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CICLO

XXVII

CHARACTERIZATION OF THE ROLE OF AGEING ON MECHANICAL AND THERMAL NOCICEPTION AND NOCIFENSIVE RESPONSE TO FORMALIN TEST IN C57BL/6 MICE

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SOMMARIO

Il dolore cronico, che riduce notevolmente la qualità della vita, interessa un vasto segmento della popolazione globale (il 25% della popolazione Europea e più di 100 milioni di cittadini Americani), diventando anche più frequente negli anziani (> 65 anni). In questa popolazione, il dolore cronico spesso viene incompreso e non opportunamente trattato (Bruckenthal & D'Arcy, 2007), anche a causa della resistenza alla maggior parte degli analgesici di elezione. Pertanto, questo progetto di dottorato è stato condotto con l'obiettivo di caratterizzare un modello di dolore cronico che potesse essere un valido strumento rappresentativo della condizione di dolore cronico nell'anziano ed il ruolo dell'invecchiamento nella nocicezione meccanica e termica e nella risposta nocifensiva in topi C57BL/6. Topi C57BL/6 di due mesi di età sono stati sottoposti al *Test della Formalina* (Dubuisson & Dennis, 1977): oltre a studiare il comportamento nocifensivo di *licking/biting/flinching* indotto dalla formalina, l'allodinia meccanica e l'edema sviluppati dagli animali in seguito alla somministrazione di formalina sono stati rispettivamente investigati tramite il *Test di Von Frey* (Chaplan *et al.*, 1994) e l'utilizzo di un calibro, nel 1°, 4°, 7°, 9°, 11° e 14° giorno successivo alla somministrazione di formalina. Inoltre, è stata studiata l'efficacia del gabapentin, uno dei trattamenti di scelta per gli stati di dolore cronico come la neuropatia diabetica e la nevralgia postherpetica (Backonja *et al.*, 1998; Rowbotham *et al.*, 1998), sia nella risposta nocifensiva al *test della formalina* sia nella conseguente allodinia meccanica, fino a 4 giorni dopo l'iniezione di formalina. I risultati ottenuti hanno dimostrato la presenza di allodinia meccanica nella zampa ipsilaterale già due ore dopo la somministrazione di formalina, con il picco nel 4° giorno. Questa allodinia meccanica a lungo termine (fino a 14 giorni), nel 4° giorno successivo

all'iniezione di formalina, interessa anche la zampa controlaterale, confermando l'esistenza di meccanismi centrali ritenuti alla base della seconda fase del *test della formalina* ed escludendo la possibilità che tale allodinia possa essere causata dall'edema evidenziato. Il gabapentin è risultato molto efficace nella seconda fase, lievemente efficace nella prima fase e nell'allodinia meccanica indotta dalla formalina 2 ore dopo la somministrazione di formalina. Tale attività non è stata evidenziata nel primo e nel quarto giorno successivo all'iniezione di formalina, verosimilmente a causa dell'emivita del farmaco. I nostri risultati caratterizzano il *test della formalin* come un modello rappresentativo di uno stato di dolore cronico che racchiude sia le caratteristiche infiammatorie del dolore articolare, spesso resistente al trattamento (Scaglione *et al*., 2014), sia gli aspetti centrali del dolore neuropatico, perciò molto utile per studiare le condizioni di dolore cronico che affliggono un crescente numero di pazienti anziani. Inoltre, il quadro fisiopatologico del dolore nell'anziano è stato approfondito per comprendere come l'invecchiamento potesse influenzare prima la nocicezione e, successivamente, lo sviluppo ed il mantenimento del dolore, dal momento che i cambiamenti nella nocicezione indotti dall'età non sono ancora ben noti (Taguchi *et al*., 2010). Pertanto, topi C57BL/6 giovani (2 mesi) e più anziani (6, 12 e 18 mesi) sono stati sottoposti a test comportamentali per determinare la sensibilità basale meccanica e termica (*test di Von Frey* (Chaplan *et al*., 1994), *Pin-prick* test (Chan *et al.,* 1992), *test di Hargreaves* (Hargreaves *et al*., 1988), test dell'acetone (Choi *et al*., 1994)). Come dimostrato anche da studi longitudinali, soprattutto la sensibilità meccanica ed anche quella termica (sia al caldo che al freddo) sono risultate incrementate. I primi cambiamenti evidenti, in particolare per la sensibilità al freddo, sono stati osservati intorno ai 6 mesi di età. Fra l'età di 12 e di 18 mesi, i livelli di sensibilità hanno mostrato una sorta di *plateau*, con un incremento molto più lento. Poiché la nocicezione è risultata essere modificata negli animali

anziani, si è deciso di esaminare l'effetto dell'età sulla risposta nocifensiva al dolore evocato (*test della formalina*). La risposta nocicettiva, espressa in secondi di *licking/biting/flinching* e come totale di eventi di *licking/biting/flinching*, è risultata differente nei topi anziani (6, 12, 18 mesi), presentando non più solo 2 ma 3 fasi di picco, uno spostamento temporale ed un'ampiezza variata. Inoltre, lo stato allodinico formalina-indotto è risultato più pronunciato nei topi anziani, nei quali l'allodinia meccanica ha continuato ancora a rimanere stabile al picco, mentre nei topi di due mesi il recupero stava già cominciando. Anche il processo di guarigione e l'edema della zampa sono risultati influenzati dall'età dell'animale. A livello molecolare, non sono state evidenziate differenze significative nei livelli della proteina p62, di LC3-I e di LC3-II, mentre solo i livelli di espressione di Beclin 1 sono risultati progressivamente ridotti con l'invecchiamento, in maniera statisticamente significativa. Sebbene siano necessari ulteriori esperimenti, questi risultati sembrano suggerire che la *pathway* dell'autofagia sia modificata e che il flusso autofagico sia probabilmente ridotto. Di fondamentale importanza è l'osservazione che il principale *marker* di dolore cronico, la subunità del canale del Ca^{2+} α2δ-1, è risultata quasi assente nei topi di 2 mesi e notevolmente *up-regolata*, seguendo un andamento a campana, nei topi di 6 e di 12 mesi. Questo fenomeno potrebbe essere alla base della ridotta soglia meccanica dei topi più anziani. Inoltre, una nuova banda a basso peso molecolare, che abbiamo chiamato α2δ-1*, è risultata altamente presente nei topi più anziani rispetto ai topi di due mesi, come la banda nota α2δ-1. I nostri risultati hanno dimostrato che anche l'efficacia del gabapentin (10 e 100 mg/Kg) nel *test della formalina* e nell'allodinia meccanica indotta dalla formalina, testato in topi C57BL/6 di 2 e 6 mesi, è risultata influenzata dall'invecchiamento. In particolare, nei topi giovani, solo la dose di 100 mg/Kg è risultata efficace in tutte le fasi del *test della formalina*, mentre, sia la dose più alta che la dose più bassa sono state efficaci nei topi di 6 mesi. A

differenza dei topi di due mesi, i topi di 6 mesi non hanno mostrato cambiamenti significativi fra la soglia meccanica dei topi trattati con veicolo e quella dei topi trattati con il farmaco due ore dopo la somministrazione di formalina. Tuttavia, la somministrazione acuta di gabapentin è risultata più efficace sull'allodinia meccanica indotta dalla formalina nei topi di 6 che nei topi di 2 mesi, suggerendo che nei topi anziani, anche una dose più bassa di gabapentin risulta terapeutica ma l'effetto terapeutico ha più breve durata. Questo fenomeno potrebbe essere dovuto all'*up-regulation* della subunità α2δ-1 evidenziata nei topi anziani. In conclusione, i risultati ottenuti durante questo progetto di ricerca di dottorato caratterizzano il *test della Formalina* (Dubuisson & Dennis, 1977) come un valido modello di dolore cronico e dimostrano che il processo di invecchiamento influenza la soglia nocicettiva agli stimoli sia di natura meccanica che termica ed il comportamento nocifensivo in tale modello sperimentale di dolore cronico. I nostri risultati sono dotati di un notevole valore traslazionale per rispondere al bisogno, non ancora soddisfatto, di un migliore sfruttamento degli strumenti farmacologici già disponibili e di nuovi trattamenti terapeutici per la gestione del dolore cronico nei pazienti anziani.

ABSTRACT

Chronic pain, which remarkably reduces the Quality of Life (QoL), affects a big segment of the population all over the world (25% of the European popolation and more than 100 millions of American citizens), becoming even more frequent in the older adults (> 65 years). In this population, chronic pain is often misunderstood and not appropriately treated (Bruckenthal & D'Arcy, 2007), even owing to the resistance to most of the current painkillers of choice. Therefore, this PhD project was carried out with the purpose of characterizing a chronic pain model that could be a valid tool representative of the chronic pain condition in the elderly and the role of ageing on mechanical and thermal nociception and nocifensive response in C57BL/6 mice. Two month-old C57BL/6 mice were subjected to the *Formalin test* (Dubuisson & Dennis, 1977): apart from studying the formalin-induced *licking/biting/flinching* nocifensive behaviour, the mechanical allodynia and the oedema developed by the mice after formalin administration were investigated respectively through the *Von Frey's test* (Chaplan *et al.*, 1994) and a caliper on the 1st, 4th, 7th, 9th, 11th and 14th day after formalin administration. Moreover, the effectiveness of gabapentin, one of the treatments of choice for chronic pain states such as painful diabetic neuropathy and postherpetic neuralgia (Backonja *et al.*, 1998; Rowbotham *et al.*, 1998), was studied both on the the nocifensive response to the *formalin test* and on the following mechanical allodynia, up to 4 days after formalin injection. The obtained results demonstrated the presence of mechanical allodynia already two hours after formalin administration in the ipsilateral hindpaw with the peak on the 4th day. This long-lasting (up to 14 days) mechanical allodynia affected even the contralateral hindpaw on the 4th day following formalin injection, thus confirming the existance of the central

mechanisms believed to be at the root of the *formalin test* second phase and excluding the possibility that this allodynia could be caused by the detected oedema. Gabapentin resulted very effective on the second phase, slightly effective on the first phase and on formalin-induced mechanical allodynia 2 hours after the formalin administration. Its activity was not shown on the first and on the fourth day after formalin injection because of the drug half life. Our results characterize the *formalin test* as a model representative of a chronic pain state including in itself both the inflammatory features of joint pain, often resistant to treatment (Scaglione *et al.*, 2014), and the central aspects of neuropathic pain, and so very useful to study the chronic pain conditions affecting an increasing number of old patients. Furthermore, the physiopathological picture of pain in the elderly was deepened in order to understand how ageing could influence first the nociception and, later, the development and the maintainance of pain, since the nociception age-induced changes are not well known yet (Taguchi *et al.*, 2010). Therefore, young (2 month-old) and older (6, 12 and 18 month-old) C57BL/6 mice underwent behavioural tests to assess basal mechanical and thermal sensitivity (*Von Frey's test* (Chaplan *et al.*, 1994), *Pin-prick* test (Chan *et al.*, 1992), *Hargreaves'test* (Hargreaves *et al.*, 1988), acetone test (Choi *et al.*, 1994)). As demontrated also by longitudinal studies, mainly the mechanical and even the thermal (to heat and cold) sensitivity resulted increased. The first evident changes, in particular for the cold sensitivity, were observed around 6 months of age. Between the age of 12 and of 18 months, the sensitivity levels showed a sort of *plateau*, with a much slower increase. Since the nociception resulted to be modified in the older animals, it was decided to examine the effect of age on the nocifensive response to evoked pain (*formalin test*). The nociceptive response, expressed in seconds of *licking/biting/flinching* and as total of events of *licking/biting/flinching*, resulted different in the aged mice (6, 12, 18 months), presenting no more only 2 but 3 peak phases, a shift in

time and a varied amplitude. Moreover, the formalin-induced allodynic state resulted more pronounced in the older mice, in which the mechanical allodynia still kept to remain stable at the peak, while in the mice of two months the recovery was already beginning. Even the healing process and the oedema of the hindpaw resulted influenced by the age of the animal. At the molecular level, no significant differences were observed in the levels of the protein p62, LC3-I, LC3-II while, only Beclin 1 expression levels resulted progressively reduced with ageing, in a statistically significant way. Even though further experiments are needed, these results seem to suggest that the autophagic pathway is modified and that autophagic flux is possibly reduced. Of pivotal importance is the observation that the main chronic pain marker, the Ca^{2+} channel subunit α2δ-1, resulted almost absent in 2 month-old mice and markedly upregulated, following a bell-shaped trend, in 6 and 12 months mice. This phenomenon could be at the root of the lowered mechanical threshold of the older mice. Furthermore, a novel low molecular weight band, which we named α2δ-1*, resulted highly present in the older mice, compared to the 2 month-old mice, as the classic α2δ-1. Our results demonstrated that also the effectiveness of gabapentin (10 and 100 mg/Kg) on the *formalin test* and on the formalin-induced mechanical allodynia, tested in 2 and 6 month-old C57BL/6 mice, resulted affected by ageing. In particular, in the young mice, only the 100 mg/Kg dosage resulted effective in all of the phases of the *formalin test*, while, both the higher and the lower dosage were effective in the 6 months mice. Unlike the two month-old mice, the mice of 6 months did not show significant changes between the mechanical threshold of the vehicle-treated and the one of the drug-treated mice two hours after formalin administration. However, the acute administration of gabapentin resulted more effective on formalin-induced mechanical allodynia in the 6 than in the 2 months mice, thus suggesting that in older mice, even a lower dose of gabapentin results therapeutic but the therapeutic effect is shorter-lasting. This

phenomenon could be due to the upregulation of the α2δ-1 subunit highlighted in the aged mice. In conclusion, the results obtained during this PhD research project characterize the *Formalin test* (Dubuisson & Dennis, 1977) as a valid chronic pain model and demonstrate that the ageing process affects the nociceptive threshold to the stimuli both of mechanical and of thermal nature and the nocifensive behaviour in this experimental model of chronic pain. Our results are endowed with a remarkable translational value to answer the still unmet need of a better exploitation of the pharmacological devices already available and of novel therapeutic treatments for the management of chronic pain in the aged patients.

LIST OF ABBREVIATIONS

- ✓ α2δ-1: voltage-dependent calcium channel of L type subunit
- ✓ A: Adult
- ✓ AD: Alzheimer's Disease
- ✓ BBB: Brain Blood Barrier
- ✓ CNS: Central Nervous System
- ✓ Contra: Contralateral
- ✓ DRG: Dorsal Root Ganglia
- ✓ EGF: Epidermal Growth Factor
- ✓ G: Gabapentin
- ✓ GABA: γ-aminobutyric acid
- ✓ GAPDH: glyceraldehyde-3-phosphate dehydrogenase
- ✓ GBPT: gabapentin
- ✓ IASP: International Association for the Study of Pain
- ✓ i.p.: intraperitoneal
- ✓ Ipsi: Ipsilateral
- ✓ LC3: microtubule-associated protein 1 light chain 3
- ✓ NK1: neurokinin 1 receptor
- ✓ O/N: over night
- ✓ PVDF: Polyvinylidene Difluoride
- ✓ RT: room temperature
- ✓ s.c.: subcutaneous
- ✓ SNL: Spinal Nerve Ligation
- ✓ TRP: Transient Receptor Potential
- ✓ V: vehicle
- ✓ VEH: vehicle
- ✓ vWF-A: von Willebrand's factor

- ✓ Y: Young
- ✓ YA: Young Adult

INTRODUCTION

1. INTRODUCTION

1.1. *Pain and Ageing*

So far, the term **Pain** has been considered expecially as "suffering", likely from the Latin acceptation of *poena*. However, this definition is not complete. Actually, pain, initially considered only a symptom, has emerged as a much more complex pathological entity. Indeed, over the past years, the basic and clinical research have been dealing with this issue in a much more scientific rigorous way. Pain is a physiological mechanism of defense since, from an evolutionary perspective, it is a perceived threat or damage to one's biological integrity and has sensory and emotional features (Chapman & Gavrin, 1999). For instance, we can think of what would happen if we did not feel pain after having put a hand on a hot object. According to the description of the *International Association for the Study of Pain* (IASP), "pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage". The nociceptive response evoked by a tissue lesion or by other inflammatory insults such as mechanical, chemical or thermal noxious stimuli represents the so called **acute pain.** Moreover, it is characterized by a spontaneous rapid recovery without the development of *sequelae* that could compromise the functional activity of the affected individual. Acute pain has a central role in the physiological homeostasis since it protects the tissue by a further damage; the sensory awareness of tissue trauma represents crucial information for adaptation and survival (Chapman & Gavrin, 1999). However, pain can turn into a pathological condition when it does not resolve spontaneously after the end of the nociceptive stimulus and lasts more than 3 months becoming **chronic pain.** The IASP defines chronic pain as "pain which has persisted beyond normal tissue healing time" (IASP., 1986). So, chronic pain is that kind of pain that persists even after the complete healing of the lesion or that

the insult that provoked its onset is over (Merskey & Bogduk, 1994) and it lasts for at least 3 months. It can even be established without a recognized and identifiable cause (Arneric *et al*., 2013). Apart from the distinction between acute and chronic, pain can be classified in several types according to its peculiar characteristics. Since nociception is "the neural process of encoding noxious stimuli", according to IASP, "**nociceptive pain** arises from actual or threatened damage to non-neural tissue and is due to the activation of nociceptors". On the contrary, IASP defines **neuropathic pain**, the "pain caused by a lesion or disease of the somatosensory nervous system". In this regard, it is fundamental to define **allodynia** and **hyperalgesia**. In agreement with the IASP definition, allodynia is the "pain due to a stimulus that does not normally provoke pain" and hyperalgesia is the "increased pain from a stimulus that normally provokes pain." Therefore, in the case of allodynia the painful response is unexpected since it is induced by a stimulus that should normally not provoke such response, while, in the case of hyperalgesia the stimulation is suprathreshold but the painful response is increased. Chronic pain notably reduces the **Quality of Life** (QoL), a parameter on which recent clinical trials focus their attention much more than in the past. Furthermore, chronic pain still represents the main reason for which patients come to clinic observation. Indeed, the pain response of the lymbic system and attitude to put up with pain are influenced by the patients' personal pain perception that can be different according to their personality, education and ethnical factors. Therefore, pain perception is affected by the individual's personality, but, even the personality of the suffering patient is modified by pain itself: chronic pain can compell the individual to several important changes in his life style. Some **patients** experiencing a state of sustained unrelieved pain **suffer because pain changes who they are:** chronic pain promotes a destructive stress response characterized by neuroendocrine dysregulation, fatigue, dysphoria, myalgia, and impaired mental and physical performance that,

together with the induced functional limitations can induce negative thoughts and create a vicious cycle of stress and disability because they become incapable of sustaining productive work, a normal family life and social interactions (Chapman & Gavrin, 1999). Indeed, the concepts of pain and suffering are frequently used as if they were one synonymous with the other, but, suffering and pain are distinct phenomena (Chapman & Gavrin, 1999). Patients affected by chronic pain can no longer perform as usual with their family or in the workplace. As shown in figure 1.1, the disparity between self-expectations and actual performance results in a damage to the self which extends into the projected life trajectory and that represents the essence of suffering (Chapman & Gavrin, 1999).

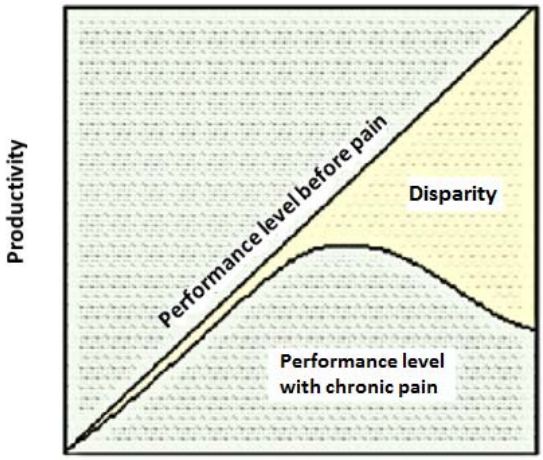

Figure 1.1. Disparity between past and actual performances caused by chronic pain (adapted from Chapman & Gavrin, 1999)

These effects are induced by a complex network of pain-induced adaptive responses. The pain-induced activity occurring in the spinohypothalamic and pontine hypothalamic pathways, *via* excitation of the hypothalamus paraventricular nucleus, gives rise to an adaptive neuroendocrine response in

the hypothalamo-pituitary-adrenocortical axis (Stratakis & Chrousos, 1995), that remains short-term if pain does not persist.

Altough for centuries people have been trying to avoid it through long-life elixirs and looking for the "fountain of youth", ageing is inevitable (Grayson, 2012). Due to the modern medicine progresses of the lat 150 years and to the improved standards of living, life expectancy at birth results increased of three months per year (Scully, 2012). However, this longevity predisposes to chronic conditions. A survey on 10.000 50 year-old people in England reported an increase of health problems with age (Scully, 2012). Indeed, the segment of the worldwide population that results to be the most affected by chronic pain is represented by adults older than 65 years old. Pain in these patients is significantly misunderstood and consequently not adequately treated (Bruckenthal & D'Arcy, 2007). Therefore, according to the American Geriatric Society, pain is not just highly prevalent but also extensively undertreated in the older adult population (AGS Panel on Persistent Pain in Older Persons, 2002) and this is the reason why its management is a very complex matter. This observation can be explained by the presence of comorbidities such as cancer, osteoporosis, arthritis, post-herpetic and diabetic neuropaties, carpal tunnel syndrome etc. However, even though pain in the absence of disease is not a normal part of ageing, unfortunately the majority of older adults usually experience it (Jakobsson, Klevsgard, Westergren, & Rahm Hallberg, 2003). This situation is made even more difficult when patients are affected by mild cognitive impairment. Patients suffering from Alzheimer disease (AD), or from other kinds of dementia, Parkinsonian (PD) patients and so on present a hurdle that should not be underestimated in communicating their pain experience both for what concerns the kind of pain which they are perceiveing and about the relief

obtained after pharmacological treatment (Matthews & Dening, 2002). Apart from the objective difficulty of aged people in conveying the degree of pain, it is necessary to consider the possible difference of pain sensitivity that can be shown in adults of advanced age (Helme & Gibson, 2001). It is very likely that ageing affects pain threshold, although it has not been well understood how this occurs. There are a lot of data supporting the hypothesis that ageing can cause a prevalence of excitatory processes on inhibitory processes leading to an overall insufficient endogenous pain control (Lautenbacher, 2012). It is believed that the key event is the modification of the systems of pain modulation (Arneric *et al*., 2013). For example, AD patients show a reduced inhibitory tone of the transmission pathway of painful stimuli due to a reduced activity of the opioid system (Risser *et al*., 1996; Yakovleva *et al*., 2007; Mathieu-Kia *et al*., 2001). The possibility that ageing induces a lowering of pain threshold with a resulting increase in sensitivity is strengthened by the severe reduction of other sensorial functions such as sight and hearing and of the capability of resolving inflammatory processes, that can be at the basis of post-herpetic neuropathy. Also basic research working in this field demonstrated that aged animals present an increased production of reactive oxygen species (ROS) that could foster an increased infiammatory tone and therefore a decreased pain threshold and an increased nociceptive sensitivity (Raut & Ratka, 2009). Furthermore, it was observed that ageing induces the activation of subsets of microglia in the CNS (Barron, 1995) and that causes the increase of OX-42 immunoreactivity, already in the absence of injury (Stuesse *et al*., 2000). Some studies highighted a hyporesponsivity of older animals to painkillers and, in particular, to opioids. It is still necessary to deepen the study of chronic pain in aged animals in a very rigorous way. A very important matter in the management of pain in the elderly is represented by its assessment. As for any other pathological condition, pain assessment consists in the preliminar construction of the patient's clinical history. Later,

the kind of perceived pain is characterized based on: quality, position, intensity and (if traceable) etiology to distinguish the nociceptive from the non nociceptive one. The choice of proper outcome measures is fundamental for the scientifically valid evaluation of pain and the demonstration of treatments efficacy. The individual report of pain experience is very useful if the interview is carried out with terms that the patient can easily understand. Some other assessment tools are:

- ✓ Numeric Rating Scale
- ✓ Verbal Rating Scale
- ✓ Visual Analog Scale
- ✓ FACES Pain Rating Scale
- ✓ General Health Questionnaire (GHQ-12)
- ✓ Brief Pain Inventory (BPI) questionnaire
- ✓ *Douleur Neuropathique en* 4 Questions (DN4)
- ✓ Leeds Assessment of Neuropathic Symptoms and Signs (LANSS) (Haroun *et al*., 2012)
- ✓ Quantitative Sensory Testing (QST) (Backonja *et al*., 2013).

