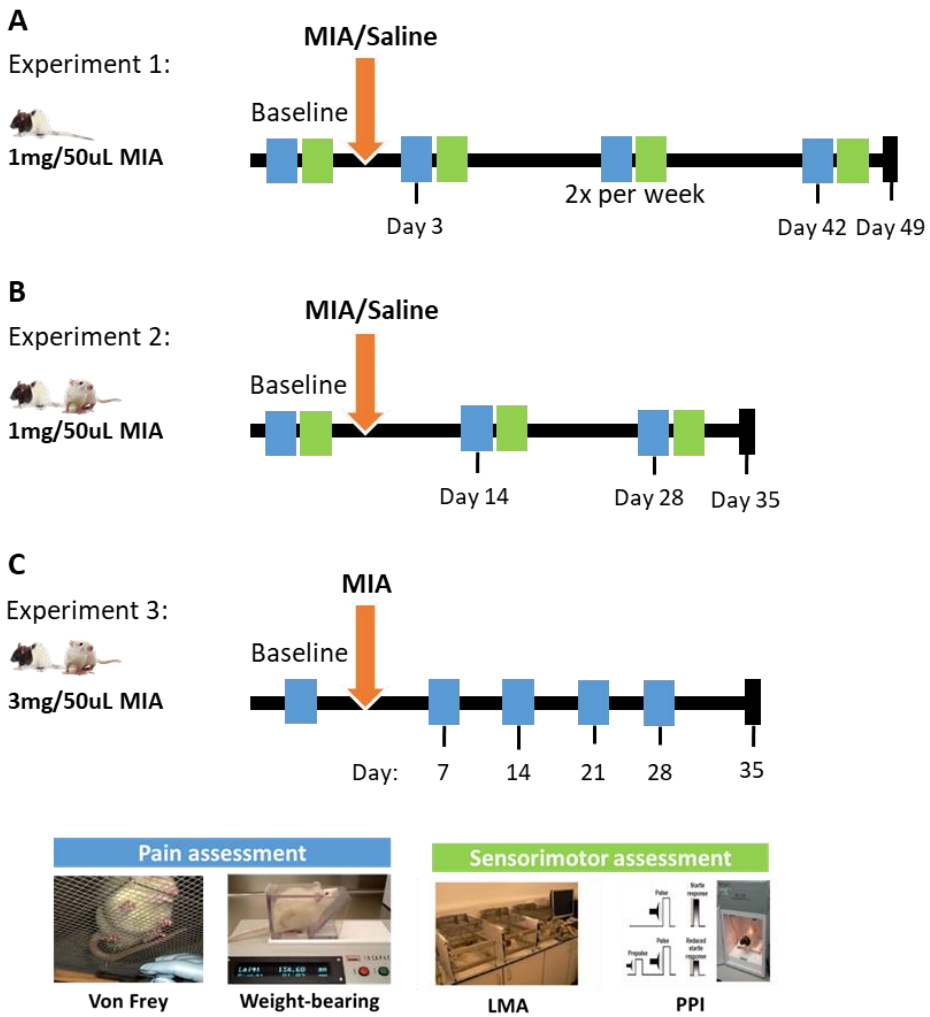

FIGURE 3.1. – TIME COURSE OF THE MIA-INDUCED OA-LIKE KNEE PAIN IN LISTER HOODED RATS STUDY. 48 LH rats and 24 SD rats were used in this study. Rats were first handled and habituated to the experimenter and pain behaviour test apparatus. Pain and sensorimotor activity baseline measurements were collected before model induction and then at specific time points after MIA (1mg or 3mg /50µl) or saline intra-articular injection as indicated in the scheme. 3 experiments were carried on in this study (A, B and C).

3.3. RESULTS

At this point, data from experiments 1 and 2 are presented together.

Rats from both SD and LH strains with approximately the same age were used to ensure the same degree of brain and bone development. At the equivalent age, adult SD rats were heavier than adult LH rats (Fig.3.2.), SD MIA-injected rats showed a trend to gain less body weight than saline control SD, but no significant differences were observed (group: $F_{(1,18)}$=1.45; p=0.24). This trend was not evident in LH rats (group: $F_{(1,18)}$=0.50).

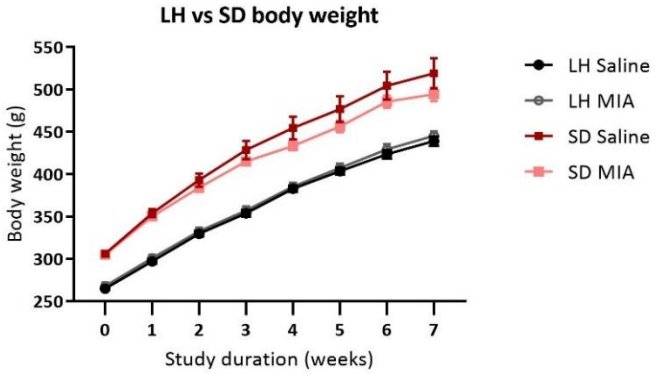

FIGURE 3.2. – BODY WEIGHT OF SPRAGUE-DAWLEY (SD) AND LISTER HOODED (LH) RATS AFTER MONOSODIUM IODOACETATE (MIA) OR SALINE INJECTIONS. Rats were injected with either 50ul of 1mg of MIA (□, ○) or saline (▪, ●) in the left knee (n=10 in each group). Data are presented as mean±SEM.

3.3.1. PAIN BEHAVIOUR – 1 MG MIA

The standard dose of MIA (1 mg) was only able to induce weak pain phenotypes in both LH and SD rat strains, reflected by small changes in the weight bearing asymmetry and no significant changes in the paw withdrawal thresholds.

LH rats injected with 1mg in the experiment 1 exhibited a decrease in weight bearing compared with control animals (group: $F_{(1,19)}$=15.74; p=0.0008) (Fig.3.3.A), showing that MIA LH rats place less weight on the injured paw compared with LH saline. Bonferroni post hoc analysis showed this difference was only statistically significant at day 3 after model induction, and likely reflects an acute inflammatory response. Main effect of time was observed ($F_{(11, 209)}$=2.22; p=0.01), however, no interaction involving group was observed ($F_{(11, 209)}$=1.26; p=0.25). No differences in hindpaw withdrawal thresholds were observed for MIA versus saline injected rats (group: $F_{(1,20)}$=3.22; p=0.09) (Fig.3.3.B). Both the MIA and control groups showed a decrease in paw withdrawal threshold after intra-articular injection, reflecting habituation to the test (time: $F_{(10,200)=}$2.09; p =0.27). No significant interaction between group and time was found(time x group: $F_{(10, 200)}$=0.88).

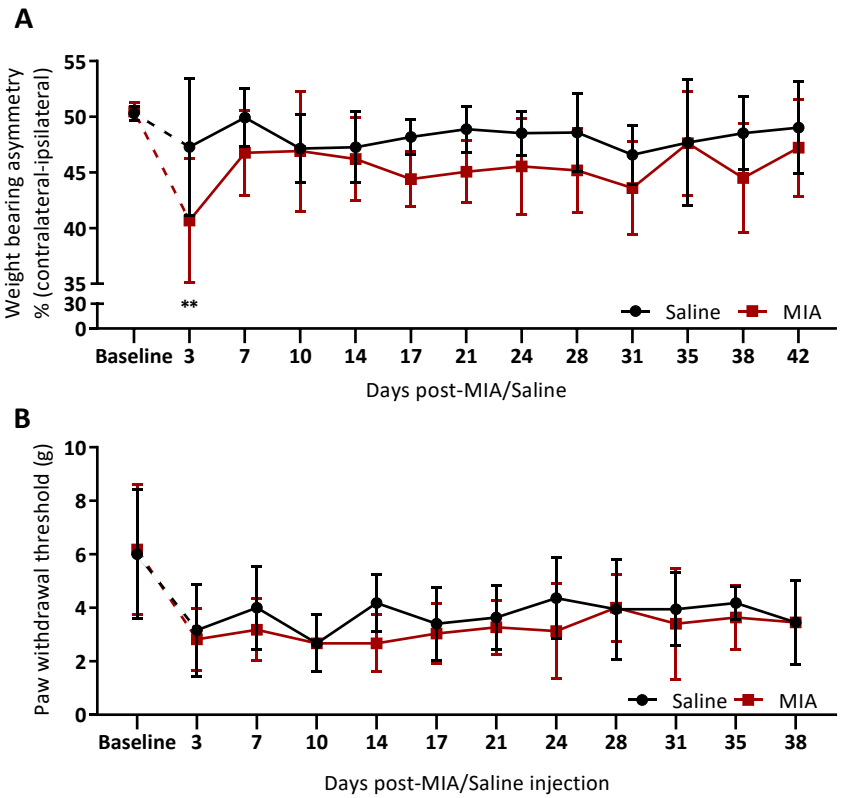

FIGURE 3.3. – MONOSODIUM IODOACETATE (MIA) INDUCED WEAK ASYMMETRY IN (A) WEIGHT BEARING AND NO CHANGES IN (B) MECHANICAL ALLODYNIA IN LISTER HOODED (LH) RATS. LH rats were injected with either 50ul of 1mg of MIA (▪; n=10 in A and n=11 in B) or saline (●; n=11) in the left knee. Data are presented as mean±SEM. 2-way ANOVA with Bonferroni post hoc testing. ** p=0.002

In experiment 2, a slight decrease in weight bearing was evident after MIA injection in both SD and LH rats (group: $F_{(3,36)}$=6.57; p=0.001) (Fig.3.4.A). However, post hoc analysis showed that only MIA LH rats displayed a statistically significant difference in weight bearing compared with controls rats of the same strain, SD MIA did not statically differ from SD saline at any timepoint. All rats in experiment 2 showed a slight trend to reduce PWT after baseline, however no main effects of group and time were observed (Fig.3.4.B). The 2-away ANOVA using MIA group as between-subjects factor and test day as repeated-measures factor showed no statistically significant interaction effect between group and time for both WB and PWTs (time: $F_{(1,36)}$=1.20; p =0.28; time x group: $F_{(3,36)}$<1.31; p>0.29).

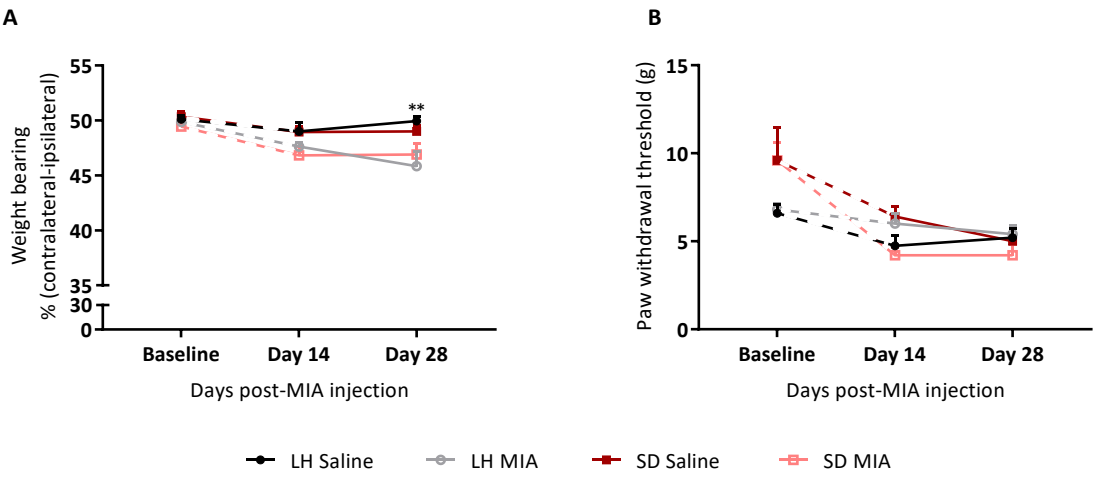

FIGURE 3.4. – MONOSODIUM IODOACETATE (MIA) INDUCED ASYMMETRY IN WEIGHT BEARING IN BOTH LISTER HOODED (LH) AND SPRAGUE-DAWLEY (SD) RATS (A), BUT ONLY INDUCED CHANGES IN MECHANICAL ALLODYNIA IN SD RATS (B). Rats were injected with either 50ul of 1mg of MIA (□, ○) or saline (▪, ●) in the left knee (n=10 in each group). Data are presented as mean±SEM. 2-way ANOVA with Bonferroni as post hoc testing. ** p=0.003

3.3.2. SENSORIMOTOR ACTIVITY – 1 MG MIA

Locomotor activity, startle response and prepulse inhibition were not affected by the intra-articular injection of the standard dose of MIA (1 mg) in either adult LH or SD rats.

Neither the horizontal (group: $F_{(1,21)}$=3.81; p=0.06) nor vertical (group: $F_{(1,21)}$=2.69; p=0.12) (data not shown) activity measured were affected by 1 mg of MIA treatment in LH rats in experiment 1 at any timepoint. 3-way ANOVA using group as between-subjects factor and test day and blocks as within-subjects was run to evaluate any potential effects of MIA-injections on locomotor activity during the time of session and time point in experiment 1, showed no triple interaction effect ($F_{(28,280)}$=1.08; p=0.36). In addition, there were no impairments in startle response (group: $F_{(1,21)}$=2.36; p=0.14) or prepulse inhibition (group: $F_{(1,21)}$=0.05; p=0.83) in the LH rats following induction of the MIA model (data not showed). 3-way ANOVA using MIA group as between-subjects factor and test day and startle amplitude or pulse intensity as within-subjects also showed no triple interaction effect regarding startle habituation ($F_{(22,262)}$=1.52; p=0.06) or % prepulse inhibition ($F_{(33,693)}$=0.83; p=0.73).

In experiment 2, injection of 1 mg of MIA also did not affect the horizontal locomotor activity of SD or LH strains (Fig.3.5.). 3-way ANOVA showed a main effect of group (group: $F_{(3,36)}$=3.72; p=0.02), however, post hoc analysis showed that this only reflected a difference between strains (LH saline > SD saline, p=0.02). The 3-way ANOVA showed no triple interaction effect of groups, blocks and days ($F_{(12,144)}$=1.229; p=0.27). Interaction between

group and blocks ($F_{(6,72)}$=6.01; p<0.0001) or days ($F_{(6,72)}$=3.10; p=0.01) and between blocks and days ($F_{(4,144)}$=10.94; p<0.001) were statistically significant, which reflects a habituation to the apparatus during the 30min-session and to the test itself. Looking in particular to the locomotor activity in the first block of each session, when the rats are less habituated to the apparatus and show a higher exploratory activity, there was only a main effect of days in both horizontal ($F_{(2,72)}$=48.81; p<0.0001) and vertical activity ($F_{(2,72)}$=40.4; p<0.0001, data not shown), reflecting habituation to the arena.

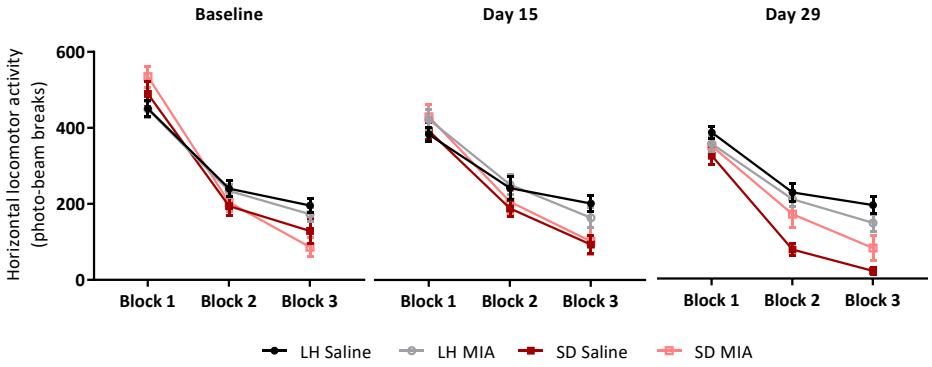

FIGURE 3.5. – MONOSODIUM ACETATE (MIA) DOES NOT AFFECT THE HORIZONTAL LOCOMOTOR ACTIVITY IN BOTH LISTER HOODED (LH) AND SPRAGUE-DAWLEY (SD) RATS. Rats were injected with either 50ul of 1mg of MIA (○, □) or saline (●, ▪) in the left knee (n=10 in each group). Data are presented as mean±SEM.

The injection of 1 mg of MIA did not affect the startle or PPI measures when compared with saline-injected rats in both strains (Fig.3.6.). 3-way ANOVA showed a main effect of group (group: $F_{(3,36)}$=19.29; p<0.001) in the startle response. However, post hoc analysis showed that this only reflected a difference between strains. Startle response is overall markedly enhanced in LH compared with SD rats (LH saline vs SD saline, p<0.0001). For this reason, different 3-way ANOVAs were performed to evaluate the startle response of both strains, no main effect of group treatment in both LH (group: $F_{(1, 18)}$=0.587; p=0.45); and SD rats (group: $F_{(1, 18)}$=0.679; p=0.42) or triple interactions between days, startle habituation across trials and group was observed.

Regarding the prepulse inhibition (%PPI), 3-way ANOVA showed a main effect of group (group: $F_{(3,36)}$=6.52; p=0.001), however, post hoc analysis showed that this only reflected a difference between strains (LH vs SD, p<0.04). Similar with startle, rats exhibit habituation to the test across days (time: $F_{(2, 72)}$=8.08; p<0.001), but no triple interaction effect of groups, pulse intensity and days after model induction was observed ($F_{(18, 216)}$=115.36; p=0.65).

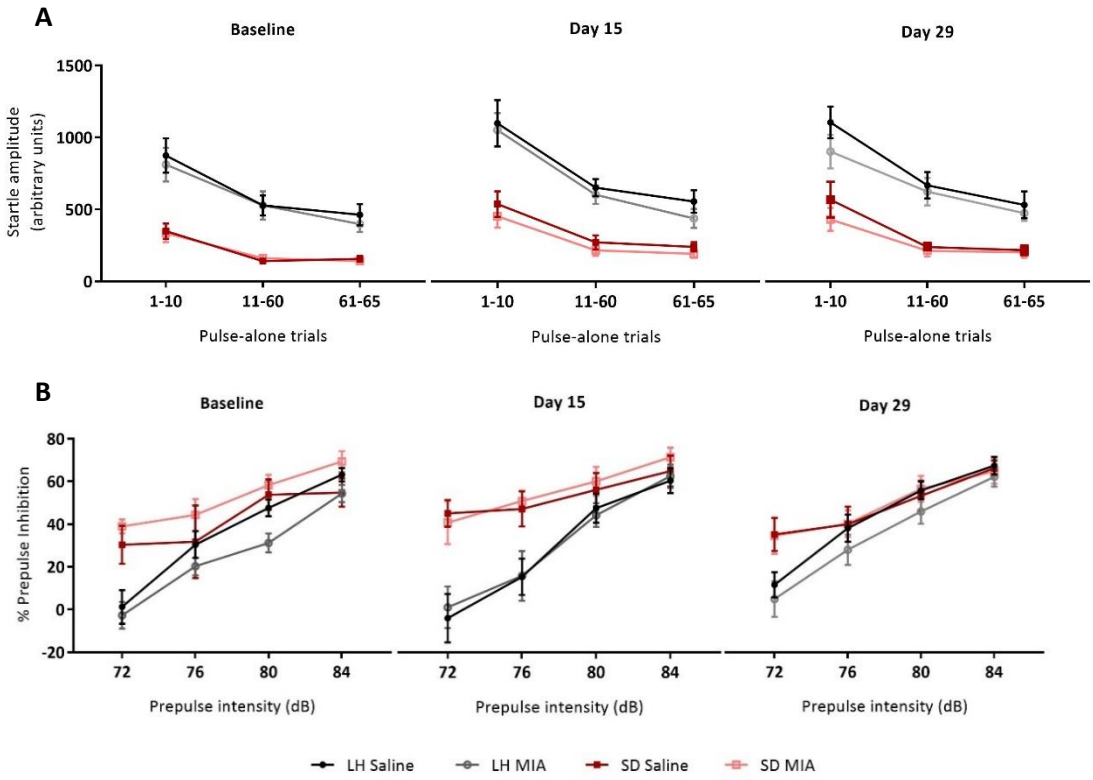

FIGURE 3.6. – MONOSODIUM IODOACETATE (MIA) DOES NOT AFFECT THE STARTLE HABITUATION (A) AND THE % OF PREPULSE INHIBITION (B) IN BOTH SPRAGUE-DAWLEY AND LISTER HOODED RATS. Rats were injected with either 50ul of 1mg of MIA (□, ○) or saline (▪, ●) in the left knee (n=10 in each group). Data are presented as mean±SEM.

3.3.3. KNEE HISTOLOGY – 1 MG MIA

Knee joints from all animals from experiment 2 were collected at day 35 after model induction and processed for pathology scoring to assess the knee pathology induced in both SD and LH rats after intra-articular injection of 1 mg of MIA (Fig.3.7. and 3.8.). This dose of MIA only induced some loss of cartilage integrity and synovium inflammation in a small number of animals, and no significant differences were observed ($H<5.26$, $p>0.15$; Fig.3.9.A and B). However, both proteoglycan loss ($H=24.44$, $p<0.0001$; Fig.3.9.C) and decreased number of chondrocytes ($H=24.52$, $p<0.0001$; Fig.3.9.D) were observed in knee joints 35 days after injection of 1 mg MIA. There were no osteophytes present in any rats in this study. One SD MIA-injected rat was not considered in this analysis because it was impossible to access the tibial plateau due to bad angle split during knee joint processing.

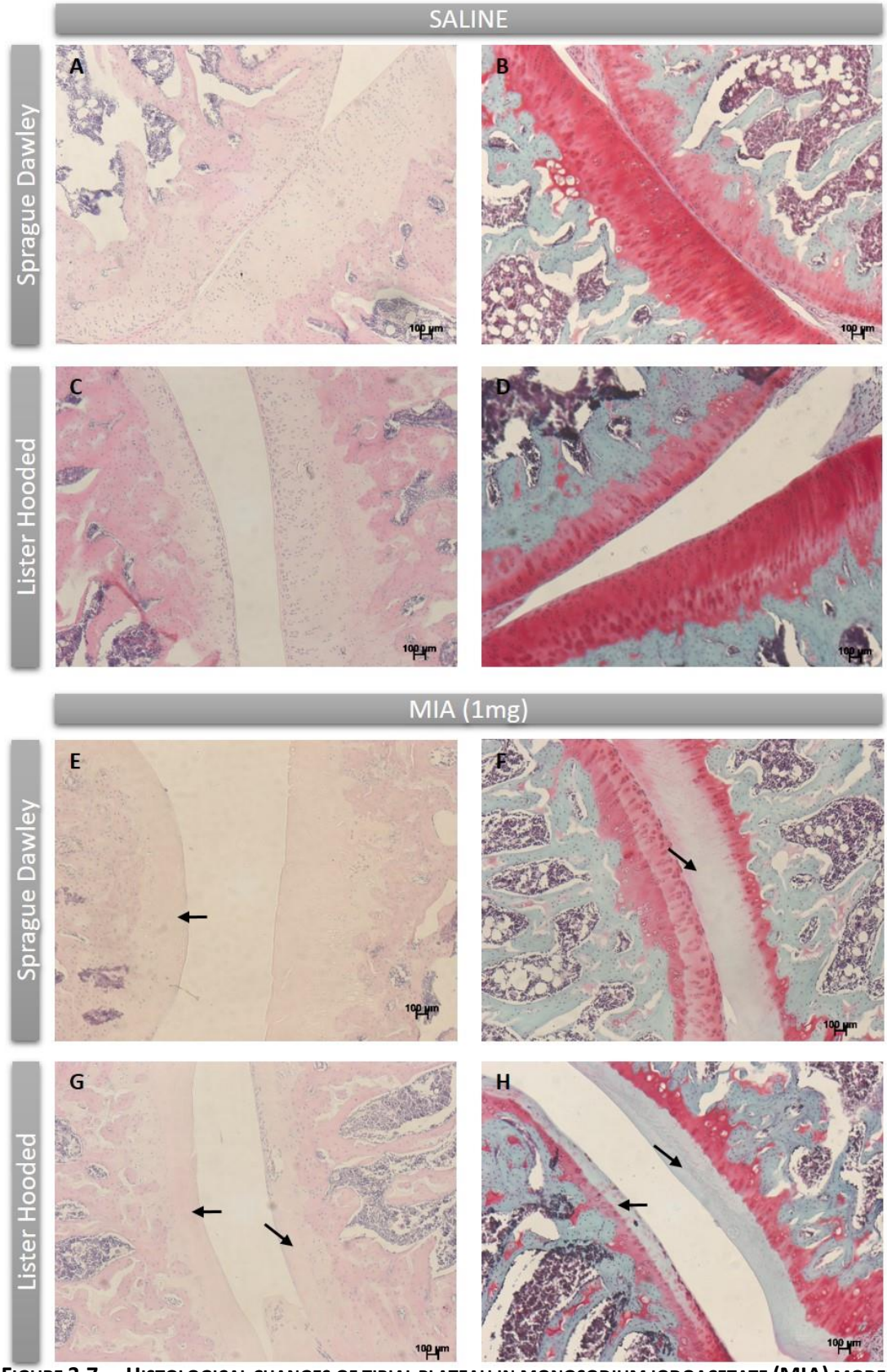

FIGURE 3.7. – HISTOLOGICAL CHANGES OF TIBIAL PLATEAU IN MONOSODIUM IODOACETATE (MIA) MODEL IN BOTH SPRAGUE-DAWLEY (SD) AND LISTER HOODED (LH) RATS. Representative coronal sections of medial and lateral tibial plateau stained with Haematoxylin and eosin (H&E) (A,C,E,G) and Safranin-O-Fast green (B,D,F,H), 10x. Rats were injected with either 50ul of saline (A-D) or 1mg MIA (E-H). 1mg MIA was only able to induce chondrocyte loss in the cartilage (black arrows) in both SD and LH rats, 35 days after model induction.

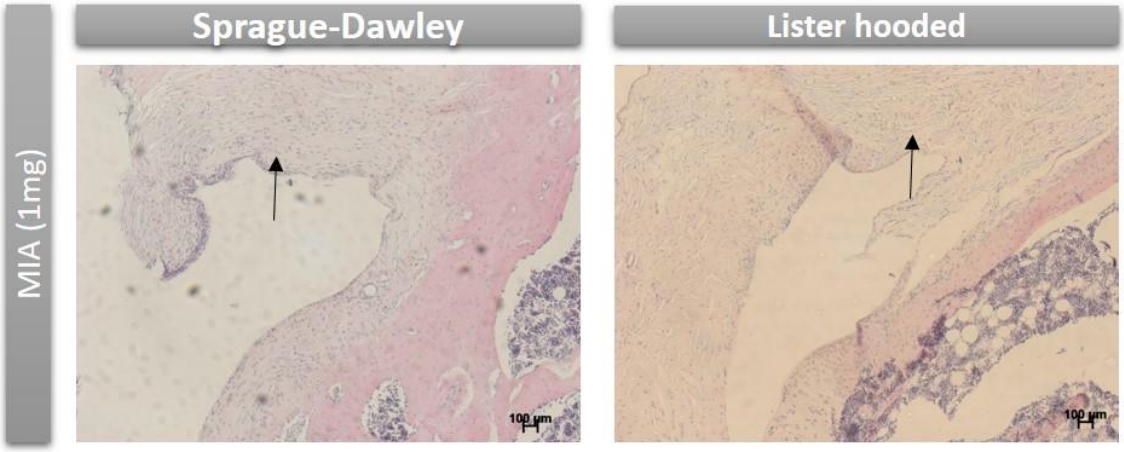

FIGURE 3.8. – HISTOLOGICAL CHANGES IN SYNOVIAL LINING LAYER THICKNESS-CELLULARITY IN THE 1 MG MONOSODIUM IODOACETATE (MIA) MODEL IN BOTH SPRAGUE-DAWLEY (SD) AND LISTER HOODED (LH) RATS. Representative coronal sections of tibial plateau haematoxylin and eosin-stained in both SD and LH rats, 10x. Black arrows indicate severe increase in cellularity in the synovium.

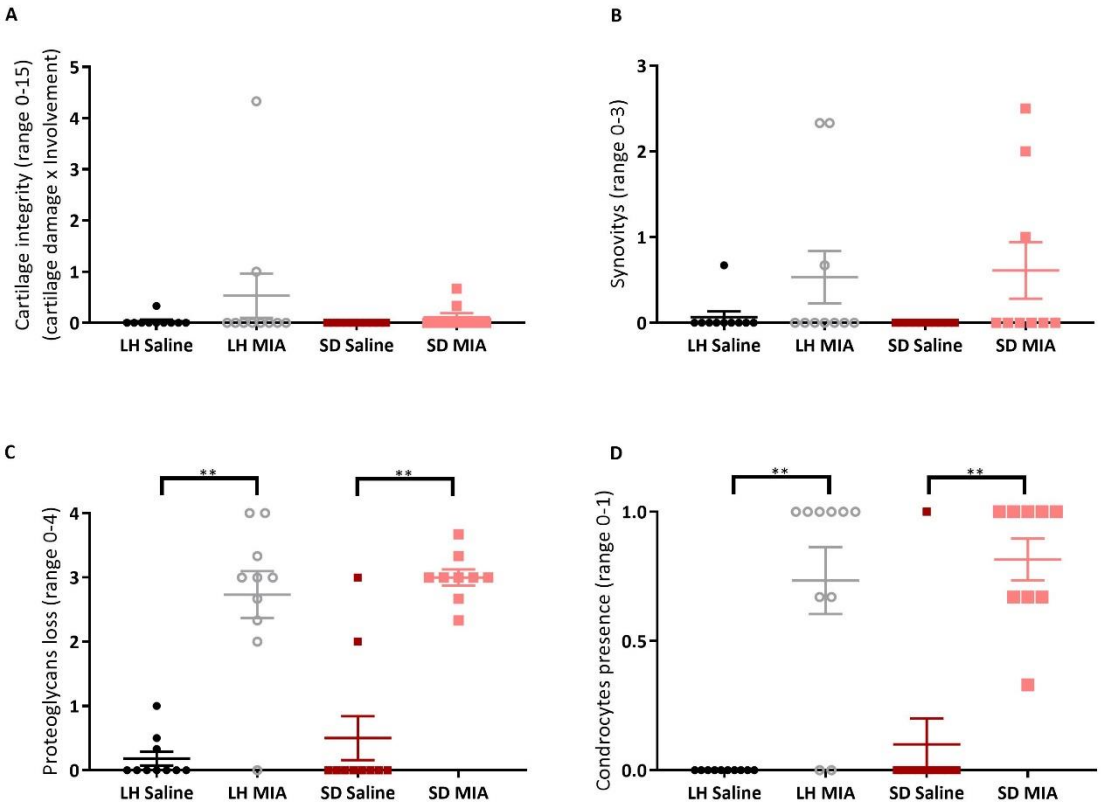

FIGURE 3.9. – MICROSCOPIC QUANTIFICATION OF HISTOLOGICAL CHANGES OF TIBIAL PLATEAU IN 1 MG MONOSODIUM IODOACETATE (MIA) MODEL IN BOTH SPRAGUE-DAWLEY (SD) AND LISTER HOODED (LH) RATS. Average scores for medial and lateral tibial plateau. Rats were injected with either 50ul of 1mg of MIA (○, □) or saline (●, ▪) in the left knee (n=10 in each group). Knees were collected and processed for scoring at day 35 after model induction. 1 mg MIA was not able to induce cartilage damage (A) or synovial inflammation (B). On the other hand, proteoglycan loss (C) and chondrocytes absence (D) were observed in both SD and LH rats. Data are presented as mean±SEM. **p<0.01.