1.2.*Epidemiology*

The problem of chronic pain affects a big segment of the population all over the world. About 25% of the European popolation and more than 100 millions of American citizens are affected by chronic pain and it becomes even more frequent in the older adults (<65 years). Indeed, more than 20% of these individuals take analgesics for a period of time superior to 6 months. The condition of chronic pain is widespread in aged people because of the often comorbidity with other pathologies such as osteoarthritis, diabetes (Bruckenthal & D'Arcy, 2007), fibromyalgia, deficit of B vitamin (Hanks-

Bell *et al*., 2004) and others that can be at the root of these chronic pain states. In 2010, the Center for Disease Control and The Global Burden of Disease Study highlighted that life expectancy is in continuous growth (Wang *et al*., 2012) and, in particular, people over age 85 are the fastest growing segment of the population in the United States so that they are projected to amount to 7.5 million by 2020 and to 14 million by 2040 (U.S. Census Bureau, 2004).

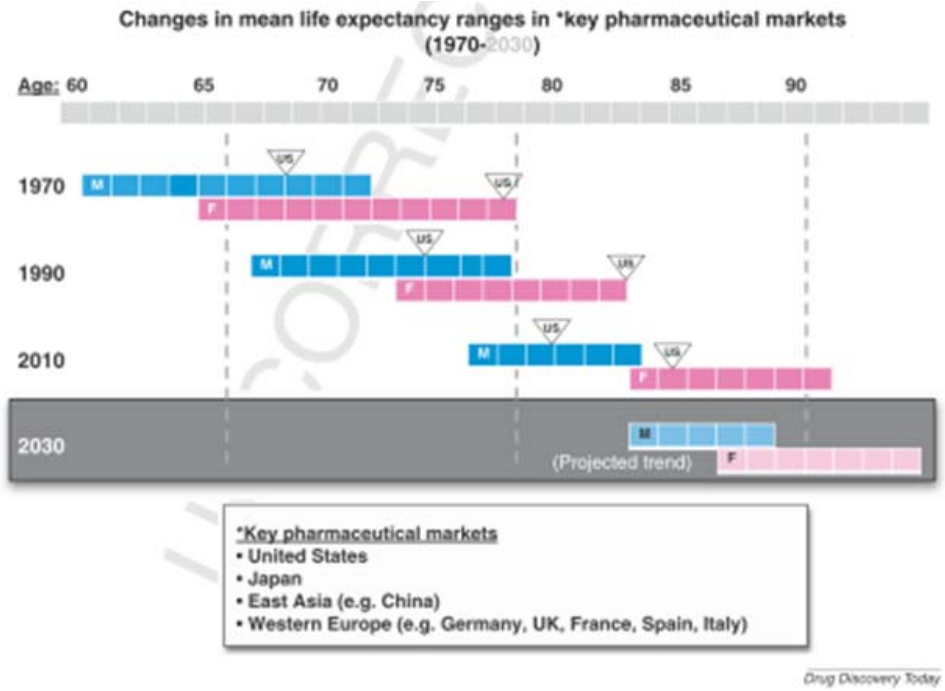

Figure 1.2. Average life expectancy for men and women in the key pharmaceuticals markets (Arneric *et al*., 2013)

A lot of aged patients live in long-term care facilities and about 80% of these suffer from chronic pain; also the 25-50% of older patients who live in the community are affected by chronic pain (AGS Panel on Persistent Pain in Older Persons., 2002). Therefore, the social and economic burden of this condition is noteworthy, as demonstrated by the fact that it ranks 4th in the classification of the 11 causes of years lived with disability (Vos et *al*., 2012).

Indeed, the costs of chronic pain account for about 600 billions of dollars and over, even more than the costs linked to cardiovascular events, cancer and diabetes (IOM., 2011).

1.3. *Chronic pain etiology*

The identification of the etiology of a chronic pain condition is very complex issue, since such a state can often arise without a precise apparent reason. Apart from a simple traumatic event, pain can be caused by: diabetic polineuropathy (Veves *et al*., 2008), post-herpetic neuropathy, cancer (Allen, 1998), acquired immunodeficiency syndrome (HIV) (Hewitt *et al*., 1997), trigeminal nevralgia, carpal tunnel syndrome and so on.

1.4. *Physiopathology of nociception*

The nervous cell responsible for the transduction of painful stimuli initiating the perception of pain are called **nociceptors**: *noci-* derives from Latin and means "hurt". The nociceptors have cell bodies located in dorsal root ganglia, or in the trigeminal ganglion that send one axonal process to the periphery and the other one into the spinal cord or brainstem. The somatic sensory receptors that transmit innocuous mechanical stimuli are associated with myelinated axons characterized by relatively rapid conduction velocities. By contrast, the axons of nociceptors are only lightly myelinated or, more commonly, unmyelinated and, therefore, they conduct relatively slowly. In particular, nociceptors belong either to the Aδ group of myelinated axons, conducting at about 20 m/s, or to the C fiber group of unmyelinated axons, conducting at velocities generally less than 2 m/s. It is possible to distinguish three major classes of nociceptors in the skin, according to the kind of stimulus that activates them: Aδ mechanosensitive nociceptors, Aδ mechanothermal nociceptors, and polymodal nociceptors (Purves *et al*.,

2001). A-δ fibers which project in the lamina I of Rexed and in the most external section of the lamina II of spinal cord and the C fibers ending in the lamina II or substantia gelatinosa are responsible for the transmission of painful stimuli through glutamate, aspartate, ATP and substance P. The inhibitory tone is provided by the neurons of the lamina II through morphine-like endogenous peptides such as enkephalin, dynorphin and other endorphins and by the noradrenergic and serotoninergic descendent systems. The transmission of pain is represented in figure:

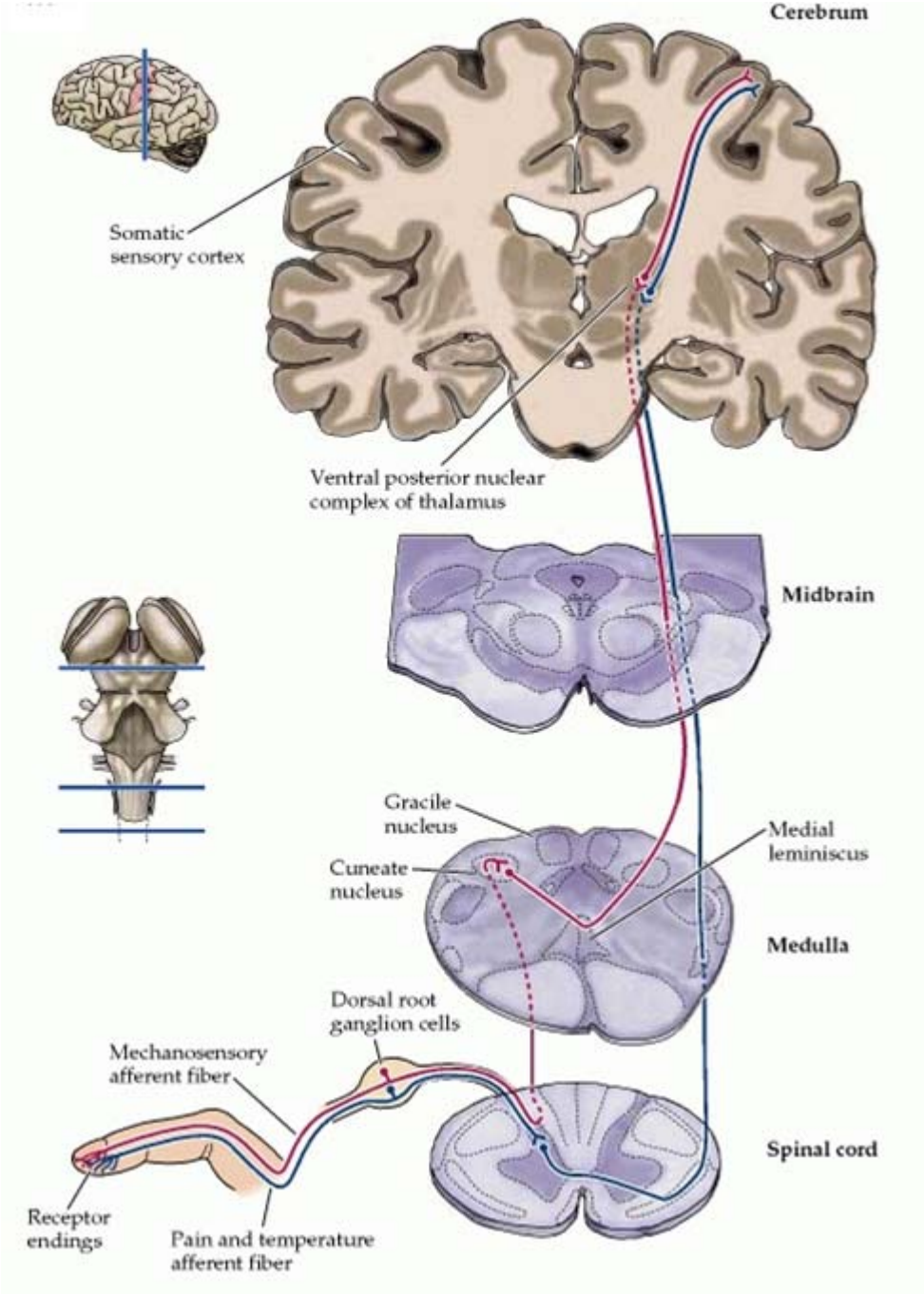

Figure 1.3. The transmission of painful signals (Purves *et al.*, 2001)

There are a lot of possible pathogenetic mechanisms that could be at the root of chronic pain such as the dysregulated activity of one or more peripheral or central structures that can become autonomously iperactive both for an

unknown cause and because of a continous exposition to painful stimuli. It is likely that also central sensitization mechanisms contribute to maintain pain states configuring a chronic condition of pain. According to the IASP, sensitization is the increased responsiveness of nociceptive neurons to their normal input, and/or recruitment of a response to normally subthreshold inputs. Sensitization lowers the threshold of activation of the nociceptors and can be supported by neuropeptides and inflammatory mediators released both through Ca^{2+}-dependent exocitosis (substance P, CGRP (Calcitonin Gene-Related Peptide), bradykinin, NGF (Nerve Growth Factor), neuropeptide Y etc…) and through not exocitotic way (NO, prostaglandins o H^{+}) (Dray, 1995). The major cellular mechanisms include ectopic or spontaneous nerve activity (as hypothesized in the phantom limb pain) and peripheral and central hyperexcitability, phenotypic changes in pain conducting pathways, and morphological reorganization (Campbell *et al*., 2006). It is also recognized that episodic inflammation, and chronic inflammatory conditions, cause nerve injury (Dray, 2008) and a changed expression and activity of several voltage-gated sodium, potassium, and calcium channels was highlighted after nerve injury (Dray, 2008). Anyway, the main pathophysiological mechanisms at the root of chronic pain always involve abnormal excitability and central sensitization. A painful stimulus induces the release of inflammatory and neuropeptidic mediators responsible for peripheral sensitization. The consequent amplification of the impulses relayed to the soma in the dorsal root and trigeminal ganglia provokes the central sensitization (Dolly & O'Connell, 2012).

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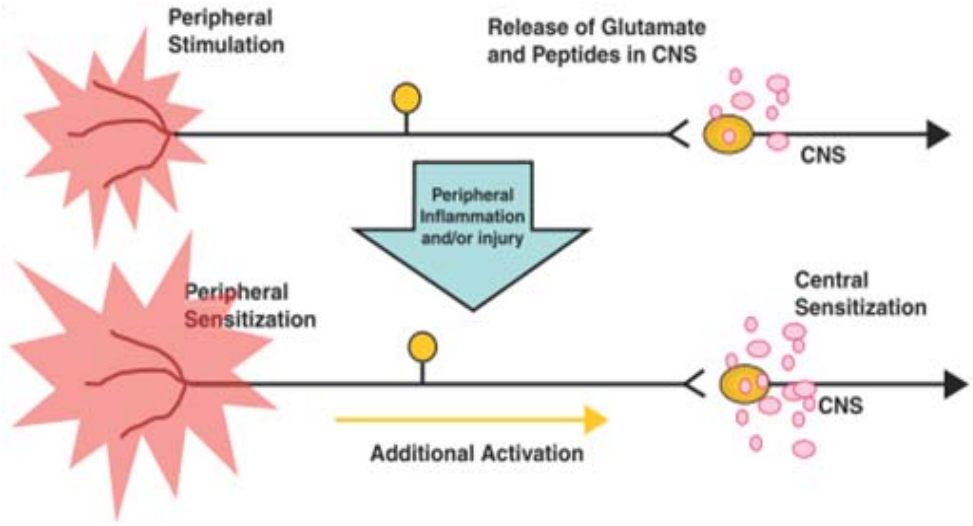


Figure 1.4. Central and Peripheral Sensitization (adapted from Dolly & O'Connell, 2012)

1.5. *Experimental pain model: the Formalin test*

The *formalin test* (Dubuisson & Dennis, 1977) in mice is a reliable model of nociception and it is also sensitive for various classes of analgesic drugs active in humans (Hunskaar & Hole, 1987). This model was introduced in 1977 as a method that allowed nocifensive behaviors to be studied with a continuous rather than transient source of stimulation (Li *et al*., 2010). Indeed, while most of the traditional nociceptive models provide short stimuli or a condition of pain developing only after some days, the *formalin test*, characterised by a first and a second phase, (Tjølsen et al., 1992) results very useful since it gives information already after one hour. This test consists in the subcutaneous (s.c.) administration of a small volume of fresh formalin into the left hindpaw of the mouse. Since formalin is a powerful algogen and inflammatory agent, this procedure induces in the animal a painful behaviour named *licking/biting* and another manifestation called *flinching*. The nociceptive response to this test consists of two distinct periods: an early

phase and a late phase after the injection of formalin (Hunskaar & Hole, 1987). The first phase corresponds to the first 5-10 min and the second phase occurs 30 min after the s.c. administration of the solution of formalin. Between the two phases, there is an interphase of weakened pain. It seems that the first phase results from direct chemical activation of nociceptive primary afferent fibers (Abbadie *et al*., 1997). In inbred mice, the response both to the early and to the late phase of the *formalin test* was shown to be highly strain-dependent (Mogil *et al*., 1999). It was recently demonstrated that formalin administration activates TRPA1 (Transient Receptor Potential), a cation channel that plays an important role in inflammatory pain (McNamara *et al*., 2007). Interestingly, marked differences in long-term mechanical sensitivity of aged and young mice were highlighted and it was suggested that TRPA1 may be involved in the transition from acute to chronic inflammatory mechanical pain and in the nociceptor sensitization shown in aged mice (Garrison & Stucky, 2014). On the contrary, the mechanisms underlying the second phase have not been completely understood yet, but, sensitization of the dorsal horn neurons was suggested to be involved (Abram & Yaksh, 1994; Ji *et al*., 1999). It was also shown that the *formalin test* induces the development of tactile allodynia on the 3rd and 7th day after the administration of formalin (Guida *et al*., 2012) that lasts up to 14 days (Lin *et al*., 2007). These findings support that subcutaneous formalin injection induces a long-lasting sensitization (Fu *et al*., 2001).

1.6.*Pharmacological treatments*

Chronic pain is generally insensitive to most of the currently used drugs such as non-steroidal anti-inflammatory drugs and it is relatively resistant to opioids. Treatments of choice, or treatments that have received regulatory approvals, include ion channel blocking drugs such as the anticonvulsants

gabapentin (Neurontin) and pregabalin (Lyrica) (Owen, 2007) together with the serotonin–norepinephrine reuptake inhibitor (SNRI) duloxetine, mainly used in cases of comorbidity pain/depression (Dray et al., 2008). Overall, some 10–30% of pain patients are responsive to the gabapentinoid drugs (Dray et al., 2008). These drugs represent a highly validated approach and target the α2δ-1 calcium channel subunit, important for channel assembly and whose overexpression was associated with allodynia in a number of specific pain models (Kim *et al*., 2001; Luo *et al*., 2002).

1.7.*Gabapentin*

The gabapentin is a cyclic analogue of GABA (γ-aminobutyric acid) (Figure 1.4.). It is an "adding up" drug used in the treatment of partial epilepsy and the Lennox-Gastaut's syndrome. In february 2001, gabapentin obtained the registered indication for pain treatment. Its pharmacocinetic is characterized by a 5-6 hours half life, with the reaching of the Tmax within 2-3 hours, and a 59% biodisponibility. It is highly liposoluble, and this favoures the crossing of the brain blood barrier (BBB). Gabapentin does not bind plasmatic proteins and is eliminated through the kidneys at the 80-100% immodified. This feature is very interesting since makes of gabapentin a very useful drug in clinic because of the absence of metabolic drug-interactions.

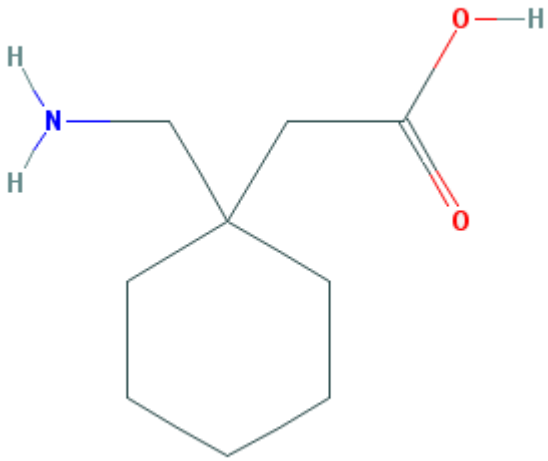


Figure 1.5. Gabapentin: structure (adapted from PubChem)

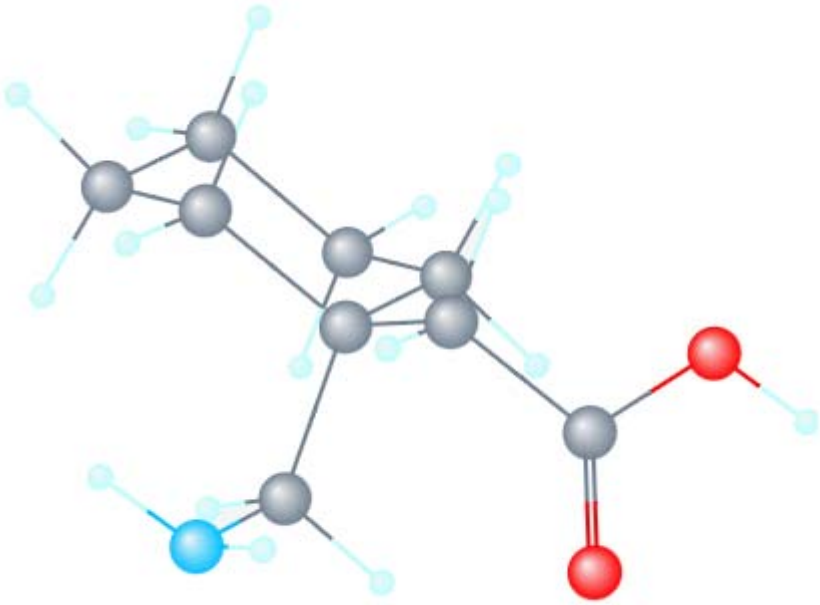


Figure 1.6. Three-dimensional conformation of gabapentin (adapted from PubChem)

From a pharmacodynamic point of view, gabapentin was initially designed as centrally acting GABA agonist, but, actually the exact mechanism of its therapeutic action has not been completely understood yet. Its pharmacological target is the L-type voltage-gated calcium channel subunit α2δ-1, temporally up-regulated in chronic pain states (Kim *et al*., 2001; Luo *et al*., 2002). Furthermore, it was demonstrated that gabapentin is able to bind both the α2δ-1 and the α2δ-2 calcium channel subunits, but not the α2δ-3. The Kd value of gabapentin binding to α2δ-2 resulted of 153 nM, compared with the higher affinity binding to α2δ-1, shown by a Kd of 59 nM (Marais *et al*., 2001). Indeed, it was demonstrated that the α2δ family includes three different genes. The α2δ-2 and α2δ-3 subunits, identified later than the α2δ-1(Klugbauer *et al*., 1999), were found to be 56 and 30% homologous to α2δ-1. The α2δ-1 subunit is made up of a highly glycosylated α2 protein, anchored to the cell membrane by a smaller δ protein (Brickley *et al*., 1995), to which it is bound by disulfide bridges (Marais *et al*., 2001). These proteins origin from the post-translational cleavage of a polypeptidic precursor encoded by a single gene (De Jongh *et al*., 1990).The overexpression of the α2δ-1 subunit after nerve injury is thought to be key for the mechanism of action of the gabapentinoid drugs (Field *et al*., 2006; Dolphin,2012). The mechanism of action of gabapentin can be various, because of the multiple functions of the α2δ-1 subunit. Therefore, gabapentin could reduce calcium currents (Gee *et al*., 1996). Some voltage sensitive calcium channels regulate substance P release from small primary afferents (Takasusuki & Yaksh, 2011). This subunit is also important in channel assembly (Dray, 2008), it was observed to increase synaptic transmission (Hoppa *et al*., 2012) and the trafficking of CaV2.1 calcium channels in hippocampal neurons to presynaptic terminals (Hoppa *et al*., 2012). Moreover, it was recently demonstrated that the α2δ-1 subunit is a neuronal receptor for thrombospondin, a protein secreted by astrocytes responsible for synaptogenesis in the CNS (Eroglu *et al*., 2009).

The vWF-A (von Willebrand's factor) domain of the α2δ-1 subunit interacts with the repetitions EGF-like (epidermal growth factor) domain of all thrombospondins. Therefore, gabapentin could compete with the binding of the thrombospondin to the α2δ-1 and the inhibition of excitatory synapses could represent one of the mechanisms underlying gabapentin effectiveness in chronic pain.

AIM OF THE RESEARCH

2. AIM OF THE RESEARCH

Growing evidence shows that aged people are often afflicted by chronic pain states resistant to most of the common-use painkillers such as non-steroidal antiflogistics and, as highlighted by epidemiology, there is a strong unmet need to develop new therapeutic strategies for a better management of pain in the elderly patients (Gagliese, 2009). The work of this PhD project aims at studying and deepening the molecular processes and the biochemical pathways involved in the development and in the maintainance of the conditions of chronic pain both in the young and in the old animal. In order to achieve this, this research intended to characterize a chronic pain model that could be a valid experimental tool representative of the clinical chronic pain conditions experienced by the ever-increasing population of the elderly. To clarify this complex physiopathological picture, this research project pointed at understanding how the process of ageing can affect, first, the nociception and, later, the development of pain or influence the maintainance of a pre-existing condition of pain. Therefore, this research intended to examine the signal pathways and the degenerative processes at cellular level that can be responsible for aberrant pathologial neuronal function and, in this way, for chronic pain. Another important purpose of this study was to find out if this cellular machinery at the root of chronic pain undergoes important changes in old animals. Therfore, it was necessary to point out the eventual age-induced modifications of the basal mechanical and thermal (both to heat and to cold) sensitivity and, consequently, of the nocifensive response of animals subjected to an experimental model of pain identified as reliable and predictive of a relevant chronic pain condition. In the future perspective, it will be useful to understand how to translate the acquired knowledge about the effect of ageing on pain into clinical practice in order both to improve the use of the pharmacological tools that result available at the moment and to

develop novel therapeutic treatments for the management of chronic pain in the aged patients.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. *Animals*

All experiments were performed on male C57BL/6 mice (Charles River, Italy) belonging to four different groups of age (2 month-old, 6 month-old, 12 month-old and 18 month-old mice) at the beginning of the experiment. Mice were housed in groups of about 4 per cage on a 12 h: 12 h light dark cycle at constant room temperature of 22 ± 1 °C and in conditions of relative humidity of the 65% and provided with food and water *ad libitum*. All of the experimental procedures used followed the guidelines of the Italian Ministry of Health (D.M. 116/92; E.U. Directive 2010/63) minimizing the conditions of pain and using only the strictly necessary number of animals to get statistically significant results.

3.2. *Experimental pain model*

In this research project, the pain model used was the *Formalin Test* (Dubuisson & Dennis., 1977). Before the test, mice were allowed to acclimatize in a plexiglas box (30 x 30 x 35 cm^3) for up to 60 min maintaining the room temperature (23-25°C) and humidity stable. Following acclimatation, the mice were gently placed in the *restrainer*, a device used to immobilize the animal without hurting it, in order to expose the posterior left hindpaw. This test consists in the subcutaneous (s.c.) administration of 20 µl of a 5% solution of fresh formalin into the left hindpaw of the mouse. The 5% solution of formalin was obtained from a solution of saturated formaldheyde at 36.5-38% (Sigma F8775-500ML) and it was prepared on the same day of the test. Since formalin is a powerful algogen and inflammatory agent, this procedure induces in the animal a painful behaviour named *licking/biting* and another manifestation called *flinching*. The *licking time* is the cumulative

value in seconds of the time that the animal spent to lick the paw after each application (Staaf *et al*., 2009). The mouse presents the *licking/biting* behaviour when it licks and bites intensively the hindpaw which underwent formalin administration. On the other hand, *flinching* consists in the vigorous shaking of the injected hindpaw which represents a characteristic expression of the *formalin test*. In order to evaluate the entity of perceived pain through the *formalin test*, mice were observed for 1 hour (and in some experimental settings for 90 min) and the seconds of *licking/biting/flinching* behaviour were counted at respectively 12 or 18 intervals of 5 min. The peculiarity of *formalin test* is that it develops in two main phases: the first phase corresponds to the first 5-10 min and the second phase occurs 30 min after the s.c. administration of the solution of formalin. It is believed that the first phase is supported by the local inflammatory process induced by the administration of formalin into the paw of the animal. On the contrary, it is thought that mechanisms of central sensitization typical of chronic pain underlie the second phase. It was also shown that the *formalin test* induces the development of tactile allodynia on the 3rd and 7th day after the administration of formalin (Guida *et al*., 2012) that lasts up to 14 days (Lin *et al*., 2007). Another important feature of the *formalin test* is that it reproduces a condition of pain that mirrors the most widespread clinical conditions, expecially in the continuously growing old population. This is the reason why this test was performed on mice from 2 to 18 months of age to be sure to cover almost all the range of age in which humans experience chronic pain conditions.

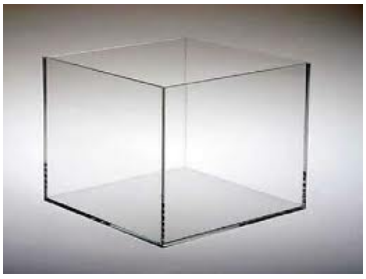



Figure 3.1. Plexiglas box and *restrainer* used in *formalin test* (Dubuisson & Dennis, 1977)

3.3. *Behavioural tests*

The thermal and mechanical sensitivity and the eventual chronic pain states were evaluated through behavioural tests.

- ✓ ***Mechanical sensitivity.*** The methods used for the assessment of mechanical sensitivity were *Von Frey's test* (Chaplan *et al*., 1994) and *Pin-prick test* (Chan *et al*., 1992). In particular, the *Von Frey's test* was used to evaluate mechanical allodynia and the *Pin-prick test* was used to evaluate mechanical hyperalgesia (Chan *et al*., 1992). These behavioural tests were performed on C57BL/6 mice of four different groups of age (2 month-old, 6 month-old, 12 month-old and 18 month-old mice) to investigate any possible modifications in their basal mechanical sensitivity according to ageing process. The basal measures were carried out at intervals of 4/6 weeks to be sure to highlight all of the remarkable changes. During the behavioural tests the room temperature and humidity remained stable.

  Von Frey's test. For acclimatation, mice were placed inside Perspex chambers (75 mm x 90 mm) on a wire mesh floor for up to 60 min. This test uses Von Frey's hairs (Ugo Basile, Comerio, Italy) that are calibrated filaments from 0.04 to 12.75 g and providing forces of 0.392 to 124.95 mN through the *up-down* method (Chaplan *et al*., 1994; Dixon, 1980) that allows to determine the value corresponding to the 50% of the *withdrawal threshold*. Von Frey's filaments provide a calibrated pressure with incremental stiffness against the hairless skin of the hindpaws (Su *et al*., 2011). Von Frey's filaments were applied on the posterior left hindpaw and then on the right one for 3-5 seconds: in

this way the *withdrawal latency* of the hindpaw was determined in seconds. The first hair applied to the center of the hindpaw surface of the mouse is the number 7 (0.6 g; 5.9 mN) since it represents a filament of intermediate strength corresponding to the 50% threshold of withdrawal in basal conditions: if the mouse responded positively to such stimulation, the other hairs were tested in descending order. On the contrary, if the mouse did not present a nociceptive response, the other hairs were tested in ascending order. The test was carried on until at least 6 measures around the value of the 50% threshold of withdrawal for each hindpaw were got, so avoiding misunderstandings in the obtained results. Indeed, two measures per hindpaw were carried out at an interval of 30 min to be sure that phenomena of sensitization could not occur and every measure was performed at a distance of 3 min from the following to avoid that the eventual response could be due to the continuous stimulation.

Figure 3.2. Representation of *Von Frey's test* (Chaplan *et al.*, 1994)

Apart from the assessment of the threshold of basal mechanical sensitivity in C57BL/6 mice of different ages, according to the literature stating the development of a formalin-induced mechanical allodynia that peaks around 3 days and lasts for 2 weeks after the administration of formalin (Guida *et al.*, 2012; Lin *et al.*, 2007), the *Von Frey's test* was used even to evaluate this allodynia. To this aim, the test was performed before the administration of formalin in order to

get the baseline threshold and in several time points after the execution of *formalin test*. In particular, mice underwent *Von Frey's test* 2 hours after the administration of formalin and on the 1st, 4th, 7th, 9th, 11th and 14th day following the *formalin test*. At every time point, the measures were executed twice at an interval of 30 min. The time sheme that was used is shown in figure:

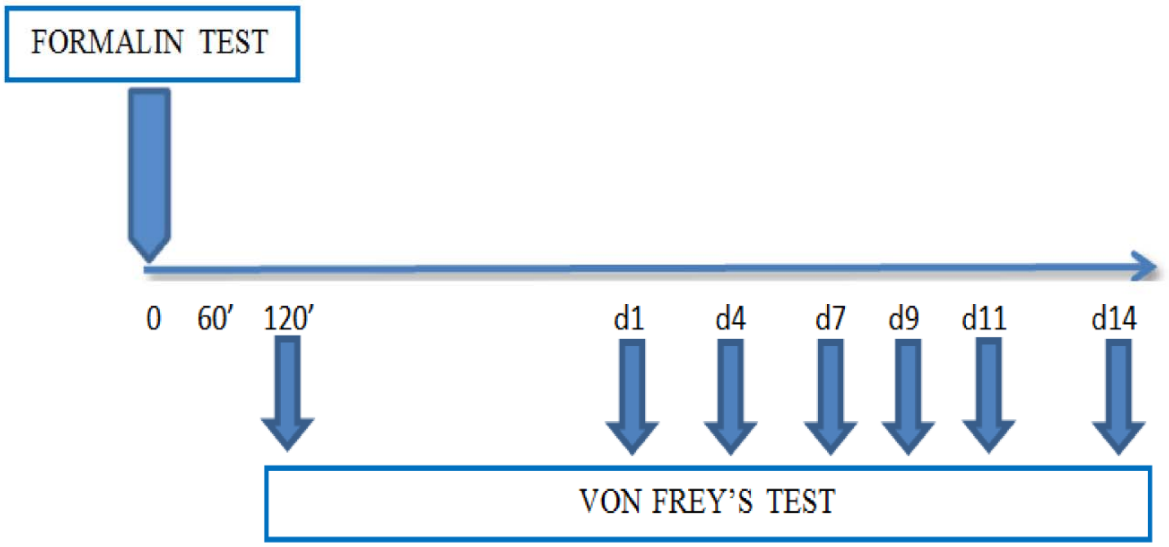


Figure 3.3. Time points in which the *Von Frey's test* was performed

Moreover, the *Von Frey's test* was used to determine the effect of a pharmacological treatment with gabapentin on this condition of pain. Indeed, α2δ-1 ligands gabapentin and pregabalin are effective in the treatment of a range of chronic pain conditions (Moore et al., 2009, 2011). Since the *formalin test* reproduces a state very similar to the most common clinical conditions, it resulted interesting to understand whether the mechanical allodynia induced by formalin administration finds relief with a treatment with gabapentin. Moreover, it was investigated the effect of this treatment on mice of different ages to find out if the effect of gabapentin was influenced by age. For this purpose,

mice of 2 different groups of age were treated with two different doses of gabapentin and a vehicle after having been subcutaneously administered with formalin. The *Von Frey's test* was performed before the administration of formalin in order to get the baseline threshold and then in several time points after formalin administration and treatment with gabapentin. The behavioural results obtained through the *Von Frey's test* were plotted in two different types of graph. As a matter of fact, to get the best possible visualization of the data, some results were shown as 50% threshold normalized for the baseline threshold vs time and other results were shown as – LOG (50% threshold) vs time. The logaritmic scale allows to see even the nearest points of a graph in a clearer way. The logaritmic scale allows to see even the nearest points of a graph in a clearer way, a very important feature for graphs representing pharmacological studies. Since this kind of graph does not need the normalization, the baseline threshold is visualized as point zero so that the comparison between the threshold measured at the baseline level and the threshold detected during the other time points following the *formalin test* only or the *formalin test* preceded by the pharmacological treatment results to be much easier.

Pin-prick test. For the determination of mechanical hyperalgesia the *Pin-prick test* (Chan *et al.,* 1992) was set up. Mice were allowed to acclimatize inside Perspex chambers (75 mm x 90 mm) on a wire mesh floor for 30 min. For the assessment of mechanical hyperalgesia the tip of a safety pin with blunt end was applied to the surface of the left posterior hindpaw of the mouse at latero-medial level, impressing with a force that could not provoke a lesion of the skin, and the seconds of *licking/biting* and of *lifting/clutching* behaviour were monitored with a cut-off of 30 seconds.

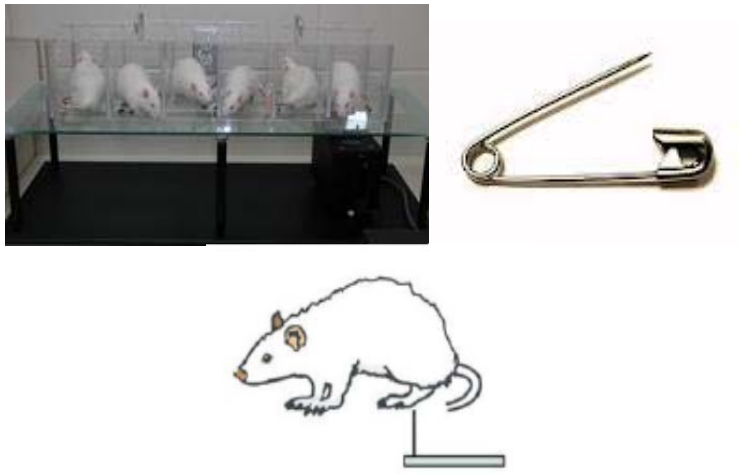


Figure 3.4. Representation of the *Pin-prick* test

- ✓ ***Thermal sensitivity.*** Mice underwent two different tests for the evaluation of thermal sensitivity: for the assessment of withdrawal threshold to noxious heat it was used the *Hargreaves' test* (Hargreaves *et al*., 1988) and for the assessment of cold sensitivity it was carried on the *acetone test* (Choi *et al*., 1994). As previously seen for the tests of mechanical sensitivity, even these behavioural tests were performed on C57BL/6 mice of four different groups of age (2 month-old, 6 month-old, 12 month-old and 18 month-old mice) to highlight changes in their basal thermal sensitivity induced by ageing. Also in this case, the basal measures were made at regular intervals of 4/6 weeks. During the behavioural tests the room temperature and humidity remained stable.

  Hargreaves' test. After 1 hour of acclimatation inside a perspex box, a source of radiant heat (Plantar Test, model 7650, Ugo Basile, Italy) was applied to the surface of both the left and the right hindpaw of the mouse using an intensity of 20% and a cut-off of 20 seconds. This test

allows to measure the latency time of reaction of the animal to thermal stimulation If the animal did not withdraw the hindpaw after 20 seconds, the exposition to the source of heat was interrupted to avoid any eventual lesions of the skin tissue. The latency time was counted in seconds. It was important to wait for some min among the various measures to be sure that the response was not due to sensitization.

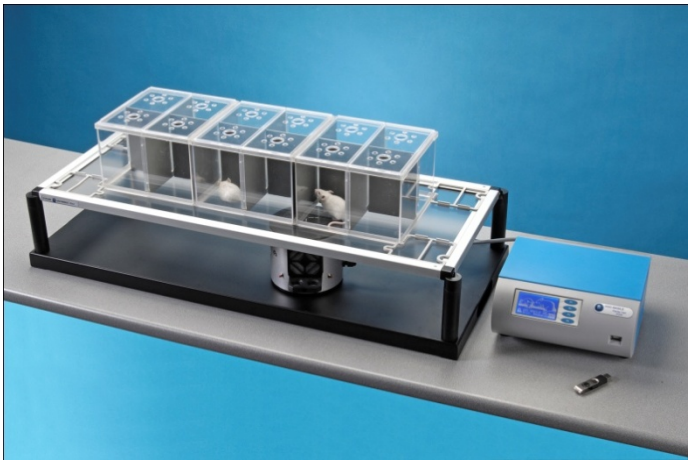


Figure 3.5. Representation of the *Hargreaves'test*

Acetone test. Mice were allowed to acclimatize inside Perspex chambers (75 mm x 90 mm) on a wire mesh floor for 30 min. For the assessment of cold sensitivity a 50 µl drop of acetone (Sigma-Aldrich 179124) was applied on the center of the plantar surface of the posterior left hindpaw through a modified 0.5 ml syringe linked to a rounded polythene tubing device. This device allows to place the drop of acetone without mechanically stimulating the skin of the mouse. The seconds of *licking/biting/lifting/clutching* behaviour were monitored with a cut-off of 30 seconds.

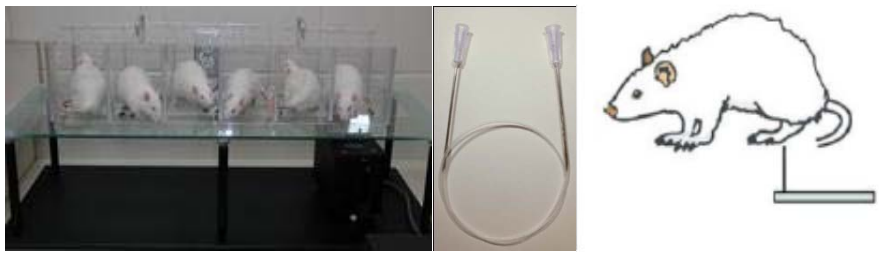


Figure 3.6. Representation of the acetone test

3.4. *Measure of oedema of the hindpaw*

The thickness of the hindpaw of the mice that underwent the *formalin test* was measured both immediately before the execution of this test (in order to obtain the basal value) and in the same following time points in which the *Von Frey's test* was performed to assess the mechanical allodynia induced by formalin. Indeed, the measure of oedema was performed 2 hours after the administration of formalin and on the 1st, 4th, 7th, 9th, 11th and 14th day following the *formalin test*. The time scheme is shown in figure:

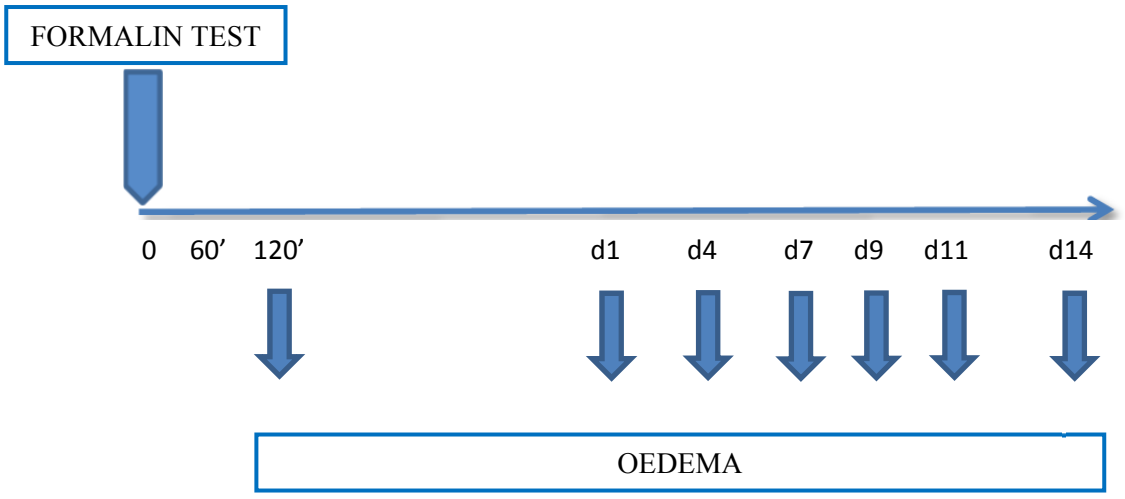


Figure 3.7. Time points of measure of oedema

The evaluation of oedema was made using a caliper of the sensitivity of 0,5 mm.

Figure 3.8. Representation of the caliper used to measure the hindpaw thickness

3.5. *Chemicals*

For the acetone test (Choi *et al*., 1994) a 50 µl drop of acetone (Sigma-Aldrich 179124) was applied to the plantar surface of the posterior left hindpaw of the mouse. For the *formalin test*, 20 µl of a 5% solution of fresh formalin were subcutaneously (s.c.) administered into the left hindpaw of the mouse. The 5% solution of formalin was prepared from a solution of saturated formaldheyde at 36.5-38% (Sigma F8775) on the same day of the test. For the biochemical analyses, these primary antibodies and dilutions were used: anti-LC3 1:3000 O/N (over night) (MBL, Japan), anti-Beclin 1 1:4000 O/N (MBL, Japan), anti-p62/SQSTM1 1:2000 O/N (MBL, Japan), anti-α2δ-1 1:2000 O/N (Sigma-Aldrich, Milan, Italy), anti-GAPDH 1:40000 (Applied Biosystem, Carlsband, CA, USA).