3.3.4. PAIN BEHAVIOUR AND KNEE HISTOLOGY – 3 MG MIA

To briefly evaluate if the lack of robust pain phenotype is related to the dose of MIA used, a pilot study was performed. Four rats of each strain (LH and SD) were injected with 50 µL of 3 mg MIA and tested for pain phenotype at baseline, 7, 14 and 28 after MIA injection. As our objective was only to ascertain whether a higher dose of MIA could induce a robust behavioural pain phenotype, saline controls were not included this experiment.

In contrast with the two previous studies using the standard 1 mg dose of MIA, intra-articular injection of 3 mg dose of MIA induced pain phenotype in both strains of rats (Fig.3.10), as reflected by reduced weight bearing symmetry. Both MIA-injected LH and SD rats showed a significant weight bearing asymmetry after model induction (time: $F_{(2,12)}$=13.44; p=0.0009), no main effect of group or interaction between group and time were observed (F<13.44, p>0.5) (Fig.3.10.A). Additionally, ipsilateral hindpaw withdrawal thresholds were also decreased after model induction (time: $F_{(2,12)}$=11.82; p=0.002) (Fig.3.10.B). No main effect of group, time or interaction of time x group were observed in the contralateral paw thresholds (F<2.54, p>0.12).

Knee joints were again collected at day 35 after model induction and processed for histology. Injection of 3 mg of MIA in both LH and SD rats was associated with proteoglycan loss, decreased chondrocyte presence and pronounced cartilage surface damage (Fig.3.11).

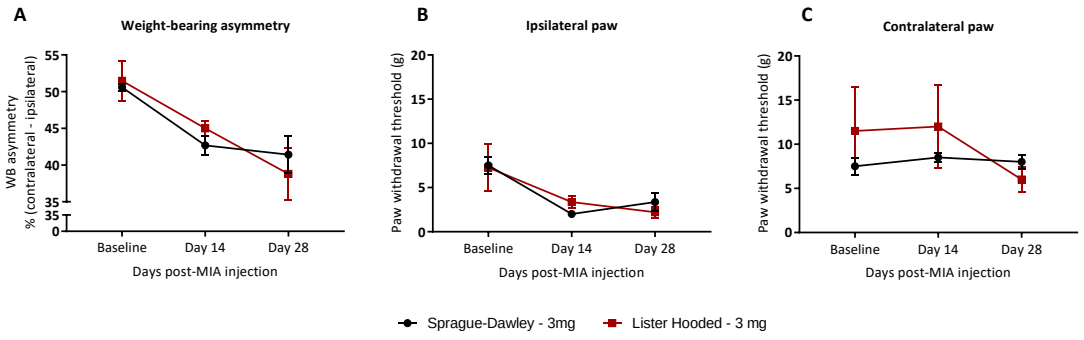

FIGURE 3.10. – 3 MG OF MONOSODIUM ACETATE (MIA) INDUCED CHANGES IN WEIGHT BEARING (A) AND IN MECHANICAL ALLODYNIA IN THE IPSILATERAL PAW (B), NOT IN THE CONTRALATERAL PAW (C) IN BOTH LISTER HOODED (LH) AND SPRAGUE-DAWLEY (SD) RATS. SD (●, n=4) and LH (▪, n=4) were injected with 3 mg of MIA. Data are presented as mean±SEM.

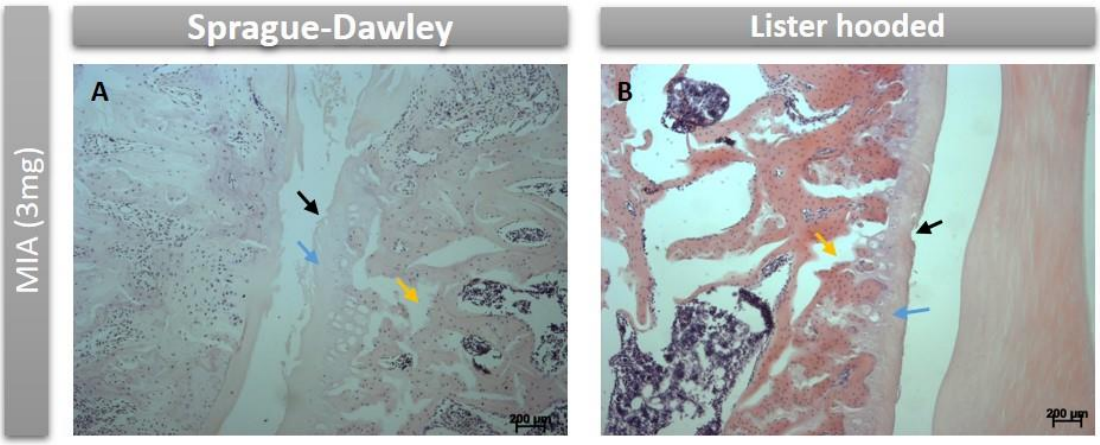

FIGURE 3.11. – HISTOLOGICAL CHANGES OF TIBIAL PLATEAU IN 3 MG MONOSODIUM IODOACETATE (MIA) MODEL IN BOTH SPRAGUE-DAWLEY (SD) AND LISTER HOODED (LH) RATS. Representative coronal sections of medial and lateral tibial plateau haematoxylin and eosin-stained, 35 days after model induction, 10x. SD (A, n=4) and LH (B, n=4) rats were injected with 50ul of 3mg MIA in a pilot study. 3mg MIA was able to induce cartilage integrity damage (black arrows), chondrocyte loss in the cartilage (blue arrows) and subchondral bone changes (yellow arrows) in both SD and LH rats.

3.4. DISCUSSION

The MIA model, as previously mentioned, is a well-established model for albino strains in our and other labs. The 1mg dose of MIA is usually enough to induce a robust pain behaviour and joint pathology across albino rat strains. Therefore, no problems were anticipated with transferring the chemical induced model to LH rats. However, the pain and joint pathology phenotypes after the standard 1 mg dose of MIA were not robust in LH rats or age matched SD rats. Increasing the dose to 3 mg MIA overcame this limitation and induced robust weight bearing asymmetry and knee pathology in LH and SD rats.

3.4.1. PAIN BEHAVIOUR IN MIA-INDUCED LISTER HOODED RATS

In a first study, only LH rats were tested with the dose usually used at our lab in albino rats (1mg) (Sagar et al., 2011; Gowler et al., 2020). 1mg MIA has been reported to induce weight bearing asymmetry and mechanical allodynia with reduced paw withdrawal thresholds during von-Frey testing in both albino strain, Sprague Dawley (Sagar et al., 2010; Nwosu et al., 2016b) and Wistar Han rats (Bove et al., 2003). Unexpectedly, the intra-articular injection of 1 mg MIA in LH rats was not associated with a robust pain phenotype. MIA injected rats showed a trend to have lower weight bearing asymmetry values, however it was not significantly different from saline-control rats.

Similar with control rats, MIA-injected LH rats showed a decrease in the paw withdrawal thresholds after baseline, showing a normal habituation to the test, which remained almost constant across the study duration. Importantly, the baseline paw withdrawal threshold measures of LH rats were much lower than albino strains (Abaei et al., 2016; Gowler et al., 2020), consistent with a previous report in LH rats (Moriarty et al., 2016b). However, different approaches were used between this study and Moriarty's study, in this study "up-down" Von Frey was used while "percentage response" method was used in Moriarty's study. These lower baseline values might be due to the fact that LH rats are naturally more inquisitive and curious than albino strains; during Von Frey testing measures should not be taken while animals are grooming and it requires exploratory behaviours to be kept to a minimum to avoid false negatives or positive responses (Deuis et al., 2017). In fact, during assessments LH rats did not show reduced activity in the Von Frey cages over the entire session. Additionally, a study to evaluate nociceptive sensory profiles using the Von Frey test conducted in 5 different strains of albino rats showed strain-dependent differences in hind

paw threshold in the absence of injury and different profiles after injury, results that may be linked with to the inherent stress profiles (Hestehave et al., 2019).

In this study, strain was not the only difference from the previous studies conducted in our lab, but also the age of the rats. Thus a second experiment was conducted comparing age matched SD and LH rats. At this experiment, pain behaviour was only assessed at three different time points, to avoid potential habituation to handling and to the test apparatus, since during the first study we observed that rats have more tendency to keep less still day after day, increasing the difficultly in assessing behavioural measures over time. In this study, both SD and LH MIA-injected rats showed a trend to decrease weight bearing asymmetry, but this was not significantly different from control rats. Since the lack of robust results was comparable between pigmented and albino strains, strain does not seem the issue for this weak pain phenotype and mild knee pathology results.

The age and the size of rats was another difference between this study and previous studies conducted in our lab. Indeed, during the intra-articular injection it was noticeable that knee joints were bigger in this age of rats, than previous SD rats used. This lead to the question of whether the dose of MIA used was not sufficient to induce the knee joint damage and consequently pain behaviour features. Intra-articular injection of 3mg MIA was associated with a considerable increase in weight bearing asymmetry in both SD and LH rats from day 7 until the end of the study. This higher dose of MIA also induced a slight mechanical allodynia in both strains, with SD- and LH-injected rats exhibiting a decrease in the hindpaw withdrawal thresholds, however this effect could reflect a time effect and not pain effect so appropriate comparison with saline controls is needed to address this.

3.4.2. SENSORIMOTOR ACTIVITY IN MIA-INDUCED LISTER HOODED RATS

Consistent with previous studies, LH exhibited higher locomotor activity than SD rats, confirming previous reports that LH rats are more inquisitive and active than albino stains. Accordingly, Weiss and colleagues (Weiss et al., 2000) previously reported that LH rats have an increased locomotor activity, in comparison with two albino strains, SD and Wistar rats. McDermott and Kelly(McDermott and Kelly, 2008) compared locomotor activity between LH, SD and Wistar rats using two different methods, no differences were reported during the 5 minutes open field session, however, LH rats showed decreased nocturnal activity and increased day-time activity when compared with both albino strains in the 24-hour home cage monitoring. In my study, startle response and prepulse inhibition were not altered

following induction of the MIA model in either strain of rats. However, as reported previously (Varty and Higgins, 1994), startle responses were higher in LH rats compared with SD rats, while prepulse inhibition was lower in LH than SD rats. Nevertheless these baseline values were not altered by the model of OA pain.

3.4.3. KNEE PATHOLOGY IN MIA-INDUCED LISTER HOODED RATS

In line with the behavioural results, microscopic histological analysis showed limited morphological alterations on the affected knee joint in animals injected with 1 mg MIA. The absence of chondrocytes in the cartilage in the MIA-injected rats shows that MIA was properly delivered in the knee joint and inhibited the glycolysis, reducing the number of chondrocytes. However, this effect was not enough to induce the other OA features usually observed with 1mg MIA, i.e., cartilage damage, synovium inflammation and osteophyte formation. Only a small number of rats showed these features in the knee joint.

As previously mentioned, young SD rats have been largely used in our lab, and strong and consistent pain phenotype and knee pathology have been reported (Guingamp et al., 1997; Marker and Pomonis, 2012). The major difference between the SD previously used and the SD rats used in this chapter is the age, here we were using young adult and not juvenile/young rats. Since at the same time of these studies, the same batch of MIA drug was being used for other researchers and robust pain behaviour and knee histology were being detected in those studies, the issue was not the drug.

To test the hypothesis that 1mg of MIA was not enough to induce cartilage damage and consequently a robust pain phenotype possibly due to a bigger knee joint in our older animals, a third study was conducted. In this pilot study, adult SD and LH rats were tested for pain behaviour MIA injection.

3mg MIA induced not only chondrocyte death but also substantial cartilage damage, subchondral bone changes and synovitis. 3mg MIA was associated with a pronounced pain phenotype, with decreased weight bearing asymmetry and increased hindpaw mechanical hypersensitivity after model induction. As previously mentioned in table I, 1 mg MIA in younger albino strains displays chondrocyte death, loss of cartilage integrity, osteophytes and synovitis (OA histologic features).

3.5. CONCLUSION

The aim of this work was to transfer the MIA model of OA pain to the adult Lister Hooded rats. In the first instance the transfer of the 1mg MIA model to LH rats did not result in significant pain behaviour or joint pathology. This likely reflects the difference in age and size of LH rats required for the cognitive tests. This was confirmed to not be strain specific as this was also evident in older SD rats. Increasing the dose of MIA to 3mg produced robust pain behaviour and joint pathology in the LH rats. It was therefore deemed that this dose of MIA was suitable for future studies in LH rats in this thesis.

CHAPTER 4

HIPPOCAMPUS-DEPENDENT MEMORY IN MIA-INDUCED OSTEOARTHRITIS KNEE PAIN IN LISTER HOODED RATS

4.1. INTRODUCTION

Chronic pain has been associated with a range of comorbidities, such as depression (Bair et al., 2003), anxiety (Gerrits et al., 2014) and cognitive impairments (Teodoro et al., 2018), including memory impairments.

4.1.1. HIPPOCAMPUS AND MEMORY

The hippocampus is traditionally associated with specific learning and memory functions, particularly aspects of place and declarative learning and memory (with 'declarative' referring to memory that can be consciously recalled and, in humans, be 'declared'), but is also related with emotional, motivational and sensorimotor functions (Bast, 2007). Additionally, a study in healthy people showed hippocampal activation when a painful stimuli was applied, indicating hippocampal involvement in pain processing (Bingel et al., 2002).

As Tulving described "memory is many things", and there are different memory stages - encoding, storage, consolidation and retrieval – and memory types, differing with respect to the type of information that is stored, the way it is learned (e.g., slowly or rapidly) and the duration for which it is stored (Spence, 1996). The hippocampus is particularly important for the rapid encoding and subsequent retrieval of spatial and declarative memory (Bast, 2007).

4.1.2. MEMORY IMPAIRMENTS AND CHANGES IN THE HIPPOCAMPUS IN CHRONIC PAIN PATIENTS

Memory impairments have been reported by patients with chronic pain, with nearly 70% of chronic pain patients reporting memory deficits (Dick and Rashiq, 2007; Berryman et al., 2013). Some of the subjectively reported memory impairments, problems with everyday

type spatial memory or episodic memory (the aspect of declarative memory storing information about personally experienced events and their spatio-temporal context), may be related to hippocampal dysfunction (Bast, 2007).

Chronic pain patients also show poor performance on tasks related with spatial, verbal and recognition memory and impaired perceptual motor coordination and long-term spatial memory, when compared with healthy people. Chronic pain patients may also be more susceptible to interference with memory. For example, fibromyalgia patients, when presented with a distraction, showed impaired short-term memory compared with healthy volunteers (Leavitt and Katz, 2006).

Modern brain imaging methods allow to link hippocampal volume and connectivity alterations with chronic pain. One neuroimaging study conducted in older patients with no dementia reported a correlation between hippocampal volume and all-cause pain: severe acute pain and chronic pain were associated with smaller hippocampal volume (Zimmerman et al., 2009). Another study reported an association between chronic pain with loss of volume in selective hippocampal subfields, but only in female patients (Ezzati et al., 2014). Hippocampal volume reduction was also shown in patients with fibromyalgia (McCrae et al., 2015). Also, altered hippocampal connectivity was associated with chronic pain. Mutso and colleagues followed patients with subacute back pain and back pain and reported increases in hippocampal connectivity compared to controls (Mutso et al., 2014). Interestingly, they also reported a longitudinal reorganization of the connectivity between the hippocampus and medial pre-frontal cortex. No correlation between hippocampal volume and connectivity was observed in this study.

4.1.3. MEMORY IMPAIRMENTS AND CHANGES IN THE HIPPOCAMPUS IN RODENT MODELS OF CHRONIC PAIN

During the last decade several studies have reported changes in the hippocampus using rodent models of persistent and chronic pain. Hippocampal plasticity changes were also reported in rodent models of neuropathic pain. Peripheral nerve injury was not only associated with disruption of long-term potentiation and frequency facilitation at hippocampal regions, but also with working and short-term memory deficits (Ren et al., 2011). Furthermore, in this study Ren and colleagues reported a positive correlation between plasticity change and memory deficits.

Altered hippocampal cytokine expression during chronic pain has been reported in a neuropathic mice model and dependent of the pain phenotype (del Rey et al., 2011). Peripheral nerve injury in rodents has also been associated with molecular changes in the hippocampus, with the alteration in the microtubules stability (You et al., 2018) and structural synaptic and morphological changes in the hippocampal neurons (Liu et al., 2017b).

Neuropathic pain induced by a diabetic model caused a reduced learning rate in the Morris water maze (Moriarty et al., 2016b). The possible effects of inflammatory pain on the hippocampus and its function is less known. Acute and chronic inflammatory pain in rodents was associated with an increased hippocampal volume in rat models of acute and chronic inflammatory pain, (Duric and McCarson, 2005), the opposite to the changes reported in the human pain studies mentioned above.

4.1.4. TRANSLATIONAL ASSESSMENT OF HIPPOCAMPUS-DEPENDENT MEMORY USING THE WATERMAZE DELAYED-MATCHING-TO-PLACE TASK

Watermaze tasks are a key tool to study hippocampal place learning and memory in rodents. The watermaze is a circular pool containing a submerged platform, onto which the rodents can escape. In the standard reference memory version, the platform is fixed in the same location across trials and days, therefore allowing for slow incremental place learning (Morris, 1984). While in the delayed-matching-to-place (DMP) task, modified version of the watermaze, the platform location is changed daily, which allows to evaluate rapid, 1-trial, place learning.

The DMP variant of the watermaze task is highly sensitive to hippocampal dysfunction (Bast et al., 2009; da Silva et al., 2013). More specifically, DMP performance is markedly impaired by disrupting hippocampal plasticity or by partial hippocampal lesions, whereas these manipulations can leave performance on the standard reference place memory task in the watermaze relatively intact and even rats with complete hippocampal lesion can come to show good performance on the standard reference place memory task when overtrained with many trials (Morris, 1984; Steele and Morris, 1999; Bast et al., 2009; Pezze and Bast, 2012). In this task, long-term memory consolidation is not required, and animals learn within one trial the daily changing place. Therefore, on the DMP task, the animal's ability to escape efficiently from the water depends on the rapid acquisition of place information and its subsequent retrieval a few minutes later.

The DMP task mimics everyday problems like the car park problem. Imagine you drive to work and you do not have an allocated car park, so the location where you park your car would usually be different each day. By the end of the working day you would need to remember where the car is parked, but this would be a different location every day, which you had to learn in the morning.

Importantly, the DMP watermaze task has also been reverse-translated into a human task, using a virtual environment on a computer screen (Buckley and Bast, 2018), and the task also appears to be closely associated with hippocampal function in human participants, individual differences in theta-band oscillations in a spatial memory network revealed by EEG predict rapid place learning (Bauer et al., 2020).

4.1.5. CHAPTER AIMS

In sum, there is evidence that chronic pain may affect significantly the hippocampus and cause memory impairments in both humans and rodents. However, the understanding of these changes and the mechanisms behind it are not completely understood and there is a lack of knowledge if, and how OA in particular affects this cognitive function. Some evidence indicates that the impact of chronic pain on the hippocampus and its functions may depend on the condition and, specifically, that OA may have less impact on the hippocampus than other chronic pain conditions (Mutso et al., 2012).

To test if OA impacts hippocampus-dependent memory, in this chapter, MIA-injected adult Lister hooded (LH) rats were subjected to the watermaze DMP task (Bast et al., 2009) to longitudinally evaluate the impact of OA-chronic knee pain on hippocampus-dependent rapid place learning performance. Pain and sensorimotor testing were also assessed across the study.

4.2. METHODS

Refer to Chapter 2 for general methodology.

4.2.1. ANIMALS

For this study, 16 (n=8 per group) adult male Lister hooded (LH) rats (Charles Rivers, UK), weighing between 250-280g and approximately 2-3 months old at the beginning of the experiment, were used. The target sample size for this study was 32 (n=16 per group), so group differences corresponding to an effect size of Cohen's d=1 could be detected with a power of about 80%, using an independent t-test (2-tailed, $p < 0.05$). However, the first series of the experiments clearly indicated that there were no substantial group differences in the main memory measures, and that completion of the second series to achieve the target sample size would not reveal significant group differences. Therefore, the study was terminated due to futility after only completion of the first series (Neumann et al., 2017). 1 rat (MIA) was excluded from the study due to a physiological abnormality, not related with MIA model, preventing the collection of behaviour data in the last two time points.

4.2.2. PAIN BEHAVIOUR AND SENSORIMOTOR ACTIVITY

The model of OA pain was induced with a single intra-articular injection of MIA (as described in 2.2.). Rats were injected with either 50 µL of MIA (3 mg/50 µL; n=8) or the same volume of sterile saline solution (n=8) as control. Rats were initially allocated to the treatment groups before model induction based on the pain and sensorimotor activity baseline measurements to match the prospective treatment groups for their baseline measurements as closely as possible. In each cage half of the animals received treatment and the other half saline. The experimenter (S.G.) was blinded to the treatment allocations throughout the data collection and analysis.

Nociceptive pain behaviours were assessed using weight-bearing and Von-Frey tests, as described in 2.3., and sensorimotor measures, locomotor activity and startle/prepulse inhibition, were taken as described in 2.4. Rats were first handled for a few days and habituated to the pain test apparatus. Baseline pain behaviour and sensorimotor activity measurements were collected before model induction with one day apart. After MIA/saline injection (day 0), pain measurements were taken on day 3, 14, 28, 50 and 84; and

sensorimotor processes were assessed on day 15, 29, 51 and 85 after model induction as described in Fig.4.1.

4.2.3. WATERMAZE DELAYED MATCHING-TO-PLACE TEST

To evaluate the effects of OA-like knee pain on hippocampus-dependent memory, the watermaze DMP task was used in this study. Animals were pretrained for 8 consecutive days in this task prior to MIA induction and then tested at several time points across the study, using established protocols (Steele and Morris, 1999; da Silva et al., 2013; McGarrity et al., 2016).

4.2.3.1. APPARATUS

The watermaze apparatus (Fig.4.1) consisted of an open-field circular white pool (2 m in diameter and 60 cm height) filled with water at 25±1°C made opaque by the addition of children white paint (Go Create). Four start points were equally spaced along the circumference of the pool (north [N], east [E], south [S], and west [W]).

Hidden in the watermaze pool, was an escape platform (1–3 cm below the water surface), which rats had to find to escape from the water. Rats are naturally very good swimmers, however they do not particularly like water which makes them swim to escape from it. Therefore, watermaze tasks are an excellent test to evaluate place memory without food restriction. The “Atlantis platform” used can be withheld at >30 cm below the water surface by a computer-controlled electromagnet a predetermined time, making it unavailable to the rats.

The lighting of the room was kept constant at about 200 lux at water level. The room was filled with spatial cues, including a traffic cone, lampshades hanging from the wall and different geometric 2D and 3D shapes. Cues were carefully kept in the same place during the study and between studies.

The rats’ behaviour was monitored and collected by an overhead video camera connected to a video recorder and a computer with EthoVision XT 8.5 software in an adjacent control room.

4.2.3.2. PROCEDURE

Rats performed four daily trials. During trial 1, animals could rapidly learn the novel location of the hidden platform using the prominent visual cues on the room, and then on subsequent

trials they use the place memory to efficiently locate the hidden platform. To start the test, rats were placed into the water facing the pool walls at one of the four start positions in a predetermined and arbitrary sequence to prevent egocentric strategies. Egocentric navigation is based on direction strategies, for example, memorising routes (such as distances, directions and sequential turns) instead of using environmental cues. Platform location was changed daily, but remained constant during the four consecutive daily trials. Rats were tested with a novel goal location each day. Each trial had the maximum duration of 120 s, after which the animal was guided to the platform by the experimenter in case of failing to find it. Following each trial, rats were allowed 30 s on the platform before they were dried gently on a towel and returned to a carrier box, resulting in an inter-trial interval of around 10-30 s.

Trial 2 was occasionally run as "probe trial". During this probe, the platform was withheld for the first 60 s, to monitor the animals' search preference for the zone containing the platform (the target zone). After the 60 s, the platform was automatically released allowing the animal to find and climb onto it. Between trial 1 and the probe trial the inter-trial interval was about 20 min, instead of 10-30 s, as this would render the task more sensitive to any impairment in hippocampal plasticity mechanisms (Steele & Morris, 1999).

4.2.3.3. PERFORMANCE MEASURES

The overhead video camera connected to the video recorder and the computer with the Ethovision tracking software captured all the trials that digitized the rats' paths and several behavioral measures, including latencies and path lengths to reach the platform location, and times spent in the different pool zones.

Latency and path length to reach the platform location, swim speed, the percentage time spent in all the zones and in the target zone are analysed. To measure search preference for the correct zone, eight virtual 20 cm diameter zones were considered (Bast et al., 2009). These extended platform zones were symmetrically arranged and positioned on virtual inner circle or outer ring. Percentage of time searching the correct platform position or previous day's zone were calculated as: $\frac{\text{time in correct zone or in previous } day}{\text{total time in all the eight zones}} * 100.$

The main measure of hippocampus-dependent rapid place learning performance was the search preference for the correct zone during probe trials. This measure has been shown to be the most robust measure of hippocampal rapid place learning performance, where latencies and path length measures are more variable and less dependent on hippocampal

function (Bast et al., 2009; da Silva et al., 2013) (also see Bauer et al., 2020, for related findings in human participants).

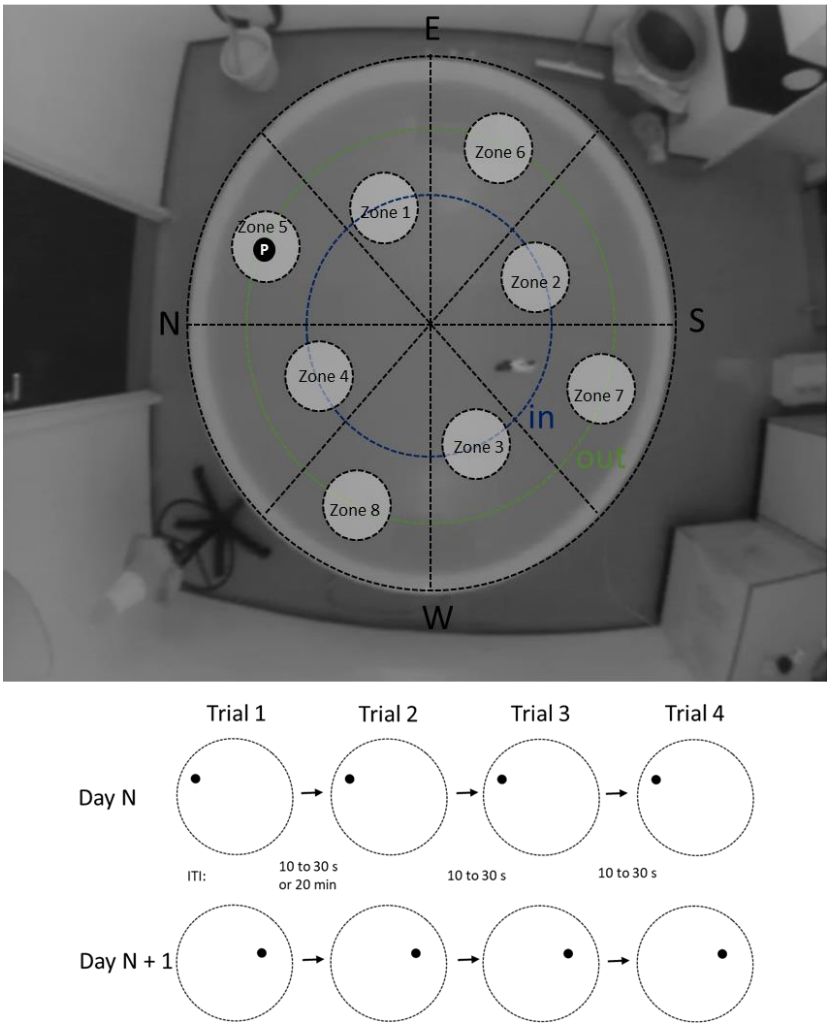

FIGURE 4.1. – WATERMAZE DELAYED MATCHING-TO-PLACE (DMP) TASK. The DMP task allows to evaluate the "everyday" memory. Rats learn to escape from the water to a hidden platform. Platform is moved to a new location each day but remains in the same position during the four consecutive daily trials. In probe days, the platform is unavailable during the first 60 sec of the trial. Inter-trial interval (ITI) is generally 10-20 sec, on probe days between T1 and T2 ITI is 20 min. Performance is followed across many days/weeks.

4.2.4. EXPERIMENTAL DESIGN

Before model induction, rats were pre-trained for 8 consecutive days in the watermaze DMP task. Then, after model induction, rapid place memory was evaluated testing the rats for 4 consecutive days in the watermaze DMP task at week 2, 4, 7 and 12 (Fig. 4.2).

At day 93 after MIA/saline injection, rats were anesthetized with a lethal dose of sodium pentobarbitone and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA). Brains, spinal cords, DRGs and knees were carefully excised,

preserved and stored. Knee joint sections were stained with haematoxylin and eosin- or safranin-O/fast green and then scored for overall joint morphology and proteoglycan loss. Total cartilage joint damage, osteophyte, proteoglycan loss, synovial inflammation and chondrocyte presence were scored to evaluate the severity of the knee joint pathology as described in 2.7.

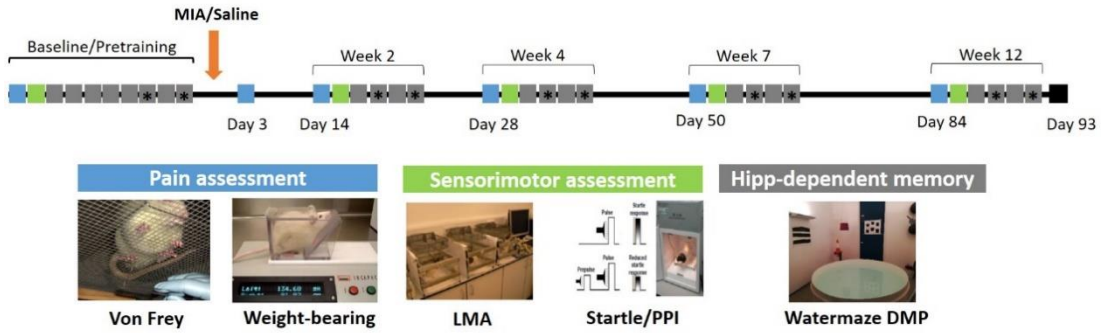

FIGURE 4.2. – TIME COURSE OF THE HIPPOCAMPUS-DEPENDENT MEMORY STUDY. 16 adult male LH rats were used in this study. Animals were either injected with MIA (3mg/50µl, n=8) or saline (n=8). *indicates probe days.

4.2.5. STATISTICAL ANALYSIS

GraphPad Prism 8 and IBM SPSS Statistics 24 were used to prepare the graphs and the statistical analysis. Results from the pain behaviour studies were analysed using an analysis of variance (ANOVA), with group as between-subjects and time as repeated measures/within-subjects variable of testing day. Results from the sensorimotor behaviour studies were analysed using a mixed design factorial ANOVA test with testing day and task blocks (or pulse intensity in the PPI test) as within-subjects factors and group as between-subjects factor. When main effect of group or interaction involving group was observed, 2-way ANOVAs were conducted at each time point. Watermaze DMP results (search preference, latencies and path lengths) were analysed using a 2-way ANOVA with testing time points as within-subjects factors and group as between-groups factor. Bonferroni multiple comparison was used as *post-hoc* testing.

Knee pathology was analysed with an independent t test or with Mann-Whitney test when D'Agostino-Pearson test showed that assumption of normality was violated. $P<0.05$ was considered to represent a significant difference and all results were expressed as mean±standard error (SEM). Baseline measures were not included in the ANOVA analysis, t-tests were conducted at baseline to ensure no significant difference between groups at this stage.

4.3. RESULTS

MIA injection did not cause any obvious distress to the animals, and regular weight measurements showed no significant difference between groups at any time point during the study (group: $F_{(1,14)}$=1.89, p=0.19) (Fig.4.3).

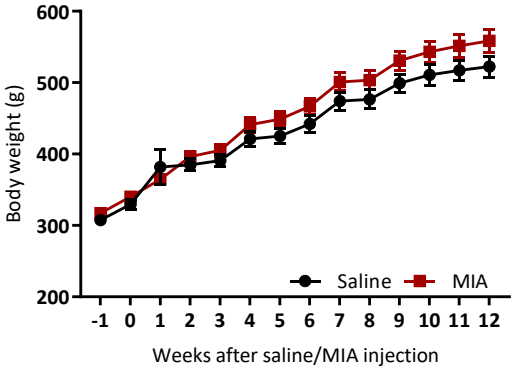

FIGURE 4.3. – BODY WEIGHT OF LISTER HOODED (LH) RATS AFTER MONOSODIUM IODOACETATE (MIA) OR SALINE INJECTIONS. Rats were injected with either 50ul of 3mg of MIA (▪, n=8) or saline (●, n=8) in the left knee at week 0. Data are presented as mean±SEM.

4.3.1. PAIN BEHAVIOUR

Weight bearing asymmetry significantly increased after MIA injection, MIA-injected rats showed weight bearing asymmetry placing less weight on the injured leg compared with saline controls (group: $F_{(1,13)}$=66.22, p<0.0001) (Fig.4.4). This effect was observed from week 1 until the end of the study (time: $F_{(4,52)}$=3.32, p<0.0001). No interaction effects were observed between time and group (time x group: $F_{(4,52)}$=0.42, p=0.80).

No evidence for mechanical allodynia was observed in the ipsilateral paw withdrawal threshold in the MIA-injected rats (group: $F_{(1,13)}$=0.63, p=0.44) (Fig.4.5A). However, a significant effect of group was observed on the contralateral hindpaw thresholds (group: $F_{(1,13)}$=14.78, p=0.002), with saline rats showing a lower response compared with MIA rats (Fig.4.5B). To explore these results, a 2-way ANOVA was performed considering the difference between the contralateral and the ipsilateral withdrawal threshold. While the control animals showed difference scores around 0, with very little fluctuations, the MIA rats showed a significant increase in the difference score after model induction (group: $F_{(1,13)}$=28.4, p=0.0001) (Fig.4.5C), with the group difference tending to decrease with time after model induction, although there was neither a main effect of time ($F_{(5,65)}$<1.74, p>0.15) nor an interaction of time and group ($F_{(5,65)}$<1.52, p>0.21).

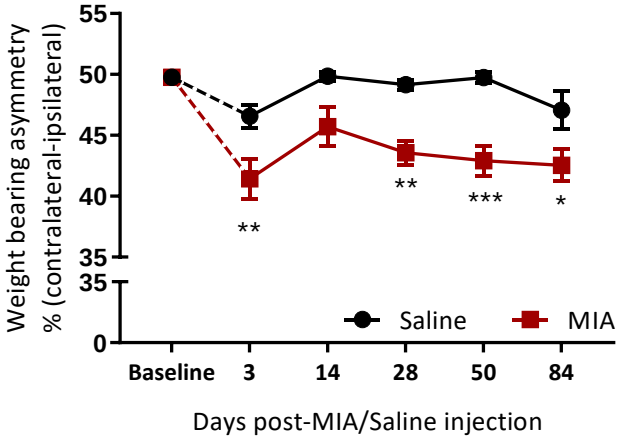

FIGURE 4.4. – MONOSODIUM IODOACETATE (MIA) INDUCED ASYMMETRY IN WEIGHT BEARING IN LISTER HOODED (LH) RATS. LH rats were injected with either 50ul of 3mg of MIA (▪; n=7) or saline (●; n=8) in the left knee. Data are presented as mean±SEM. * p<0.05, ** p<0.004, *** p<0.0003, 2-way ANOVA with Bonferroni multiple comparisons *post-hoc* testing.

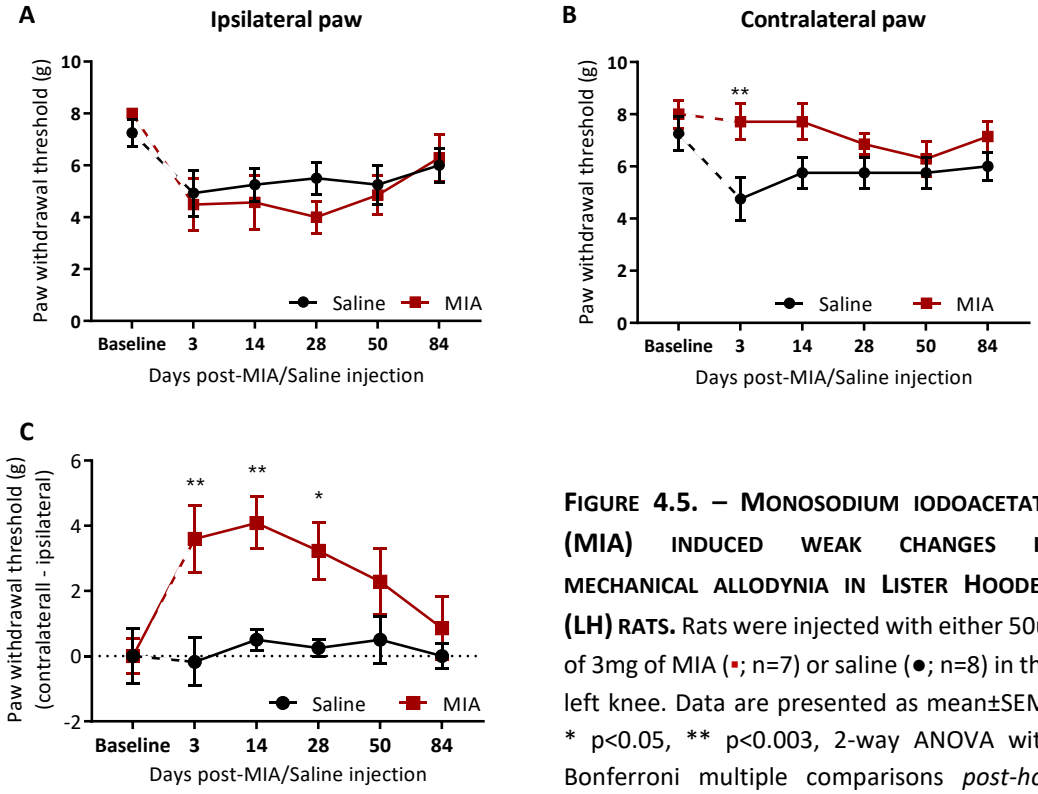

FIGURE 4.5. – MONOSODIUM IODOACETATE (MIA) INDUCED WEAK CHANGES IN MECHANICAL ALLODYNIA IN LISTER HOODED (LH) RATS. Rats were injected with either 50ul of 3mg of MIA (▪; n=7) or saline (●; n=8) in the left knee. Data are presented as mean±SEM. * p<0.05, ** p<0.003, 2-way ANOVA with Bonferroni multiple comparisons *post-hoc* testing.

4.3.2. SENSORIMOTOR MEASURES

Locomotor activity was not substantially affected by MIA model induction. No changes in horizontal activity were observed in MIA-injected rats, compared to control rats (group: $F_{(1,13)}$=0.02, p=0.88) (Fig.4.6A). However, MIA-injected rats showed less rearing than controls (group: $F_{(1,13)}$=4.86, p=0.046) (Fig.4.6B). All animals showed habituation to the apparatus within the individual open field test sessions (block: $F_{(2,26)}$>146.45, p<0.0001) and across test days (time: $F_{(4,52)}$>6.05, p<0.0001). No interaction involving group was observed in the horizontal activity (time x block: $F_{(4,52)}$=1.61, p=0.39). No interaction of block/testing day x group was observed in the vertical activity (block x group: $F_{(2,26)}$<1.84, p>0.23), but the interaction block x time was significant in the vertical locomotor activity (time x block: $F_{(8,104)}$=11.36, p<0.0001).

Startle response was not affected by MIA injection (Fig.4.7A), but MIA-injected rats showed some evidence of lower PPI at higher prepulse intensities (Fig.4.7B). No main effect of group was observed on startle response (group: $F_{(1,13)}$=0.168; p=0.69) or prepulse inhibition (group: $F_{(1,13)}$=2.079; p=0.17). Animals showed habituation to the pulse-alone (pulse-alone: $F_{(2,26)}$=70.45, p<0.0001) and increasing PPI with increasing prepulse intensity (pulse: $F_{(3,39)}$=208.30, p<0.0001) during the trials within the session. In addition, main effect of day was observed, startle increased across days possibly reflecting that animals were getting bigger (time: F>11.35, p<0.0001). 3-way ANOVA using MIA group as between-subjects factor and test day and startle block as within-subjects factors showed no triple interaction effect on startle responses (group x pulse-alone x time: $F_{(8, 104)}$=0.60, p=0.77). Both groups showed relatively comparable PPI, but there was some evidence for different PPI at some prepulse intensities (prepulse x group: $F_{(3,39)}$=3.42; p=0.03). No interaction between prepulse intensity, day and group was observed (group x prepulse x time: $F_{(12, 156)}$=1.20, p=0.29), however there was some evidence that MIA rats had a lower PPI at higher prepulse intensities (prepulse x day: $F_{(12,156)}$=2.056; p=0.02), but not between day and group (time x group: $F_{(4,52)}$=0.82; p=0.52).

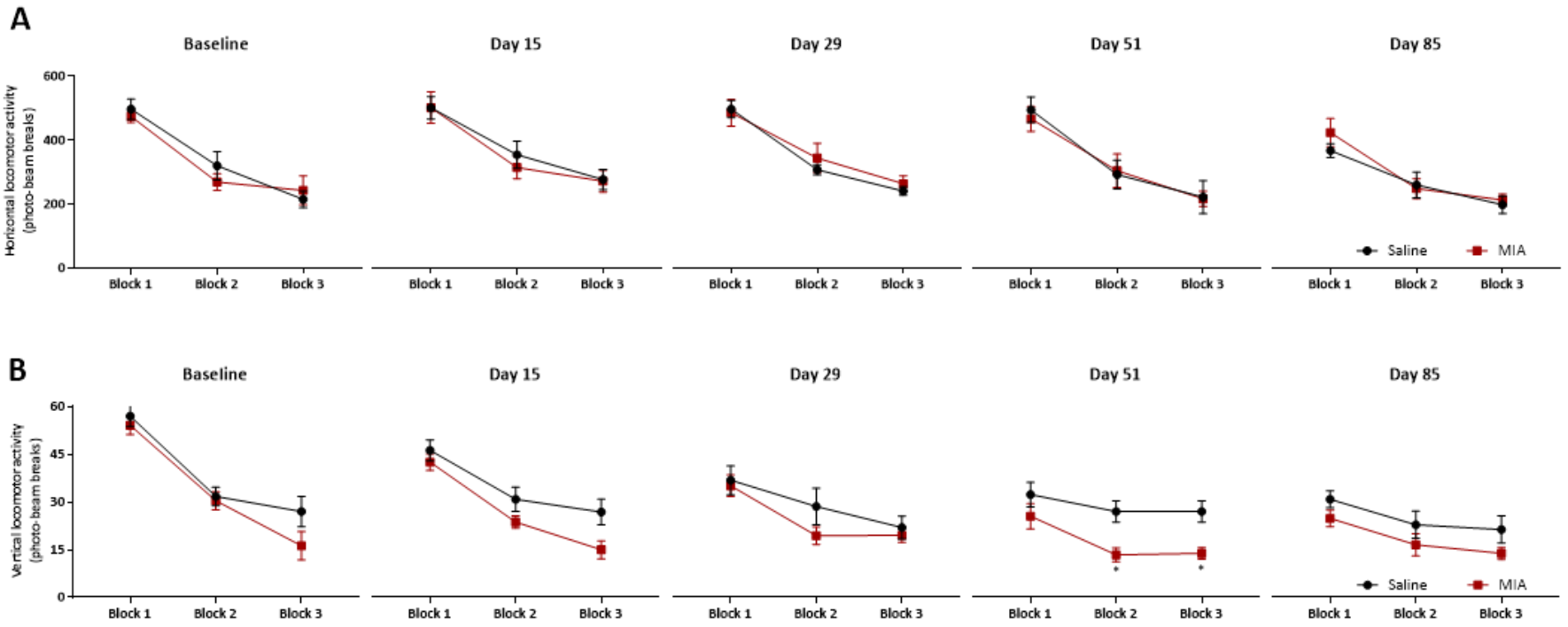

FIGURE 4.6. – MONOSODIUM ACETATE (MIA) EFFECT IN THE HORIZONTAL LOCOMOTOR ACTIVITY (A) AND VERTICAL ACTIVITY (B) IN LISTER HOODED (LH) RATS. Rats were injected with either 50ul of 3mg of MIA (▪, n=7) or saline (●, n=8) in the left knee. Data are presented as mean±SEM. * P<0.05, mixed factorial ANOVA and independent 2-way ANOVA in each time point with Bonferroni multiple comparison *post-hoc* testing.

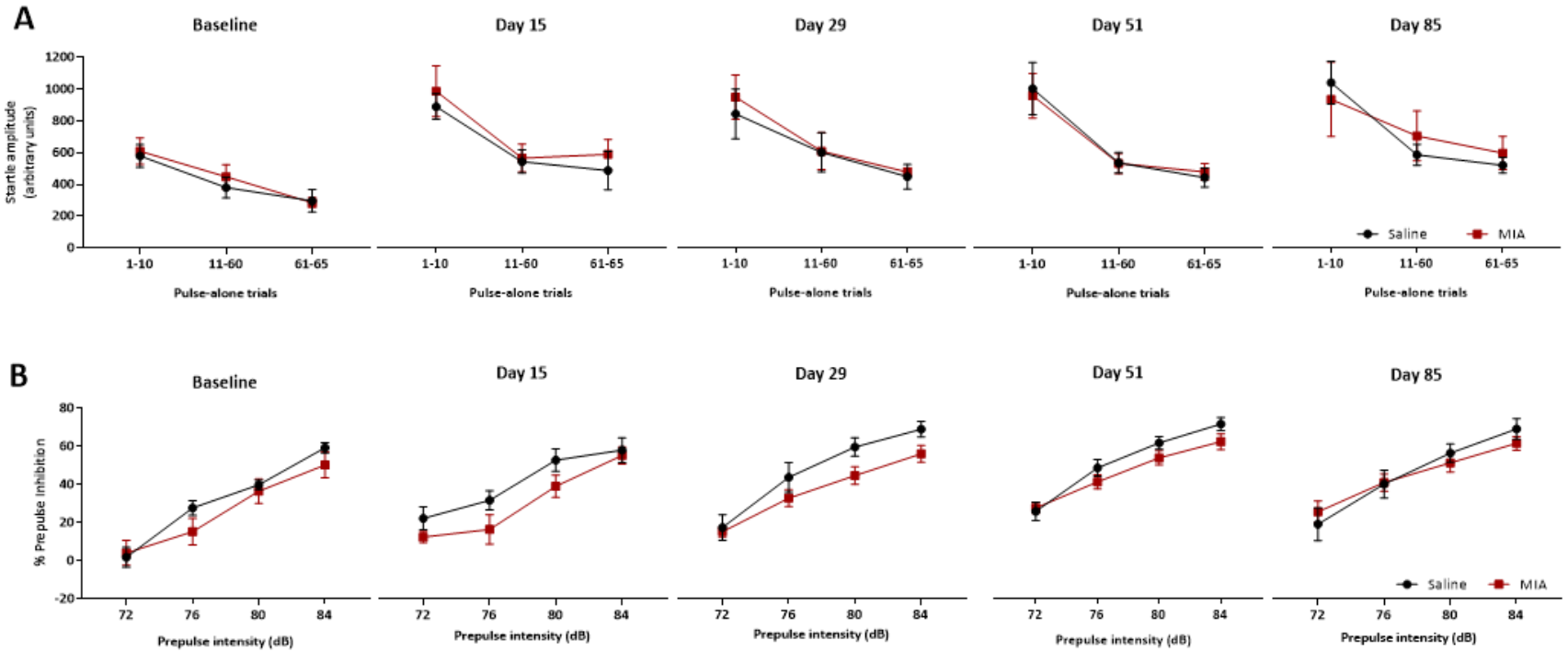

FIGURE 4.7. – STARTLE HABITUATION (A) AND PREPULSE INHIBITION ACTIVITIES (B) IN LISTER HOODED RATS INDUCED WITH MONOSODIUM IODOACETATE (MIA) MODEL. Rats were injected of MIA (▪, n=7) or saline (●, n=8) in the left knee. Data are presented as mean±SEM. Mixed factorial ANOVA with Bonferroni multiple comparison *post-hoc* testing.

4.3.3. HIPPOCAMPUS-DEPENDENT MEMORY

To investigate the effects of OA-like chronic knee pain on hippocampal rapid place learning performance, MIA and saline animals were tested on the watermaze DMP task. Both prospective groups learned the task similar to what was reported in previous studies (Steele&Morris, 1999; Bast et al., 2009), showing the characteristic reductions in the latency to reach the hidden platform from trial 1 (encoding) to trial 2 (retrieval) and improvement across days (day x trial: $F_{(21,294)}$=4.32; p<0.0001) (Fig.4.8.). There were no differences between the prospective groups (group: $F_{(1,14)}$=1.08; *p*=0.32; interactions involving group: F<1). Also, on the probes conducted on days 6 and 8 during the pretraining, no differences between the prospective groups were found in the time spent exploring the target zone, previous day's zone, the total eight zones or in the swim speed (all p>0.22).

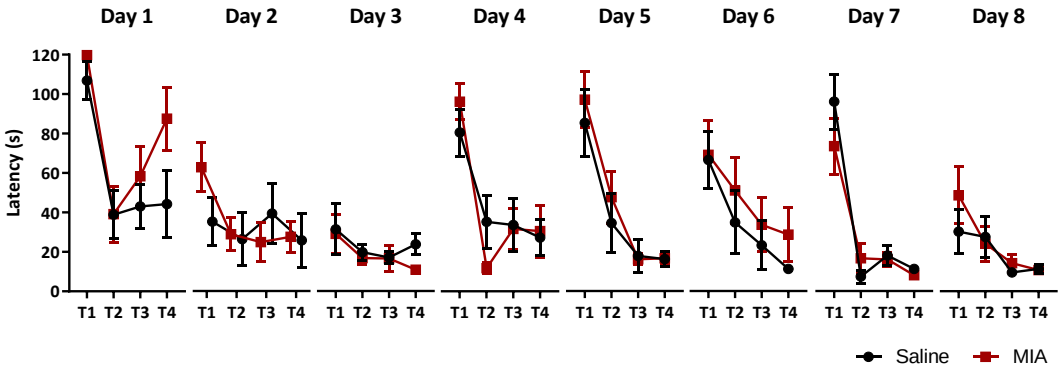

FIGURE 4.8. – LATENCIES TO REACH THE PLATFORM DURING PRETRAINING. Rats were trained in the watermaze delayed-matching-to-place for 8 consecutive days before model induction. Data are presented as mean±SEM.

After model induction, both groups showed virtually identical performance patterns in terms of latencies and also of path length (Fig.4.9). There was no main effect or interaction involving the factor group in both latencies and path length measures (main effect of group and interaction involving group: all F<1). There was a significant main effect of trial, reflecting rapid place learning, on latency to reach the platform (trial: $F_{(3,174)}$=170.70; p<0.0001) (Fig.4.9A) and on the path length (trial: $F_{(3,84)}$=80.19; *p*<0.0001) (Fig.4.9B).

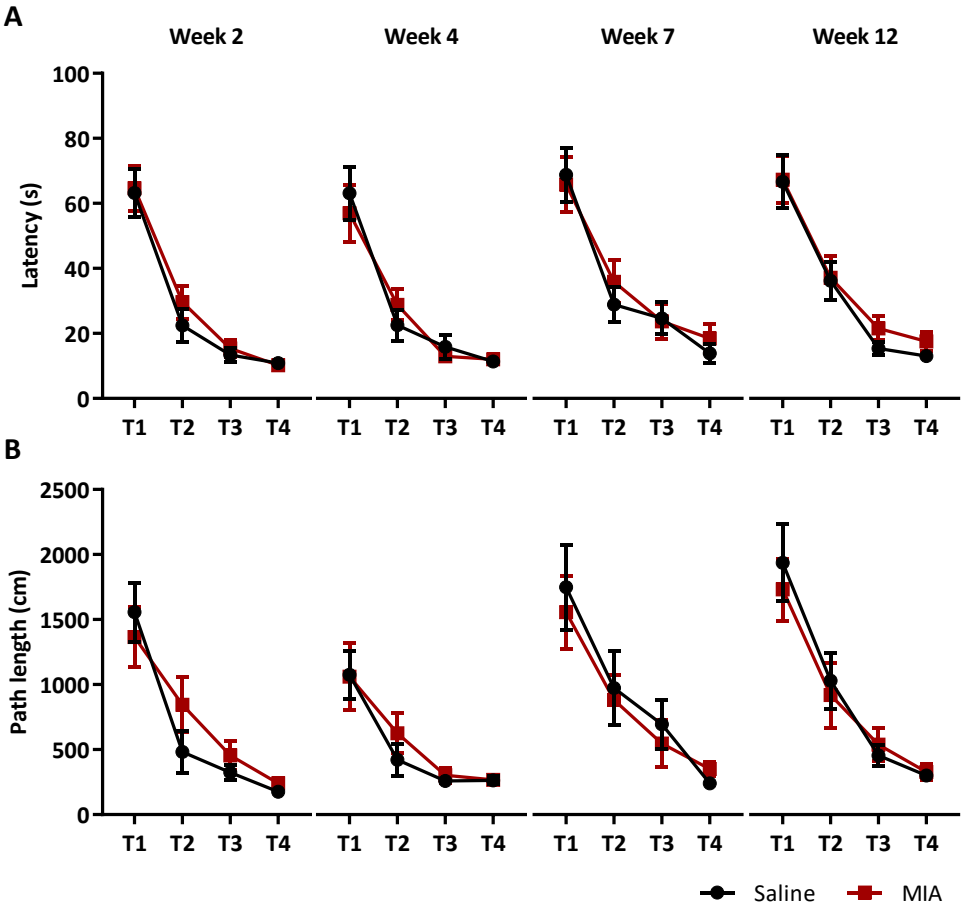

FIGURE 4.9. – LATENCY (A) AND PATH LENGTH (B) TO REACH THE PLATFORM WERE NOT AFFECTED BY MIA INJECTION. Rats were injected with either 50ul of 3mg of MIA (▪, n=7) or saline (●, n=8) in the left knee. Rats were tested in the watermaze DMP task four times in each week, trial data are presented as average. Data are presented as mean±SEM.

During the probe trials, when the platform was unavailable during the first 60 seconds, rats were tested for search preference (Fig.4.10). MIA-injected rats, compared with control rats, spent a similar percentage of time exploring the target zone (group: $F_{(1,28)}$=1.72; p=0.20) (Fig.4.10A) and the previous day's zone (group: $F_{(1,28)}$<1) (Fig.4.10B) and spent a similar total time exploring the eight zones (group: $F_{(1,28)}$=2.17; p=0.15) (Fig.4.10C). There was a main effect of time on the percentage of time spent exploring the previous day's zone ($F_{(3,84)}$=4.63; p=0.005), reflecting that both groups spent more time in the previous day's zone at the last two testing time points (probably reflecting that goal locations were not fully counterbalanced across test days, but only across groups). No other main effect of time and interaction effects were observed (F<1).

Regarding the swim speed, overall MIA-injected rats and controls did not show significant differences (group: $F_{(1,28)}$= 1.58; p=0.22) (Fig.4.10D). However, there was a significant

interaction of group X time point ($F_{(3,84)}$=8.87; p<0.0001), reflecting that MIA rats showed lower swim speed than control rats at the last two testing time points. More specifically, MIA rats showed a stable swim speed throughout the study, whereas control animals slightly increased their swim speed from week 7.

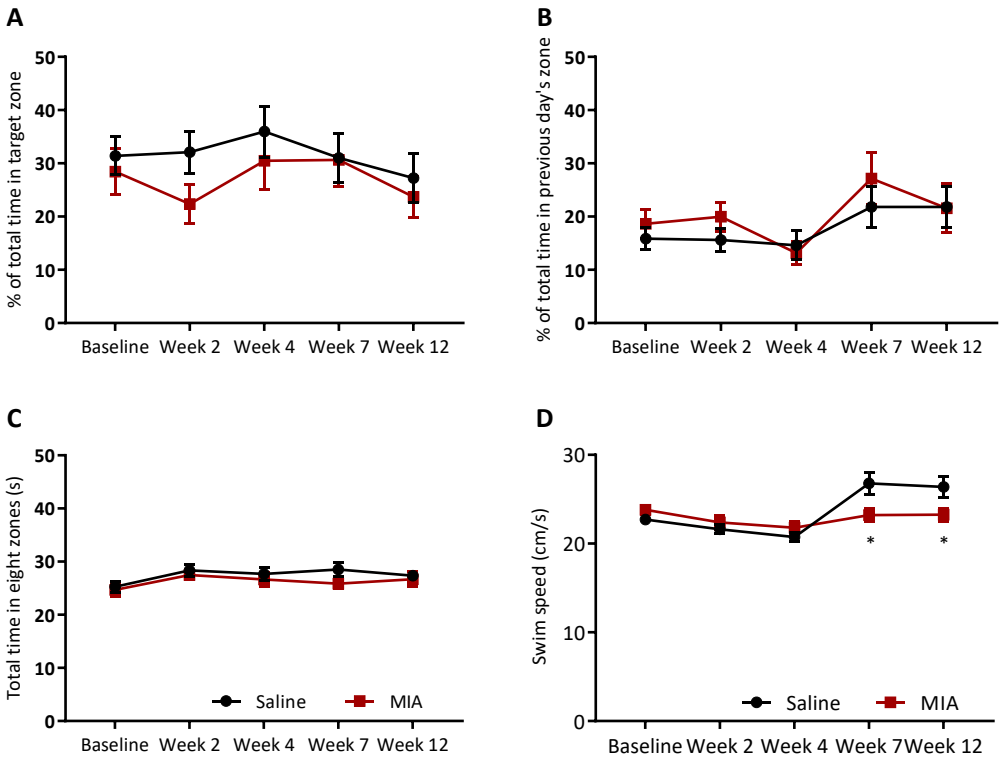

FIGURE 4.10. – EFFECTS OF MONOSODIUM IODOACETATE (MIA) ON THE PLACE MEMORY OF YOUNG ADULT LISTER HOODED RATS. Rats injected with either 50ul of 3mg of MIA (▪, n=7) or saline (●, n=8) in the left knee performed a hippocampus-dependent memory task. Twice per week, rats were tested in a probe task during the second trial when the escape platform was unavailable to evaluate the search preference. MIA-injected rats did not show differences in the total time exploring the target zone (A), the previous day's zones (B) or the total eight zone (C) when compared with control saline. Control rats increased their swim speed in the last time points (D). Data are presented as mean±SEM. * p<0.05, 2-way ANOVA with Bonferroni multiple comparisons *post-hoc* testing.

4.3.4. KNEE PATHOLOGY

At the end of the study, knees were collected and processed for pathology scoring as described in section 2.7. Joints were scored for cartilage integrity (cartilage damage x involvement), synovial inflammation and osteophyte formation on three different levels of both lateral and medial tibial plateau (Fig.4.11). Results are showed as mean between medial and tibial plateau values, as no differences were observed between tibial parts. During the

splitting process, one of the saline animals was split with the wrong angle making it impossible to score.

MIA injected rats showed increases in both lateral and medial tibial plateau loss of cartilage integrity (Fig.4.11A; t=4.63, *p*=0.0005) and in synovial inflammation (Fig.4.11B; U=0, *p*=0.0003) compared with control animals. There was a trend for MIA-injected rats to show a higher number of osteophytes (U=14, *p*=0.08), with 4 out of 8 rats in the MIA group showing osteophytes, but not a single control rat (Fig.4.11C).