3.6. *Pharmacological treatment*

Chronic pain treatments of choice include ion channel blocking drugs such as the anticonvulsants gabapentin (Neurontin) and pregabalin (Lyrica) (Owen., 2007). Therefore, it was investigated both whether gabapentin were effective in the treatment of formalin-induced mechanical allodynia and, if this was the case, whether this effect were influenced by age since chronic pain states more and more often affect aged patients. In order to answer these questions, 2 month-old and 6 month-old mice were treated with two different doses of gabapentin and a vehicle after having been subcutaneously administered with formalin. In particular, this two age groups of mice were divided into three subgroups for each age: one subgroup was treated with vehicle, another subgroup was treated with gabapentin 10 mg/Kg and the last subgroup was treated with gabapentin 100 mg/Kg. The gabapentin (Mylan generics) was taken in capsules as a pharmaceutical formulation available on the market. Since every capsule contains 100 mg of active principle gabapentin, the dose of 100 mg/Kg was prepared dissolving the content of 1 capsule in 100 ml of depurated water and the dose of 10 mg/Kg was prepared by diluition of the dose of 100 mg/Kg. As gabapentin was dissolved in depurated water, this same water was used as vehicle. The *Von Frey's test* was performed before the administration of formalin in order to get the baseline threshold. Later, mice were allowed to acclimatize in a plexiglas box (30 x 30 x 35 cm^3) for 30 min maintaining the room temperature (23-25°C) and humidity stable. Following acclimatation, the mice underwent intraperitoneal (i.p.) administration of one of the two doses of gabapentin or of the vehicle and, after 15 min, they were gently placed in the restrainer so that the 5% formalin solution could be subcutaneosly administered into their left posterior hindpaw according to the *formalin test* (Dubuisson & Dennis., 1977). The *licking/biting/flinching* behaviour was monitored for 90 min. To assess the effect of gabapentin on formalin-induced mechanical allodynia on mice of

different ages, the *Von Frey's test* was performed 2 hours after the administration of formalin and on the following day. On the 4th day after *formalin test*, mice underwent a second i.p. administration of gabapentin/vehicle; on this day the first measure of the *Von Frey's test* was performed before the second i.p. administration of gabapentin/vehicle, while the second measure of the *Von Frey's test* was performed after the second i.p. administration of gabapentin/vehicle. The treatment schedule is shown in figure:

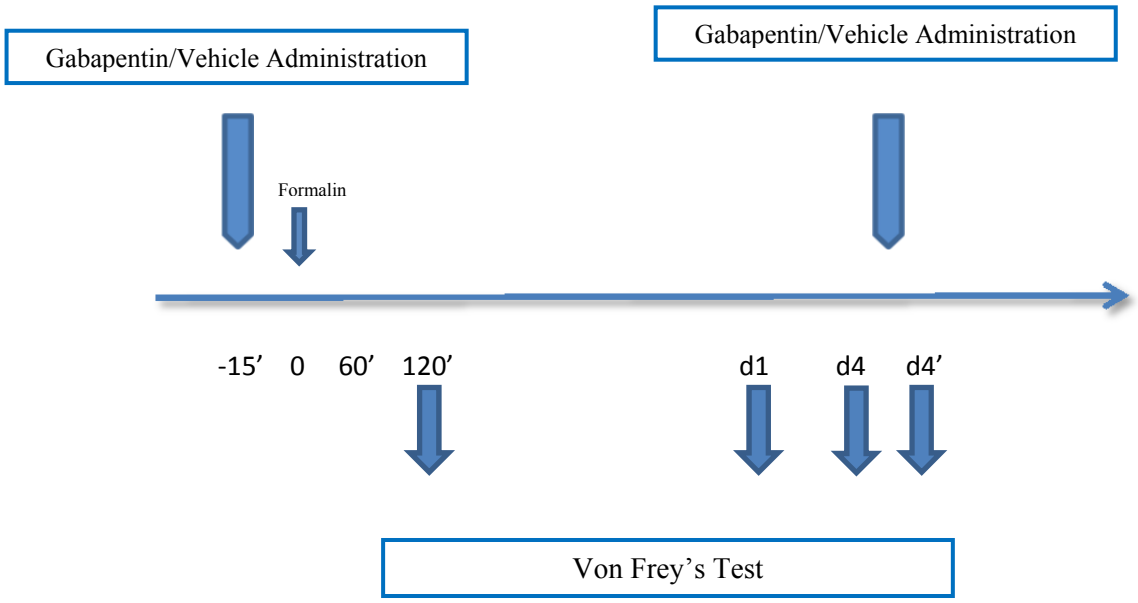


Figure 3.9. Schedule of administration of gabapentin

3.7. *Biochemical analysis*

The group of naïve differently aged mice, that underwent the assessment of mechanical and thermal basal sensitivity only, were anaesthetized with 2-3% isoflurane and sacrificed on the 15th day after formalin administration, in order to extract the tissues to analyze. Samples of spinal cord, in particular of

the L4-L5 portion since this is the one involved in chronic pain states, were rapidly removed and dissected in ipsilateral (ipsi) and contralateral (contra) so that they could have the functions to give an image of the proteic arrangement in the sections of spinal cord that are subjected to changes during ageing, and so, to allow the comparison with groups of different age. The term "ipsi" refers to the side of the injury that is conventionally the left side. Since, in the group which underwent *formalin test* the formalin solution was administered into the left hindpaw, even in the naïve group "ipsi" represents the left side. By contrast, the term "contra" refers to the right side. The samples of L4-L5 sections of spinal cord were subjected to western blotting anlysis.

- ✓ ***Western blotting***

The ipsi- and the contra-lateral sides of each spinal cord were dissected, snap-frozen and stored at -80°C until further processing. For protein extraction, each single hemi-cord segment was homogenized in ice-cold lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0,1% SDS, 1% IGEPAL, 0,5% Na-deoxycholate) in the presence of protease inhibitors (Sigma-Aldrich, Milan, Italy) and incubated on ice for 40 min. Then, the samples were centrifuged at 14,000 × g for 15 min at 4 °C. Total protein content was determined in the supernatants by the Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Milan, Italy). An equal amount of total proteins was separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 15%) and transferred onto PVDF (polyvinylidene difluoride) membranes (Immobilon-P, Sigma-Aldrich, Milan, Italy). After blocking for 1 hour at RT (room temperature) in Tris-buffered saline containing 0.05% Tween 20 (TBST) and 5% non-fat milk, the membranes were incubated overnight at 4°C with the primary antibody directed against the protein of interest. After several washes, an appropriate HRP-conjugated secondary

antibody (goat IgG; Pierce Biotechnology, Rockford, IL, USA) was applied for 1 hour at RT. Signal intensity was measured using ImageJ software (NIH, Bethesda, MD, USA). For quantitative analysis, the Beclin 1, LC3-I, LC3-II, p62 and α2δ-1 signals of each sample were normalized versus the corresponding GAPDH signal.

3.8. *Statistical analysis*

The statistical analysis of the results of behavioural tests expressed as Mean ± SEM was implemented by Student's t tests or by the analysis of the variance at one way (One-way ANOVA) followed, when necessary, by a test for multiple comparisons as Tukey's test. All of the differences that presented a value of $P<0.05$ were considered statistically significant. Microsoft Office Excel, GraphPad Prism, Image Quant 5.0 and Adobe Illustrator were used to make graphs of the results and GraphPad Prism was used to make the statistical analysis of the data.

RESULTS

4. RESULTS

4.1. CHAPTER 1: CHARACTERIZATION OF THE *FORMALIN TEST* AS A CHRONIC PAIN MODEL

During the last years, epidemiology has highlighted that aged people often suffer from chronic pain states and, therefore, a better management of pain in these patients is needed (Gagliese, 2009). For this reason, this PhD project intended to study and characterize a model of pain that can mirror a chronic pain condition comparable to the painful states experienced by an increasing number of old patients. In particular, the focus of our attention was on the *Formalin test* (Dubuisson & Dennis, 1977), a reliable nociception model consisting of two distinct phases (Hunskaar & Hole, 1987). Studies with different classes of analgesic drugs showed that these two phases can have different nociceptive mechanisms (Hunskaar & Hole, 1987): direct activation of nociceptive primary afferent fibers (Abbadie *et al*., 1997) for the first phase and, likely, central sensitization for the second phase (Abram & Yaksh, 1994; Ji *et al*., 1999). Such central sensitization appeared to be confirmed by the cortical somatosensory evoked potentials observed after formalin injection (Lebrun *et al*., 2000). It was demonstrated that protein synthesis is important for the behavioural hypersensitivity induced by the injection of formalin into mice hindpaw (Kim *et al*., 1998). Moreover, according to literature two hours after formalin administration, hyperalgesic responses were observed: such response resulted to be enhanced 1 to 3 days after injection and lasted for 3 – 4 weeks (Fu *et al*., 2001). Other studies show that the *formalin test* induces the development of tactile allodynia on the 3rd and 7th day after the administration of formalin that lasts up to 14 days (Lin *et al*., 2007; Guida *et al*., 2012). These findings support that subcutaneous formalin injection induces a long-lasting sensitization (Fu *et al*., 2001). Therefore, it was

decided to investigate whether the *formalin test* could be used, not only as acute inflammatory pain model, but also as a chronic pain model. The nocifensive response to this test was studied in 2 month-old C57BL/6 mice and, according to literature, was underlined the presence of the first and the second phase spaced out by an interphase of reduced pain behaviour. The nocifensive behaviour highlighted consisted of *licking/biting/flinching*. The presence of a second phase of more extensive amplitude than the first one demontrates the existance of central sensitization processes at the root of this phenomenon. The development of a central sensitization process suggests that the formalin test, apart from being a test for the assessment of the acute nociceptive response, can represent a chronic pain model. This is the reason why the mechanical sensitivity of the mice after formalin administration was examined through the *Von Frey's test* (Chaplan *et al.*, 1994) in order to detect the development of tactile allodynia. The *Von Frey's test* was performed two hours after formalin injection and on the 1st, 4th, 7th, 9th, 11th and 14th day following the *formalin test*. Our results underlined the onset of mechanical allodynia already two hours after formalin administration in the hindpaw ipsilateral to the insult. The complete development of this allodynia occurred on the 1st day and it peaked on the 4th day following formalin injection. Starting from the 4th day following formalin injection, also the contralateral hindpaw presented mechanical allodynia. Later, allodynia decreased progressively and the recovery occurred around the 14th day following the *formalin test*. Furthermore, a lesion of the ipsilateral hindpaw was observed on the 9th day. Therefore, the *Von Frey's test* on the 11th and 14th day following the *formalin test* was performed only on the contralateral hindpaw. Two hours after formalin administration, the thickness of the injected paw was measured. The paw presented an identifiable degree of oedema that increased up to the 4th day and remained quite stable during the following days, beginning to recover around the 11th and 14th day following the *formalin*

test. Since gabapentin is one of the treatments of choice for chronic pain states (Owen, 2007), it was administered 15 min before formalin to verify if it was effective on the formalin-induced nociceptive behaviour. According to the literature (Gustafsson *et al.*, 2003), it resulted very effective on the *licking/biting/flinching* behaviour of the second phase, thus demonstrating that this phase is depending on sensitization mechanisms. Moreover, even if in a less extent, it resulted effective also on the first phase. This effect could be due to a different mechanism of action of gabapentin. Indeed, it acts on the α2δ-1 calcium channel subunit, that increases synaptic transmission (Hoppa *et al.*, 2012) and is involved in the trafficking machinery (Hendrich *et al.*, 2008; Tran-Van-Minh & Dolphin, 2010). Therefore, the action of gabapentin on multiple α2δ-1-mediated trafficking processes likely could be at the root of one of the mechanisms of action of gabapentin (Hendrich et al., 2008; Tran-Van-Minh & Dolphin., 2010). According to the literature, gabapentin was used in previous work (mainly) at two different doses: 10 mg/Kg and 100 mg/Kg. Measuring the formalin-induced nociceptive response as total of events of *licking/biting/flinching* for each phase, it resulted very clear that only the 100 mg/Kg dose was effective in all of the *formalin test* phases. In this way, it was possible to evaluate gabapentin effect even on the 3rd phase of formalin test, a phase sometimes considered together with the 2nd phase as late phase because it respresents the end of the events of *licking/biting/flinching* of the 2nd phase and the beginning of the recovery. The effectiveness of gabapentin was also evaluated on formalin-induced mechanical allodynia. Indeed, gabapentin was administered 15 min before formalin administration and two hours after the *formalin test* the assessment of mechanical allodynia was carried out performing the *Von Frey's test*: when compared with a vehicle, gabapentin resulted effective on formalin-induced mechanical allodynia. These data support the hypothesis that the *formalin test* can be used as a chronic pain model since gabapentin, which is active in

reducing the allodynic states induced by neuropathic pain models, is also effective on formalin-induced mechanical allodynia. Because of the drug half life, on the 1st day and on the 4th day its effect resulted used up. Therefore, our results suggest that the *formalin test* can be used as chronic pain model and the remarkable advantage of this model is that, unlike other experimental chronic pain models, it is not very invasive and it provides valid information more rapidly. The *formalin test* can turn into a very useful tool for the study of ongoing chronic pain in the elderly in order to improve the clinical treatment of this ever-increasing population.

4.1.1. The nocifensive response to the *Formalin test* in 2 month-old C57BL/6 mice

The *Formalin test* (Dubuisson & Dennis, 1977) is a widely used experimental pain model for the assessment of the acute inflammatory nociceptive response. It was investigated whether the formalin test could be used also as a chronic pain model in 2 month-old C57BL/6 mice. As expected, in agreement with previous data, the characteristic two phases of *licking/biting/flinching* behaviour were observed, with the 1st phase lasting for the first 5 min and the 2nd phase lasting from 20 to 30 min after the injection of formalin (Hunskaar & Hole, 1987) (Figure 4.1.).

Figure 4.1.

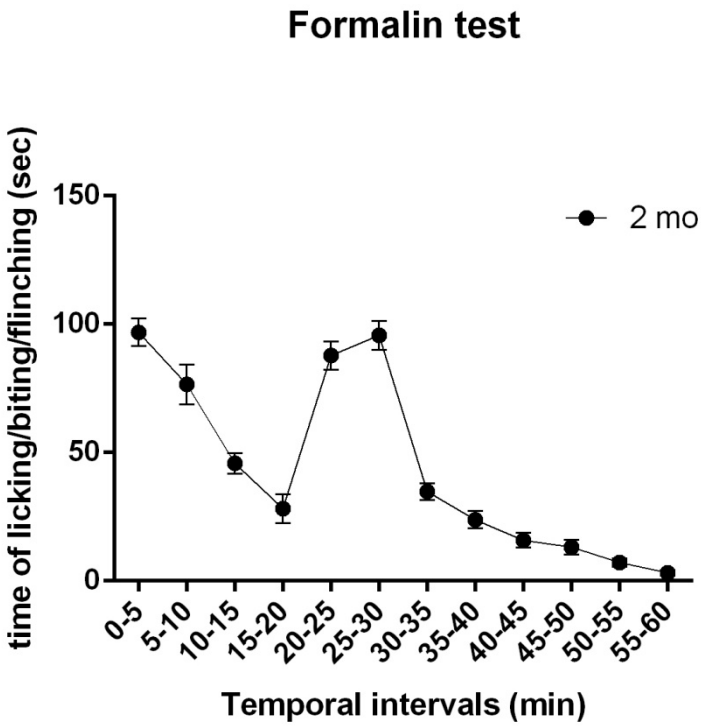


Figure 4.1. *Formalin test* in 2 month-old C57BL/6 mice. Nociceptive reaction of 2 month-old C57BL/6 mice to the *formalin test*, expressed as time of *licking/biting/flinching*. The curve shows a 1st phase, lasting 5 min, and a 2nd phase, lasting from 20 to 30 min after the injection of formalin. The period of decreased nocifensive behavior represents the interphase. Data are expressed as mean ± SEM of the duration of the nociceptive reaction characterized by *licking/biting* and *flinching*. *p* values < 0.05 were considered statistically significant. (2 month-old mice: n=6).

4.1.2. Formalin-induced mechanical allodynia in 2 month-old C57BL/6 mice

The 2 month-old C57BL/6 mice that were subjected to the *formalin test* underwent the assessment of mechanical sensitivity through the *Von Frey's test* (Chaplan *et al.*, 1994). This test was performed in order to verify whether the formalin injection could induce the development of long-lasting tactile allodynia and so if the formalin test could be used as an experimental chronic pain model. The *Von Frey's test* measures were performed following a particular time course: two hours after formalin injection and on the 1st, 4th, 7th, 9th, 11th and 14th day following the *formalin test*. These time points were established to obtain a clear complete picture of the development of formalin-induced mechanical allodynia from its onset to the recovery of the basal sensitivity levels. Mechanical allodynia resulted present already two hours following formalin administration in the hindpaw ipsilateral to the insult, keeping to increase on the 1st day. The peak of allodynia was reached on the 4th day following formalin injection. Interestingly, on 4th day, also the contralateral hindpaw showed mechanical allodynia levels comparable to the levels of tactile allodynia of the ipsilateral hindpaw (Figure 4.2.). These results suggest that formalin-induced mechanical allodynia may be due to central mechanisms and not only to the inflammatory nociceptive response evoked by formalin injection. Indeed, in the case of a exclusively peripheral component, only the ipsilateral hindpaw that received the insult would have developed mechanical allodynia. From the 9th day following formalin injection, a lesion was observed on plantar surface of the ipsilateral hindpaw. The mechanical sensitivity turned back to the basal levels around the 14th day following the *formalin test* (Figure 4.2.). Our results suggest that the *formalin test* includes in itself the features both of an acute and of a chronic pain model.

Figure 4.2.

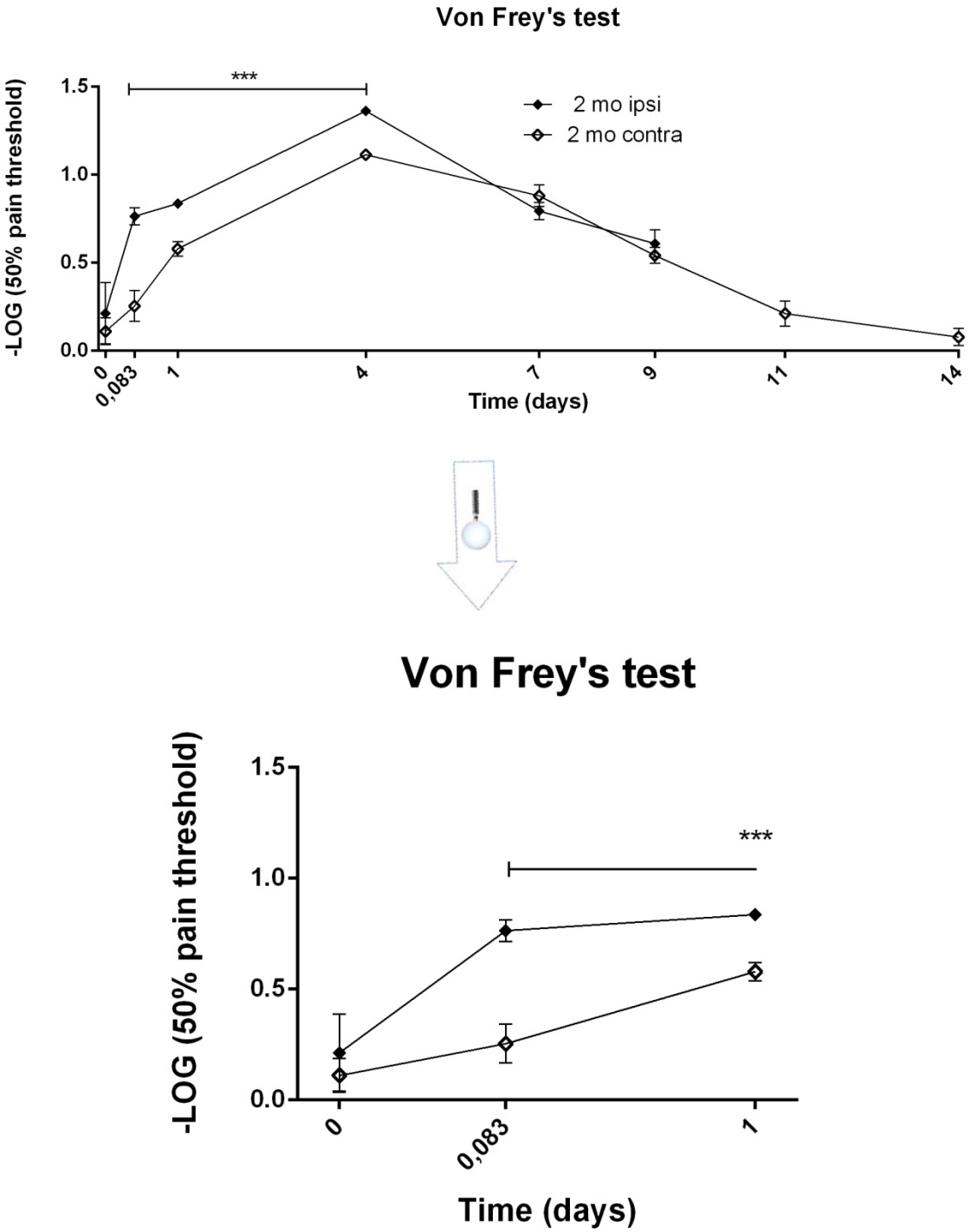


Figure 4.2. Formalin-induced mechanical allodynia in 2 month-old C57BL/6 mice. The development of the tactile allodynia after the formalin administration was earlier in the ipsilateral hindpaw. On the 4th day after the formalin test, even the contralateral hindpaw showed mechanical allodynia. The mechanical allodynia reverts to the basal levels around the 14th day after formalin injection. Data are expressed as -LOG of the mean ± SEM of the 50% pain threshold. p values < 0.05 were considered statistically significant. p values < 0.03: *; p values < 0.002: **; p values < 0.001: ***. For

iconographic reasons, the time points interval between 0 and 1is enlarged compared with the real dimensions: an enlargement is shown. 0,083 day represents the time point of two hours after the *formalin test*. (2 month-old mice: n=6).

4.1.3. Formalin-induced oedema in 2 month-old C57BL/6 mice

It is known that the inflammatory response induced by formalin intraplantar administration induces paw swelling (Lee & Crosby, 1999). In order to monitor this oedema evolution in time, the hindpaw size was measured before and two hours, 1, 4, 7, 9, 11 and 14 days after the algogen stimulus through a caliper, as previously described (Burke *et al*., 2010). The paw presented a high size of oedema that increased up to the 4th day and began to recover starting from the 11th and 14th day (Figure 4.3.), thus suggesting that several molecular changes, other than the early-inflammatory response, occur in the paw after formalin administration. It could seem that the observed increased mechanical sensitivity could be caused by the developed oedema of the hindpaw (Vierck *et al*., 2008) but, it is independent from the oedema since, on the 4th day following formalin injection, both the injured ipsilateral and the uninjured contralateral hindpaw present mechanical allodynia. The statistical analysis for the time course data of figure 4.3 is shown in Table. 4.1.

Figure 4.3.

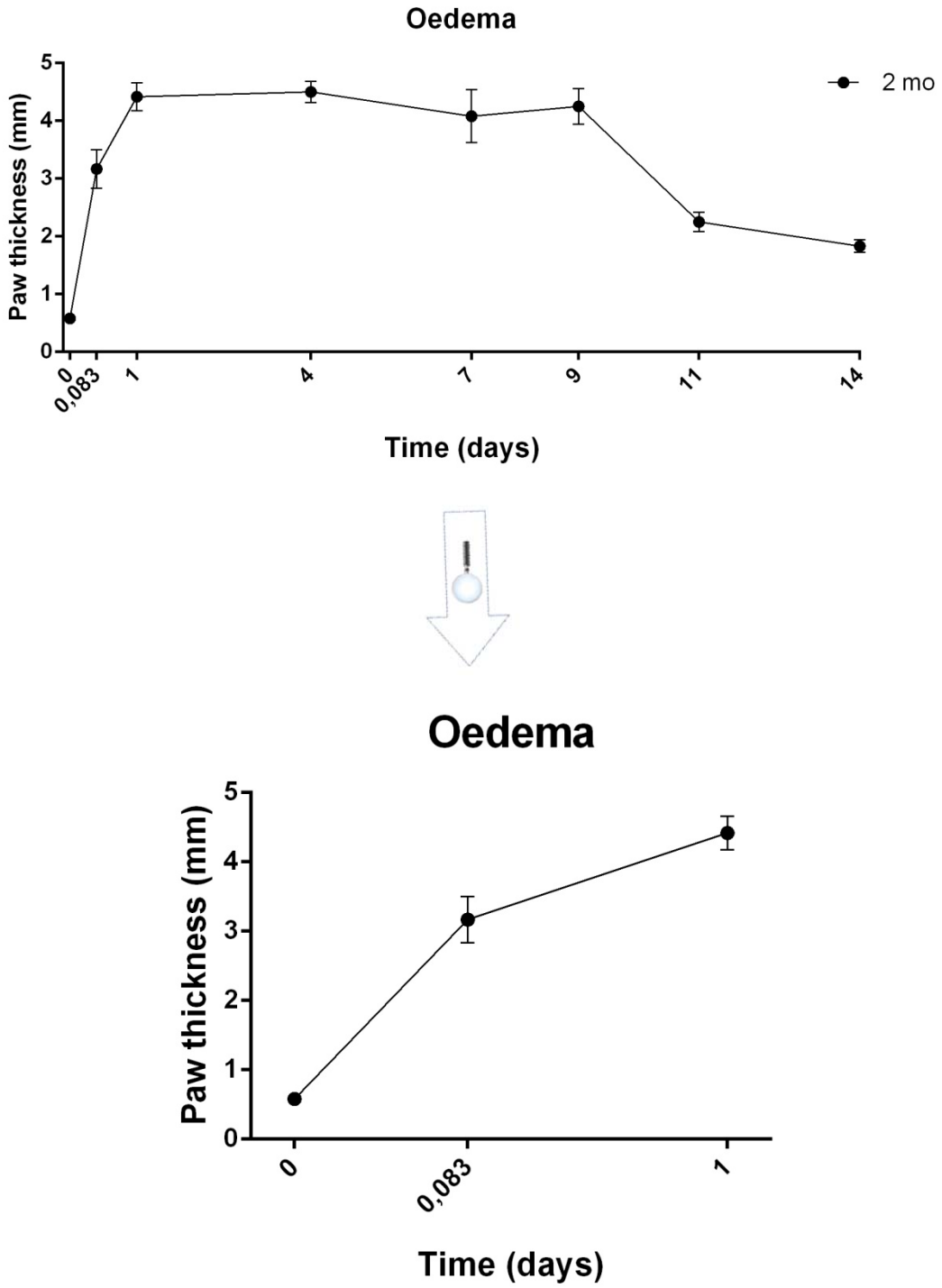


Figure 4.3. Formalin-induced oedema in 2 month-old C57BL/6 mice

Two hours after formalin administration, the mice subjected to the paw thickness measures showed an increase of odema. This oedema size increased progressively over the 1st day. The established thickness level began to decrease from the 11th day. Data are expressed as mean ± SEM of hindpaw thickness**.** *p* values < 0.05 were considered statistically significant. For iconographic reasons, the time points interval between 0

and 1is enlarged compared with the real dimensions: an enlargement is shown. 0,083 day represents the time point of two hours after the *formalin test*. (2 month-old mice: n=6).