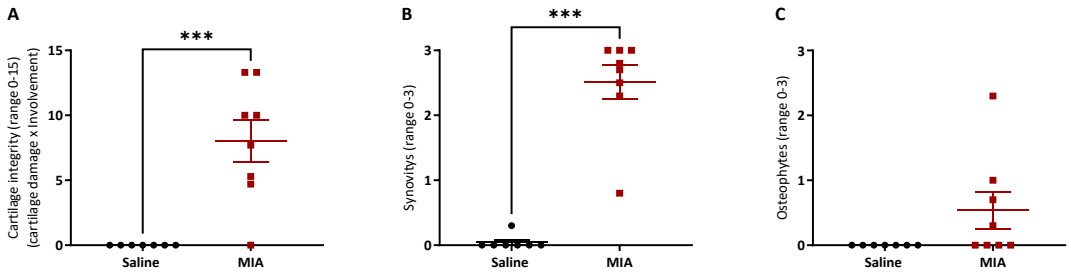

FIGURE 4.11. – MICROSCOPIC QUANTIFICATION OF HISTOLOGICAL CHANGES OF TIBIAL PLATEAU IN LISTER HOODED RATS INJECTED WITH EITHER 3 MG MONOSODIUM IODOACETATE (MIA) MODEL OR SALINE. Average scores for medial and lateral tibial plateu. Rats were injected with either 50ul of 3mg of MIA (▪, n=8) or saline (●, n=7) in the left knee. Knees were collected and processed for scoring at day 93 after model induction. 3 mg MIA were able to induce cartilage damage (A). Data are presented as mean±SEM *** p<0.001. Unpaired t-test. 3 mg MIA were also able to induce synovial inflammation (B) and only a few animals injected with MIA showed presence of osteophytes (C). Data are presented as median±IQR, ***<0.001. Mann-Whitney U test.

4.4. DISCUSSION

In this study, MIA-injected LH rats were tested on the watermaze DMP task to investigate the impact of OA on hippocampus-dependent memory. No evidence of impaired hippocampal memory was found after MIA model induction. MIA injected rats showed robust weight bearing asymmetry, slightly decreased rearing activity and features of knee joint pathology. Also, there was some evidence for MIA rats to show reduced PPI at higher prepulse intensities compared with saline rats.

4.4.1. PAIN BEHAVIOR, SENSORIMOTOR ACTIVITY AND KNEE PATHOLOGY

Pain behaviour was assessed across this study, with MIA-injected rats showing a robust pain phenotype. As in the pilot study conducted in chapter 3, 3 mg of MIA caused asymmetry in the weight bearing distribution and did not induce mechanical allodynia. Interestingly, the paw withdrawal threshold difference between paws was significantly different between MIA-injected and control rats. Saline animals showed a similar drop across the study in both contralateral and ipsilateral paws, whereas, in MIA rats, the threshold only dropped in the ipsilateral paw. Apart from the finding that MIA did not induce mechanical allodynia compared with saline controls, this abnormal behaviour may reflect an extra protective and careful behaviour regarding the paw on the leg with the injured knee by the MIA-injected rats. This type of behaviour was not observed in chapter 3, or in previous studies, as far as we know. We found marked knee pathology at the end of the study, with MIA-injected rats showing similar features to chronic OA in humans, such as cartilage degradation and synovial inflammation.

Sensorimotor measures were mildly affected by MIA model induction. MIA-injected rats did not show changes in horizontal locomotor activity, but results show that rats with an injured knee tend to rear slightly less than controls. Decreased rearing activity may reflect spontaneous ongoing pain. It is important to note that all the animals were housed in IVC cages with two floors, and, based on visual inspection, no rats showed marked issues in jumping to the top floor. MIA injection also did not affect the startle response. However, there was some evidence for sensorimotor gating processes, as reflected by PPI of the acoustic startle response, to be mildly affected by the MIA injections. PPI was mildly reduced in MIA-injected rats at the higher prepulse. Several forebrain areas, such as the medial PFC, nucleus accumbens and basolateral amygdala are involved in PPI regulation (Koch, 1999). Additionally, abnormal hippocampus activity has been associated with altered PPI (Zhang et

al., 2002). The reduced PPI observed in this study may indicate possible impacts of MIA in some of the forebrain regions involved in this task. Therefore more studies are needed to unveil areas and/or mechanisms leading to the changes observed.

4.4.2. HIPPOCAMPUS-DEPENDENT MEMORY IN MIA LH RATS

The main aim of this chapter was to evaluate if OA-like chronic pain affected hippocampus-dependent memory in young adult LH rats. Before induction of the chronic pain model, all animals were pre-trained to the watermaze DMP task. The key feature of this watermaze task is the fact that the platform is moved to a different location daily, allowing to assess "everyday"-type rapid place learning. After MIA model induction, DMP task performance was assessed at different time points to evaluate if and how the progression of the disease affected hippocampus-dependent memory. No deficits were observed during the watermaze DMP task after MIA model induction in LH rats. On standard testing days, the latencies or path lengths to reach the platform were not altered by the MIA model at any time point. Rats from both groups decreased latencies and path lengths across daily trials, showing that MIA did not affect the rapid encoding and subsequent retrieval of place information.

Search preference during the probe trial was also not affected by MIA model induction. MIA-injected rats spent a similar percentage of time exploring the target zone and the previous day's zone compared with control animal. Animals from both groups spent a similar time exploring the total eight zones of the pool. Interestingly, control rats slightly increased the swim speed at the two last time points of the study.

Altogether, our results suggest that OA-like chronic knee pain did not affect hippocampus-dependent memory in young adult LH rats. A study conducted in Wistar Han rats with spared nerve injury reported no impairments in Morris water maze performance (Leite-Almeida et al., 2009). Rats of three different ages were tested (3-, 10- and 22-months old), general performance was not affected by chronic neuropathic pain; however, young adults only showed deficits during the reversal phase of the Morris watermaze. Similarly, a different study in young adult Sprague Dawley rats with spinal nerve ligation reported impairments in the reversal task of the Morris watermaze (Moriarty et al., 2016a). Moreover, no reports of memory impairment were reported in Lister hooded rats with diabetic neuropathic pain in the acquisition phase of the Morris watermaze task (Moriarty et al., 2016b); these animals showed deficits in spatial learning with slower acquisition of the task, as reflected by reduced

latency improvements across training days (which may reflect reduced swim speed), but memory performance during a probe trial, as reflected by search preference was not affected. Although these studies used different tasks, these are consistent with our findings in showing limited evidence for impairment of place learning and memory in the watermaze in rat models of chronic pain. It is important to note that these studies used neuropathic pain models, which have different pathological mechanisms.

Importantly, mechanical allodynia development was reported in the studies above. In this chapter no evidence of mechanical allodynia were observed, so the possibility that our MIA-injected rats do not show central sensitization should also be considered. Central nervous system may not be impacted and consequently not impacting the cognitive function.

4.4.3. HIPPOCAMPUS-DEPENDENT MEMORY IN CHRONIC OA PAIN?

As previously mentioned, there is some evidence that chronic pain is associated with changes in the hippocampus in both rodents and humans. Additionally, clinical observations indicate that chronic pain is associated with memory impairments (Berryman et al., 2013). However, no previous studies have focused on OA yet. Our findings in this chapter indicate that hippocampal memory is not impaired by OA-like chronic pain in the MIA model in LH rats.

In order to study the impact of persistent pain on the hippocampus, Mutso and colleagues measured the hippocampal volume in human patients in three different pain conditions – chronic back pain, complex regional pain syndrome and OA (Mutso et al., 2012). In accordance with previous studies, they found robust decreases in the hippocampus volume in chronic back pain (CBP) and complex regional pain syndrome (CRPS), but not in OA patients, compared with controls. CBP and CRPS seem to have a higher impact on hippocampal volume than OA. These findings may indicate that the hippocampus may be less affected in OA than in other chronic pain conditions, which may explain the results obtained in this chapter.

Other factors associated with chronic pain in humans may account for why chronic pain patients show memory impairments, but MIA-injected rats may not show these. Cognitive decline is most usually associated with ageing (Deary et al., 2009), a factor that can also be related or occur at the same time but independent of some of the most popular chronic diseases such as osteoarthritis (Vos et al., 2016). Age is one of the most important factors that can be

influencing these reported cognitive deficits. From the human studies exploring the impacts of pain on cognition, only a few of them take in consideration the impact of dementia and medication for example. Ageing and memory loss are strongly linked, and as reported the majority of chronic pain conditions such as OA are more commonly find in older people. Chronic pain treatments, such as opioids, can also be associated with cognitive declines – this will be discussed in detail on chapter 6. Other factors might also be associated with cognitive/memory deficits reported, as for example sleep deprivation and alcohol consumption (Yeung et al., 2017; McCrae et al., 2018). In fact, a relationship between alcohol consumption and hippocampus volume has been reported in patients with fibromyalgia (Boissoneault et al., 2017).

Life style seems to play an important role in the implications of chronic pain. Rheumatoid arthritis patients self-reported with poor memory, word finding and concentration but those who were physically active reported less cognitive dysfunctions (Shadick et al., 2019). Chronic pain patients who feel a higher sense of inclusion and engagement with others also reported lower impact of pain in daily life (Karayannis et al., 2019), which may result in less self-reported comorbidities associated with chronic pain.

4.5. CONCLUSION

In sum, our results showed that hippocampus-dependent memory was not affected by chronic OA knee-like pain in male young adult MIA-injected Lister hooded rats.

Previous studies have shown that changes in the hippocampus are associated with chronic pain in both rodents and humans. Also, clinical observations reported memory impairments in chronic pain conditions. However, the knowledge regarding OA and memory remains unclear, and even less is known regarding hippocampus-dependent memory in particular. OA pain may be associated with less brain changes in hippocampus than in other types of pain (Mutso et al., 2012) and consequently may have less impact on hippocampal functions, which may explain the absence of hippocampus-dependent memory impairments observed in this chapter.

In accordance with findings in this chapter, rodents with different pain models and using a different version of the watermaze showed limited evidence of spatial memory deficits. However, in these models, there was some evidence for impairments in the acquisition and reversal phases of place learning tasks in the watermaze. These deficits in the reversal task

may indicate that behavioural flexibility is compromised in this condition, a possibility that will be addressed in the next chapter.

Other types of memory may also be impaired, in fact recognition memory deficits were observed in rodents with OA (Negrete et al., 2017). This will be also addressed in the next chapter.

CHAPTER 5

COGNITIVE FLEXIBILITY AND NOVEL OBJECT RECOGNITION MEMORY IN LISTER HOODED RATS WITH MIA-INDUCED OSTEOARTHRITIS-LIKE KNEE PAIN

5.1. INTRODUCTION

5.1.1. RECOGNITION MEMORY

Recognition memory is a subtype of declarative memory which allows to know or remember a familiar person, object or experience (Brown et al., 2010). In humans recognition memory is usually assessed using visual-paired comparison memory tasks, while in rodents the test used is the novel object recognition(NOR) test (Cohen and Stackman Jr., 2015). In this test, animals are presented with a familiar and a novel object, and the time exploring both objects is quantified and the discrimination index between objects is calculated (more details about the protocol in 5.2.3.) (Ennaceur and Delacour, 1988). Rodents have the innate tendency to explore novel items, so there is no need for extensive training or external motivation in this test. Animals without any impairment in recognition memory are expected to spend more time exploring the novel item/object.

Preclinical studies have shown impairments in recognition memory in rodents under chronic pain condition. Neuropathic rats showed reduced novel exploration time in the NOR test after nerve injury surgery compared with controls (Moriarty et al., 2016a). Furthermore, a study conducted in mice induced with MIA model of OA-pain also showed that OA may be linked to deficits in recognition memory (Negrete et al., 2017). Negrete and colleagues reported that wild-type MIA mice had a lower discrimination index compared with the wild-type saline mice in the NOR test, showing that OA pain may in fact affect this type of memory.

5.1.2. BEHAVIOURAL FLEXIBILITY

Cognitive flexibility refers to the ability to switch and adapt behaviour in response to emergent changes in the internal or external environment. Cognitive flexibility cannot be directly observed; however, it is possible to observe and study the associated behavioural change, behavioural flexibility. Both terms are often used as synonymous (Mikhalevich et al., 2017), but behavioural flexibility refers to the adaptive behaviour reflecting the cognitive change.

Some researchers argue that cognitive flexibility involves more than one major component (Martin and Rubin, 1995). In a first instance the person needs to be aware that there is another alternative, then the individual must be willing to adjust and finally the individual needs to feel confident and able to adapt the behaviour (Laureiro-Martínez et al., 2009). Altogether, this complex cognitive function involves attention, motivation and decision-making.

Behavioural flexibility is commonly assessed using questionnaire measures, attentional shifting tests, rule switching and reversal learning (Lange et al., 2017). Standardized tests such as card sorting or gambling tests – such as the Wisconsin Card Sorting test, the intra-dimensional/extra-dimensional set-shift task of the Cambridge Neuropsychological Test Automated Battery or the Trail-Making test have been widely used in humans (Brown and Tait, 2014).

5.1.3. BEHAVIOURAL FLEXIBILITY ASSESSMENT IN RATS

In rodents, to investigate the relationship between cognitive flexibility and diseases, reversal and set-shifting tasks have been used. Reversal learning consists in a change in response strategy while shifting strategy refers to a change in the stimulus dimension.

Floresco and colleagues have developed an automated strategy shifting and reversal task using operant chambers that allows to test the same animals in both tasks (Brady and Floresco, 2015). In contrast with the digging task, a well described rodent task to assess attentional set-shifting (Birrell and Brown, 2000), this automated method allows to test several animals at the same time and, most importantly, the analysis is not manually controlled by the experimenter, making the test less subjective to interpretation and/or human error. Furthermore, Brady and Floresco have showed that similarly with previous digging and maze tasks, this automated task is sensitive to disruptions in the PFC and

subcortical circuits (Brady and Floresco, 2015). This task was used in this chapter, and more detail about the protocol can be found below in the methods.

5.1.4. BEHAVIOURAL FLEXIBILITY IN RODENTS WITH CHRONIC PAIN

Evidence suggests that behavioural flexibility is impaired in rodent models of chronic pain (Brown and Tait, 2014; Murray et al., 2015; Moriarty et al., 2016a; Cowen et al., 2018). Cowen and colleagues showed impairments in the cognitive flexibility induced by neuropathic pain in rats when tested in an operant protocol (Cowen et al., 2018). Moriarty et al, also reported impaired cognitive flexibility in neuropathic rats (Moriarty et al., 2016a). In this study the same rodent model was used, spinal nerve ligation, but this time animals were tested in a reversal task during the Morris watermaze. Neuropathic rats showed worse performance than controls in the reversals (Moriarty et al., 2016a).

These results indicate that chronic neuropathic pain may affect and impair cognitive flexibility; however, there is no evidence so far that similar results would occur with other types of pain including OA.

5.1.3.1. PREFRONTAL CORTEX, PAIN AND COGNITION

The PFC is known for its important role in pain processing, including in knee OA pain (Parks et al., 2012). Several studies conducted in both humans and animal models have been showing the involvement of PFC in both acute and chronic pain; structure, anatomical and connectivity changes in PFC have been reported in chronic pain condition (Ong et al., 2019). Brain imaging studies have showed significant decreases in the volume of grey matter in the PFC in patients with chronic pain (Kelley and Domesick, 1982; Kuchinad et al., 2007; Moriarty et al., 2011), and this morphological alteration was suggested to contribute to cognitive impairments (Luerding et al., 2008).

Moreover, this forebrain region has also been reported to have a major role in several different cognitive functions (Hiser and Koenigs, 2018; Parnaudeau et al., 2018), including, in NOR and cognitive flexibility. While, pain processing was suggested to be more localized in the dorsal mPFC, cognitive processing seems to be more ventrally localized (Jahn et al., 2016).

Impairments in decision-making have been linked to chronic pain patients (Apkarian et al., 2004), decision-making deficits were correlated positively with cortical grey matter volume changes in chronic pain patients (Elvemo et al., 2014).

Several studies have assessed the role of the rodent hippocampus in NOR memory, besides some conflicting and diverse results the hippocampus seems important for the NOR memory at least with a delay greater than 10 minutes between sample and testing (for review see (Cohen and Stackman Jr., 2015)). However, recent works have indicated that other areas as the perirhinal cortex (Brown and Aggleton, 2001) and the PFC have a crucial role in NOR (see for review (Morici et al., 2015)).

Additionally, one key function associated with the PFC, although not exclusively, is cognitive flexibility (Kim et al., 2011; Brown and Tait, 2014; Brady and Floresco, 2015). There is evidence that pain severity negatively correlates with behavioural flexibility in patients with chronic pain (Karp et al., 2006). Interestingly, there is evidence that PFC pharmacological manipulations of both dopamine and serotonin systems altered behavioural flexibility (Winter et al., 2009; Nilsson et al., 2019).

5.1.6. CHAPTER AIMS

In sum, there is evidence that indicates that chronic pain may disrupt the PFC and consequently associated functions, including cognitive flexibility. Unfortunately, the knowledge behind the relationship between chronic pain and cognitive flexibility remains unclear, even less is known regarding if, and how OA in particular affects this cognitive function.

In the previous chapter, we focused only on the possible impact of chronic OA knee pain on hippocampus-dependent rapid place learning performance and no significant impairments were detected. However, as discussed above chronic pain has been also associated with recognition memory deficits. Therefore, in this chapter we tested MIA-injected LH rats in the NOR test to investigate the possible impacts of OA-chronic knee pain on NOR memory.

To test if OA causes cognitive flexibility deficits, in this chapter, the same cohort of MIA-injected adult LH rats were subjected to an automated set-shifting task (Brady and Floresco, 2015) to evaluate the impact of OA-chronic knee pain on behavioural flexibility.

5.2. METHODS

Refer to Chapter 2 for general methodology.

5.2.1. ANIMALS

A total of 32 adult male Lister hooded (LH) rats weighing between 253-283g, approximately 2-3 months old, (Charles Rivers, UK) at the beginning of the experiment were used in this study. The target sample size for this study was 32 (n=16 per group), so group differences corresponding to an effect size of Cohen's d=1 could be detected with a power of about 80%, using an independent t-test (2-tailed, $p<0.05$).

5.2.2. PAIN BEHAVIOUR AND SENSORIMOTOR ACTIVITY

The model of OA pain was induced with a single intra-articular injection of MIA (as described in 2.2.). LH rats were injected with either 50 µL of MIA (3 mg/50 µL; n=16) or the same volume of sterile saline solution (n=16) as control. Rats were initially allocated to the treatment groups before model induction based on the pain and sensorimotor activity baseline measurements to match the prospective treatment groups for their baseline measurements as closely as possible. In each cage half of the animals received treatment and the other half saline. The experimenter (S.G.) was blinded to the treatment allocations throughout the data collection and analysis.

Nociceptive pain behaviours were assessed using weight-bearing and Von-Frey tests, as described in 2.3., and sensorimotor measures, locomotor activity and startle/prepulse inhibition, were taken as described in 2.4.. Rats were first handled for a few days and habituated to the pain test apparatus. Baseline pain behaviour and sensorimotor activity measurements were collected before model induction with one day apart. After MIA/saline injection (day 0), pain measurements were taken on day 3, 14, 28, and 63; and sensorimotor processes were assessed on day 15, 29, and 63 after model induction.

5.2.3. NOVEL OBJECT RECOGNITION TEST

To assess if NOR memory was impaired in MIA-injected LH rats, animals were submitted to the NOR test at baseline, i.e. 10 days prior to model induction, and 30 days after model induction, adapting NOR testing procedures described in (Pezze et al., 2015).

5.2.3.1. NOR APPARATUS

Animals were tested in groups of 4. The animals were tested in individual plastic rectangular arenas (38 x 40 x 54 cm high walls) with an opaque plastic lid. Objects consisted of duplicate copies of bottles of glass or plastic with different shapes, colour and sizes. These objects were filled with water to make them too heavy to be displaced by the animal. Objects were counterbalanced across groups and placement (right or left of arena). Sessions were recorded using an overhead camera and later analysed. Arenas and objects were cleaned with 20% ethanol before each trial/session to remove odour cues.

5.2.3.2. NOR PROCEDURE

The NOR task consists of three major phases: habituation to the apparatus, sampling and testing phase. On the first day, animals were placed into the empty arena for 1h for acclimatisation (habituation). On the following day, animals were placed in the empty arenas for 3 min for re-acclimatisation, returned to the home cage for 30 – 45 s while arenas were cleaned and objects placed, and then placed in the arenas again for the sampling phase. The two copies of the object were placed in opposite corners of the arena as in Fig.5.1. and animals were then allowed to explore them for 5 min (familiar/sampling phase), after which the animal was returned to the home cage. 24 h after the sampling phase, animals were

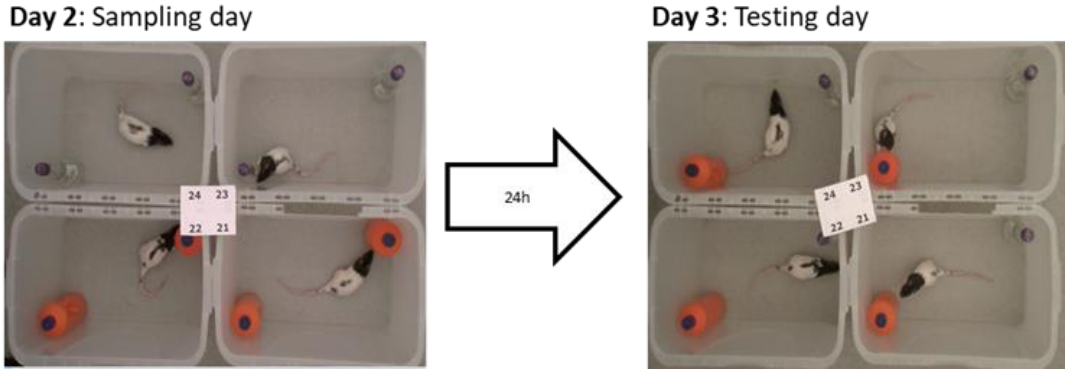

FIGURE 5.1. – NOVEL OBJECT RECOGNITION (NOR) TEST. Rats were allowed to explore two identical objects (sampling day) and after 24 hours one of the objects was replaced by a novel one (testing day).

replaced into the arena for 3 min; at this point, the arena contained one of the objects used in the sampling phase on the previous day (the familiar object) and one novel object (testing phase). Both sampling phase and testing phase were recorded and later analysed. The duration of sampling and test phase were selected based on previous studies (Pezze et al., 2015).

Time exploring each object was defined as only direct contact/active exploring the object by directing the nose at the object at a distance of less than 1 cm, e.g. sniffing and or interacting with the object. Contact with the object, but facing it or sitting next to it was not scored as exploration time (Ennaceur and Delacour, 1988; Pezze et al., 2015). The discrimination ratio was calculated using the following equation: $total\ time\ exploring\ Novel\ Object / total\ time\ exploring\ Novel + Familiar\ Object$.

5.2.4. BEHAVIOURAL FLEXIBILITY TEST

To assess the behavioural flexibility, an automated strategy shifting and reversal task was used in this study. The protocol used was based on a previously established protocol (Brady and Floresco, 2015). Animals were food-restricted during this test to provide better control of food intake. Food-restriction was gradually introduced a few days prior to the beginning of the pretraining. Moreover, 10-20 sugar pellets (Purified rodent tablet 5TUL, TestDiet) per animal were placed in the animal's home cage to familiarise animals with the pellets on the day before the pretraining. The target animal weight was 85-90% of the free feeding weight; animals were carefully weighed every day before the task and only fed with their normal food after the task.

5.2.4.1. APPARATUS

The task was conducted in individual operant chambers (MED-Associate Operant Chambers). Each chamber was equipped with a house-light, two retractable levers, two stimulus lights above the levers and a reinforcement pellet dispenser located between the levers. Each animal was assigned to an operant chamber. Chambers were cleaned with 20% ethanol between tests to prevent odour cues. The stimuli presented, lever operation and data collection were controlled via an interface with the computer and using custom software (MED-PC software) (Brady and Floresco, 2015).

5.2.4.2. PRETRAINING

Phase 1: Food restricted rats were initially trained to respond by lever pressing under a fixed-ratio (FR1) schedule of reinforcement. In this phase 1, only one lever (right or left) was extended and one reward pellet was delivered for each lever press. Phase 1 had the duration of 4 days. In the first 2 days animals were trained with only one lever (right or left) and on the next 2 days animals were trained on the opposite lever, with half of the animals first trained on the right lever and the other half on the left lever. On the first day only, two reward pellets were placed in the magazine cup and crushed pellets on the top of the extended lever. Minimum trial criterion was 50 lever presses in a maximum of 30 min.

Phase 2: Animals were then trained on the retractable lever to familiarise them with the extension and retraction of the levers and the respective sound. Levers were pseudorandomly extended, but the same lever was not presented more than two consecutive times. The program started with the house-light off, then both stimulus light turned on, 3s after house-light came on and one of the levers extended for 10 seconds. Pressing the lever resulted in its retraction, release of a reward pellet and switching off of all lights, no lever press was considered an omission. Each session was composed of 90 trials. Phase 2 lasted 5 days, and on the 5^{th} day rats should be making fewer than 5 omissions over the session to proceed to the next phase.

Phase 3: On the 5^{th} day, a side preference test was conducted immediately after the end of phase 2 - rats were not removed from the chamber after phase 2. Both levers were extended into the chamber on each trial and no light stimulus was presented during this phase. On the first trial a press on either levers was rewarded with a sugar pellet, on second trial (20 seconds after) press on the opposite lever was rewarded, choosing the same lever as the first trial was not rewarded. The opposite levers should be pressed 7 times, so 7 + 7 presses in total. The test only ended after 7 pairs of rewarded trials were achieved. In the response phase animals were trained to press the levers opposite the one they showed preference during this side preference training.

5.2.4.3. VISUAL CUE DISCRIMINATION

Phase 4: This was the first testing phase. Both levers extended into the chamber and only one cue light was illuminated (Fig.5.2.A). To receive a reward, the animal needed to press the

levers with the cue light illuminated above it. The program stopped after the rat reached the criterion – 10 consecutive correct trials – or after a maximum of 150 trials.

The pre-exposure to stimulus lights presented during phase 2 reduces the novelty and, therefore, may also reduce the salience of the stimulus lights, and consequently increase the difficulty and increase the number of trials required to achieve criterion performance on this visual cue discrimination phase (Floresco et al., 2008). Animals may require multiple days to learn this rule under these conditions. If rats did not reach the criterion on day 1, the rat was tested again on the following day up to a maximum of 3 days. Rats that did not reach the criterion on the 3rd day were excluded from further behavioural testing.

5.2.4.4. SET SHIFT TO SPATIAL RESPONSE STRATEGY

Phase 5: At the beginning of phase 5 and just on the first day, the program ran the first 20 trials identical to phase 4, i.e. with responding according to the cue rule being rewarded (Fig.5.2.B). These trials served to measure retrieval/expression of the cue discrimination rule. On trial 21 the program shifted to the spatial response strategy. Either the left or right stimulus light was pseudorandomly illuminated for 3 s, then both levers extended into the chamber for 10 seconds or until a response occurred. The reward was delivered only when the rat pressed the opposite lever of the side bias defined during side preference training, independent of the position of the light. The program again stopped after the rat reached the criterion - 10 consecutive correct choices and only after it completed a minimum of 30 trials - or after a maximum of 180 trials on the first set shift day and a maximum of 150 trials on subsequent set shift days. Rats performed 3 consecutive days of this task. A few days later,

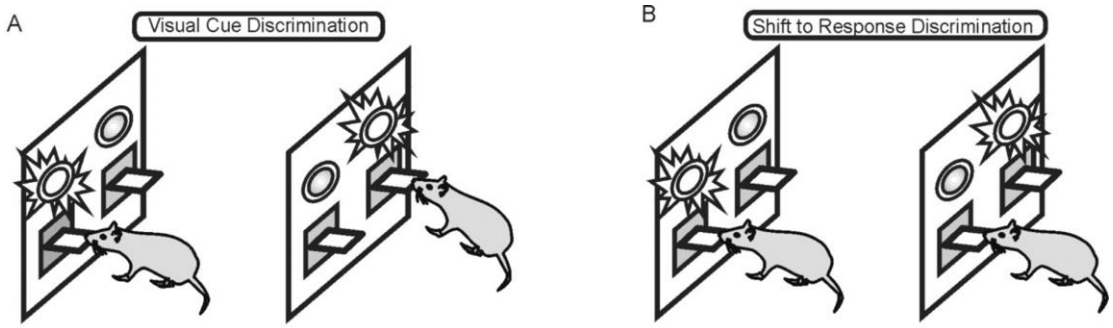

FIGURE 5.2. – DISCRIMINATION TASKS. A) During visual cue discrimination task (phase 4), rats are reinforced for a response on the lever under the illuminated stimulus light. **B)** During shift to response discrimination task (phase 5), rats are reinforced for a response on one lever (either left or right) regardless of the position of the stimulus light. Adapted from (Brady and Floresco, 2015).

a fourth day as a reminder of phase 5 was presented to the rats, to ensure robust performance on the spatial response task, followed, on the next day, by reversals.

5.2.4.5. REVERSAL TASK

The reversal task was run similar to phase 5. Either left or right stimulus light were pseudorandomly illuminated for 3 seconds, then both levers were extended into the chamber for 10 seconds or until a response occurred. However, here the correct response was 'reversed'. So, if in phase 5 the designated lever for a particular rat was the right lever, now the left lever was the designated lever to receive a reward pellet, and vice versa. The criterion was again 10 consecutive correct responses, with maximum of 150 trials. All rats performed this task for 4 consecutive days, lever only changed on the first day.

5.2.4.6. BEHAVIOURAL PERFORMANCE MEASURES

The analysis of this test focused on trials to criterion, the percentage of correct responses and percentage of omissions. Errors were also counted and analysed during the shifting and reversal phases.

Two types of errors were taken in consideration: perseverative error - when rats responded incorrectly, but in accordance with the rule that was correct on the previous task, and never-reinforced errors – when rats responded incorrectly on the task with a response that was not correct either according to the current or previous rule.

5.2.5. EXPERIMENTAL DESIGN

This experiment was conducted in two batches; half of the animals were in batch 1 and the other half in the batch 2. Both batches were ran with only 1 day of delay between them to facilitate the behaviour testing, so all animals could be tested during the morning.

Study was conducted as described in Fig 5.3.. Before model induction, rats were tested for NOR, followed by pain behaviour and sensorimotor activity (baseline). Then, after model induction (day 0), pain measurements were taken on day 3, 14, 28, and 63; and sensorimotor processes were assessed on day 15, 29, and 63 after model induction as described in Fig.5.2.. NOR was also re-assessed at day 30 (sample phase) and 31 (testing phase) after model

induction. Then, food restriction started on the day after NOR testing animals. Pretraining of automated set shifting task started on day 37 after model induction.

At day 70 after MIA/saline injection, rats were anesthetized with a lethal dose of sodium pentobarbitone and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA). Brains, spinal cords, DRGs and knees were carefully excised, preserved and stored. Knee joint sections were stained with haematoxylin and eosin- or safranin-O/fast green and then scored for overall joint morphology and proteoglycan loss. Total cartilage joint damage, osteophyte, proteoglycan loss, synovial inflammation and chondrocyte presence were scored to evaluate the severity of the knee joint pathology as described in 2.7.

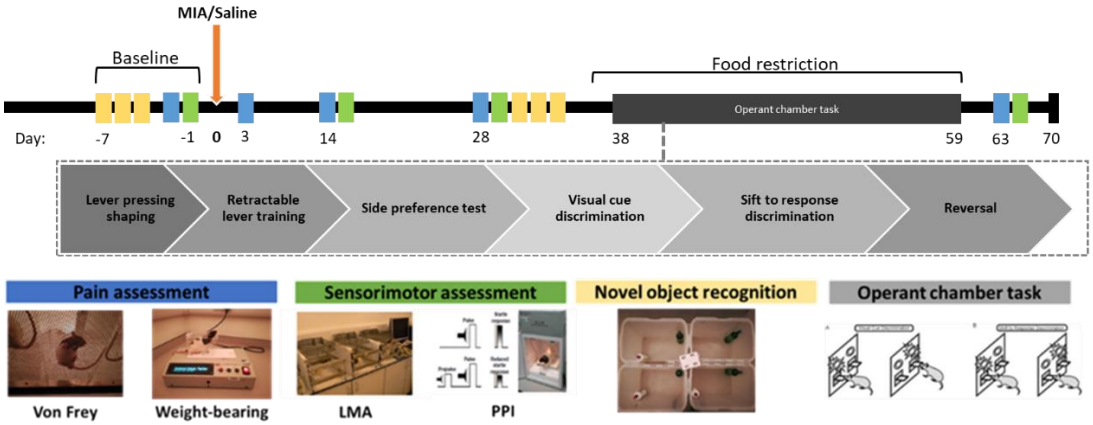

FIGURE 5.3. – TIME COURSE OF THE BEHAVIOURAL FLEXIBILITY AND RECOGNITION MEMORY STUDY. 32 adult males Lister hooded rats were used in this study. Animals were either injected with MIA (3mg/50µl, n=16) or saline (n=16).

5.2.6. STATISTICAL ANALYSIS

GraphPad Prism 8 and IBM SPSS Statistics 24 were used to prepare the graphs and the statistical analysis.

Results from the pain behaviour studies were analysed using an analysis of variance (ANOVA), with group as between-subjects factor and days from model induction as repeated measures/within-subjects variable. Results from the sensorimotor behaviour studies were analysed using a mixed design factorial ANOVA test with days from model induction and task blocks (or pulse intensity in the PPI test) as within-subjects factors and group as between-subjects factor. NOR results were analysed also using an ANOVA test with testing day and object as within-subjects factors and group as between-subjects factor.

From the cognitive flexibility test, results of the Cue Acquisition phase were analysed using unpaired t-test or the Mann-Whitney when normality could not be assumed. For the shifting and reversal phase that involved four sessions of trials, a mixed design factorial ANOVA test was used with testing day as within-subjects factors and group as between-subjects factor.

Knee pathology was analysed with an independent t test or with Mann-Whitney test when normality was violated.

$P<0.05$ was considered to represent a significant difference and all results were expressed as mean±standard error (SEM). Normality was tested using D'Agostino-Pearson test. Baseline measures were not included in the ANOVAS analysis, t-tests were conducted at baseline two ensure no significant difference between groups at this stage. Bonferroni multiple comparison was used as *post-hoc* testing.

5.3. RESULTS

5.3.1. PAIN BEHAVIOUR

Rats showed weight-bearing asymmetry, placing less weight on the injured leg, after MIA injection, compared to saline injection (group: $F_{(1,30)}$=33.89, p<0.0001) (Fig.5.4). No main effect of time and interaction between time and group were observed (time: $F_{(3,90)}$=0.71, p=0.55; group x time: $F_{(3,90)}$=0.21, p=0.89).

Paw withdrawal measures did not differ between MIA and control rats (Fig.5.5). No differences were observed between MIA and controls in the paw withdrawal threshold of the ipsilateral (group: $F_{(1,30)}$=1.24, p=0.27; Fig.5.5A) and the contralateral paw (group: $F_{(1,30)}$=0.03, p=0.87; Fig.5.4B). No difference between the contralateral and the ipsilateral withdrawal threshold was observed as well (group: $F_{(1,30)}$=0.70, p=0.41; Fig.5.5C). No main effect of time or interaction involving group were observed (time: $F_{(3,90)}$<2.67, p>0.07; group x time: $F_{(1,90)}$<0.43, p>0.0.33).

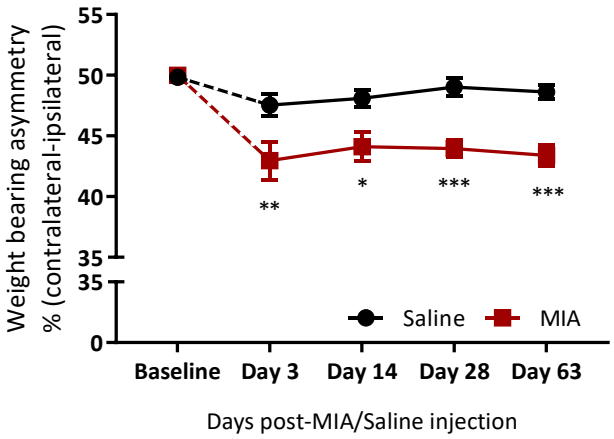

FIGURE 5.4. – MONOSODIUM IODOACETATE (MIA) INDUCED ASYMMETRY IN WEIGHT BEARING IN LISTER HOODED (LH) RATS. LH rats were injected with either 50ul of 3mg of MIA (•; n=16) or saline (●; n=16) in the left knee. Data are presented as mean±SEM. * p<0.05, ** p<0.003, *** p<0.0006, 2-way ANOVA with Bonferroni multiple comparisons *post-hoc* testing.

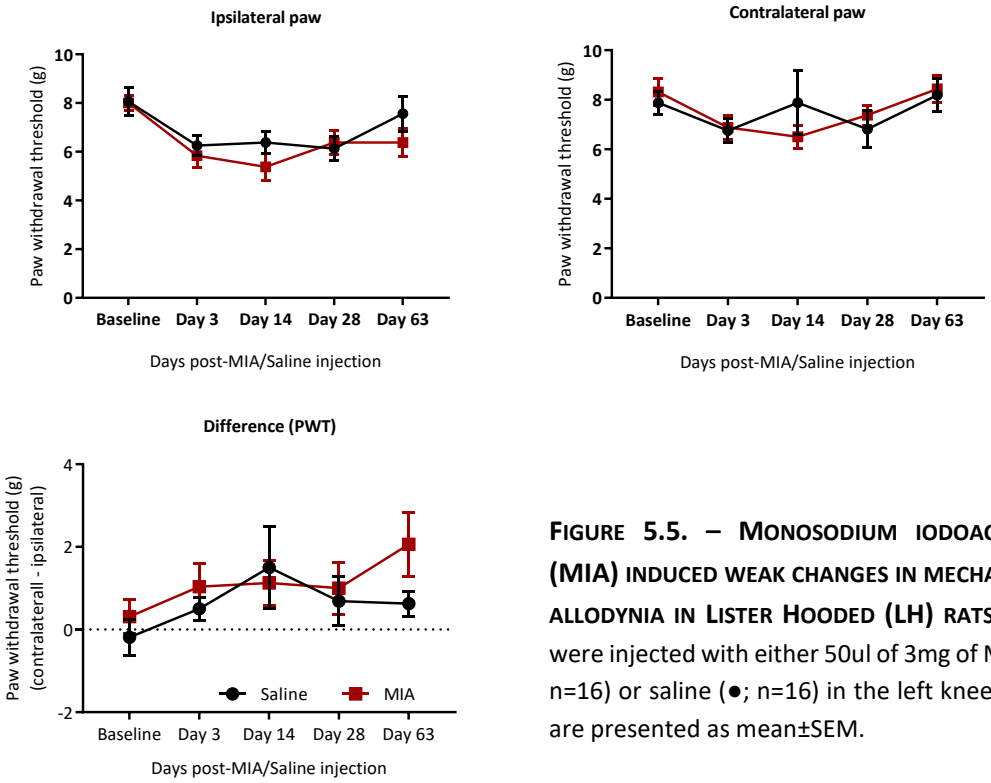

FIGURE 5.5. – MONOSODIUM IODOACETATE (MIA) INDUCED WEAK CHANGES IN MECHANICAL ALLODYNIA IN LISTER HOODED (LH) RATS. Rats were injected with either 50ul of 3mg of MIA (▪; n=16) or saline (●; n=16) in the left knee. Data are presented as mean±SEM.

5.3.2. SENSORIMOTOR ACTIVITY

MIA injection did not affect horizontal locomotor activity (Fig.5.6A), however rearing (vertical locomotor activity) was significantly reduced in these animals compared with saline injected controls (Fig. 5.6B). A 3-way ANOVA of horizontal locomotor activity, using group as between-subjects factor, and day from model induction and 10-min blocks of testing as within-subjects factor, revealed no significant main effect of group (group: $F_{(1,30)}$=3.65, p=0.07). However, MIA rats tend to be a little less active than saline, but this is likely to reflect a pre-existing difference, unrelated to the MIA injection, as a similar difference was already present at baseline. However, significant difference in the vertical activity was observed (group: $F_{(1,30)}$=5.08, p=0.03), MIA injected rats stand on the rear paws less than controls.

In both horizontal and vertical activity, no interactions of block or testing day with group were observed (time/block x group: $F_{(2,60)}$<1.05, p>0.36), nor triple interaction of block, testing day and group (time x block x group: $F_{(4,120)}$<0.92, p>0.45). For both horizontal and vertical activity, animals showed habituation to the apparatus within the individual open field test sessions, reflected by a main effect of 10-min block (block: $F_{(2,60)}$=319.19, p<0.0001; $F_{(2,60)}$=95.38, p<0.0001) and across test days, reflected by a main effect of days (time: $F_{(2,60)}$=3.42, p=0.04; $F_{(2,60)}$=3.22, p=0.047).

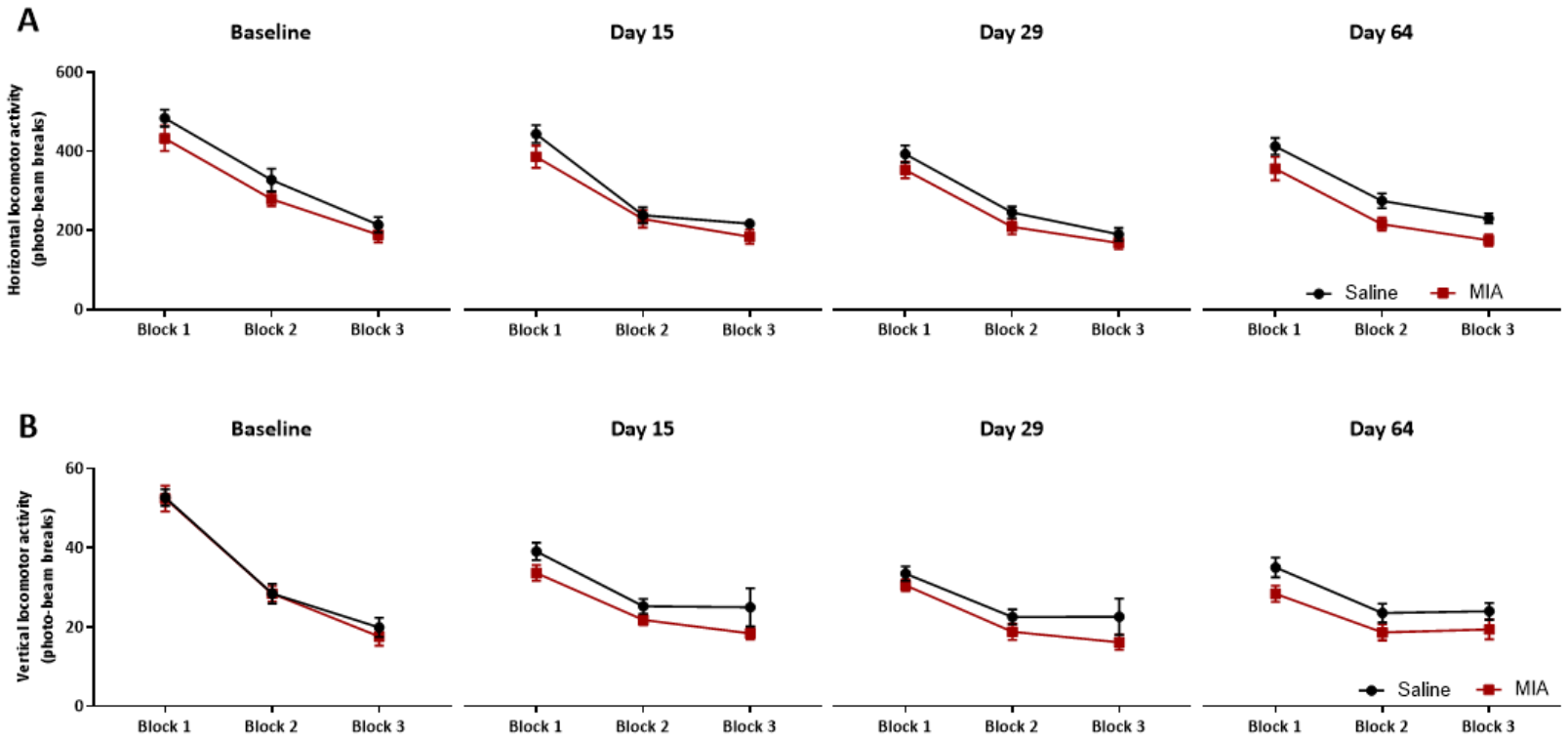

FIGURE 5.6. – HORIZONTAL (A) AND VERTICAL (B) LOCOMOTOR ACTIVITY. Rats were injected of MIA (▪) or saline (●) in the left knee (n=16 in each group). Data are presented as mean±SEM.

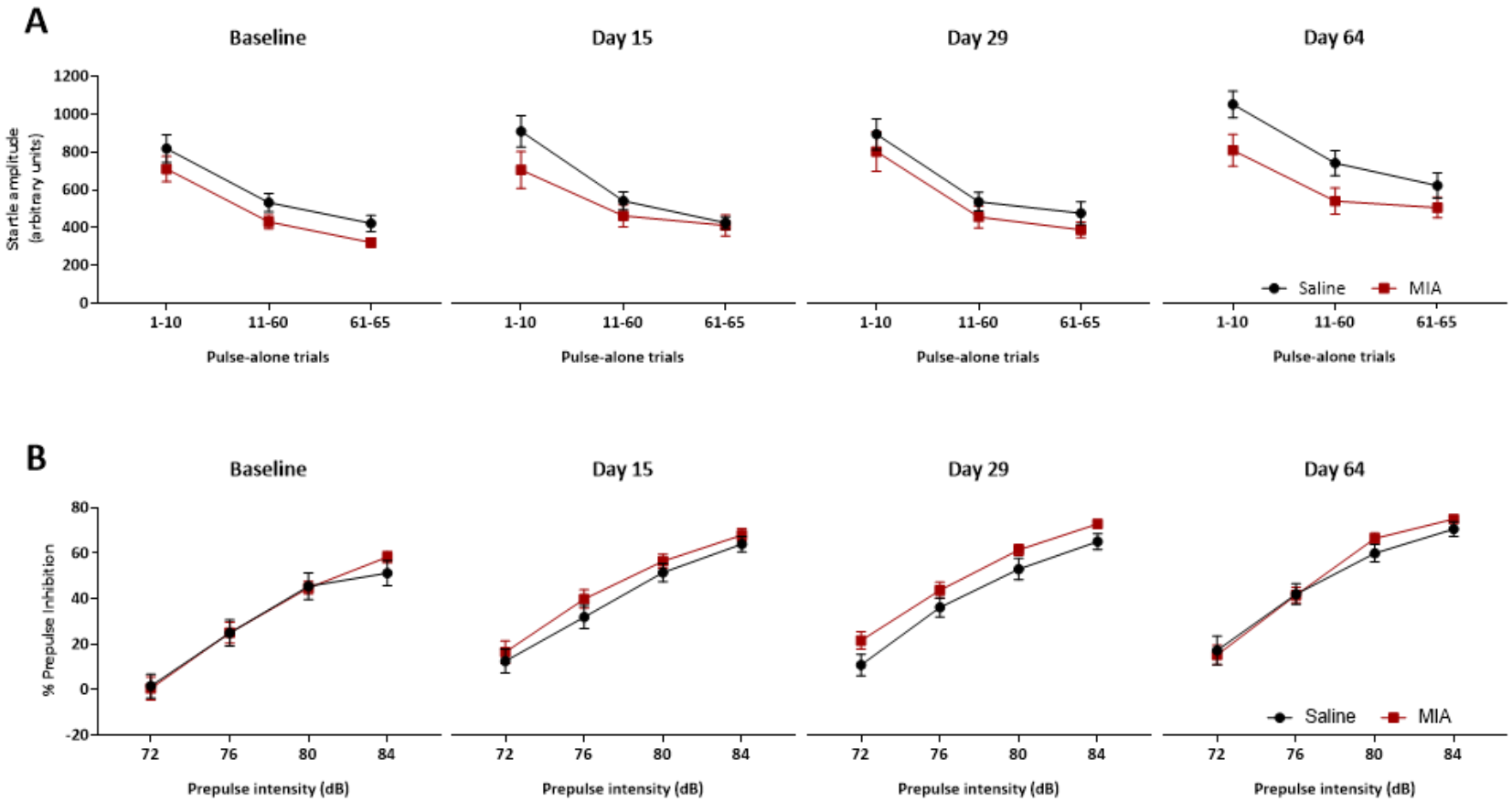

FIGURE 5.7. – STARTLE HABITUATION (A) AND PREPULSE INHIBITION ACTIVITIES (B) WERE NOT AFFECTED BY MONOSODIUM IODOACETATE (MIA). Rats were injected of MIA (▪) or saline (●) in the left knee (n=16 in each group). Data are presented as mean±SEM.

Startle response and prepulse inhibition were not affected by MIA injection (Fig.5.7). Animals have showed habituation to the pulse-alone ($F_{(2.60)}$=113.06, p<0.0001) and pulse intensity ($F_{(3,90)}$=320.68, p<0.0001) during the trials within the session. In addition, rats showed habituation to the test during the study ($F_{(2.60)}$>3.20, p<0.04). MIA model induction did not affect the startle response ($F_{(1,30)}$=2.91; p=0.10) (Fig.5.7A) or prepulse inhibition ($F_{(1,30)}$=2.14; p=0.15) (Fig.5.7B). No interaction effect with group was observed in the startle response ($F_{(2,60)}$<1.88, p>0.16) and in the prepulse inhibition, also no double interaction ($F_{(3,90)}$<1.07, p>0.38).

5.3.3. RECOGNITION MEMORY

Novel object recognition memory was not affected after MIA model induction (Fig. 5.8). Before model induction, exploration time was similar in both groups (group: $F_{(1,30)}$=0.41, p=0.52) and animals from both groups showed a similar preference for the novel object, i.e. there was a significant main effect of object (object: $F_{(1,30)}$=41.26, p<0.0001). MIA model induction did not affect exploration time (group: $F_{(1,30)}$=0.02) and both groups showed a similar preference for the novel object ($F_{(1,30)}$=27.26, p<0.0001).. The discrimination ratio between familiar and novel object was not affected by MIA injection (group: $F_{(1,30)}$=2.27, p=0.14) compared with saline controls. No interaction effect between group and object was observed at baseline, post model induction or in the discrimination ration analysis (object x group: $F_{(1,30)}$<1.03, p>0.32).

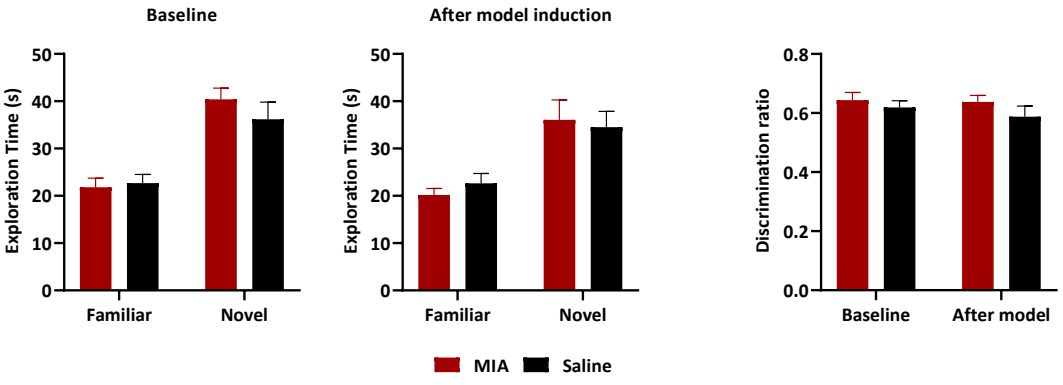

FIGURE 5.8. – RESULTS FROM THE TESTING PHASE FROM THE NOVEL OBJECT RECOGNITION MEMORY TEST. LH rats were injected with either 50ul of 3mg of MIA (▪; n=16) or saline (●; n=16) in the left knee. Data are presented as mean±SEM.

To ensure that the possible preference for the novel object did not decline rapidly over the 3-min session the results were also scored and analysed in 1-min bins using object and bin as within-subjects and group as between-subjects (data not showed). The 3-way ANOVA at baseline showed decrease of exploration time over time during the 3-min session (time: $F_{(2,60)}$=8.94, p>0.0001). Animals spent more time exploring the novel object (object: $F_{(1,30)}$=41.3, p>0.0001), but no main effect of group (group: $F_{(1,30)}$=0.42, p=0.5) or interaction involving group ($F_{(1,60)}$<1.03, p>0.3) was observed. Similar results were obtained post model induction, decrease of exploration time over time session (time: $F_{(2,60)}$=28.88, p>0.0001). Animals spent more time exploring the novel object (object: $F_{(1,30)}$=27.26, p>0.0001), but no main effect of group (group: $F_{(1,30)}$=0.02, p=0.9) or interaction with group ($F_{(2,60)}$<1.15, p>0.2) were observed. No minute by minute alteration in object exploration over the 3-min session was observed by the 3-way ANOVA interaction between group, object and 1-min bins, at both baseline ($F_{(2,60)}$=0.74, p=0.5) and post model induction ($F_{(2,60)}$=2.52, p=0.09).

5.3.4. BEHAVIOURAL FLEXIBILITY

5.3.4.1. CUE ACQUISITION

MIA did not impair acquisition of the cue discrimination task. An unpaired t-test showed that MIA (mean=119±34.12) and saline (mean=169.6±38.45) rats did not differ in trials to reach the criterion (t<1, df=30) (Fig.5.9A). No changes were also observed in terms of % of correct responses (t=1.35, df=30, p=0.19) (Fig.5.9B) and % of omissions (U=109.5, p=0.46) (Fig.5.9C) between MIA and saline controls. At this phase, 4 animals (3 saline + 1 MIA) failed to make streak of 10 correct responses within the three 150-trial sessions and failed to reach the criterion so they were excluded from further testing (signed in grey ellipses in Fig.5.9.A).

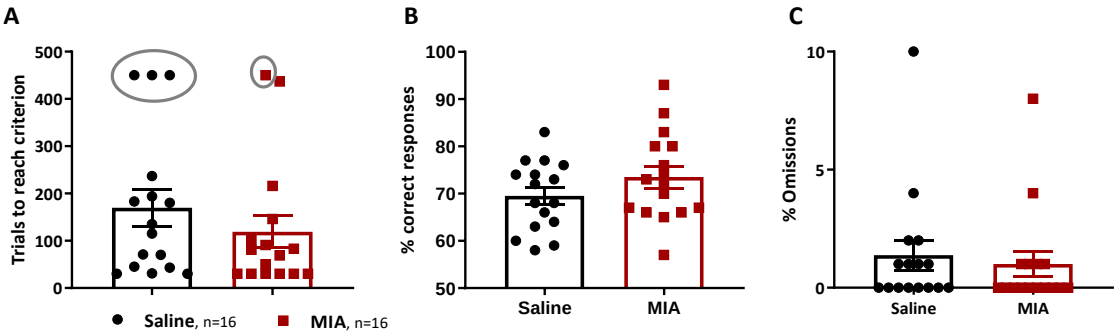

FIGURE 5.9. - TRIALS TO CRITERION (A), % OF CORRECT RESPONSES (B) AND % OF OMISSIONS (C) ON THE INITIAL CUE DISCRIMINATION TASK (SET). Cue Acquisition was not affected after MIA injection compared with controls. Animals in the grey ellipses did not reach criterion on the 3 days of testing and were excluded from the further testing phases. LH rats were injected with either 50ul of 3mg of MIA (▪; n=16) or saline (●; n=16) in the left knee. Data are presented as mean±SEM.

5.3.4.2. SHIFT TO RESPONSE STRATEGY

Shift from visual cue discrimination to response strategy was not affected by the MIA injection (Fig.5.10.). Trials to reach criterion decreased across days (time: $F_{(3,78)}$=123.8, p<0.0001) (Fig.5.10A) and % of correct responses increased across days ($F_{(3,78)}$=38.73, p<0.0001) (Fig.5.10B), however in both parameters there was no main effect of group (group: $F_{(1,26)}$<1) or interaction involving group (time x group: $F_{(3,78)}$>1.77, p<0.1). Omissions were never greater than 1 and did not differ between groups (data not shown). Perseverative (Fig.5.10C) and never-reinforcement errors (Fig.5.10D) decreased across days (time: $F_{(3,78)}$=14.21, p<0.0001; $F_{(3,78)}$=9.60, p<0.0001), however there was no main effect of group (group: $F_{(1,26)}$=0.22, p=0.6; $F_{(1,26)}$=0.004,p=0.9) or interaction involving group (time x group: $F_{(3,78)}$=1.23, p=0.3; $F_{(3,78)}$=0.92, p=0.4).

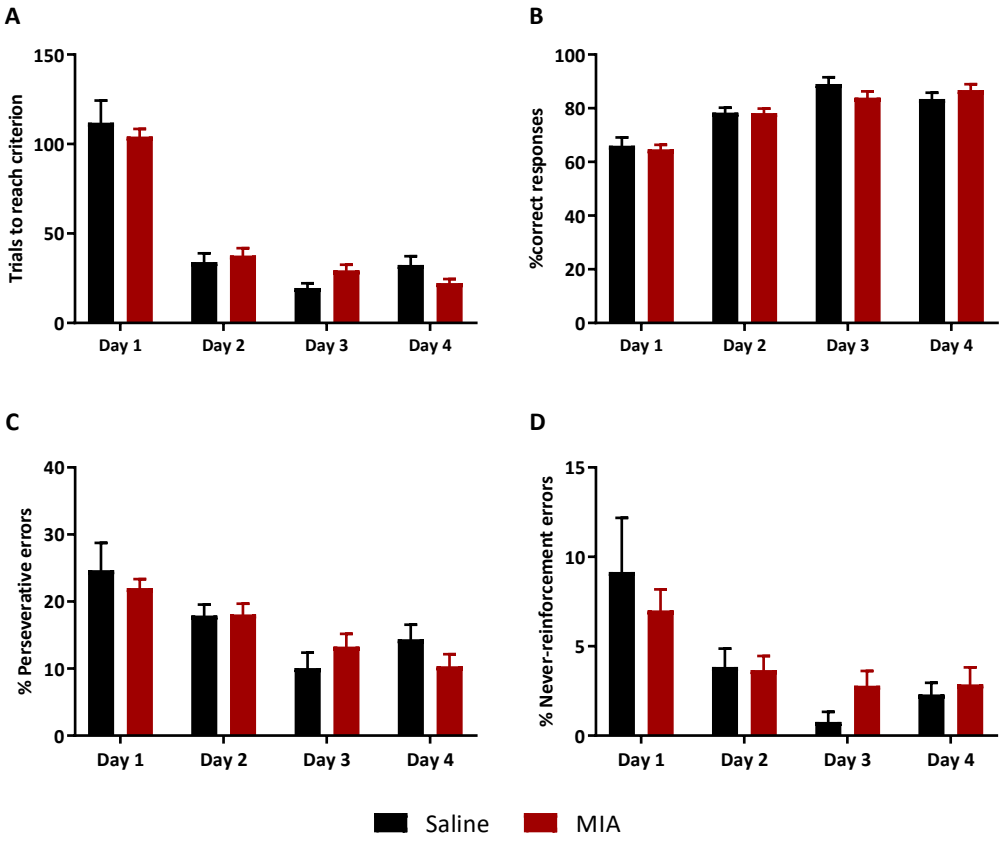

FIGURE 5.10. - TRIALS TO CRITERION (A), % OF CORRECT RESPONSES (B), % OF PERSEVERATIVE (C) AND NEVER-REINFORCEMENT (D) ERRORS ON THE SHIFT TO RESPONSE TASK (SHIFTING PHASE). Shift was not affected after MIA injection compared with controls. LH rats were injected with either 50ul of 3mg of MIA (▪; n=13) or saline (▪; n=15) in the left knee. Data are presented as mean±SEM.

5.3.4.3. REVERSALS

MIA did not impair the ability to reverse the spatial response rule (Fig.5.11). Both groups decreased the number of trials to reach criterion across days (time: $F_{(3,78)}$=96.82, p<0.0001) (Fig.5.11A) and slightly increased the % of correct responses (time: $F_{(3,78)}$=130.5, p<0.0001) (Fig.5.11B). However, in both parameters there was no main of group (group: $F_{(1,26)}$<1) or interaction involving group (time x group: $F_{(3,78)}$>1.67, p<0.2). Omissions were never greater than 1 and did not differ between groups (data not shown).