For a better comparison of the oedema changes over time, the most relevant time points have been selected and represented in Figure 4.4.

Figure 4.4.

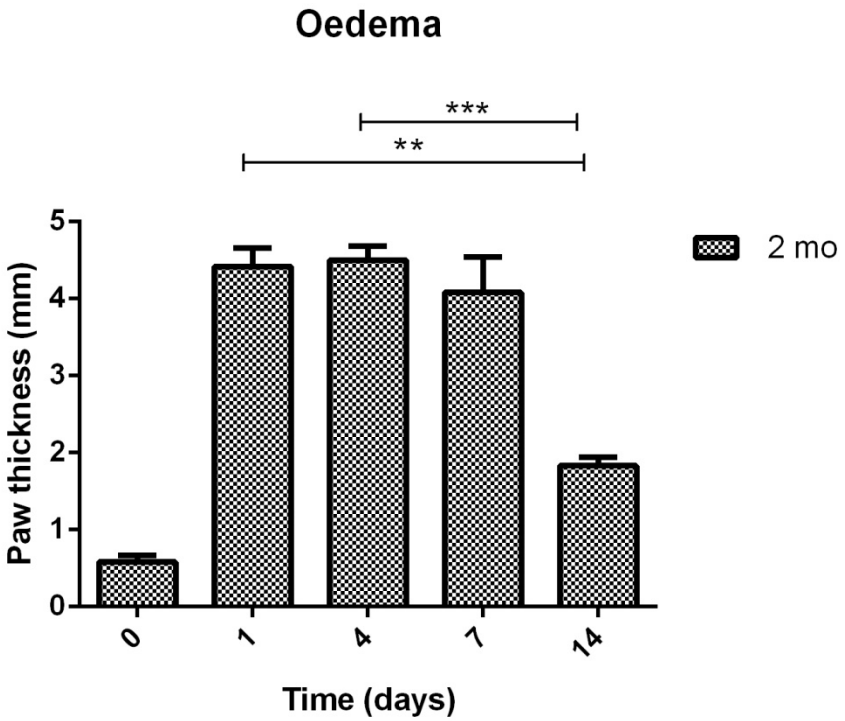


Figure 4.4. Formalin-induced oedema in 2 month-old C57BL/6 mice: the most remarkable time points. The oedema size of 2 month-old C57BL76 mice subjected to the *formalin test* increased on the 1st-4th day. The established thickness level began to decrease in the following days (11th -14th day). Data are expressed as mean ± SEM of hindpaw thickness**.** *p* values < 0.05 were considered statistically significant. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: ***. 0,083 day represents the time point of two hours after the *formalin test*. (2 month-old mice: n=6).

Table 4.1.

STATISTIC SIGNIFICANCE			
0 vs 1 0 vs 4 0 vs 7 0 vs 14	*** *** *** ***	1 vs 14 4 vs 14 7 vs 14	*** *** ***

Table 4.1. Statistical analysis of all the time points of the formalin-induced oedema measure in 2 month-old C57BL/6 mice. *p* values < 0.05 were considered statistically significant. 0,083 day represents the time point of two hours after the *formalin test*. Only the statistically significant points are shown. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: ***.

4.1.4. Gabapentin is effective on the *licking/biting/flinching* behaviour in the *Formalin test*

Gabapentin is an anticonvulsant that was synthesized as a structural analog of the γ- aminobutyric acid (GABA) (Yoon & Yaksh, 1999) and represents an adjunctive drug for the treatment of resistant partial seizures (Dixit *et al*., 1999). Some anticonvulsants, such as gabapentin, have a neuromodulatory effect on pain perception mechanisms (Paudel *et al*., 2011), and so they are used in the treatment of chronic pain conditions. The effect of gabapentin was tested on 2 month-old mice subjected to *formalin test* (Dubuisson & Dennis, 1977) to identify the doses effective on this behaviour and that could be tested for their effectiveness also in reducing the later mechanical allodynia. Based on previous data (Rode *et al*., 2006; Paudel *et al*., 2011; Miranda *et al*., 2013; Taneja *et al*., 2014), the dose of 100 mg/Kg was selected and compared with a second 10-fold lower dose (10 mg/Kg) and a vehicle. Our results demonstrated that the high dose (100 mg/Kg) of gabapentin is effective both on the 1st and on the 2nd phase of formalin-induced *licking/biting/flinching* behaviour. In particular, it resulted much more active on the 2nd phase than on

the 1st phase. The effectiveness of gabapentin was identifiable also on the intermediate phase. On the contrary, the low dose did not display the same efficacy since the only effect could be seen on the 2nd phase and it resulted very poor in reducing pain behaviour when compared with the high dose effectiveness. The results are shown in figure 4.5. Therefore, the obtained results demonstrate that gabapentin is as effective in the *formalin test* as it is in chronic pain models induced, for instance, by peripheral nerve injury, like the Spinal Nerve Ligation (SNL) (Kim & Chung, 1992).

Figure 4.5.

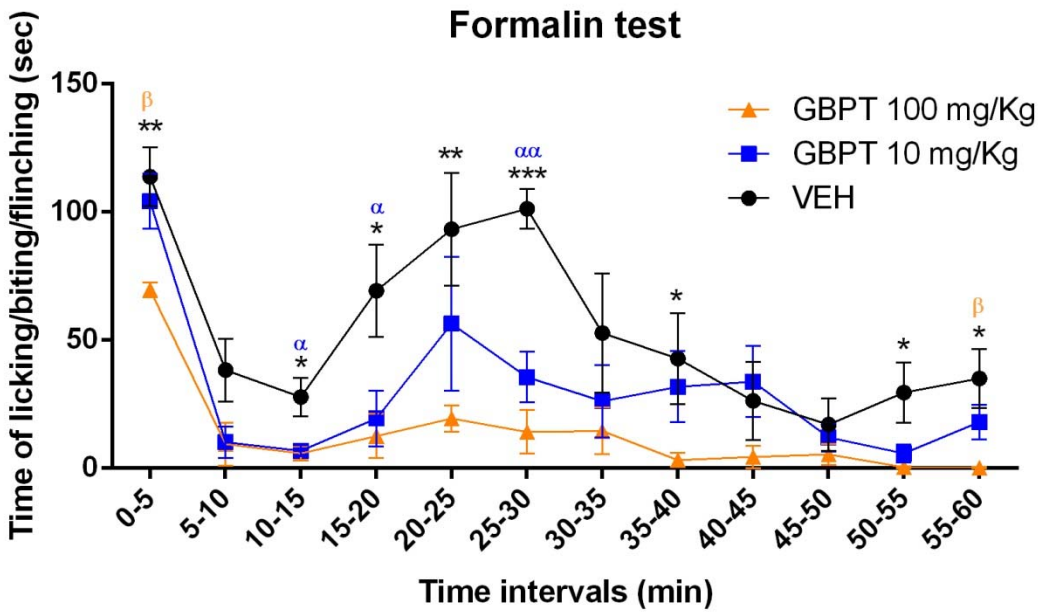


Figure 4.5. Effect of gabapentin on 2 month-old mice subjected to the *formalin test*. The high dose (100 mg/Kg) of gabapentin is effective both on the 1st and on the 2nd phase of formalin-induced *licking/biting/flinching* behaviour (much more effective on the 2nd than on the 1st phase). It resulted effective also on the interphase. Data are expressed as mean ± SEM of the nociceptive reaction**.** *p* values < 0.05 were considered statistically significant. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: ***. (VEH (vehicle) n=4; GBPT (gabapentin) 10 n=5; GBPT (gabapentin) 100 n=5). For the statistical analysis: *= VEH vs GBPT 100; **α**= VEH vs GBPT 10; **β**= GBPT 10 vs GBPT 100.

4.1.5. The total events of licking/biting/flinching shown by 2 month-old C57BL/6 mice in the *Formalin test* are markedly reduced after treatment with gabapentin

The nociceptive response to the *formalin test* (Dubuisson & Dennis, 1977) was represented also as total of events of *licking/biting/flinching*. This representation is very useful to understand the effectiveness of a pharmacological treatment in each phase of the formalin test, even on the 3rd phase of formalin test. During this phase, going from 45 to 60 min after formalin administration, the events of *licking/biting/flinching* weaken and the animal begins to recover the basal behaviour. The obtained data showed the effectiveness of gabapentin 100 mg/Kg both on the 1st and, mainly, on the 2nd phase of the *formalin test*. This dose of gabapentin resulted effective even on the interphase and on the 3rd phase of recovery (Figure 4.6.).

Figure 4.6.

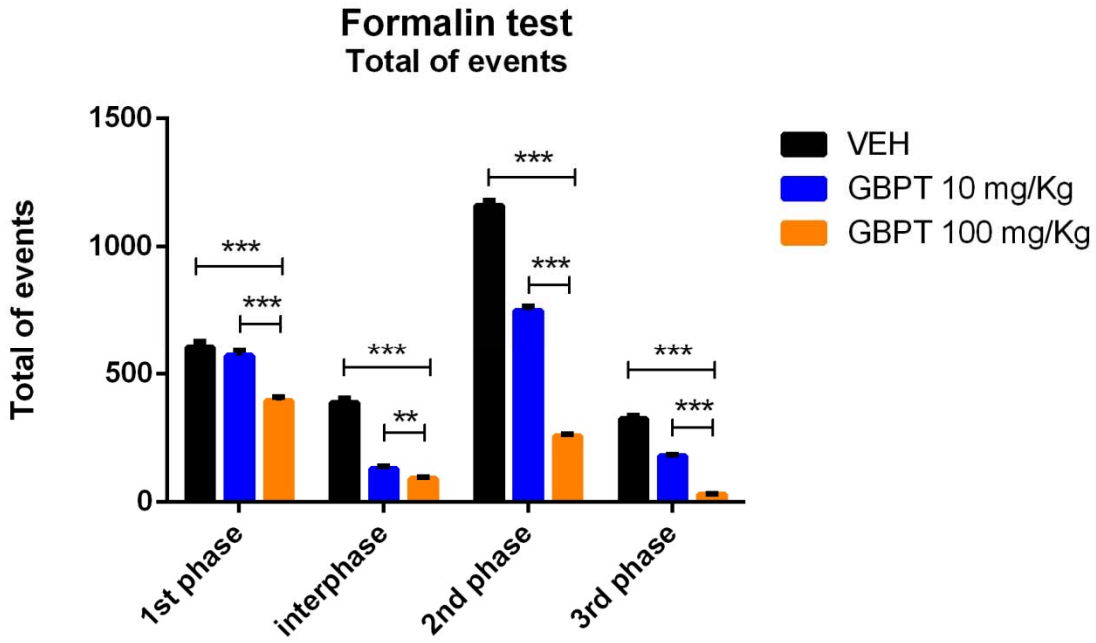


Figure 4.6. Effect of gabapentin on 2 month-old mice subjected to the *formalin test*: total of events of *licking/biting/flinching*. The high dose (100 mg/Kg) of gabapentin resulted effective on each phase of the *formalin test*. Data are expressed as mean ± SEM of the nociceptive reaction assessed as total of events of *licking/biting/flinching*. *p* values < 0.05 were considered statistically significant. *p* values < 0.05 were considered statistically significant. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: ***. (VEH (vehicle) n=4; GBPT (gabapentin) 10 n=5; GBPT (gabapentin) 100 n=5).

The correspondence between each phase of the *formalin test* and the real time points is shown in Table 4.2.

Table 4.2.

PHASES OF THE *FORMALIN TEST*	
1st phase	0-10 min
interphase	10-20 min
2nd phase	20-40 min
3rd phase	45-60 min

Table 4.2. Phases of the *formalin test*

4.1.6. Gabapentin is effective on the formalin-induced mechanical allodynia

The formalin injection induces a state of long-lasting mechanical allodynia that reaches its maximum on the 4th day: the effectiveness of gabapentin was also evaluated on this allodynia developing after the formalin test (Dubuisson & Dennis, 1977). Gabapentin was administered intraperitoneally 15 min before formalin, at two different doses (10 and 100 mg/Kg) and compared with vehicle, resulted effective on formalin-induced mechanical allodynia assessed through the *Von Frey's test* (Figure 4.7.). With a 5-6 hours half life, gabapentin, which does not bind plasmatic proteins, reaches the Tmax in 2-3 hours. Therefore, on the 1st and on the 4th day following formalin

administration, its effect resulted used up. The recovery of the basal sensitivity was slower for the ipsilateral hindpaw most probably because of the absence of a direct peripheral insult (formalin injection) at the contralateral side. The effect of gabapentin on the persistent allodynic state induced by formalin injection suggests that also the *formalin test* shares some typical features of more classical chronic pain models.

Figure 4.7.

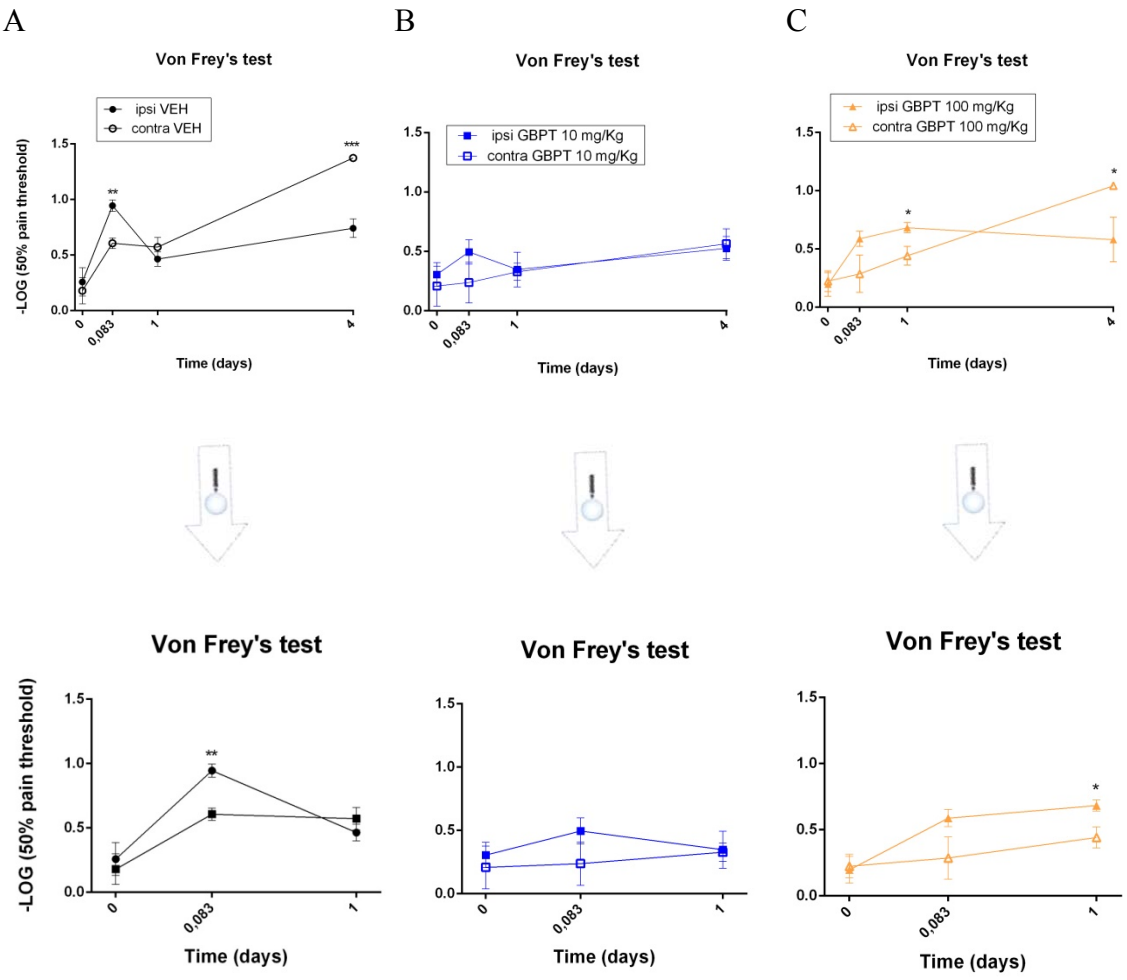


Figure 4.7. Effect of gabapentin on formalin–induced mechanical allodynia in 2 month-old C57BL/6 mice. Gabapentin (GBPT) resulted effective on formalin-induced mechanical allodynia assessed through the *Von Frey's test*. Data are

expressed as -LOG of the mean ± SEM of the 50% pain threshold. *p* values < 0.05 were considered statistically significant. *p* values < 0.05 were considered statistically significant. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: ***. For iconographic reasons, the time points interval between 0 and 1is enlarged compared with the real dimensions: an enlargement is shown. 0,083 day represents the time point of two hours after the *formalin test*. (VEH (vehicle) n=4; GBPT 10 n=5; GBPT 100 n=5.)

The statistical analysis of the time course data (Figure 4.7.) are shown in Table 4.3.

Table 4.3.

STATISTIC SIGNIFICANCE			
0,083 ipsi VEH vs contra VEH	**	0,083 ipsi VEH vs ipsi GBPT 10	**
4 ipsi VEH vs contra VEH	***	0,083 ipsi VEH vs ipsi GBPT 100	**
1 ipsi GBPT 100 vs contra GBPT 100	*	1 ipsi VEH vs ipsi GBPT 100	*
4 ipsi GBPT 100 vs contra GBPT 100	*	4 contra VEH vs contra GBPT 10	***
		4 contra VEH vs contra GBPT 100	***
		4 contra GBPT 10 vs contra GBPT 100	**

Table 4.3. Effect of gabapentin on formalin–induced mechanical allodynia in 2 month-old C57BL/6 mice: statistical analysis of all time points. *p* values < 0.05 were considered statistically significant. 0,083 day represents the time point of two hours after the *formalin test*. Only the statistically significant points are shown. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: ***.

4.2. CHAPTER 2: INFLUENCE OF AGEING ON BASAL NOCICEPTION AND ON NOCEIFENSIVE BEHAVIOUR

Aged population is ever-increasing thanks to the progress of modern medicine in the last 150 years and the improved life standards have been fostering life expectancy increase (Scully, 2012). Therefore, the ageing-related research is gainig increasing interest and is becoming more and more complex, acquiring scientific rigor (Grayson, 2012). When people live long enough, they are more likely to develop long-term conditions (Abdulla *et al*., 2013), such as chronic pain. Furthermore, the changes occurring in nociception with age have not been well understood yet (Taguchi *et al*., 2010). This PhD project aimed at understanding the effects of ageing on mechanical and thermal sensitivity and on the development and maintainance of a condition of pain that can be assimilated to a clinically relevant state in C57BL/6 mice. In order to achieve this purpose, behavioural tests to assess mechanical and thermal sensitivity were performed on C57BL/6 mice belonging to an interval of ages that covers a remarkable segment of the whole human life from young to old adult. First, these measures were made in basal conditions to establish the basal sensitivity levels both to mechanical and to thermal (heat and cold) stimuli. Mechanical and thermal sensitivity seemed to increase with age. The variations of mechanical sensitivity were clearer than the changes of thermal sensitivity. The response threshold to mechanical stimuli, assessed through the *Von Frey's test* (Chaplan *et al*., 1994) and the *Pin-prick* test (Chan *et al.,* 1992), gradually lowered from 2 to 6, 12 and 18 month-old mice. The threshold of thermal sensitivity to heat, evaluated using the *Hargreaves'test* (Hargreaves *et al*., 1988), was subjected to a reduction with the increased age of the animal, following the same trend of mechanical sensitivity. The first evident increase of cold sensitivity, assessed through the acetone test (Choi *et*

al., 1994), could be seen already at 6 months of age. Between the age of 12 and the one of 18 months, the sensitivity reached a sort of *plateau*, increasing in a less pronounced manner. Longitudinal studies performed on young (1 month-old), young adult (6 month-old) and adult (12 month-old) mice confirmed that groups of young and young adult mice that get older present an increase in mechanical and thermal sensitivity as older mice. Later 2, 6, 12, 18 month-old mice underwent the *Formalin test* (Dubuisson & Dennis., 1977) since this test has become very used during the past years because it is not so invasive as surgical experimental pain models and provides valid reliable information with a short time expense. Indeed, we decided to subject four different age-groups mice (2, 6, 12, 18 months) to the *formalin test*, based on our previous results (shown in chapter 1) and according to literature (Abram & Yaksh, 1994; Ji *et al*., 1999; Lebrun *et al*., 2000; Fu *et al*., 2001; Lin *et al*., 2007; Guida *et al*., 2012) suggesting this model to represent a very useful chronic pain model. The obtained results showed that the nocifensive behaviour induced by the *formalin test* is affected by ageing process. The 2 months mice responded classically to the *formalin test,* presenting a 1st (5-10 min) and a 2nd phase (20-30 min). On the contrary, adult (12 month-old) and older (18 month-old) mice showed a shift of the time spans, with no more only 2 but 3 peak phases. These 3 peaks differ both for height and for amplitude between younger and older animals. Six month-old mice nocifensive behaviour resulted quite intermediate. In order to be able to detect all the ageing-prompted modifications of the *formalin test*, the nociceptive behaviour was expressed as total of events of *licking/biting/flinching*. Formalin injection induces central sensitization phenomena, responsible for the *formalin test* 2nd phase, and the development of tactile allodynia lasting at least for about 14 days and reaching its peak about 3 days after the execution of *formalin test*. Therefore, mechanical allodynia was monitored in the C57BL/6 mice subjected to *formalin test* 2 hours after the s.c. formalin