Perseverative (Fig.5.11C) and never-reinforcement errors (Fig.5.11D) decreased across days (time: $F_{(3,78)}$=90.20, p<0.0001; $F_{(3,78)}$=79.17,p<0.0001), however no main effect of group (group: $F_{(1,26)}$=0.0.05, p=0.8; $F_{(1,26)}$=4.07, p=0.054) and interaction involving group (time x group: $F_{(3,78)}$=0.46, p=0.7; $F_{(3,78)}$=0.92,p<=0.4) were observed.

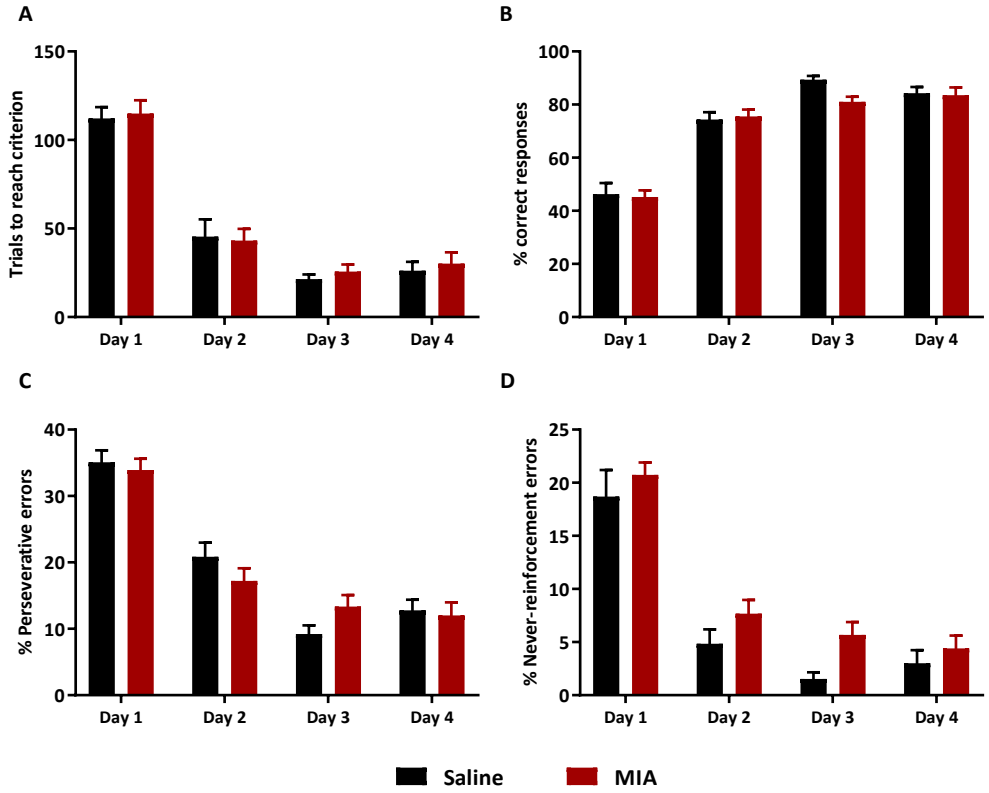

FIGURE 5.11. - TRIALS TO CRITERION (A), % OF CORRECT RESPONSES (B), % OF PERSEVERATIVE (C) AND NEVER-REINFORCEMENT (D) ERRORS ON THE REVERSALS. Reversal task was not affected after MIA injection compared with controls. LH rats were injected with either 50ul of 3mg of MIA (▪; n=13) or saline (▪; n=15) in the left knee. Data are presented as mean±SEM.

5.3.5. KNEE PATHOLOGY

Knees were collected and processed for pathology scoring at the end of the study as described in section 2.7. Joints were scored for cartilage integrity (cartilage damage x involvement), synovial inflammation and osteophyte formation on three different levels of both lateral and medial tibial plateau (Fig.5.12). Results are shown as mean between medial and tibial plateau values, as no differences were observed between medial and lateral tibial parts. During the splitting process, one knee joint from a saline animal was split with the wrong angle making it impossible to score.

MIA injected rats lost cartilage integrity (Fig.5.12A; $U=24$, $p<0.0001$), developed synovial inflammation (Fig.4.12B; $U=48$, $p=0.0002$) and showed a higher number osteophytes (Fig.5.12C; $t=2.51$; $df=29$, $p=0.02$), compared with control animals.

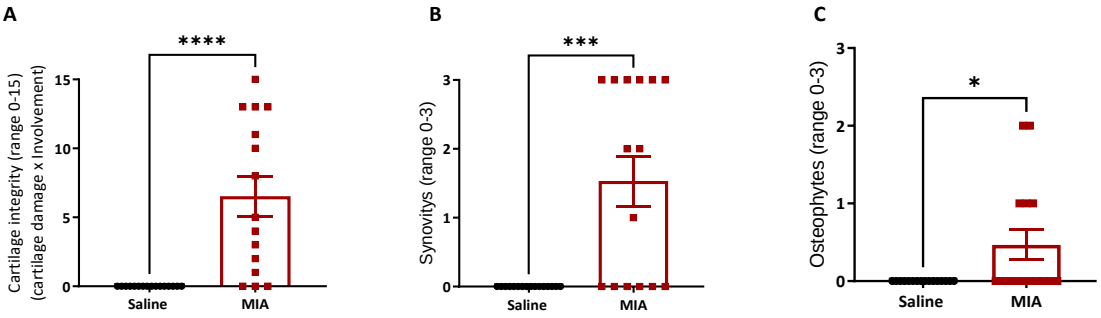

FIGURE 5.12. – MICROSCOPIC QUANTIFICATION OF HISTOLOGICAL CHANGES OF TIBIAL PLATEAU IN LISTER HOODED RATS INJECTED WITH EITHER 3 MG MONOSODIUM IODOACETATE (MIA) MODEL OR SALINE. Average scores for medial and lateral tibial plateau. Rats were injected with either 50ul of 3mg of MIA (▪, n=15) or saline (●, n=16) in the left knee. Knees were collected and processed for scoring at day 70 after model induction. 3 mg MIA was able to induce cartilage damage (A), synovial inflammation (B) and osteophyte formation (C). Data are presented as mean±SEM. **** $p<0.0001$; *** $p<0.001$; * $p<0.05$.

5.4. DISCUSSION

In this study, MIA-injected LH rats were subjected to a novel object recognition test and to an automated set shifting task to investigate the impacts of OA on recognition memory and behavioural flexibility. No evidences of impaired recognition memory and behavioural flexibility after induction of chronic MIA were observed.

5.4.1. PAIN BEHAVIOUR, SENSORIMOTOR ACTIVITY AND KNEE PATHOLOGY

Pain behaviour was assessed across this study. 3 mg of MIA injected into the knee, as in the previous experiments (chapter 2 and 3), produced a pain phenotype, reflected by weight bearing asymmetry. As observed in previous chapters, there was no evidence for mechanical allodynia, so the possibility that our MIA-injected rats do not show central sensitization should also be considered. Both MIA and control animals showed a slight decrease in ipsilateral paw withdrawal threshold after MIA/saline injection into the knee, with values then remaining at this lower level throughout the rest of the study.

Locomotor activity was slightly affected after MIA injection. As in chapter 3, in this study, MIA injected rats showed a significant decrease in the vertical activity compared with saline. As in chapter 4, there was no evidence for changes in startle response. Nevertheless, the PPI was mildly affected in MIA-injected rats at the higher prepulse in chapter 4, these effects were not observed in this chapter.

Similarly to the knee pathology results in chapter 3 (at day 35 after MIA injection) and 4 (at day 93), knee histology conducted at the end of this study at day 70 confirmed that MIA induced cartilage damage, synovial inflammation, and some animals developed osteophytes.

5.4.2. RECOGNITION MEMORY

MIA injected rats did not show impairments in novel object recognition memory compared with saline controls. Both sham and MIA rats spent more time exploring the novel object, the discrimination ratio was not affected after induction of the model, indicating that memory may not be affected by MIA.

Previous studies using a neuropathic pain model in both young adult (7weeks old) (You et al., 2018) and mid-aged (41 weeks old) male Sprague Dawley rats (Moriarty et al., 2016a),

showed that chronic neuropathic pain may be associated with recognition memory deficits. Impaired recognition memory was also reported in MIA-induced OA model in mice (Negrete et al., 2017). However, in contrast with these previous findings in rodents, in this study chronic knee OA-like pain in adult LH rats did not affect novel object recognition memory.

On top of the different models and animals used between studies, the principal barrier when comparing recognition memory results between studies is the protocol used across labs. First, the test is usually conducted in an open rectangular arena with high walls as in this study, however some labs use Y-mazes (Negrete et al., 2017). Second, in the study by Negrete et al. (2017), as in the present study, mice were tested 30 days after MIA induction and the interval time between sampling and testing phase was 24h.

Beside the evidence previously mentioned that chronic pain may be affecting recognition memory, there are also a few human studies that seem to be in accordance with our finding, indicating a limited impact. Once more, this conflicting data shows the importance to further investigate this relationship. A study conducted in middle-aged male patients with fibromyalgia showed no impairments in recognition memory compared with pain free groups when tested in the Camden Topographical Recognition Memory test (Lee et al., 2010). In addition, chronic back pain patients also did not show deficits in recognition memory when tested in the Cambridge Neuropsychological Test Automated Battery test (Schiltenwolf et al., 2017).

5.4.3. BEHAVIOURAL FLEXIBILITY

Behavioural flexibility was not affected in adult LH rats after induction of chronic OA like knee pain. Both MIA-injected rats and saline controls were able to learn and perform operant rules, and shift and reverse them similarly well during the automated set shifting test.

Cowen and colleagues conducted a study in adult Sprague Dawley rats with neuropathic pain showing weak impairments in behavioural flexibility in an operant chamber task with choice testing and progressive ratio tasks (Cowen et al., 2018). Rats with spinal nerve ligation did not show deficits in terms of learning and motivation, however, when compared with controls (both naïve and sham rats) chronic pain animals were slower to adapt to a new and more challenging task. However, impairments may have been less specific, since the in rats seem to show a non-specific impairment in operant learning, rather than specific impairments in behavioural flexibility.

As previously observed in chronic pain patients that tend to stay longer on the previous rule even when less proficient than healthy controls (Verdejo-Garcia et al., 2009; Indart et al., 2017), these neuropathic rats also seem to take longer to revert to an optimal choice. Interestingly, these neuropathic rats pressed the lever in bursts followed by long delays. This unique and possible adaptive coping strategy lead the authors to hypothesize and suggest that chronic neuropathic pain may drive a preference for familiar situations.

Leite-Almeida et al. showed during a spatial navigation reversal task in the watermaze test that only adult neuropathic rats had difficulties to shift from the old task compared with both young and old neuropathic Sprague Dawley rats. Furthermore, old sham rats also failed to adapt the behaviour to the new rule in this task, showing a relevant age-related impairment in behavioural flexibility (Leite-Almeida et al., 2009). This study points out once more the importance of considering the relationship between ageing, pain severity and cognition. In both human and principally in rodent studies, the age range is restricted which may be a translational issue for both pain and cognition research. Increasing the age range may increase clinical utility/relevance of the findings.

Cognitive flexibility in chronic pain patients has been very poorly investigated. Karp and colleagues showed that pain severity may be associated with mental flexibility (Karp et al., 2006), importantly, the sample of older adults appears to indicate that mental flexibility was more impaired as pain severity increased. Interestingly, Weiner et al (Weiner et al., 2006), demonstrated that older adults suffering from OA with chronic low back pain performed worse in a mental flexibility task compared with pain-free OA subjects. These results lead to hypothesise that OA by itself is not sufficient to affect neurocognitive function, but that chronic pain associated with OA is critical for the neurocognitive impairment.

5.5. CONCLUSIONS

In sum, our results showed that in contrast with some evidence in other rodent models of pain, chronic OA knee-like pain in MIA-injected Lister hooded rats does not seem to be associated with recognition memory and behavioural flexibility deficits.

Previous studies in rodents to investigate the impacts of pain on recognition memory and behavioural flexibility have been used mainly neuropathic models of chronic pain. As previously mentioned, OA pain may be associated with less brain changes compared with other types of pain (Mutso et al., 2012) and consequently has less impact in the respective cognitive functions which may explain the lack of cognitive impairments in our MIA model. However, Negrete's study conducted a study in MIA mice and showed recognition memory impaired in these animals. Despite, the species and dose difference, this result difference was not expected since MIA is a well-established model across species due to its chemical induction nature.

As briefly mention in chapter 4, another point that should be considered is the possibility that our MIA-injected rats do not show evidence of central sensitization. In contrast with the rodent studies mentioned, in this discussion including Negrete's study, our MIA-injected rats did not show mechanical allodynia changes which may indicate that their central nervous system may not be impacted and consequently not impacting the cognitive function observed in the other studies.

Several factors may mediate the relationship between chronic pain and cognitive functions, such as life style, age and medication. In fact, some studies have shown evidence that chronic pain patients under long-term opioid treatments perform worst in cognitive tasks than patients undergoing non-opioid treatments (Schiltenwolf et al., 2017; Richards et al., 2018). Opioid use for analgesics effects in patients under chronic pain states have rapidly escalated with wide ranging consequences. Furthermore, the long-term use of opioids can induce hyperalgesia (Angst and Clark, 2006), i.e. central nervous system changes and by itself cause cognitive function deficits. This topic will be studied and discussed in the next chapter.

CHAPTER 6

SUSTAINED OPIOID TREATMENT EFFECTS ON MEMORY IN MIA-INDUCED LISTER HOODED RATS

6.1. INTRODUCTION

Chronic pain has been associated with cognitive function impairments. However, the relationship between osteoarthritis (OA) and such impairments remains unclear. Results in chapters 4 and 5 suggest that chronic OA-like pain behaviour in a rat model does not affect memory and cognitive flexibility. This finding may reflect the relatively short duration of OA-like pain behaviour in the rat model compared to the clinical situation, or may reflect the combined impact of other factors with OA pain on cognitive function. For example, the development of cognitive deficits in humans is associated with life style (alcohol intake, lack of sleep, etc.), comorbidities (anxiety, stress, etc.), age and medication. In particular, there is evidence that some drugs used to treat chronic pain can contribute to cognitive deficits, such as opioids (Schiltenwolf et al., 2017; Richards et al., 2018).

6.1.1. TREATMENTS FOR OSTEOARTHRITIS PAIN

OA is a progressive and degenerative disease and a major public health burden. Pain is the main symptom of OA, but this disease is also associated with other comorbidities (Moriarty et al., 2011). Unfortunately, effective medication to either prevent or treat this disease has not been found yet, and, in order to improve the quality of life and joint mobility, current OA treatments are mainly focused on symptomatic relief of pain and/or inflammation. Surgical intervention is only indicated when pharmacological treatments do not work or when the degree of pathology is high (Hunter and Bierma-Zeinstra, 2019). Non-pharmacological methods are recommended as a first key management strategy; such methods include exercise, weight loss if overweight, patient education and self-management (Hunter and Bierma-Zeinstra, 2019). Pharmacological treatments are mainly focused on

analgesics, such as paracetamol, opioids, duloxetine; and/or anti-inflammatory agents, such as non-steroidal anti-inflammatory drugs (NSAIDs) (Hunter and Bierma-Zeinstra, 2019).

The first-line of the pharmacological treatments is focused on the use of NSAIDs (oral or topical administration). Although NSAIDs have beneficial effects in some people (da Costa et al., 2014; Bannuru et al., 2015), several adverse effects in particular in the gastrointestinal tract or renal effects have been reported in some patients with long-term treatment (Pelletier et al., 2016). Intra-articular injection of corticosteroids produces beneficial pain relief and improves joint mobility in knee OA in patients not responding to oral analgesics/anti-inflammatory agents (Hunter and Bierma-Zeinstra, 2019). Several other pain management therapies have been used and several new pain treatments are currently in study and development (Tive et al., 2019; Gowler et al., 2020). Strong opioids are prescribed for moderate to severe pain in patients unresponsive to NSAIDS.

6.1.2. OPIOIDS AND OSTEOARTHRITIS

The huge increase in opioid prescription, misuse and abuse has been considered as an epidemic, particularly in North America (Shipton et al., 2018). In England, the long-term prescription of opioids to manage chronic pain increased around 34% (or 127% after correcting for total oral morphine equivalency) between 1988 and 2016 (Curtis et al., 2019). Between 2016 and 2017 there was a decline in the prescription of opioids, however the number is still high compared with the previous decade (Curtis et al., 2019).

Opioid drugs are a common treatment to manage OA pain despite lack of supporting evidence regarding its efficacy (Krebs et al., 2018). Krebs and colleagues (2018) conducted a randomised trial including 240 patients with moderate to severe chronic back pain or hip or knee pain. Half of the patients were under opioid treatment and the other half under non-opioid analgesics. Opioids had no superior improvements in pain-related effect over non-opioid treatments over 12 months. These recent findings demonstrated that opioids are not more effective than the non-opioid treatments, and recently the Osteoarthritis Research Society International (OARSI) updated the guidelines for the management of OA and strongly recommended against the use of opioids (Bannuru et al., 2019). Increasing awareness has been raised concerning the use and the consequences of opioids (Yip and Oettinger, 2020).

The most common types of opioids prescribed are codeine and its combinations, followed by the strong opioid tramadol, and then buprenorphine, morphine and oxycodone (Ashaye

et al., 2018). There are three main types of opioid receptors: mu (μ), delta (δ), and kappa (κ), which are G-protein coupled receptors and activate inhibitory G-proteins (Al-Hasani and Bruchas, 2011). μ-receptors are essential for the analgesic action of opioids (Williams et al., 2013) and are expressed throughout the central nervous system, including both spinal cord and supraspinal areas (Al-Hasani and Bruchas, 2011).

6.1.3. OPIOID TOLERANCE AND HYPERALGESIA

Opioid use to manage pain has been associated with increased adverse effects related with gastrointestinal depression (constipation, nausea and vomiting) and respiratory depression (Imam et al., 2018). Opioid use also increases risk of addiction and overdose in a dose-dependent manner (Bedson et al., 2019). The prolonged use of opioids has additionally been associated with tolerance and hyperalgesia (Colvin et al., 2019).

Opioid tolerance occurs when an increased dose is required to achieve the same amount of analgesic effect. Ongoing opioid exposure can lead to a reduction of the responsiveness to the drug, resulting in less analgesia over time (Colvin et al., 2019). Clinical loss of analgesic efficacy during opioid treatments may also result from a different phenomenon, known as opioid-induced hyperalgesia (OIH) (Chu et al., 2008).

OIH is associated with prolonged opioid treatment and has been shown mechanistically to be a pronociceptive process, leading to an increase in pain sensitivity (Silverman, 2009). OIH was reported in both humans (Chu et al., 2008) and animal models (Araldi et al., 2015). Additionally, OIH has been reported following various routes of opioid administration at both high and ultra-low doses (Angst and Clark, 2006). Several studies have been conducted to investigate the mechanisms behind OIH, the majority of which have focused on peripheral neuronal and spinal cord mechanisms, but there is some evidence for a contribution from supraspinal areas (Chu et al., 2008; Chen et al., 2010; Ferrini et al., 2013; Sun et al., 2019).

6.1.4. COGNITIVE IMPACT OF OPIOID ANALGESIA

There is evidence that some analgesic drugs, including opioids, may interfere with cognitive function [see for review (Moriarty et al., 2011)]. μ-receptor activation inhibits GABA transmission in some brain areas, such as the hippocampus (Zieglgansberger et al., 1979; Valentino and Volkow, 2018). Also, μ-receptors activate GABA inhibition in the reward brain circuits, which can lead to increased dopamine release into ventral tegmental area and

prefrontal cortex (Bull et al., 2017; Colvin et al., 2019). These results may suggest that opioids acting on μ-receptors may stimulate GABAergic inhibition and consequently this may cause inhibition of brain circuits involved in cognitive functions. Such (hippocampal and prefrontal) disinhibition may well cause cognitive impairments (Bast et al., 2017) and increased dopamine transmission may also disrupt some cognitive processes.

The impact of opioid treatment for chronic pain on cognitive function is under-studied. In some studies of chronic pain and cognition, patients under medication are excluded or the medication effect is corrected (Moriarty et al., 2011). Nevertheless, there are some studies addressing this question. Chronic pain patients taking opioids performed worse in memory tasks compared with non-opioid patients (Karp et al., 2006). In this study, no correlation between pain severity and memory was detected and pain scores between non-opioid patients and patients prescribed with opioids were also not statistically different, however patients taking opioids had more difficulty with unprompted memory compared with non-opioids subjects. Moreover, patients with non-malignant pain under long-term opioid treatment had worse spatial working memory compared with non-opioid patients (Sjøgren et al., 2000).

A study including chronic back pain patients under long-term opioid treatment versus non-opioid treatment showed an interaction of low back pain and opioid treatment with cognitive deficits. Chronic back pain patients using both treatments showed impairments in attention and cognitive flexibility compared with controls, patients who underwent opioid therapy showed a trend to perform even worse in the tasks compared with healthy controls. Interestingly, only patients who underwent opioid therapy showed impairments in short term memory and working memory compared with both patients under non-opioid treatment and healthy controls. In this study cognitive impairments were associated with pain intensity (Schiltenwolf et al., 2014).

Preclinical studies have also associated chronic opioid treatment with cognitive dysfunction. For example, chronic administration of morphine in naïve rats was associated with impaired learning during an operant chamber task (Wang et al., 2006) and in spatial memory during the probe trial in the Morris watermaze (Brolin et al., 2018). On the other hand, morphine treatment improved attention and recognition memory in a rat model of visceral pain (Millecamps et al., 2004).

6.1.5. AIMS OF CHAPTER

Opioids are widely used to manage chronic pain despite the potential for adverse effects and misuse. Opioids have wide-ranging effects on neuronal function, including in brain regions contributing to cognition. The impact of long-term exposure to opioids upon cognitive function in people suffering chronic pain is poorly understood. Evidence suggests that opioids may lead to an exacerbation of the pain phenotype and also cognitive dysfunction.

The aim of this chapter was to investigate the effects of sustained morphine treatment and withdrawal from such treatment on hippocampal rapid place learning performance and recognition memory performance in the MIA model of OA-like knee pain in LH rats.

To address this aim, I first investigated whether morphine treatment, following early acute analgesic action, induced morphine tolerance and/or OIH in LH rats following sustained morphine treatment and withdrawal from such treatment. Naïve LH were chronically treated with morphine, and pain behaviour and locomotor activity were assessed during the treatment and during drug withdrawal.

Secondly, I investigated whether sustained morphine treatment altered memory in MIA-injected LH rats using the watermaze DMP task and the novel object recognition memory task. Cognitive and pain phenotypes were assessed acutely and during withdrawal from morphine treatment.

6.2. METHODS

Refer to Chapter 2 for general methodology.

6.2.1. MORPHINE DOSE – PILOT STUDY TO EXAMINE WHETHER MORPHINE CAUSES TOLERANCE AND HYPERALGESIA IN LISTER HOODED RATS

6.2.1.1. ANIMALS

A pilot study using 10 Lister hooded (LH) (Charles Rivers, UK) rats weighing between 250-280g was conducted to evaluate pain behaviour changes in naïve LH rats during a sustained morphine treatment. Rats were housed in groups – 1 cage with 4 and 2 cages with 3 rats. The target sample size was calculated so group differences corresponding to an effect size of Cohen's d=1 could be detected with a power of about 80%, using an independent t-test (2-tailed, $p<0.05$).

6.2.1.2. MORPHINE ADMINISTRATION

Morphine (sulphate, Sigma) was dissolved in saline and administered subcutaneously (3mg/kg); based on previous studies (Babbini and Davis, 1972; Aguilar et al., 1998; Raghavendra et al., 2004; Ferrini et al., 2013; Brolin et al., 2018) and unpublished studies of our lab. Rats were allocated randomly to the treatment groups, with half of the animals in each cage receiving treatment and the other half saline (n=5 per treatment group). Animals were injected twice a day, in the morning (between 9-10 am) and in the evening (between 4-5 pm), for 7 days. Controls received an injection of saline. The experimenter (S.G.) was blinded to the drug treatment throughout the data collection and analysis.

6.2.1.3. PAIN BEHAVIOUR AND LOCOMOTOR ACTIVITY

Nociceptive pain behaviour was assessed using the Von-Frey test, as described in 2.3. and locomotor activity was assessed as described in 2.4. Rats were first handled for a few days and habituated to the Von-Frey test apparatus. Baseline locomotor activity measurements were collected one day before the treatment, and baseline pain behaviour was measured on the day of the treatment, 1h before the first injection.

6.2.1.4. EXPERIMENTAL DESIGN

As indicated in the scheme in fig 6.1., rats were first handled and habituated to the pain behaviour apparatus. Then, baseline measurements were collected prior to morphine treatment. Animals were either injected with morphine twice a day for 7 days (sc., 3mg/kg x 2 day, n=5) or saline (sc., x 2 day, n=5). Locomotor activity was measured one day before the start of the treatment and then on day 5 of the treatment (1h after the morning injection), and then during withdrawal, on days 10 and 15 after the first day of treatment. Pain behaviour was assessed using the Von-Frey test, and measures were taken 1 h before and 1 h after the morning injections from day 1 to 4, 6 and 7 of treatment days, and then on day 8 and 12. Von-Frey measures collected 1h before the injection on the first treatment day were used as baseline.

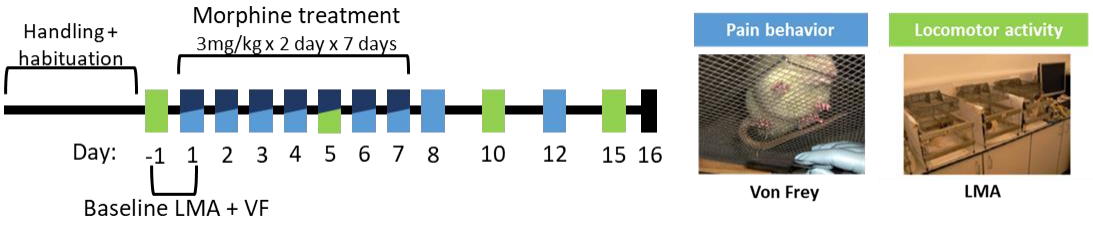

FIGURE 6.1. – TIME COURSE OF MORPHINE PILOT STUDY. 10 adult male LH rats were used in this study. Animals were injected (dark blue rectangle) with either morphine twice a day (morning and evening) (sc., 3mg/kg x 2 per day x 7days, n=5) or saline (sc. x 2 per day x 7days, n=5). Green rectangles - locomotor activity. Light blue rectangle- Von-Frey test.

6.2.2. EFFECTS OF SUSTAINED MORPHINE TREATMENT ON PAIN BEHAVIOUR AND MEMORY IN MIA-INDUCED LH RATS

6.2.2.1. ANIMALS

For this study, 12 (n=6 per group) adult male LH rats (Charles Rivers, UK) weighing between 250-280g at the beginning of the experiment were used. The target sample size for this study was 32 (n=16 per group), so group differences corresponding to an effect size of Cohen's d=1 could be detected with a power of about 80%, using an independent t-test (2-tailed, $p < 0.05$). However, the first series of the experiment clearly indicated that there were no substantial group differences in the main memory measures, and that completion of the additional series to achieve the target sample size would not reveal significant group differences. Therefore, the study was terminated due to futility after only completion of the first series (Neumann et al., 2017).

6.2.4. PAIN BEHAVIOUR AND SENSORIMOTOR ACTIVITY

The model of OA pain was induced with a single intra-articular injection of MIA (as described in 2.2.). All the LH rats were injected with 50 µL of MIA (3 mg/50 µL; n=12). Nociceptive pain behaviours were assessed using weight-bearing and Von-Frey tests, as described in 2.3., and sensorimotor measures, locomotor activity and startle/prepulse inhibition, were taken as described in 2.4.. Rats were first handled for a few days and habituated to the pain test apparatus. Baseline pain behaviour and sensorimotor activity measurements were collected before MIA injections with one day apart. After MIA/saline injection (day 0), pain measurements and sensorimotor processes were then collected as indicated in fig 6.2.

6.2.5. MEMORY ASSESSMENTS

To assess if recognition memory was impaired in MIA-injected LH rats treated with morphine, animals were subjected to the novel object recognition (NOR) test as described in 5.2.3.. Measures were collected prior to MIA injection and any morphine injection and 47-49 days after MIA injection, i.e. 7-9 days after the end of the morphine treatment, as indicated in fig 6.2.

Hippocampus-dependent rapid place learning performance was evaluated using the watermaze DMP task as described in 4.2.3.. Animals were pretrained for 8 consecutive days on this task prior to MIA injection and any morphine treatment and then tested at several time point across the study (before, during and after morphine treatment) as indicated in fig 6.2.

6.2.6. EXPERIMENTAL DESIGN

Rats were first handled and habituated to the pain behaviour and NOR apparatus, then baseline NOR measures were collected, watermaze DMP pretraining was conducted and pain behaviour and sensorimotor activity baseline measurements were collected (Fig.6.2).

The MIA model was then induced in all rats. Before the start of morphine treatment, pain behaviour measures were collected on days 7, 14 and 28 and the sensorimotor activity measures on days 8, 15 and 29. Also before the start of morphine treatment, 2 extra days of watermaze testing, including a probe day, was conducted to re-baseline. Morphine

treatment started on day 30 after MIA injection, to ensure that complete development of OA phenotype. Animals were either treated for 10 days with morphine (sc., 3mg/kg x 2 per day x 10 days, n=6) or saline (sc. x 2 per day x 10 days, n=6). During the treatment, pain behaviour was assessed using the Von-Frey test (days 1, 3 and 8 of treatment) or weight bearing (days 2 and 4 of treatment); measures were taken 1h before and 1h after the morning injections. Also during treatment, locomotor activity was measured on day 5, 1h after the morning injection. Watermaze DMP performance was assessed on days 6, 7, 9 and 10 – days 7 and 10 were probe days.

On day 50 after MIA injection, rats were anaesthetized with a lethal dose of sodium pentobarbitone and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains, spinal cords, DRGs and knees were carefully excised, preserved and stored. Knee joint sections were stained with haematoxylin and eosin- or safranin-O/fast green and then scored for overall joint morphology and proteoglycan loss. Total cartilage joint damage, osteophyte, proteoglycan loss, synovial inflammation and chondrocyte presence were scored to evaluate the severity of the knee joint pathology as described in 2.7.