administration and then on the following days: 1st, 4th, 7th, 9th, 11th, 14th. The ageing process resulted to affect formalin-induced allodynic state: mechanical allodynia was shown already two hours following the formalin administration in the hindpaw ipsilateral to the insult. The older mice presented a higher level of mechanical allodynia than the 2 months mice. This difference likely is due to the diverse basal threshold of these mice according to their age. This threshold kept to increase on the 1st day gradually from 2 to 6, 12 and 18 months mice. The peak of allodynia was reached on the 4th day in all the four groups but, on the following days, while in the mice of two months of age the recovery of the basal sensitivity level was beginning, the mechanical allodynia remained stable at the maximum level. Interestingly, on the 4th day, even the contralateral hindpaw showed mechanical allodynia levels comparable to the levels of tactile allodynia of the ipsilateral hindpaw. In the 6, 12 and 18 months mice the mechanical threshold resulted much higher than the basal one up to 14 days with a much slower recovery than the 2 month-old mice. Even the healing process seemed to undergo modifications with the age. Indeed, all of the mice subjected to formalin test developed a lesion in correspondence of the point of injection of formalin but older mice showed this lesion at an earlier time point (7th day following the *formalin test*) if compared with 2 month-old mice (9th day) and this cutaneous manifestation was more extended expecially in 12 and in 18 month-old mice. Because of the development of such lesion, the *Von Frey's test* was performed only on the contralateral hindpaw. Moreover, the degree of oedema of the hindpaw subcutaneously injected with formalin was measured to verify whether paw thickness and recovery process from oedema were influenced by ageing. These measures were made on the same time points in which the assessment of mechanical allodynia was performed. The decrease of paw thickness to the basal level resulted quicker in the 2 months mice than in the aged mice. Since we observed this decrease of pain threshold during ageing, we decided to

investigate the molecular basis of this phenomenon. For this purpose, the main autophagic markers were analyzed in 3 groups of mice of different age: 2, 6 and 12 months. No major differences were observed in levels of the autophagic proteins LC3-I, LC3-II and p62, while only Beclin 1 expression resulted progressively reduced with ageing in a statistically significant way. Although further functional experiments are needed, these results seem to suggest that the autophagic pathway is modificated and that autophagic flux is possibly reduced. This could mean that, during ageing process, a dysregulation of autophagy may occur and be involved in the increased nociceptive response observed in aged mice. Even in the SNL neuropathic pain model, although in a different way, the autophagic process was demonstrated to be dysregulated (Berliocchi *et al*., 2011). Since the voltage-dependent calcium channel of L type subunit α2δ-1 is up-regulated in the DRGs (Dorsal Root Ganglia) and in the spinal cord of animals suffering from allodynia in several chronic pain models (Kim *et al*., 2001; Luo *et al*., 2002), it represents the main marker of chronic pain. For this reason, the expression of α2δ-1 was analyzed in the spinal cord of 2, 6 and 12 months mice. While α2δ-1 was almost absent in 2 months mice, in agreement with their basal sensitivity levels, it resulted remarkably overexpressed in the older mice. In particular, the 6 month-old mice showed the highest up-regulation of the subunit α2δ-1. Therefore, it was interesting to highlight a bell-shaped trend of α2δ-1 expression with an almost absent expression in the 2 month-old mice and the peak at 6 months of age. The 12 months mice showed a slightly inferior level of expression, when compared to the 6 months mice. The up-regulation of the levels of the Ca^{2+} channel subunit α2δ-1 might explain the lowered mechanical threshold shown by the older mice and the underlined bell-shaped trend of expression could be at the root of the *plateau* of sensitivity detected in the aged mice. Moreover, a very interesting aspect was the appearance of a low molecular weight band (25-17kDa), named α2δ-1* to

be distinguished from α2δ-1, recognised by the anti α2δ-1 antibody. This band appeared highly expressed in 6 and 12 month-old mice, in comparison to 2 month-old mice; it reached a peak around 6 months of age with a trend of expression similar to the classical α2δ-1. There are several known α2δ subunits but, because of its very low molecular weight, α2δ-1* could be either a little subunit only expressed in old mice or a cleavage product occurring during ageing. In both cases, it will be relevant to further investigate its nature and involvement in the increased mechanical sensitivity shown by aged mice. Then, it was decided to investigate the effect of gabapentin, one of the pharmacological treatments of choice for chronic pain (Owen, 2007), on 2 and 6 month-old mice in order to understand whether it were effective on the formalin-induced mechanical allodynia in both of the ages and how this effect could undergo modifications according to age. Gabapentin was administered intraperitoneally in two different doses (10 and 100 mg/Kg) established according to literature, to highlight if the two doses were both effective, and this treatment was compared to the vehicle-treated group. Indeed, it was evaluated whether the effectiveness of gabapentin changed with ageing and different doses. This is a very important aspect since gabapentin, together with the antidepressants, represents one of the drugs of choice for the treatment of chronic pain (Moore *et al.*, 2009, 2011), expecially experienced by older adults. The obtained results demonstrated that, in the 2 month-old mice subjected to the *formalin test* (Dubuisson & Dennis, 1977) only the 100 mg/Kg dosage was effective in all of the phases; the 10 mg/Kg dose of gabapentin resulted slightly active on the 2nd phase only. On the contrary, both the doses resulted effective in reducing *licking/biting* and *flinching* in the 6 month-old mice. Moreover, interestingly, as shown in the graphs plotting the total of events of *licking/biting/flinching,* in the 1st phase, in the interphase and in the 3rd phase the dose of gabapentin of 10 mg/Kg presented the same efficacy as the dose of 100 mg/Kg. Only in the 2nd phase, the dose of 100

mg/Kg was more effective than the 10 mg/Kg dose. Later, it was decided to examine the effect of gabapentin on formalin-induced mechanical allodynia in 2 and 6 month-old C57BL/6 mice. In the 2 months group, the treatment with gabapentin resulted effective 2 hours after the execution of the *formalin test*. On the 1st day and on the 4th day, the effect could result used up, because of the drug half life. In the 6 months group, no significant variation between the mechanical threshold of the vehicle-treated and of the drug-treated mice was observed. This could suggest that, in older mice, even a lower dose of gabapentin is sufficient to exert a therapeutic effect but that this effect lasts for a shorter period than in 2 month-old mice. Finally, in order to point out the effectiveness of a single acute administration of gabapentin on formalin-induced allodynic states, gabapentin (10 and 100 mg/kg) was administered 30 min prior to the assessment of mechanical allodynia through the *Von Frey's test* (Chaplan *et al*., 1994), after a previous determination of the level of allodynia in 2 and 6 month-old C57BL/6 mice. This evaluation was carried out on the 4th day after the *formalin test*, since this is the time point in which allodynia peaks and the drug-treated groups were compared to a vehicle-treated group. Both age-groups treated with 10 mg/Kg gabapentin showed reduced allodynia of the hindpaw contralateral to the algogen stimulus. In particular, the 10 mg/Kg dose of gabapentin resulted more effective in the 6 month-old mice compared to the 2 month-old mice. These results concur with the central nature of the mechanisms that fostered the development of formalin-induced tactile allodynia also in the contralateral hindpaw. By contrast, this reduction of allodynia was not detected in the ipsilateral hindpaw and this can be due to the peripheral insult received by this paw. Two and six month-old mice treated with gabapentin 100 mg/Kg presented a decrease of mechanical allodynia both of the ipsilateral and of the contralateral hindpaw and also the 100 mg/Kg dose of gabapentin resulted more effective in the 6 month-old mice than in the 2 month-old mice.

Therefore, our results demonstrate that the effectiveness of gabapentin on the nocifensive response to the *formalin test* and on the formalin-induced mechanical allodynia is affected by the ageing process and this is noteworthy since it could represent a clue to study a novel therapeutic approach and treatment regimen for the management of chronic pain in the elderly.

4.2.1. Basal mechanical sensitivity in C57BL/6 mice belonging to different age groups

In order to unravel the role of ageing process on perception and transmission of mechanical stimuli, C57BL/6 mice of 2, 6, 12 and 18 months of age were subjected to behavioural tests for the evaluation of mechanical sensitivity (*Von Frey's test* (Chaplan *et al*., 1994) and *Pin-prick* test (Chan *et al.,* 1992)). The *Von Frey's test* was used to assess mechanical allodynia and the *Pin-prick* test (Chan *et al.,* 1992) was performed for the assessment of hyperalgesia. This tests were carried out at regular intervals of 4/6 weeks. Our results suggest that ageing influences mechanical and thermal nociception and, in particular, aged animals showed a reduced pain threshold indicative of an increased level of mechanical sensitivity (Figure 4.8.).

Figure 4.8.

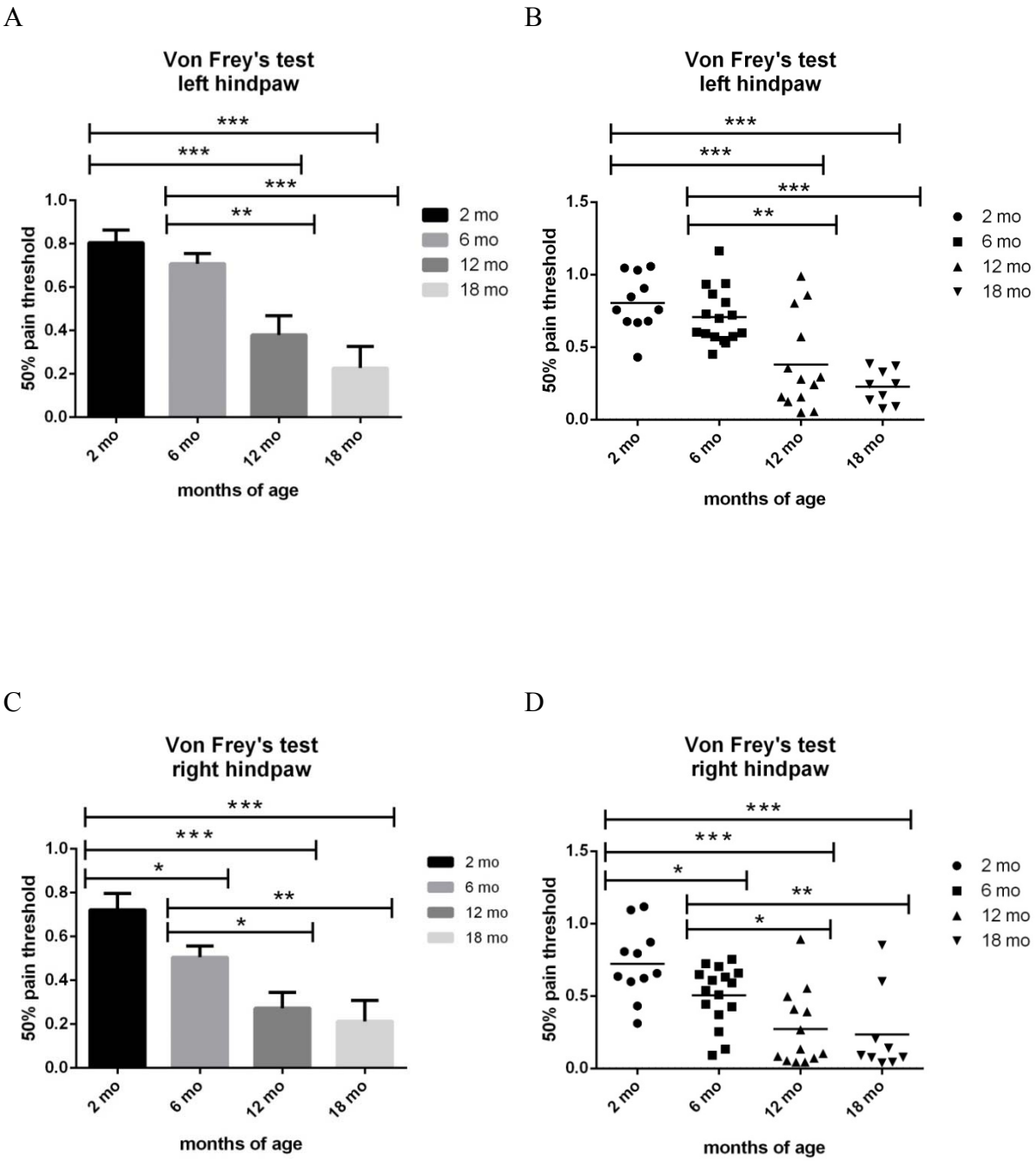


Figure 4.8. Ageing process prompts an increase in basal mechanical sensitivity in C57BL/6 mice. The response threshold to innocuous mechanical stimuli gradually lowered from 2 to 6, 12 and 18 month-old mice. Mechanical sensitivity was assessed in C57BL/6 mice *via Von Frey's test* (Chaplan *et al*., 1994) (bar graphs in fig. 4.8. A-C). Graphs in fig. 4.8. B-D show the distribution within each experimental group. Data are expressed as mean ± SEM of the 50% pain threshold. *p* values < 0.05 were considered statistically significant. *p* values < 0.05 were considered statistically significant. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: ***. (2 month-old mice: n=22; 6 month-old mice n=16; 12 month-old mice n=13; 18 month-old mice n=9).

The behavioural data suggest that basal mechanical sensitivity increased progressively with the ageing of the animal. The threshold of response to innocuous mechanical stimuli lowered progressively lowered from 2 to 6 and 12 month-old mice reaching a threshold that was kept up to 18 months. This is also shown by the homogeneous distribution within each age group as plotted in figure 4.8. B-D.

As basal mechanical allodynia, even the levels of basal hyperalgesia seemed to increase proportionally to the increase of age of the mice subjected to the test. According to the obtained results, the duration of the nociceptive response to painful mechanical stimuli decreased from 2 month-old mice to 6, 12 and 18 month-old mice (Figure 4.9.).

Figure 4.9.

A B

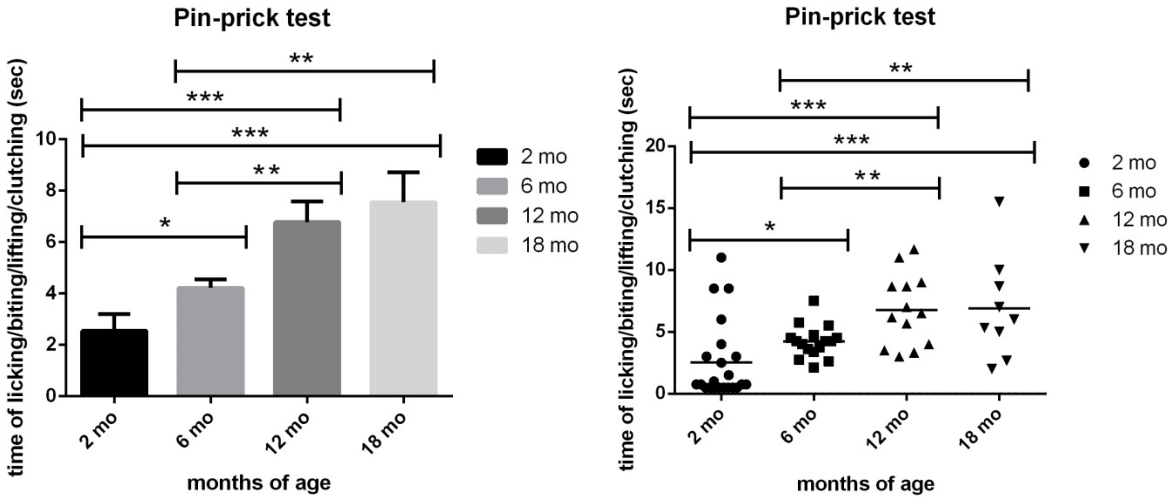


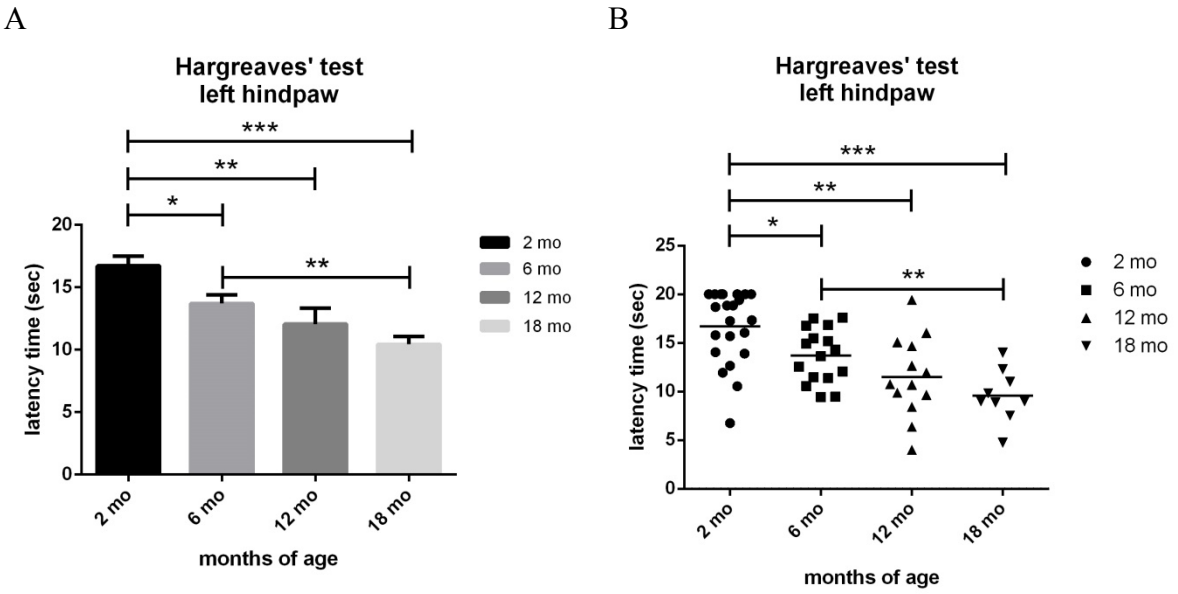
Figure 4.9. Ageing process causes an increase in basal mechanical sensitivity in C57BL/6 mice. The response threshold to painful mechanical stimuli progressively decreased from 2 to 6, 12 and 18 month-old mice. Mechanical sensitivity was assessed in C57BL/6 mice *via Pin-prick* test (Chan *et al.,* 1992) (bar graphs in fig. 4.9. A). Graphs in fig. 4.9. B show the distribution within each experimental group. Data are expressed as mean ± SEM of the duration of nociceptive reaction. *p* values < 0.05 were considered

statistically significant. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: ***. (2 month-old mice: n=22; 6 month-old mice n=16; 12 month-old mice n=13; 18 month-old mice n=9).

4.2.2. Basal thermal sensitivity in C57BL/6 mice belonging to different age groups

Based on the obtained results, also the basal thermal sensitivity, both to hot and to cold stimuli, underwent modifications during ageing of C57BL/6 mice. Indeed, the latency time following the exposition of the hindpaw of the mouse to a heat source ((*Hargreaves'test* (Hargreaves *et al*., 1988)) resulted reduced with the increased age of the animal, as shown in figure 4.10.

Figure 4.10.



C D

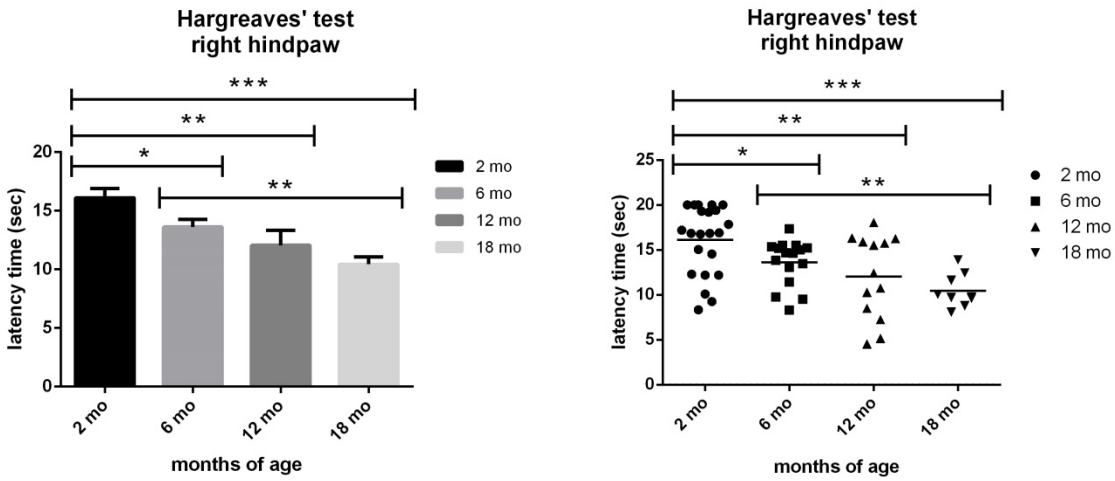


Figure 4.10. Ageing process causes an increase in basal thermal sensitivity in C57BL/6 mice. Thermal sensitivity to heat, evaluated using the Hargreaves'test (Hargreaves *et al*., 1988), was subjected to an increase with the increased age of the animal. Thermal sensitivity was assessed in C57BL/6 mice *via Hargreaves' test* (Hargreaves *et al*., 1988) (bar graphs in fig. 4.10. A-C). Graphs in fig. 4.10. B-D show the distribution within each experimental group. Data are expressed as mean ± SEM of the latency time. *p* values < 0.05 were considered statistically significant. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: ***. (2 month-old mice: n=22; 6 month-old mice n=16; 12 month-old mice n=13; 18 month-old mice n=9).

The increase in the duration of the nociceptive reaction to cold stimuli assessed through the acetone test (Choi *et al*., 1994) showed an increased perception and response to a cold stimulation in older age groups. The first amplification of the response to cold stimuli was seen already at 6 months of age.

Figure 4.11.

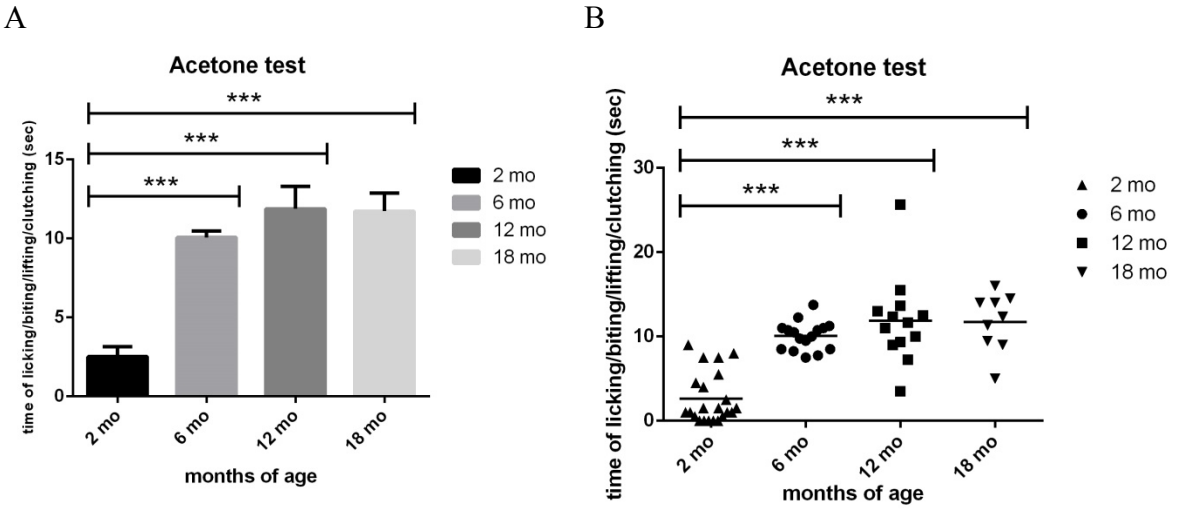


Figure 4.11. Ageing process provokes an increase in basal thermal sensitivity in C57BL/6 mice. Investigating the cold sensitivity, an increased perception of cold stimuli was detected in older age groups. Thermal sensitivity was assessed in C57BL/6 mice *via* acetone test (Choi *et al*., 1994) (bar graphs in fig. 4.11.A). Graphs in fig. 4.11. B show the distribution within each experimental group. Data are expressed as mean ± SEM of the duration of nociceptive reaction to cold stimuli. p values < 0.05 were considered statistically significant. p values < 0.03: *; p values < 0.002: **; p values < 0.001: ***. (2 month-old mice: n=22; 6 month-old mice n=16; 12 month-old mice n=13; 18 month-old mice n=9).

The means distribution graphs underline the consistency of changes in mechanical and thermal sensitivity induced by ageing process. Proceeding from 12 to 18 months of age, both mechanical and thermal sensitivity reached a sort of *plateau*. The first evident increase of cold sensitivity could already be seen at 6 months of age (Figure 4.12.).

Figure 4.12.

A

1. Allodynia
2. Hyperalgesia
3. Heat sensitivity
4. Cold sensitivity

B

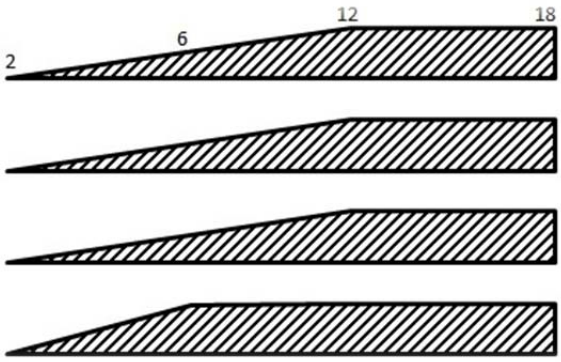


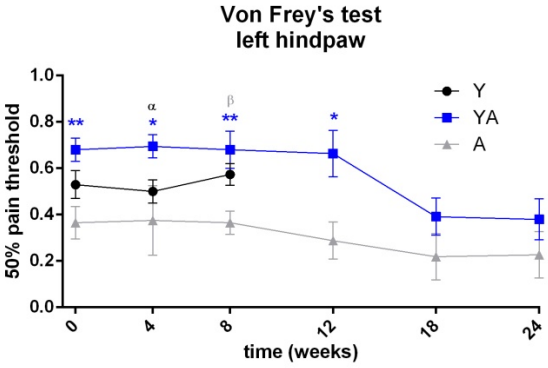
Figure 4.12. Evolution of basal sensitivity during ageing. Basal mechanical and thermal sensitivity of C57BL/6 mice increased while passing from 2 to 6 and 12 months of age, reaching a peak up to 18 months. The peak of cold sensitivity was reached already at 6 months of age.

4.2.3. Progressive change of the basal mechanical sensitivity within a same population over time: longitudinal studies

The behaviour (*Von Frey's* test (Chaplan *et al*., 1994) and *Pin-prick* test (Chan *et al.,* 1992)) of a group of Young mice (Y, 1 month-old), Young Adult mice (YA, 6 month-old) and Adult mice (A, 12 month-old) was followed at regular intervals of 4/6 weeks for a time course of 24 weeks to assess whether Young, Young Adult mice and Adult mice developed the same modifications of mechanical sensitivity that characterize the pain threshold of Older mice (18 month-old). It resulted that groups of Young, Young Adult mice and Adult mice that get older present an increase in mechanical sensitivity, assessed through the *Von Frey's test* (Chaplan *et al*., 1994) (Figure 4.13.) and through the *Pin-prick* test (Chan *et al.,* 1992) as Older mice (Figure 4.14.).

Figure 4.13.

A



B

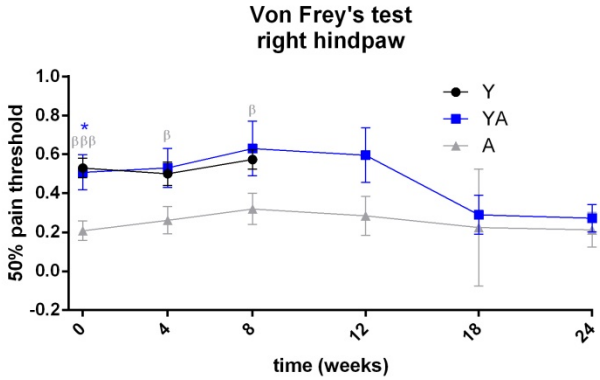


Figure 4.13. Time course of mechanical sensitivity levels in C57BL/6 mice assessed through *Von Frey's test* at 4/6 weeks intervals . During ageing, Young, Young Adult and Adult mice displayed the increase in mechanical sensitivity shown by Older mice. Graphs in fig.4.13. A show the left hindpaw mechanical sensitivity, while graphs in fig.4.13. B show the right hindpaw mechanical sensitivity. Data are respectively expressed as mean ± SEM of the 50% pain threshold**.** *p* values < 0.05 were considered statistically significant. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: *** (Y, 1 month-old mice: n=22; YA, 6 month-old mice n=13; A, 12 month-old mice n=10). For the statistical analysis:
*= YA vs A; **α**= Y vs YA; β= Y vs A.

Figure 4.14.

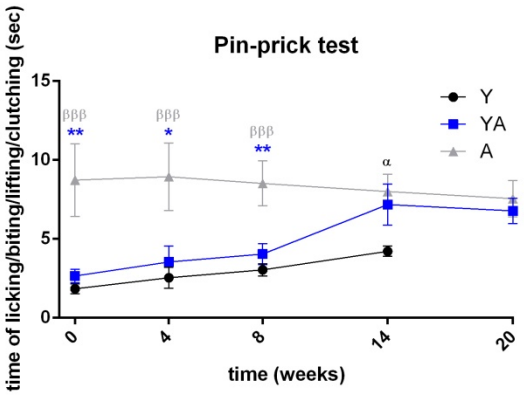


Figure 4.14. Time course of mechanical sensitivity levels in c57bl/6 mice assessed through *Pin-prick* test at 4/6 weeks intervals. During ageing, mechanical sensitivity of Young, Young Adult and Adult mice increased as it occurred for Older mice. Data are respectively expressed as mean ± SEM of the duration of nociceptive reaction**.** *p* values < 0.05 were considered statistically significant. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: *** (Y, 1 month-old mice: n=22; YA, 6 month-old mice n=13; A, 12 month-old mice n=10). For the statistical analysis: *= YA vs A; **α**= Y vs YA; β= Y vs A.

4.2.4. Longitudinal studies of the basal thermal sensitivity within a same population over time

The thermal sensitivity to hot (*Hargreaves'* test (Hargreaves *et al.*, 1988)) and to cold stimuli (acetone test ((Choi *et al.*, 1994)), Young mice (Y, 1 month-old), Young Adult mice (YA, 6 month-old) and Adult mice (A, 12 month-old) was followed at regular intervals of 4/6 weeks for a time course of 24 weeks. The obtained results suggest that, by ageing, Young, Young Adult and Adult mice developed increased thermal sensitivity recahing the same levels shown by Older mice (18 month-old) both to heat (Figure 4.15.) and to cold (Figure 4.16.).

Figure 4.15.

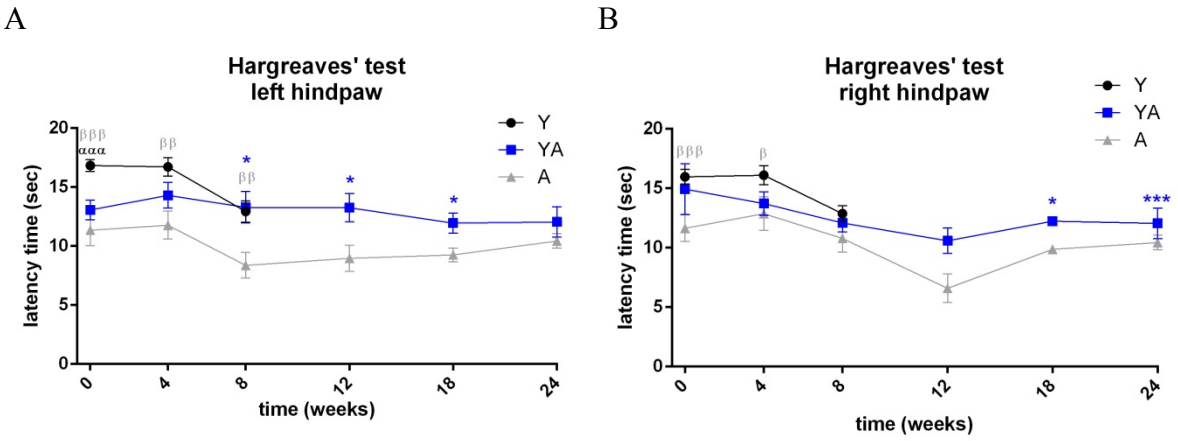


Figure 4.15. Time course of thermal sensitivity levels in C57BL/6 mice assessed through the *Hargreaves' test* at 4/6 weeks intervals . With age, Young, Young Adult and Adult mice presented the increase in thermal sensitivity to heat shown by Older mice. Graphs in fig.4.15. A show the left hindpaw thermal sensitivity, while graphs in fig.4.15. B show the right hindpaw thermal sensitivity. Data are respectively expressed as mean ± SEM of the 50% pain threshold. *p* values < 0.05 were considered statistically significant. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: *** (Y, 1 month-old mice: n=22; YA, 6 month-old mice n=13; A, 12 month-old mice n=10). For the statistical analysis: *= YA vs A; **α**= Y vs YA; β= Y vs A.

Figure 4.16.

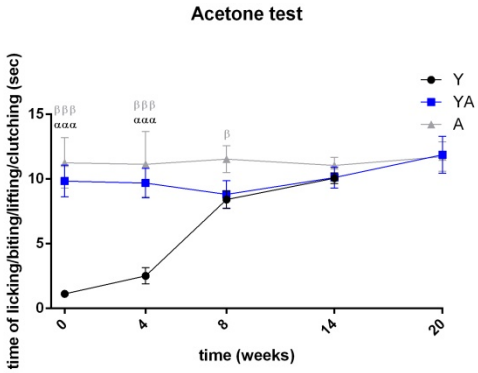


Figure 4.16. Time course of mechanical sensitivity levels in c57bl/6 mice assessed through the acetone test at 4/6 weeks intervals. During ageing, cold sensitivity of Young, Young Adult and Adult mice reached the increased levels shown by Older mice. Data are respectively expressed as mean ± SEM of the duration of nociceptive reaction**.** *p* values < 0.05 were considered statistically significant. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: *** (Y, 1 month-old mice: n=22; YA, 6 month-old mice n=13; A, 12 month-old mice n=10). For the statistical analysis: *= YA vs A; **α**= Y vs YA; β= Y vs A.

4.2.5. The duration and the intensity of the nociceptive response to *formalin test* in C57BL/6 mice are affected by ageing

To verify whether ageing could affect also the response to evoked pain, the response to an inflammatory algogen stimulus like formalin was studied in mice of 2, 6, 12 and 18 months. While 2 months mice showed the classic trend of *formalin test* characterized by a first (5-10 min) and a second phase (20-30 min), the curves of the groups of older mice showed a temporal shift with the appearance of an extra third peak and an increase in the peaks amplitude. Six month-old mice presented an intermediate behaviour. Our results are shown in figure 4.17.

Figure 4.17.

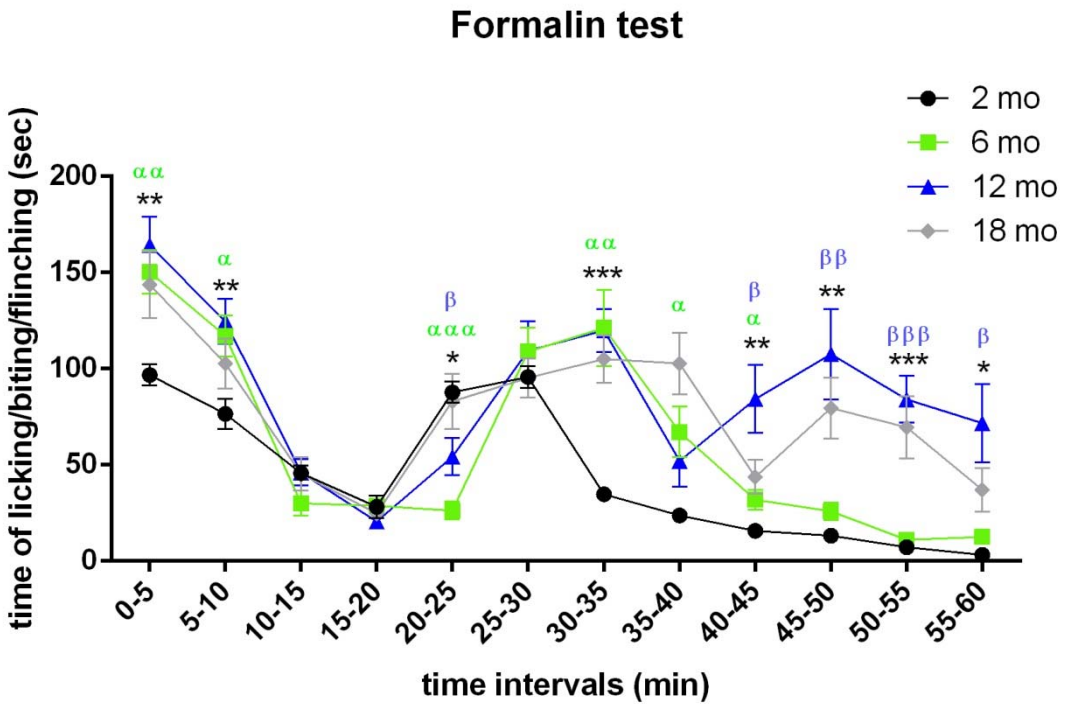


Figure 4. 17. Formalin test in C57BL/6 mice belonging to different age groups. Nociceptive reaction of C57BL/6 mice to formalin test, expressed as time of *licking/biting/flinching* is subjected to variations in its duration and intensity during ageing process. Data are expressed as mean ± SEM of the duration of nociceptive reaction characterized by *licking/biting* and *flinching***.** *p* values < 0.05 were considered statistically significant. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: ***. (2 month-old mice: n=6; 6 month-old mice n=9; 12 month-old mice n=9; 18 month-old mice n=9). For the statistical analysis: *= 2 mo vs 12 mo; **α**= 2 mo vs 6 mo; **β**= 6 mo vs 12 mo.

The statistical analysis of data displayed in Figure 4.17. is shown in Table 4.4.

Table 4.4.

STATISTIC SIGNIFICANCE			
20-25 6 mo vs 18 mo	**	45-50 2 mo vs 18 mo	**
30-35 2 mo vs 18 mo	***	45-50 6 mo vs 18 mo	**
35-40 12 mo vs 18 mo	*	50-55 6 mo vs 18 mo	**
35-40 2 mo vs 18 mo	**	50-55 2 mo vs 18 mo	**
40-45 2 mo vs 18 mo	*	55-60 2 mo vs 18 mo	*

Table 4.4. Formalin test in C57BL/6 mice belonging to different age groups: statistical analysis of all the time points. *p* values < 0.05 were considered statistically significant. 0,083 day represents the time point of two hours after the *formalin test*. Only the statistically significant points not present in figure 4.20. are shown. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: ***.

To better highlight the age-related changes observed in the *formalin test* (Dubuisson & Dennis, 1977), the nocifensive behaviour was analyzed as total of events of *licking/biting/flinching* during each phase (Figure 4.18.).

Figure 4.18.

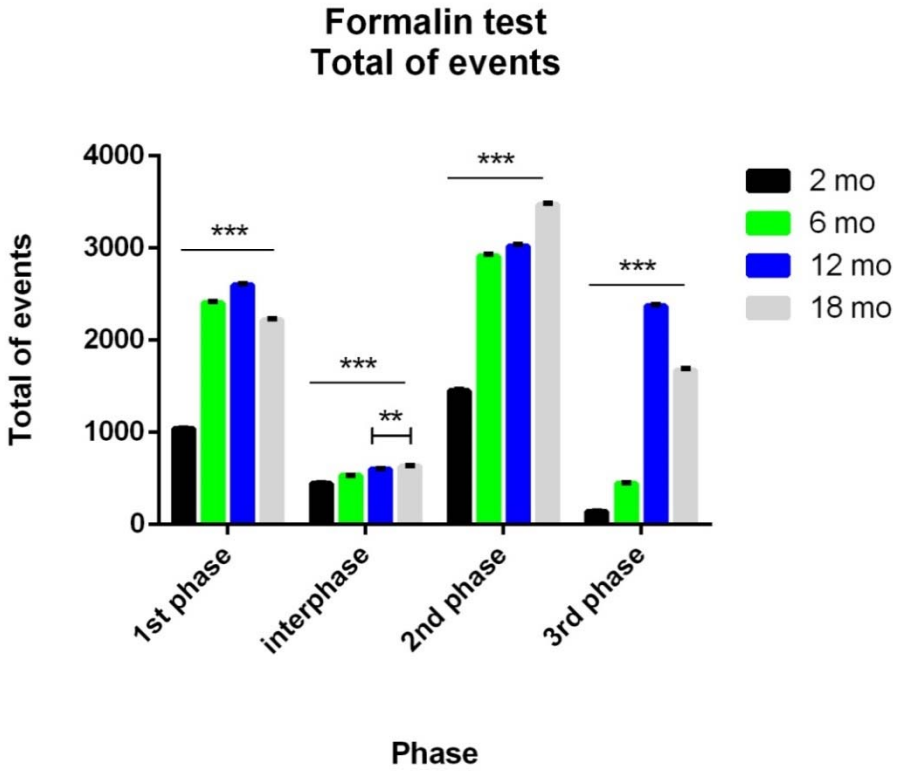


Figure 4.18. Formalin test in C57BL/6 mice belonging to different age groups
Nociceptive reaction of C57BL/6 mice to *formalin test*, analyzed and expressed as total of events of *licking/biting/flinching* is subjected to variations in its duration and intensity during ageing process. Data are expressed as mean ± SEM of the duration of nociceptive reaction characterized by *licking/biting* and *flinching*. *p* values < 0.05 were considered statistically significant. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: ***. In the statistical analysis, the line without direction boundaries represent the significance of all the bars of each phase. (2 month-old mice: n=6; 6 month-old mice n=9; 12 month-old mice n=9; 18 month-old mice n=9).

The correspondence of the time points with each phase of the *formalin test* is shown in table 4.5.

Table 4.5.

PHASES OF THE *FORMALIN TEST*	
1st phase	0-10 min
interphase	10-20 min
2nd phase	20-40 min
3rd phase	45-60 min

Table 4.5. Phases of the *formalin test*

4.2.6. Formalin administration induces mechanical allodynia in the ipsilateral and controlateral hindpaw up to 14 days

According to our results, ageing influences formalin-induced allodynic state. Groups of C57BL/6 mice of different age, already two hours after formalin administration, showed tactile allodynia of the ipsilateral hindpaw. The older mice presented a higher level of mechanical allodynia than the 2 months mice, likely also because of the different basal threshold of these mice. This allodynia increased on the 1st day progressively between 2 to 6, 12 and 18 months mice reaching the peak on the 4th day in all the four groups. On the 4th

day, even the contralateral hindpaw showed this mechanical allodynia. The allodynic state lasted up to 14 days with an initial decrease about the 11th day and a much slower recovery in the aged mice than in the 2 month-old mice (Figure 4.19.).

Figure. 4.19.

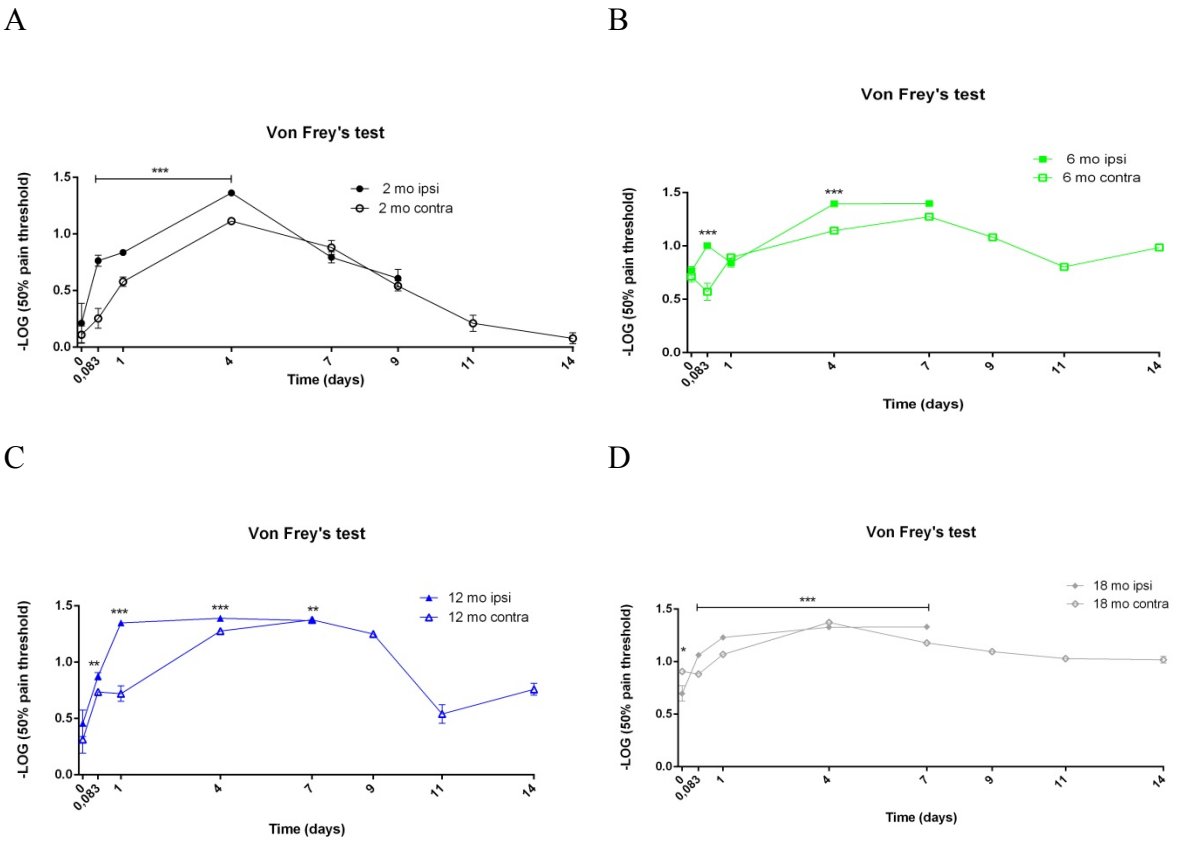


Figure 4.19. Formalin-induced mechanical allodynia in C57BL/6 mice of different age. Ageing influences formalin-induced allodynic state in its intensity and in the recovery process. . *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: ***. Data are expressed as -LOG of the mean ± SEM of the 50% pain threshold. *p* values < 0.05 were considered statistically significant. (2 month-old mice: n=8; 6 month-old mice n=9; 12 month-old mice n=9; 18 month-old mice n=9).