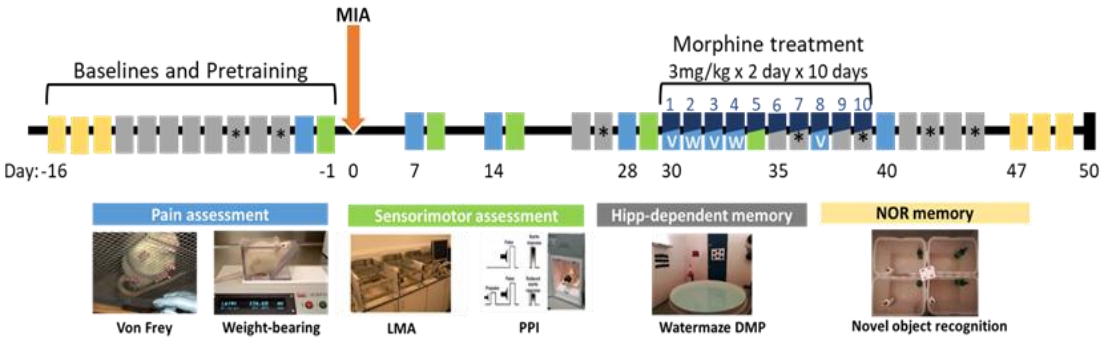

FIGURE 6.2. – TIME COURSE OF OPIOID TREATMENT EFFECTS OF SUSTAINED MORPHINE TREATMENT ON PAIN BEHAVIOUR AND MEMORY STUDY. 12 adult male LH rats were injected with MIA (3mg). At day 30 post model induction animals were either treated with morphine (3mg/kg x 2 per day, n=6) or saline (x 2 per day, n=6) for 10 days (dark blue rectangle). Yellow rectangles – novel object recognition test. Grey rectangle – watermaze delayed matching-to-place task, * indicates probe days. Light blue rectangle- weight bearing (W) and Von-Frey (V) test. Green rectangles - locomotor activity and startle prepulse inhibition. During morphine treatment days, pain behaviour measures were collected 1h before and 1 h after the morning injection, locomotor activity and watermaze measure were collected 1h after morning injection.

6.2.7. STATISTICAL ANALYSIS

GraphPad Prism 8 and IBM SPSS Statistics 24 were used to prepare the graphs and perform the statistical analysis, respectively.

Results from the pain behaviour studies were analysed using an ANOVA, with group as between-subjects factor, and testing day as repeated measures/within-subjects variable. Results from the sensorimotor behaviour studies were analysed using ANOVA, with time points and task blocks (or pulse intensity in the PPI test) as within-subjects factors and treatment group as between-subjects factor. Unpaired t-tests were used to compare activity between groups on the treatment days to evaluate acute morphine effect. Watermaze DMP results were analysed using ANOVA with testing time points and trials as within-subjects factors and group as between-groups factor. Bonferroni multiple comparison was used as *post-hoc* testing.

Knee pathology was analysed with unpaired t-tests or with Mann-Whitney test when normality was violated. NOR results were analysed using ANOVA, with testing day and object (novel vs familiar) as within-subjects factors and group as between-subjects factor. $P<0.05$ was considered to represent a significant difference and all results were expressed as mean±standard error (SEM). Normality was tested using D'Agostino-Pearson test. Baseline measures were not included in the ANOVAs, but were analysed separately, using unpaired t-tests to ensure that there were no significant difference between groups at this stage.

6.3. RESULTS

6.3.1. EFFECTS OF SUSTAINED MORPHINE TREATMENT ON PAIN BEHAVIOR AND LOCOMOTOR ACTIVITY IN LISTER HOODED RATS

Body weight of LH rats tended to gradually diverge during the first days of morphine treatment and converge again after the treatment (time x group: $F_{(13,104)}$=9.16, p<0.0001), post hoc analysis showed significant differences between control saline and morphine rats only on day 9 (p=0.016) (Fig.6.3). Visual inspection indicated hyperactivity, licking of cage walls and digging behaviour in rats, starting within about 10 min of the morphine injection and lasting for about 1 to 1.5h.

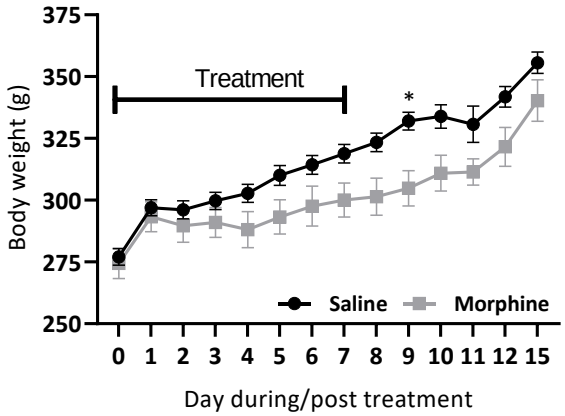

FIGURE 6.3. – BODY WEIGHT OF LISTER HOODED RATS DURING SUSTAINED MORPHINE TREATMENT AND DURING WITHDRAWAL. LH rats were treated subcutaneously with morphine (3mg/kg, twice a day, ●; n=5) or saline (▪; n=5) for 7 days. Data are presented as mean±SEM. * p<0.05, 2-way ANOVA with Bonferroni multiple comparisons *post-hoc* testing

LH rats treated with morphine for 7 days developed morphine-induced hyperalgesia, which was evident in the daily tests 1 h before the first daily morphine injection and during the tests after cessation of the morphine treatment, i.e. during withdrawal (Fig.6.4A). PWTs were lowered from day 2 until the end of the study in both morphine and saline treated rats, reflecting habituation to the test itself (time: $F_{(6,48)}$=18.96, p<0.0001). Morphine group had numerically lower PWT values from day 4 of treatment until day 7 and during treatment withdrawal, post-hoc analysis showed difference between saline and morphine group on day 12 (p=0.018), supporting development of morphine-induced hyperalgesia. Although inspection of the data indicates that morphine-treated rats did not show reduced PWTs compared to saline-injected rats before day 3 of treatment, there was only a significant main effect of treatment group (group: $F_{(1,8)}$=7.93, p=0.02), but no group x day interaction ($F_{(6,48)}$<1).

In the tests following 1 h after morphine treatment, acute antinociceptive effects were apparent, but tolerance to the drug's antinociceptive effects developed within the 7 days of treatment (Fig.6.4B). Acute morphine injection increased PWTs, but this effect decreased across days and was not evident by the 6th day of morphine injection, reflecting a tolerance to the antinociceptive effect of morphine. In support of this finding, a 2-way ANOVA using group as between subjects factor and day of morphine treatment as repeated measures factor showed an interaction of group x time: $F_{(5,40)}$=2.42, p=0.05 (alongside a significant main effect of time, $F_{(5,40)}$=7.68, p<0.0001 and a trend towards a main effect of group ($F_{(1,8)}$=3.82, p=0.09).

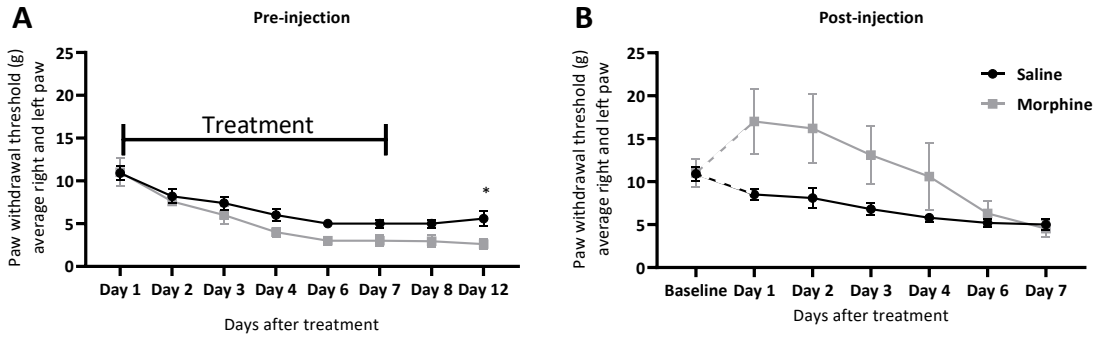

FIGURE 6.4. – PAW WITHDRAWAL THRESHOLD PRE-INJECTION AND WITHDRAWAL FROM MORPHINE (A) AND POST-INJECTION (B) OF MORPHINE/SALINE. LH rats were treated subcutaneously with morphine (3mg/kg, twice a day, ●; n=5) or saline (▫; n=5▪) for 7 days. Measurements were collected 1h prior (A) and 1h after (B) each morning injection. Data are presented as mean±SEM. * p<0.05, 2-way ANOVA with Bonferroni multiple comparisons *post-hoc* testing

Morphine treatment only affected acutely the horizontal activity (Fig.6.5). Main effect of group (group: $F_{(1,8)}$=10.02, p=0.01) and time (time: $F_{(2,16)}$=4.05, p=0.04) were observed in the horizontal activity. No interaction involving group was observed (time x group: $F_{(2,16)}$=1.44, p=0.27). Post-hoc analysis show that on day 5 of the morphine treatment (1h after morphine injection), rats showed acute horizontal locomotor hyperactivity compared to saline treated rats (p=0.03). Vertical activity did not differ between groups (group: $F_{(1,8)}$=1.79, p=0.22) no time and interaction effect were observed (time: $F_{(1.6;\ 12.5)}$=2.01, p=0.17; time x group: $F_{(1,8)}$=1.79, p=0.22).

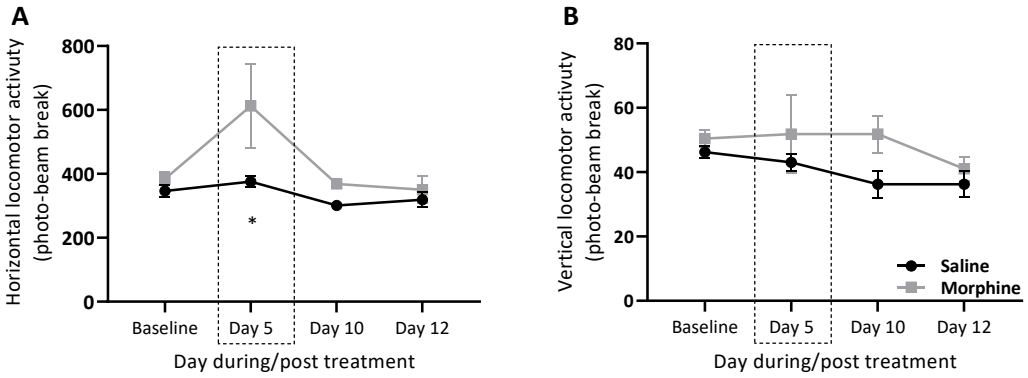

FIGURE 6.5. – HORIZONTAL (A) AND VERTICAL (B) LOCOMOTOR ACTIVITY DURING THE FIRST 10-MIN BINS. Locomotor activity was assessed at baseline, 1h after morphine/saline injection on day 5 and the on day 10 and 12 after treatment commenced. LH rats were treated subcutaneously with either morphine (3mg/kg, twice a day, ●; n=5) or saline (▪; n=5) for 7 days. Dashed rectangles indicate day when locomotor activity was assessed 1h after injection. Data are presented as mean±SEM. * p<0.05, 2-way ANOVA with Bonferroni multiple comparisons *post-hoc* testing

6.3.2. EFFECTS OF SUSTAINED MORPHINE TREATMENT IN MIA-INJECTED LH RATS

6.3.2.1. PAIN BEHAVIOUR

MIA injection caused weight-bearing asymmetry in LH rats, as in the previous studies (chapter 3, 4 and 5): rats placed less weight on the injured limb after MIA injection reflected by an increase in the asymmetry after baseline (time: $F_{(2.6,25.8)}$=20.62; p<0.0001). No main effect of group or interaction involving group was observed in the prospective groups (F<1) (Fig.6.6A). Morphine treatment had acute analgesic actions, i.e. antagonised the weigh bearing asymmetry, on day 34 (day 4 of treatment), but not day 31 (day 2 of treatment) (Fig. 6.6B). 2X2 ANOVA of weight bearing data on day 31 and 34 revealed a significant interaction treatment by day (group: $F_{(1,10)}$=15.95, p=0.003), as simple main effects analysis revealed weight bearing asymmetry was reduced in morphine treated compared to saline treated rats on day 34 (p<0.02), but not day 31. There was no evidence of morphine-induced hyperalgesia, weight bearing asymmetry did not differ between morphine and saline groups before morning injections on days 31 and 34 (no main effect or interaction involving group, F<1) or during treatment withdrawal on day 40 (t=0.38,df=10,p=0.71).

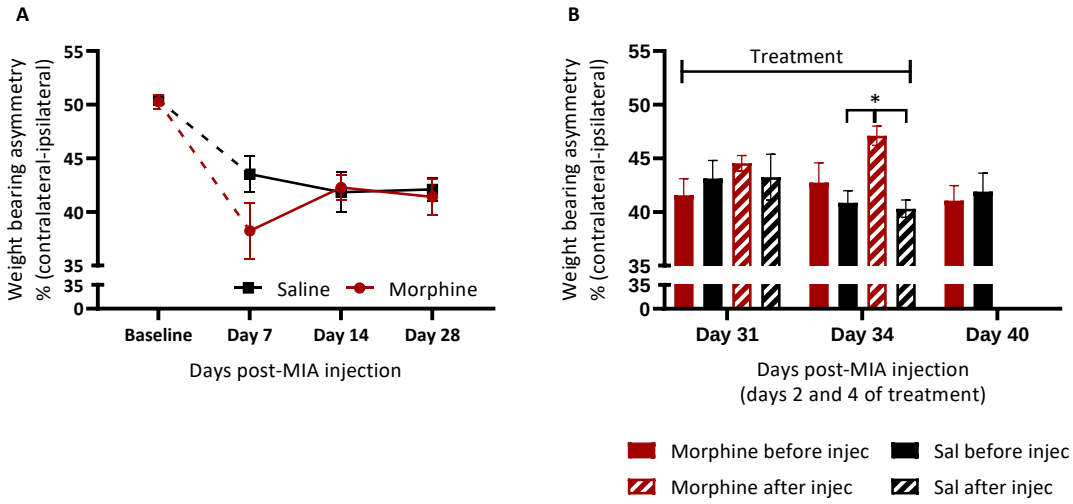

FIGURE 6.6. – WEIGHT BEARING ASYMMETRY AFTER MIA MODEL INDUCTION IN LH RATS (A) AND THE EFFECTS OF MORPHINE TREATMENT IN THIS PAIN BEHAVIOUR (B). (A) Weight bearing asymmetry was assessed at baseline and after MIA model induction in LH rats (3mg, n=12) at day 7, 14 and 28. (B) Rats were then treated subcutaneously with either morphine (3mg/kg, twice a day, ●; n=6) or saline (▪; n=6) for 10 days from day 30 post-MIA. Weight bearing was assessed 1h prior and 1 h after the morning injection. Data are presented as mean±SEM. * p<0.05, 2-way ANOVA with Bonferroni multiple comparisons *post-hoc* testing

Slight reductions of PWTs were observed in ipsilateral paw (time: $F_{(2.14,21.48)}$=16.86, p<0.0001) in the prospective treatment groups after MIA injection, reductions were also observed in the contralateral side (time: $F_{(2.35,23.57)}$=4.62, p=0.02), which may reflect sensitization (Fig. 6A and B, left panels). No differences were found between prospective groups prior to the treatment in both contra (time x group: $F_{(2,20)}$<1) and ipsilateral paw (time x group: $F_{(2,20)}$=2.07; p=0.15). Also, in both contra and ipsilateral paw no main effect of group and interaction involving group were observed (F<1).

Acute injection of morphine induced a pronounced analgesic effect across day 30, 32 and 37 (day 1, 3 and 8 of treatment), increasing PWTs compared with saline control injection in both ipsilateral ($F_{(1,10)}$=66.21; p<0.001) and contralateral paws ($F_{(1,10)}$=23.27; p=0.0007) (Fig. 6.6A and B, right). The analgesic effect was numerically, but no statically significant, attenuated across treatment days reflecting tolerance to the drug, although the interaction of day X group did not attain significance (ipsilateral: $F_{(2,20)}$=1.55, p=0.24; contralateral: $F_{(2,20)}$<1) and no main effect of day was observed. Additionally, no evidence of morphine-induced hyperalgesia was found for either hindpaws (time x group: $F_{(3,30)}$<1). PWTs measured prior to morning injection (on days 30, 32 and 37) and after the treatment was ended (on day 40) reduced across days in both ipsilateral ($F_{(3,30)}$=22.19; p<0.0001) and contralateral paws ($F_{(3,30)}$=10.39; p=0.007), but this reduction was similar between morphine and saline treated rats (time x group: F<1).

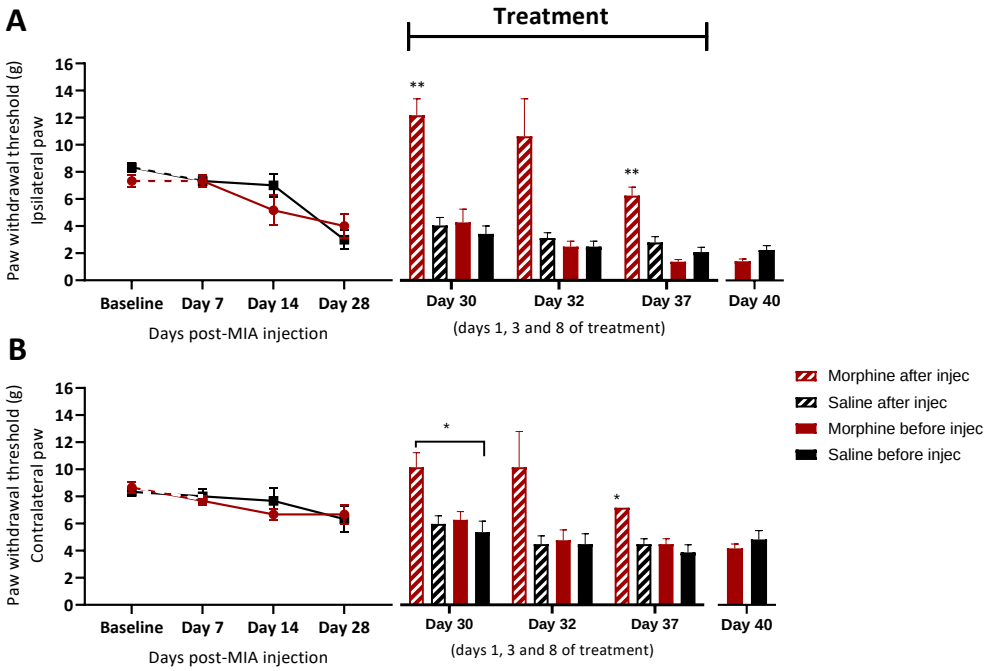

FIGURE 6.7. – PAW WITHDRAWAL THRESHOLD OF IPSILATERAL (A) AND CONTRALATERAL (B) PAWS AND THE EFFECTS OF MORPHINE TREATMENT. (A) PWTs were assessed at baseline and after MIA model induction in LH rats (3mg, n=12) at day 7, 14 and 28. (B) Rats were treated subcutaneously with morphine (3mg/kg, twice a day, ●; n=6) or saline (▪; n=6) for 10 days from day 30 after MIA injection. PWTs were measured 1h prior and 1 h after the morning injection. Data are presented as mean±SEM. * p<0.05, ** p<0.001, 2-way ANOVA with Bonferroni multiple comparisons *post-hoc* testing.

6.3.2.2. SENSORIMOTOR FUNCTIONS

As observed in the naïve LH rats in the pilot study, acute morphine injection numerically increased the horizontal locomotor activity of the MIA LH rats on day 34 (day 5 of treatment) (Fig.6.8A), although this was not significant (t=1.63, df=10, p=0.13). Interestingly, and contrasting with the findings in the naïve rats in the pilot study, morphine acutely reduced vertical locomotor activity (t=3.90, df=10, p=0.003) (Fig.6.8B).

Acute morphine injection did not affect the startle response or prepulse inhibition (Fig.6.9). Following morphine injection on day 35 (day 5 of morphine treatment), no differences were observed between morphine-treated MIA rats and saline-treated MIA rats in both the startle response (group: $F_{(1,10)}<1$) (Fig.6.9A) and prepulse inhibition (group: $F_{(1,10)}<1$) (Fig.6.9B). Animals from both groups showed habituation to startle amplitude (time: $F_{(2,20)}=41.28$, $p<0.0001$), and no pulse-alone trials x group interaction ($F_{(2,20)}<1$) were observed. Also, animals from both treatment groups showed similar increase of % prepulse inhibition with the increase of pulse intensity (time: $F_{(3,30)}=74.53$, $p<0.0001$), and there was

no pulse x group interaction ($F_{(3,30)}$<1). Before the start of morphine treatment, during testing between days 8 and 34, the prospective treatment groups did not differ in their startle response or prepulse inhibition (data not shown).

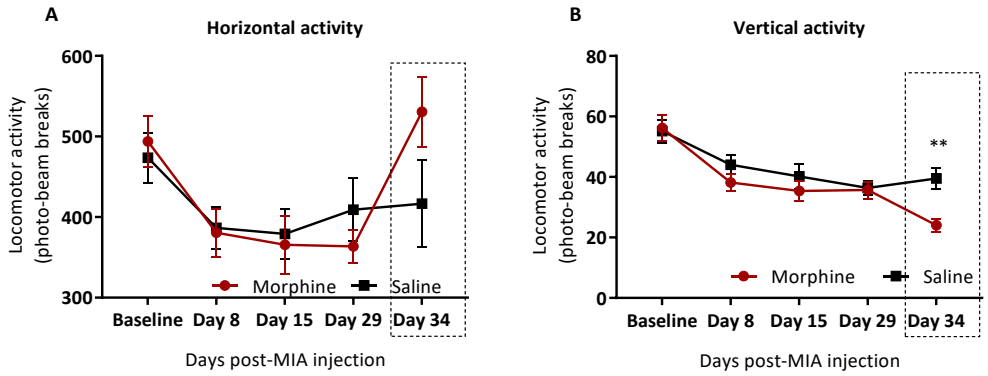

FIGURE 6.8. – HORIZONTAL (A) AND VERTICAL (B) LOCOMOTOR ACTIVITY OF MIA-INDUCED LH RATS ON THE FIRST 10-MIN BINS. LH rats were treated subcutaneously with either morphine (3mg/kg, twice a day, ●; n=6) or saline (▪; n=6) for 10 days from day 30 post-MIA. Data are presented as mean±SEM. Data are presented as mean±SEM. ** p<0.001, 2-way ANOVA with Bonferroni multiple comparisons *post-hoc* testing.

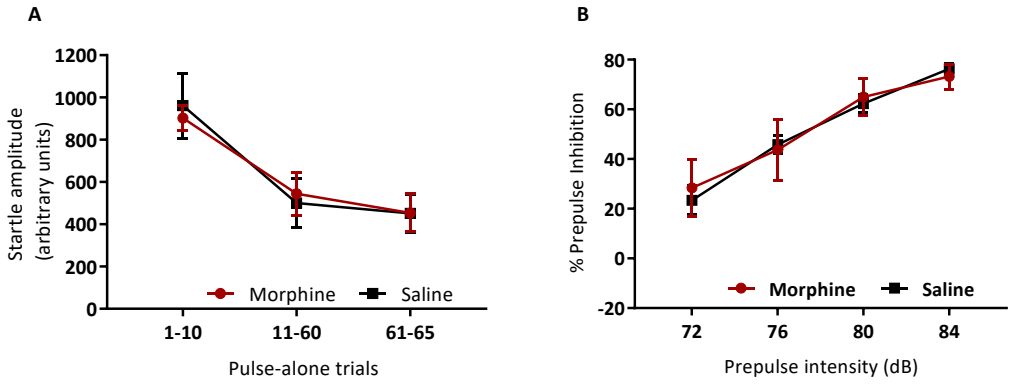

FIGURE 6.9. – STARTLE RESPONSE (A) AND PREPULSE INHIBITION (B) OF MIA-INDUCED LH RATS ON DAY 34 AFTER MIA INJECTION – DAY 5 OF THE MORPHINE/SALINE SUSTAINED TREATMENT. LH rats were treated subcutaneously with either morphine (3mg/kg, twice a day, ●; n=6) or saline (▪; n=6) for 10 days from day 30 after MIA injection. Data are presented as mean±SEM.

6.3.2.3. HIPPOCAMPUS-DEPENDENT MEMORY

6.3.2.3.1. PATH LENGTHS

At pretraining, all rats were able to learn the watermaze DMP task, no differences were observed between the prospective groups in the path length (effect of interaction involving prospective treatment group: all F<1) (data not showed). MIA model induction, as previously observed in chapter 4, did not affect performance of rats, path lengths did not differ from baseline to re-baseline on days 26 and 27 (before the sustained treatment) (day x group x trial: $F_{(21,168)}$=1.39, p=0.13) (data not showed).

Acute morphine treatment of MIA rats did not affect hippocampus-dependent memory compared with saline controls. Morphine did not affect path length of MIA LH rats across treatment days (days 35, 36, 37 and 38) and morphine withdrawal (days 41 to 44) (Fig.6.10). 3-way ANOVA showed no main effect of group or interactions involving group (F<1.32, p>0.13).

6.3.2.3.2. SEARCH PREFERENCE DURING PROBE TRIALS

During the probe trials, no evidence of impaired hippocampus-dependent memory was observed following acute morphine treatment or during withdrawal (Fig.6.11). At all time points, following acute morphine injections (day 36 and day 39) or during withdrawal from that sustained treatment (day 42 and 44), morphine treated MIA rats spent a similar percentage of time exploring the target zone (Fig.6.11A; $F_{(1,10)}$=1.33; p=0.25) and the previous day's zone (Fig.6.11B; $F_{(1,10)}$<1), compared with control rats. Also, no differences were observed between groups in the total time spent exploring the eight zones (Fig.6.11C; $F_{(1,10)}$=0.003; p=0.96). Swim speed was not affected by morphine treatment compared with saline controls (Fig.6.11D; $F_{(1,10)}$=0.31; p=0.59). Apart from swim speed, a main effect of time was observed on all parameters analysed ($F_{(3,39)}$>3.98; p<0.001) and no group x time interaction was found ($F_{(3,29)}$<0.84; p>0.27).

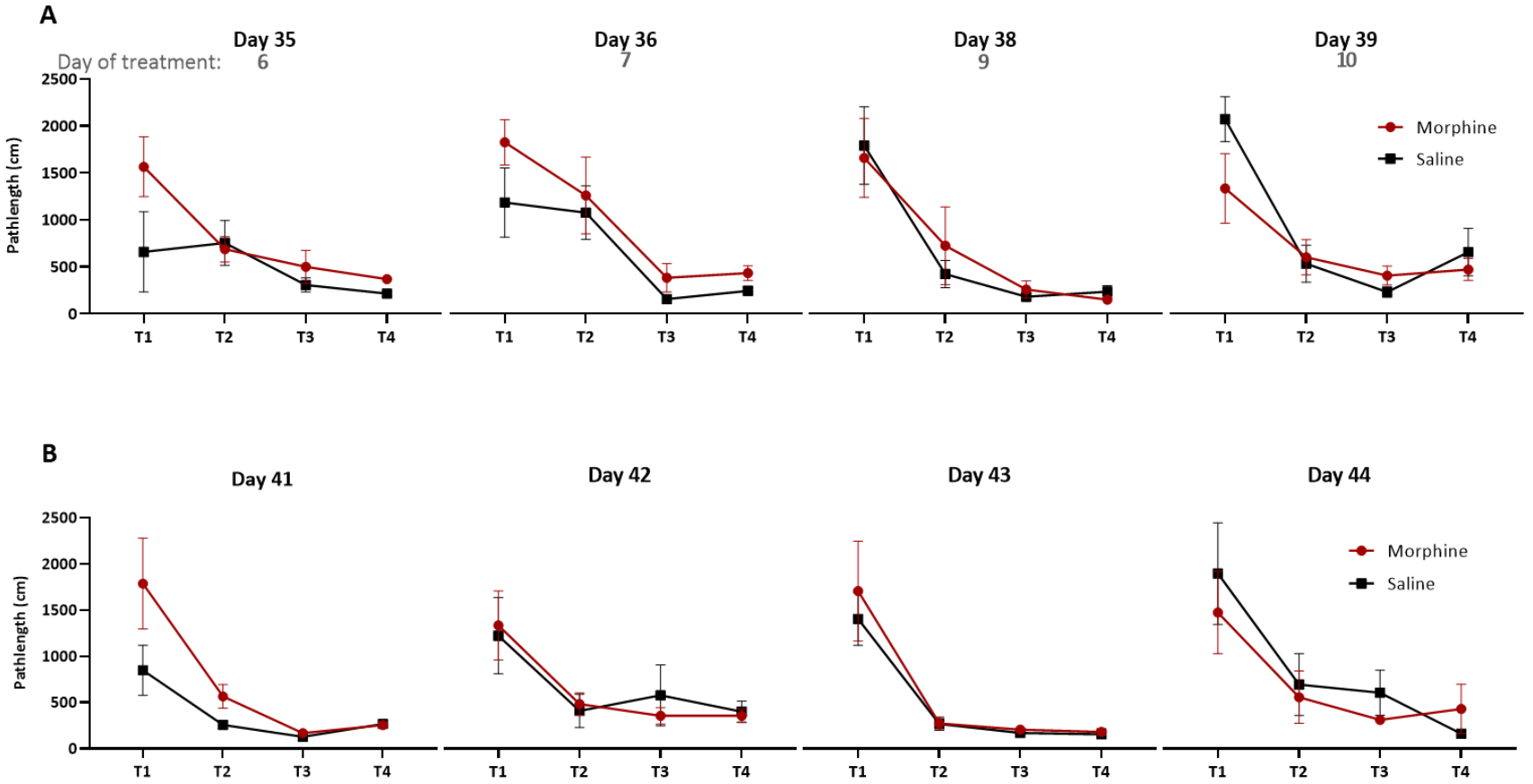

FIGURE 6.10. – PATH LENGTH OF MIA-INDUCED LH RATS 1 HOUR AFTER INJECTION WITH EITHER MORPHINE OR SALINE (A) AND DURING DRUG WITHDRAWAL (B). LH rats were treated subcutaneously with either morphine (3mg/kg, twice a day, ●; n=6) or saline (▪; n=6) for 10 days from day 30 after MIA injection. Data are presented as mean±SEM.

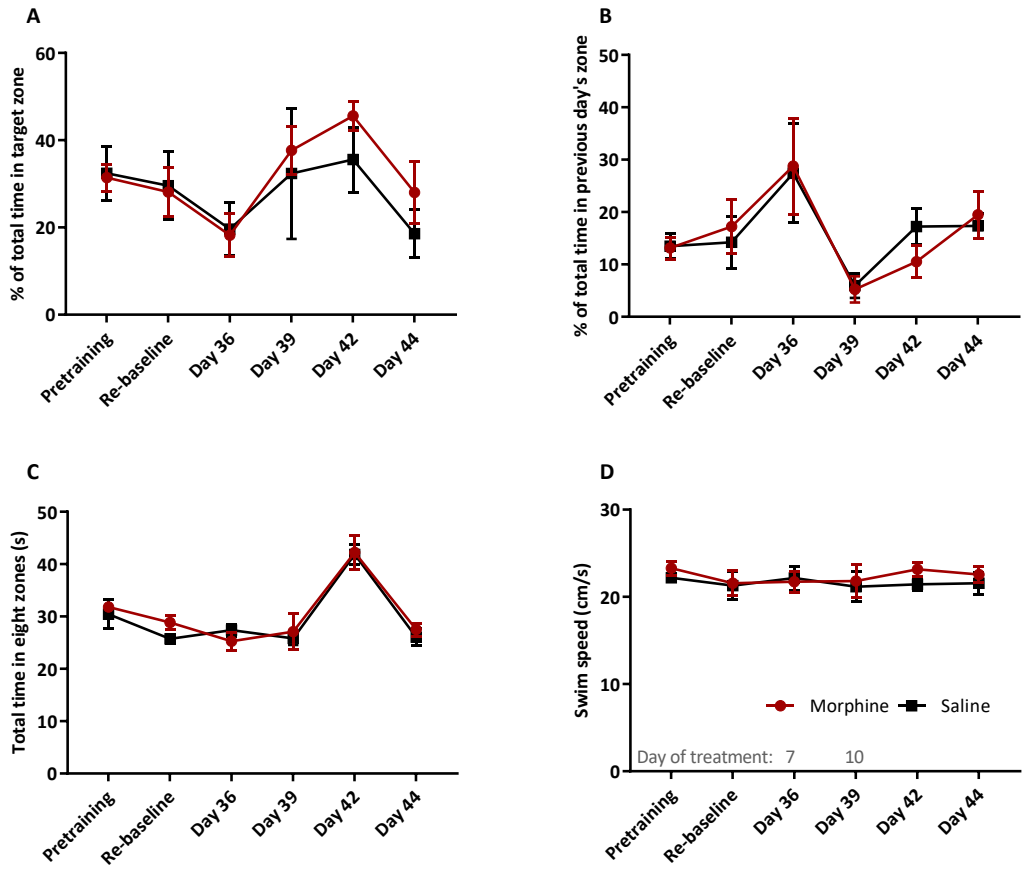

FIGURE 6.11. – PLACE MEMORY IN MIA-INJECTED LH RATS SUSTAINED TREATMENT WITH MORPHINE. LH rats were treated subcutaneously with either morphine (3mg/kg, twice a day, ●; n=6) or saline (▪; n=6) for 10 days from day 30 after MIA injection. Rats were tested on probe trials during the second trial when the escape platform was unavailable in order to evaluate the search preference at baseline, re-baseline and following morphine injection during the drug treatment period (day 36 and 39) and during drug withdrawal after the end of the sustained morphine treatment (day 42 and 44). Total time exploring the target zone (A), the previous day's zone (B), the total eight zone (C) and swim speed in the last time points (D) were analysed. Data are presented as mean±SEM.

6.3.2.4. RECOGNITION MEMORY

There was some evidence for an impaired recognition memory after MIA model induction and treatment, but this deficit was not associated with the sustained morphine treatment (Fig.6.12). Rats in both prospective treatment groups were able to recognise the novel object at baseline ($F_{(1,10)}$=20.29; p=0.001), with no differences between prospective groups (object x group: $F_{(1,10)}$=1.16; p=0.31). 49 days after MIA or saline injection into the knee (10 days after last day of morphine treatment), both groups of rats showed numerically more exploration of the novel object compared to the familiar object, but this preference was not significant ($F_{(1,10)}$=1.27; p=0.29). There was no main effect of group or group x object

interaction ($F_{(1,10)}$<1), indicating that this impairment was not associated with the morphine treatment. The discrimination ratio was not different between groups at baseline and day 49 (F<1.20; p>0.30).

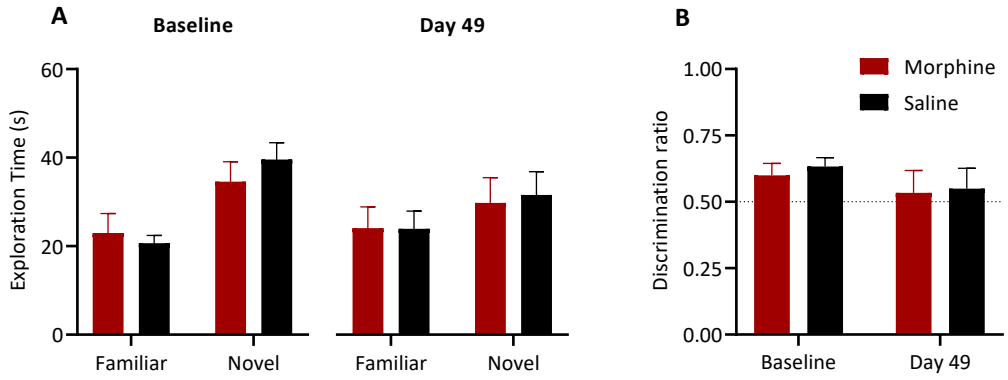

FIGURE 6.12. – EXPLORATION TIMES OF THE FAMILIAR AND NOVEL OBJECTS (A) DURING THE OBJECT RECOGNITION TEST AND DISCRIMINATION INDEX (B) OF MIA-INDUCED LH RATS AFTER A SUSTAINED TREATMENT WITH MORPHINE/SALINE. LH rats were treated subcutaneously with either morphine (3mg/kg, twice a day, red; n=6) or saline (black; n=6) for 10 days from day 30 after MIA injection. Results presented correspond to the testing phase of the novel objected recognition test conducted during the treatment withdrawal. Data are presented as mean±SEM.

6.3.2.5. KNEE PATHOLOGY

Knees were collected at the end of the study and processed for pathology and then scored as described in section 2.7. Joints were scored for cartilage integrity (cartilage damage x involvement), synovial inflammation and osteophytes formation on three different levels of both lateral and medial tibial plateau (Fig.6.12). Results are shown as mean between medial and tibial plateau values, as no differences were observed between medial and lateral tibial parts.

Similar to the results obtained in the previous chapters, MIA model induction was associated with loss of cartilage integrity and synovial inflammation. In this study, MIA-injected rats were either treated for 10 consecutive days with either morphine or saline. MIA morphine treated rats and controls showed similar degrees of cartilage damage (t=<1, df=10) (Fig.6.12A)and synovial inflammation (t=1.80, df=10, p=0.10) (Fig.6.12B), however morphine treated rats showed a trend for higher degree of synovitis compared with MIA saline treated rats. A similar degree of osteophytes were observed between morphine and control groups (U=17.50, p>0.99) (Fig.6.12C).

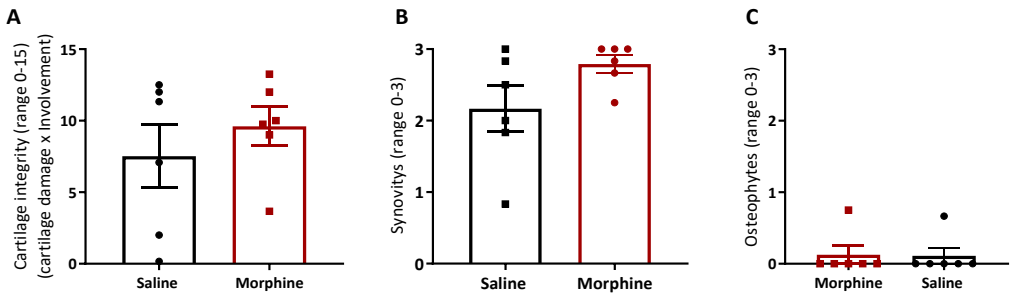

FIGURE 6.12. – MICROSCOPIC QUANTIFICATION OF HISTOLOGICAL CHANGES OF TIBIAL PLATEAU IN MIA-INDUCED LH RATS AFTER A SUSTAINED TREATED WITH MORPHINE/SALINE. Average scores for medial and lateral tibial plateau. Rats were injected with 50ul of 3mg of MIA in the left knee. Rats were treated subcutaneously with either morphine (3mg/kg, twice a day, ●; n=6) or saline (▪; n=6) for 10 days from day 30 after MIA injection. Knees were collected and processed for scoring at day 50 after model induction. Cartilage damage (A), synovial inflammation (B) and osteophytes (C) were scored. Data are presented as mean±SEM.

6.4. DISCUSSION

Morphine treatment induced antinociceptive effects in LH rats, followed by tolerance and development of morphine-induced hyperalgesia. Following induction of the model of OA pain (MIA) in LH rats, morphine had analgesic effects with no evidence of morphine-induced hyperalgesia. In both naïve and MIA LH rats, morphine acutely promoted hyperactivity. There was no evidence that the sustained morphine treatment induced any impairment in hippocampal or recognition memory in MIA-injected rats. However, in this study, MIA-injected LH rats did not show significant object recognition memory 49 days after model induction.

6.4.1. PAIN BEHAVIOUR, SENSORIMOTOR ACTIVITY AND KNEE PATHOLOGY

In this study, effects of sustained morphine treatment on pain behaviour of LH rats were first investigated. As expected, morphine acutely induced antinociception, reflected by the increase in PWTs. This effect decreased within days of treatment, and by the end of treatment, this effect was not evident. These results indicate that the LH rats developed tolerance to the antinociceptive effects of morphine. These results are in accordance with previous findings in Sprague Dawley rats treated subcutaneously with 10mg/kg twice daily for 7 days (Ferrini et al., 2013).

Morphine also induced analgesic effects in MIA LH rats, weight bearing asymmetry was reduced and PWT increased. Regarding the weight bearing, on the second day of treatment no significant analgesic effect was observed, contrasting with day 4 when analgesia was present. However, the analgesic effect observed in the mechanical withdrawal peaked on the first day of treatment, decreasing on days 3 and 8 of treatment.

The development of morphine hyperalgesia was evaluated by assessing pain behaviour 1h before the morning injection of morphine and during treatment withdrawal. No evidence of morphine-induced hyperalgesia was observed in the LH MIA rats. There was no difference in the weight bearing and PWTs data collected before the morning injection of morphine and during withdrawal between the morphine-treated rats and saline controls. An important consideration is that the measurement of PWT may be subject to a ceiling effect which may mask any further change, i.e. PWT values were already very low, so were difficult to reduce further and detect changes.

Locomotor activity was assessed half way through the sustained treatment in both naïve LH and MIA-injected LH rats. In both studies, acute morphine increased horizontal locomotor activity. Previous studies in rats have also reported that morphine at low doses, similar to the one used in the present study (3 mg/kg), can promote increased locomotor activity, although higher doses of morphine (such as 40mg/kg) can cause sedation and substantially reduce locomotor activity (Babbini and Davis, 1972; Zhang and Kong, 2017). Our results are in accordance with the locomotor hyperactivity previously observed using 3mg/kg morphine in mice (Murphy et al., 2008). With regards to rearing, morphine treatment did not affect rearing in the naïve LH rats in the pilot study, however MIA LH rats treated with morphine showed decreased rearing compared with MIA LH rats treated with saline. It should be noted that an analgesic effect of morphine would be expected to cause the opposite effect – i.e., increase rearing, as rats are able to put more weight on their hind legs. It is possible that the reduced rearing reflects that morphine-injected rats spend less time rearing because they showed other hyperactive behaviours caused by the morphine injection, but this does not explain why we found a reduction in morphine-induced rearing in MIA-injected, but not naïve, rats.

Startle response and PPI were not affected by morphine treatment showing no impairments in sensorimotor gating. Acute morphine administration in healthy humans has previously been reported to increase PPI without altering startle habituation (Quednow et al., 2008). In rats, impaired startle was reported during morphine withdrawal (Harris and Gewirtz, 2004; Rothwell et al., 2009); unfortunately, in this study we did not assess startle habituation and PPI during this period.

6.4.2. EFFECTS OF SUSTAINED MORPHINE TREATMENT ON HIPPOCAMPUS-DEPENDENT MEMORY

To evaluate if the sustained treatment of MIA with morphine caused impairments in hippocampal memory, MIA LH rats were tested in the watermaze DMP task during and after morphine treatment. In chapter 4, we showed that OA chronic pain did not affect hippocampus-dependent memory in LH rats. So, in this chapter all animals were injected with MIA, and then on day 30 post-MIA half of them were either treated with morphine or saline as control, to examine if morphine may induce memory impairments in MIA-injected rats.

No changes in DMP performance were observed during and after morphine treatment. On standard days of DMP task, the latency to reach and path length to the platform were not altered by the morphine treatment at any time point. Rats from both groups decreased time across daily trials, showing normal evidence of rapid place learning as expected on this task.

Search preference on probe trials of the DMP task has been suggested to be a more sensitive measure of hippocampus-dependent rapid place learning than latencies or path lengths (Bast et al., 2009; da Silva et al., 2013). The search preference during probe trials was also not affected by morphine treatment. Morphine did not alter the percentage of time exploring the target zone, the previous day's zone and time exploring the total eight zones of the pool. Morphine also did not affect swim speed of MIA rats.

Previous studies of the impact of morphine on place learning and memory in watermaze tasks have produced somewhat inconsistent findings. Hippocampal LTP was shown to be reduced in Sprague Dawley rats chronically treated with morphine (10 mg/kg) twice daily for 10 days (Pu et al., 2002). The same animals were then re-exposed to morphine after the 10 days treatment and tested in the Morris watermaze task, animals who showed previous severe reduction in hippocampal LTP exhibited worse performances compared with controls. Sprague Dawley rats chronically administered morphine for 28 days (17.5 mg/kg/day by osmotic mini pumps) showed impaired performance during the probe trial when tested in the Morris water maze during treatment (Brolin et al., 2018). Wistar rats chronically treated with morphine (10mg/kg, s.c.) twice per day for 12 days have also been tested in the Morris water maze task 1h after morning injection and 5 weeks after withdrawal. Chronic morphine treated rats spent less time exploring the target area compared with control, but no impairments were observed during task acquisition phase (Wang et al., 2006). This previous study also tested the animals in an operant conditioning task and showed impaired operant learning, which was alleviated 5 weeks after treatment withdrawal. Altogether these studies suggest that sustained morphine treatment might impair operant learning, but not hippocampal place learning and memory. These results hint at the possibility that higher doses of opioids and/or a longer time of treatment may be necessary for morphine induced changes in hippocampal memory impairments. Therefore, it is possible that if the morphine treatment had lasted longer in our study LH animals would present impairments in the watermaze DMP task. Additionally, testing for learning impairments might also be important to establish possible effects of prolonged exposure to opioids.

To date few studies have investigated effects of chronic opioid treatments on memory of patients with chronic pain. In both humans and animal models the results are somewhat inconsistent. However, it is really important to notice that in human studies, in some cases it is impossible for ethical reasons to have the appropriate control groups and in animal models the administration routes, strains and drug doses are not always consistent.

Clinical studies have showed that patients with non-malignant pain (Sjøgren et al., 2000) and chronic low back pain (Schiltenwolf et al., 2014) under long-term opioid treatment (at least 3 months) showed worse spatial working memory compared with non-opioid patients. However, in another study, non-cancer pain patients under long-term opioid treatment (12months) did not show cognitive dysfunction (Tassain et al., 2003).

6.4.3. EFFECTS OF SUSTAINED MORPHINE TREATMENT OF MIA LH RATS IN RECOGNITION MEMORY

Regarding recognition memory, no differences were observed regarding recognition memory between morphine- and saline-treated rats with MIA-induced knee pain. However, when tested on day 49 after MIA injection, there was no evidence for significant object recognition memory compared to the saline controls. This may indicate that MIA itself can induce recognition memory deficits. However, this was not apparent in the data presented in chapter 5. However, in chapter 5 NOR memory was assessed on day 32 post-MIA, whereas in the present study NOR memory was assessed on day 49 post-MIA. These results suggest recognition deficits may develop at later stages in the MIA model. Further studies including appropriate control groups should be conducted to investigate this question further. Furthermore it would be interesting to assess cognition at later stages of the MIA model.

NOR measures were not collected immediately after morphine injection, as we focused on the assessment of hippocampus-dependent rapid place learning performance; we only tested recognition memory during treatment withdrawal. Impaired recognition memory was previously shown in mice during morphine withdrawal, exposure to different morphine doses was associated with impairments in Y-maze recognition task (Ma et al., 2007). Our results did not speak to whether or not morphine withdrawal impairs NOR memory, because our control rats (saline-injected MIA rats) did not show NOR when tested during morphine withdrawal. Although it may be interesting to explore these questions further, it is worth noting that chronic low back pain patients with ongoing long-term opioid treatment did not show

impairments in pattern recognition memory, compared with non-opioid patients (Schiltenwolf et al., 2014).

6.5. CONCLUSIONS

Some studies have associated chronic pain with memory impairments or changes in the hippocampal morphology (Moriarty et al., 2011). However, in chapter 4 we showed that hippocampus-dependent memory was not affected in MIA LH rats and in chapter 5 that recognition memory was not affected as well. Other factors associated with chronic pain in humans may account for why human chronic pain conditions show memory impairments, such as the effects of treatment. In this chapter, we evaluated the possible impacts of morphine treatment in both hippocampal and recognition memory.

Hippocampal plasticity is believed to be altered after exposure to opioids (Valentino and Volkow, 2018). In fact, hippocampal LTP was showed to be severely reduced after chronic morphine treatment in Sprague Dawley rats (Pu et al., 2002). However, the work in this chapter presents no evidence that the sustained treatment of morphine in MIA LH rats impaired the hippocampus-dependent memory. In sum, our results indicate that a sustained morphine treatment of MIA LH rats was able to produce analgesia; however, this effect was rapidly lost due to the development of morphine tolerance. Furthermore, the sustained morphine treatment did not induce OIH or any impairment in hippocampal rapid place learning performance on the DMP task for the time period tested in this study. Interestingly, results in this chapter do suggest that memory impairments in the MIA rat model may emerge at later stages than tested herein and therefore future research should be conducted addressing this hypothesis.

CHAPTER 7

GENERAL DISCUSSION

This thesis investigated if and how chronic OA-like knee pain in the MIA rat model affects cognitive function, more specifically hippocampal one-trial place memory, recognition memory and behavioural flexibility. Additionally, we examined the impact of sustained opioid treatment on pain behaviour and memory function in the MIA rat model.

7.1. KNEE PATHOLOGY, WEIGHT BEARING ASYMMETRY AND REDUCED REARING IN MIA-INJECTED LISTER HOODED RATS

First, we transferred the MIA model to young adult Lister Hooded (LH) rats. The MIA model is a well-established model of OA pain in our and other labs. As discussed in chapter 3, The MIA model has been widely used in albino rat strains, including Sprague-Dawley (SD) rats, and in mice to mimic the development of OA and associated pain responses. Unfortunately, albino rats show comparatively poorer performances in translational tests of clinically relevant cognitive function compared with pigmented strains, such as LH rats. MIA-induced pain phenotype and join pathology, are dose and time dependent.

As described in table I in chapter 3, in young albino rats, injection of 1 mg/50 µl MIA into the knee induces pain behaviours, such as weight bearing asymmetry and mechanical allodynia, and OA-like knee damage, reflected by chondrocyte death, loss of cartilage integrity, osteophytes and synovitis. Therefore, no problems were anticipated with transferring the MIA model to LH rats. However, there were limited pain phenotype with the standard 1mg dose of MIA in LH rats or age matched SD rats. A point that should be considered is the environmental differences between the studies conducted in this thesis and the previous studies conducted in our lab using the MIA model. In the previous studies conventional cages have been used, however due to the bigger size and increased inquisitive behaviour of the LH rats, double decker ICV cages were used in this thesis. Some studies have been suggested that environmental enrichment (EE) can improve pain behaviour which may explain the attenuated pain phenotype observed compared with previous studies conducted

using conventional cages. In fact, mechanical allodynia after spared nerve injury in SD rats was shown to be alleviated in animals placed in EE cages after surgery compared with animals in standard cages (Parent-Vachon and Vachon, 2018). Similarly, EE has been shown to have a positive impact in SD rats after carrageenan-induced inflammatory model, mechanical allodynia was attenuated in animals housed in EE prior and after model induction and in animals housed in EE cages only prior to model induction (Gabriel et al., 2010). However, limited knee pathology was also observed by the dose of 1 mg. The limited pain and joint pathology phenotypes likely reflect the difference in age and size of LH rats required for the cognitive tests.

Increasing the dose to 3mg MIA in the young adult LH rats overcame this limitation and it was therefore deemed that this dose of MIA was suitable for the studies involving LH rats in this thesis. This dose produced consistent pain behaviour and knee pathology in young adult LH rats across all studies in this thesis, including robust weight bearing asymmetry and knee pathology with a loss of cartilage integrity, synovitis and occasional osteophyte formation.

Following induction of the MIA model, rats also exhibited decreased rearing activity across all studies. Reduced locomotor activity has previously been suggested as an automated test to measure non-stimulus evoked pain (Deuis et al., 2017). An advantage of this technique is that animals are unrestrained and measures are collected in a completely objective way. The reduction of rearing may indicate movement-provoked pain behaviour, in fact reduced frequency of rearing in MIA-injected Sprague Dawley rats has been reported previously (Nagase et al., 2012). No consistent MIA-induced changes were observed in horizontal locomotor activity or in the other sensorimotor processes examined in this thesis, the acoustic startle response and its PPI.

7.2. NO EVIDENCE FOR COGNITIVE IMPAIRMENTS IN MIA-INJECTED LISTER HOODED RATS

To investigate the impact of chronic OA knee pain on hippocampus-dependent rapid place learning performance, MIA-injected LH rats were tested on the watermaze DMP task (see chapter 4). Previously, changes in the hippocampus have been associated with chronic pain in both rodents (Duric and McCarson, 2005) and humans (Zimmerman et al., 2009), and clinical observations also indicate that chronic pain is associated with memory impairments (Berryman et al., 2013). However, the impact of chronic OA-related pain is poorly

understood. In this thesis, no evidence of impaired hippocampal memory was found after MIA model induction. Rats' performance on the DMP task, as reflected by path length reductions and search preference for the correct location on probe trials, was not affected by MIA model induction. Interestingly, there was evidence for reduced swim speed in MIA-injected compared to control rats at later test stages. Our findings of intact hippocampus-dependent memory in MIA-inject rats are consistent with the finding that hippocampal volume was found to be intact in patients with OA, whereas patients with other chronic pain conditions, including chronic back pain and complex regional pain syndrome, showed reduced hippocampal volume (Mutso et al., 2012).

MIA-injected LH rats were also tested on the novel object recognition test (NOR) to evaluate the impact of OA-chronic knee pain on recognition memory (chapter 5). MIA injected rats did not show impairments in NOR memory compared with saline controls. Our findings contrast with previous preclinical studies using different models, impaired recognition memory was reported in MIA-induced mice (Negrete et al., 2017), and associating chronic pain with recognition memory deficits (Moriarty et al., 2016a; You et al., 2018). However, our findings of intact recognition memory in MIA-injected rats with OA-like knee pain are in accordance with some clinical studies conducted in chronic back pain and fibromyalgia patients, indicating a limited impact of chronic pain on patients' recognition memory in some conditions (Lee et al., 2010; Schiltenwolf et al., 2017).

We also investigated the impact of OA-like knee pain on behavioural flexibility, using an automated operant assay of set-shifting and reversal learning in MIA-injected LH rats (chapter 5). Behavioural flexibility was not affected in young adult LH rats after MIA injection. There is limited evidence concerning behavioural flexibility in chronic pain patients. However, a previous study, based on findings on the spatial reversal task in the watermaze, suggested impaired behavioural flexibility in neuropathic rodent models (Leite-Almeida et al., 2009). On an operant task, neuropathic rodents also adapted more slowly to new and optimal choices (Cowen et al., 2018). Moreover, on a task involving behavioural flexibility (the Wisconsin Card Sorting Test - WCST), chronic pain patients tend to stay longer on the previous rule than healthy controls (Verdejo-Garcia et al., 2009; Indart et al., 2017). A clinical study in older adults suggested that pain severity measured by questionnaire may be associated with impaired mental flexibility measured with the Trail Making Test, interestingly cognitive impairments seemed to correlate with pain severity (Karp et al., 2006). In fact, age-related impairments in behavioural flexibility have been reported in both rodent (Leite-Almeida et al., 2009) and human studies (Weiner et al., 2006). Ageing may affect pain and its impact on

cognition. Therefore, the very restricted age range in most rodent studies of chronic pain, including in the present study, may limit the translational validity of these studies. Increasing the age range may increase the clinical relevance of the findings. It is also important to consider that ageing is a key factor contributing to cognitive decline (Deary et al., 2009) and is also associated with osteoarthritis (Vos et al., 2016). Therefore, aging may interact with chronic pain to impair cognitive functions in patients with OA.

7.3. IMPACT OF MORPHINE TREATMENT IN IN MIA-INJECTED LISTER HOODED RATS

Other factors associated with chronic pain in humans may also account for why chronic pain patients show cognitive impairments, such as medication. Drugs used to treat chronic pain, such as opioids, can also be associated with cognitive decline. In this thesis, we investigated the effects of sustained morphine treatment and withdrawal from such treatment on hippocampal rapid place learning performance and recognition memory in MIA model of OA-like knee pain in LH rats. No changes in the watermaze DMP task were observed during and after morphine treatment. In addition, no differences were observed in recognition memory between morphine- and saline-treated rats with MIA-induced knee pain. Interestingly, when tested on day 49 after MIA injection, there was no evidence for significant object recognition memory in the MIA-injected rats. As discussed in Chapter 6, these results may indicate that MIA itself can induce recognition memory deficits at later stages in the MIA model. However, further studies including appropriate control groups should be conducted to investigate this question.

7.4. LIMITATION OF THE MIA MODEL IN LISTER HOODED RATS

Although OA rodent models, including the MIA model, show knee pathology and pain behaviour comparable to human OA, it is important bear in mind that rodent models develop rapidly compared to human OA, and, therefore, the pain mechanisms might differ from human OA development (see chapter 3). Nevertheless, preclinical studies in rodent models play a crucial role in improving our understanding in several aspects of a disease. In this specific thesis, the use of a rat model allowed us to evaluate the impact of chronic knee OA pain on objective measures of cognitive function, independently of confounding factors, such as age, comorbidities and pain medication.

Importantly, pain behaviour tests can be subjective. To minimise possible bias, data collection and analysis were conducted blind to the treatment. The weight bearing test is well-suited to measure spontaneous nociception in unilateral models as the MIA model. However, the test requires that the animals freely adopt and then maintain for a few seconds the appropriate position for the measures to be taken, which can be challenging in inquisitive strains as LH rats. In fact, in chapter 4, one rat could not be tested in the weight bearing test because he did not settle into the right position. Measures in both weight bearing and von-Frey test can be very variable between rats. Also, it is important to note that the von-Frey test requires some repeated stimulation, which can lead to sensitization (Deuis et al., 2017).

An important point that should be considered is that there was very limited evidence of central sensitization in our MIA-injected LH rats. More specifically, there was limited evidence for mechanical allodynia, as indicated by reduced PWTs, which is considered an indication of central sensitisation (Merksey and Bogduk, 1994). Previous studies mentioned across this thesis that showed cognitive impairments in rodent models of chronic pain usually reported central sensitisation by showing reduced paw withdrawal threshold, and this is likely an important factor to impact the forebrain. Previous studies in the MIA model in SD rats also showed central sensitisation, as reflected by reduced paw withdrawal thresholds (see chapter 3). In contrast, MIA-injected LH rats used across this thesis did not show mechanical allodynia changes, which may indicate that their central nervous system may not be impacted, and this may explain that we did not find impairments in cognitive function. However, it is also important to bear in mind that Von-Frey test may not be sensitive to the model of OA pain in LH rats. Complementary analysis of microglia and astrocyte activation in the spinal cord of these animals might be conducted to analyse these markers of central sensitization (Sagar et al., 2014).

Although the LH rats are a challenging strain to work with in pain research, these inquisitive rats have been widely used to investigate cognitive functions, whereas albino rat strains are of limited use for cognitive tests (see chapter 3 and 4). In fact, in a recent study using nerve-injured LH rats authors reported the exclusion of one cohort of animals due to failure to collect von-Frey data (Phelps et al., 2021). In this study, animals were tested in a battery of cognitive tasks (bowl digging: reward learning and affective bias assays; sucrose preference and operant chamber: successive negative contrast task) and in the Von-Frey test (up-down method) after partial saphenous nerve injury surgery, but one cohort of animals failed to show reduced activity in the von-Frey cages to allow measurements to be collected.

7.5. SOME FUTURE DIRECTIONS

Some others points may also be interesting to further investigate. First, some previous results regarding chronic opioid use hint at the possibility that higher doses of opioids and/or a longer time of treatment may be necessary for morphine-induced impairments in hippocampal memory function (see chapter 6). Therefore, it is possible that if the morphine treatment had lasted longer in our study LH rats would have presented impairments in the watermaze DMP task.

Also, as highlighted by the absence of significant NOR memory in MIA-injected LH rats when tested on Day 49 after MIA injection (chapter 6), some cognitive deficits may emerge later in the model. This possibility may be further investigated in a study with an appropriate control group and appropriate sample sizes.

7.5. SUMMARY/CONCLUSION

The purpose of this thesis was to investigate, in a rat model, the impact of chronic OA-like knee pain on cognitive function, more specifically hippocampal and recognition memory and behavioural flexibility, and to investigate how sustained opioid treatment may affect memory function. Chronic pain has been associated with changes in forebrain regions, as well as impairments in related cognitive functions, and it has been suggested that this relationship between chronic pain and cognitive dysfunction is the reflection of the competition for the same neural network. However, specific evidence on the cognitive impact of chronic pain in patients with OA has been largely lacking.

In this thesis, we did not find evidence for cognitive impairment in the MIA model in LH rats. Also, we did not find evidence that sustained morphine treatment induces any cognitive impairment in MIA-injected LH rats. However, future studies may examine this further in a wider age range and for longer periods after model induction. On the other hand, it should be noted that present clinical evidence in OA is mainly limited to subjective reports of cognitive impairments (see chapter 1), and it remains to be demonstrated if OA patients with chronic pain show objective impairments on translational assays similar to the ones used in the present study.

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