The statistical analysis of data in Figure 4.19. is shown in Table 4.6.

Table 4.6.

STATISTIC SIGNIFICANCE			
0 - 2 mo ipsi vs 6 mo ipsi	**	7 - 2 mo ipsi vs 6 mo ipsi	***
0 - 2 mo ipsi vs 18 mo ipsi	*	7 - 2 mo ipsi vs 12 mo ipsi	***
0 - 2 mo contra vs 6 mo contra	***	7 - 2 mo ipsi vs 18 mo ipsi	***
0 - 2 mo contra vs 18 mo contra	***	7 - 2 mo contra vs 6 mo contra	***
0 - 6 mo ipsi vs 12 mo ipsi	*	7 - 2 mo contra vs 12 mo contra	***
0 - 6 mo contra vs 12 mo contra	**	7 - 2 mo contra vs 18 mo contra	***
0 - 6 mo contra vs 18 mo contra	**	7 - 6 mo contra vs 12 mo contra	***
0 - 12 mo contra vs 18 mo contra	***	7 - 6 mo contra vs 18 mo contra	***
0,083 - 2 mo ipsi vs 6 mo ipsi	***	7 - 12 mo ipsi vs 18 mo ipsi	***
0,083 - 2 mo ipsi vs 18 mo ipsi	***	7 - 12 mo contra vs 18 mo contra	***
0.083 - 2 mo contra vs 6 mo contra	*	9 - 2 mo contra vs 6 mo contra	***
0,083 - 2 mo contra vs 12 mo contra	***	9 - 2 mo contra vs 12 mo contra	***
0,083 - 2 mo contra vs 18 mo contra	***	9 - 2 mo contra vs 18 mo contra	***
0,083 - 6 mo ipsi vs 12 mo ipsi	**	9 - 6 mo contra vs 12 mo contra	***
0,083 - 6 mo contra vs 18 mo contra	**	9 - 12 mo contra vs 18 mo contra	***
0,083 - 12 mo ipsi vs 18 mo ipsi	***	11 - 2 mo contra vs 6 mo contra	***
0,083 - 12 mo contra vs 18 mo contra	***	11 - 2 mo contra vs 12 mo contra	**
1 - 2 mo ipsi vs 12 mo ipsi	***	11 - 2 mo contra vs 18 mo contra	***
1 - 2 mo ipsi vs 18 mo ipsi	***	11 - 6 mo contra vs 12 mo contra	**
1 - 2 mo contra vs 6 mo contra	***	11 - 6 mo contra vs 18 mo contra	***
1 - 2 mo contra vs 18 mo contra	***	11 - 12 mo contra vs 18 mo contra	***
1 - 6 mo ipsi vs 12 mo ipsi	***	14 - 2 mo contra vs 6 mo contra	***
1 - 6 mo ipsi vs 18 mo ipsi	***	14 - 2 mo contra vs 12 mo contra	***
1 - 6 mo contra vs 12 mo contra	*	14 - 2 mo contra vs 18 mo contra	***
1 - 6 mo contra vs 18 mo contra	***	14 - 6 mo contra vs 12 mo contra	***
1 - 12 mo ipsi vs 18 mo ipsi	***	14 - 12 mo contra vs 18 mo contra	***
1 - 12 mo contra vs 18 mo contra	***		
4 - 2 mo ipsi vs 6 mo ipsi	***		
4 - 2 mo ipsi vs 12 mo ipsi	***		
4 - 2 mo ipsi vs 18 mo ipsi	***		
4 - 2 mo contra vs 12 mo contra	***		
4 - 2 mo contra vs 18 mo contra	***		
4 - 6 mo ipsi vs 18 mo ipsi	***		
4 - 6 mo contra vs 12 mo contra	***		
4 - 6 mo contra vs 18 mo contra	***		
4 - 12 mo ipsi vs 18 mo ipsi	***		
4 - 12 mo contra vs 18 mo contra	***		

Table 4.6. Formalin-induced mechanical allodynia in C57BL/6 mice of different age: statistical analysis of all the time points. *p* values < 0.05 were considered statistically significant. 0,083 day represents the time point of two hours after the *formalin test*. Only the statistically significant points not present in figure 4.25. are shown. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: ***.

4.2.7. Formalin-induced oedema of the injected paw of C57BL/6 mice is influenced by age

Before the *formalin test,* a basal assessment of paw thickness was carried out and it was measured 2 hours, 1, 4, 7, 9, 11 and 14 days after *formalin test*. The paw presented a high level of oedema that increased up to the 4th day and began to recover around the 11th and 14th day (Figure 4.3.), thus suggesting the existance of several molecular changes, other than the initial inflammation. In particular, the aged mice presented a much slower recovery from the oedema of the hindpaw than the 2 month-old mice. It could be hypothesized that the observed increased mechanical sensitivity could be induced by the developed oedema (Vierck *et al*., 2008); on the contrary, formalin-induced tactile allodynia does not depend on the oedema, as demonstrated by the observation that, on the 4th day after formalin administration, both the ipsilateral and the contralateral hindpaw present mechanical allodynia. Therefore, these results suggest that the degree of oedema might be influenced by the age of the animal.

Figure 4.20.

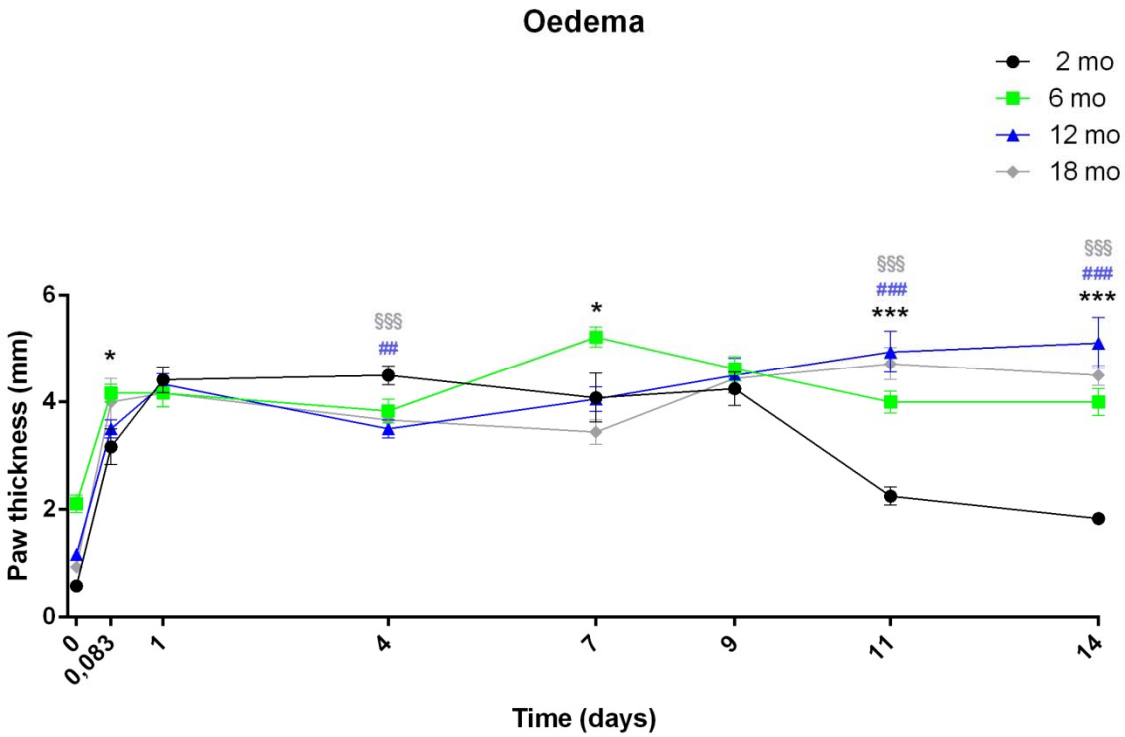


Figure 4.20. Formalin-induced oedema in C57BL/6 mice of different age. The oedema resulted influenced by the age of the animal: the aged mice presented a much slower recovery from the oedema of the hindpaw than the 2 month-old mice. Data are expressed as mean ± SEM of hindpaw thickness**.** *p* values < 0.05 were considered statistically significant. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: ***. (2 month-old mice: n=6; 6 month-old mice n=9; 12 month-old mice n=9; 18 month-old mice n=9). For the statistical analysis: *= 2 mo vs 6 mo; #= 2 mo vs 12 mo; §= 2 mo vs 18 mo.

The statistical analysis of data in Figure 4.20. is shown in Table 4.7

Table 4.7.

STATISTIC SIGNIFICANCE			
0 - 2 mo vs 6 mo 0 - 2 mo vs 12 mo 0 - 2 mo vs 18 mo 0 - 6 mo vs 12 mo 0 - 12 mo vs 18 mo 0,083 - 6 mo vs 12 mo	*** *** *** *** * *	7 - 6 mo vs 12 mo 7 - 6 mo vs 18 mo 11 - 6 mo vs 12 mo	** *** *

Figure 4.7. Formalin-induced oedema in C57BL/6 mice of different age: statistical analysis of all the time points. *p* values < 0.05 were considered statistically significant. 0,083 day represents the time point of two hours after the *formalin test*. Only the statistically significant points not present in figure 4.26. are shown. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: ***.

For a better comparison of the age-related oedema changes over time, the most relevant time points have been selected and represented in Figure 4.21.

Figure 4.21.

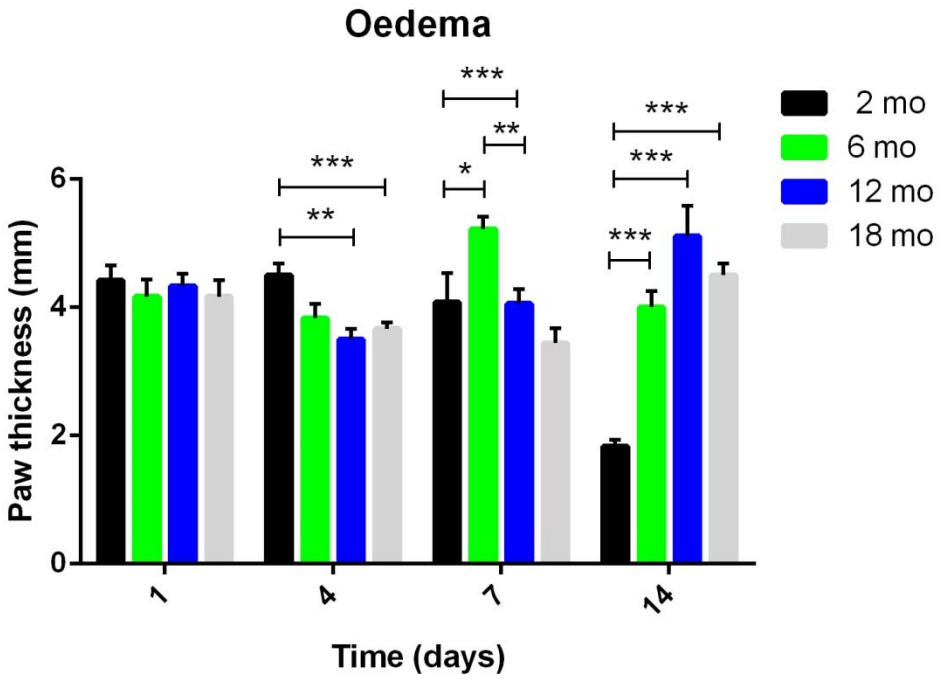


Figure 4.21. Formalin-induced oedema in C57BL/6 mice of different age: the most remarkable time points. The ageing process influences the oedema size: the older mice presented a much slower recovery from the oedema of the hindpaw on the 7^{th}-14^{th} day than the 2 month-old mice. Data are expressed as mean ± SEM of hindpaw thickness**.** *p* values < 0.05 were considered statistically significant. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: ***. (2 month-old mice: n=6; 6 month-old mice n=9; 12 month-old mice n=9; 18 month-old mice n=9). For the statistical analysis: *= 2 mo vs 6 mo; #= 2 mo vs 12 mo; §= 2 mo vs 18 mo.

4.2.8. Levels of the autophagic markers in C57BL/6 naïve mice of different age

Autophagy is an essential cellular process that has recently been involved in ageing (Morimoto & Cuervo, 2014). Also it has recently been shown that some autophagic markers are modulated at spinal level in chronic pain models like the SNL. In order to verify whether changes in the expression of some typical autophagic markers could be associated to the different pain senistivity observed in different age groups, the levels of LC3, beclin 1 and p62 were analyzed in dorsal spinal cord of naive mice from three different age groups: 2, 6 and 12 months. The representative blots and statistical analysis in (Figure 4.22.) show that only beclin 1 expression decreased with ageing, while none of the other proteins was affected. This might suggest a disregulated autophagic activity and a decreased autophagic flux, and would be in agreement with what described in different experimental systems (Berliocchi *et al*., 2011). Further functional experiments will need to address the questions to whether such a change might be relevant in the mechanism of central sensitization associated to pain processing during ageing.

Figure 4.22.

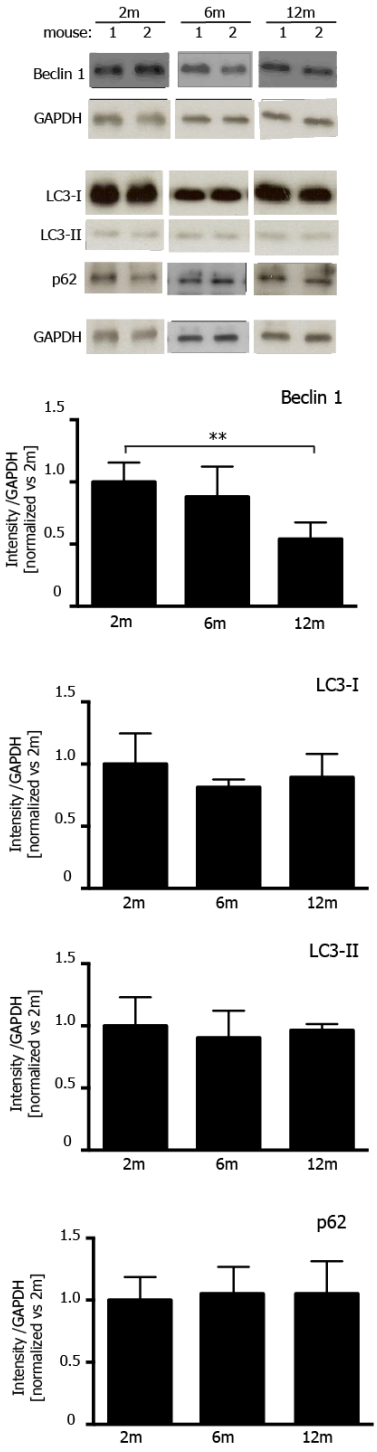


Figure 4.22. Autophagic markers in 3 groups of C57BL/6 naïve mice of different age. The expression of the main autophagic markers Beclin 1, LC3I, LC3-II and p62 was analyzed in the spinal dorsal horn. The representative blots and statistical analysis show that only beclin 1 expression decreased with ageing, while none of the other proteins was affected. For the densitometric analysis, the signal of each specific band was normalized toward the corresponding GAPDH signal. Values were expressed as mean ± SEM. (For each age group n=4, $p<0.01$: **)

4.2.9. Levels of the α2δ-1 in naïve mice of different age

The α2δ-1 subunit is up-regulated in the spinal cord of animals subjected to chronic pain models (Kim *et al*., 2001; Luo *et al*., 2002). So, because of the lowered nociceptive threshold presented by aged animals, the expression of α2δ-1 was examined in the spinal cord of 2, 6 and 12 months C57BL/6 naïve mice. In the 2 month-old mice, presenting normal basal sensitivity levels, α2δ-1 was almost absent. By contrast, it resulted remerkably overexpressed in the older mice with the 6 months mice showing the highest up-regulation of the subunit α2δ-1. The trend of α2δ-1 expression, very slightly present in the 2 month-old mice and mostly up-regulated at 6 months, can be defined bell-shaped. The 12 months mice showed inferior levels expression of α2δ-1, when compared to the 6 months mice. The up-regulation of the levels of the Ca^{2+} channel subunit α2δ-1 could be responsible for the increased mechanical sensitivity of the older mice, shown through the *Von Frey' s test* (Chaplan *et al*., 1994). Another very interesting aspect regards the presence of a low molecular weight band (25-17 kDa) highly present in the 6 and in the 12 month-old mice, compared to the 2 month-old mice. This "novel" band, named α2δ-1* reached, like the known α2δ-1 band, a peak around 6 months of age (Figure 4.23.). Because of the very low molecular weight, α2δ-1* might represent a cleavage product or an abnormal variant of the known subunit recognised by the antibody used. The α2δ-1 bell-shaped expression could explain the increased mechanical allodynia shown by 6 month-old mice and, at a later age (12 months), also other mechanisms may come into play.

Figure 4.23.

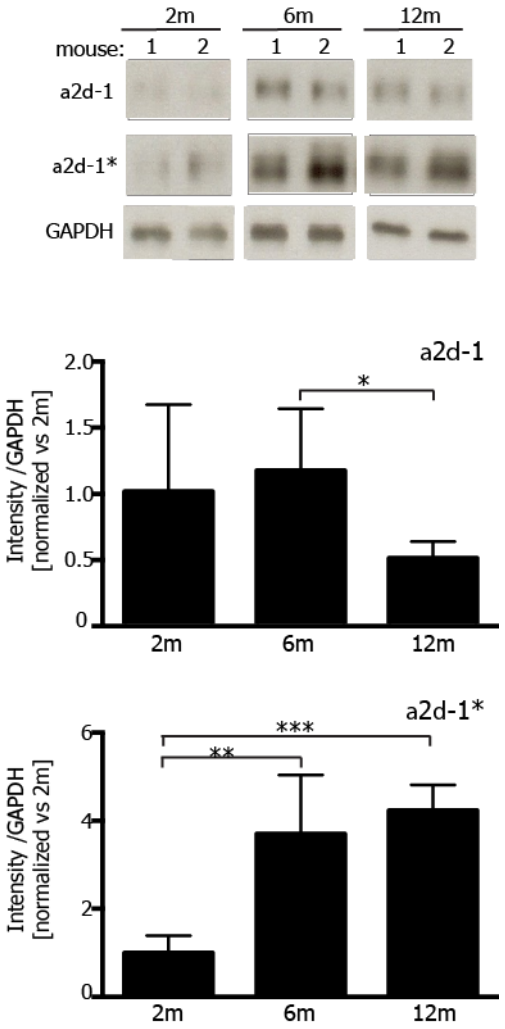


Figure 4.23. Levels of expression of the α2δ-1 subunit in the spinal dorsal horn of naïve mice of different age. The α2δ-1 showed a bell-shaped expression trend: very slightly present in the 2 month-old mice, mostly up-regulated at 6 months and less expressed in 12 months mice. The up-regulation of the α2δ-1* was very similar to that one of α2δ-1. The representative blots and statistical analysis show that only α2δ-1 expression decreased with ageing, following a bell-shaped trend. The same trend was paralleled by a α2δ-1-positive band of lower molecular weight (25-17kDa), designed α2δ-1*. For the densitometric analysis the signal of each specific band was normalised toward the corresponding GAPDH signal. Values were expressed as mean ± SEM. (For each age group n=4, $p<0.05$: *; $p<0.01$: **; $p<0.001$: ***)

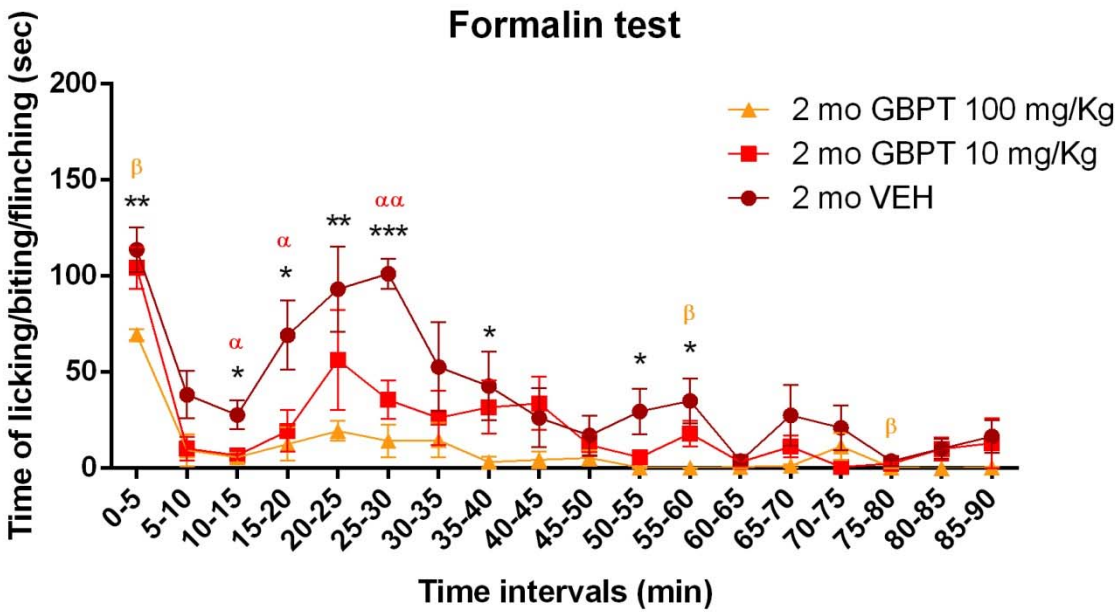
4.2.10. Gabapentin reduces licking/biting/flinching behaviour during the *formalin test*: effect of ageing on the drug effectiveness

The anticonvulsants gabapentin and pregabalin, of which the α2δ-1 calcium channel subunit is the receptor, are among the most commonly used drugs in the treatment of chronic pain (Dray., 2008). Therefore, since the first age-induced behavioural changes occur around the 6 months of age even though

they are much more evident in the following months, the effect of gabapentin was tested on 2 month-old mice and 6 month-old mice subjected to the *formalin test* (Dubuisson & Dennis, 1977) to understand if it was influenced by ageing. Based on the existing literature, the dose of 100 mg/Kg was selected and compared with a second 10-fold lower dose (10 mg/Kg) and a vehicle. As shown by our results, the *formalin test* provides a stimulus that, initially, is mainly of inflammatory nature, turning into a chronic pain trigger in the long term (chapter 1). Therefore, since we had previously seen that the nocieptive reaction to the *formalin test* was changed both in the intensity and in the duration with the increasing age of the animal, it was decided to examine the response to gabapentin of two different age groups mice subjected to the *formalin test.* In this case, due to the different duration of the nocifensive *licking/biting/flinching* behaviour, the latter was followed for 90 min, instead of the classic 60 min. Our results demonstrated that, in the 2 month-old mice just the 100 mg/Kg dosage was effective in all of the phases, with the 10 mg/Kg dose slightly active on the 2nd phase only (Figure 4.24. A). By contrast, both the doses resulted effective in the 6 month-old mice (Figure 4.24. B).

Figure 4.24.

A



B

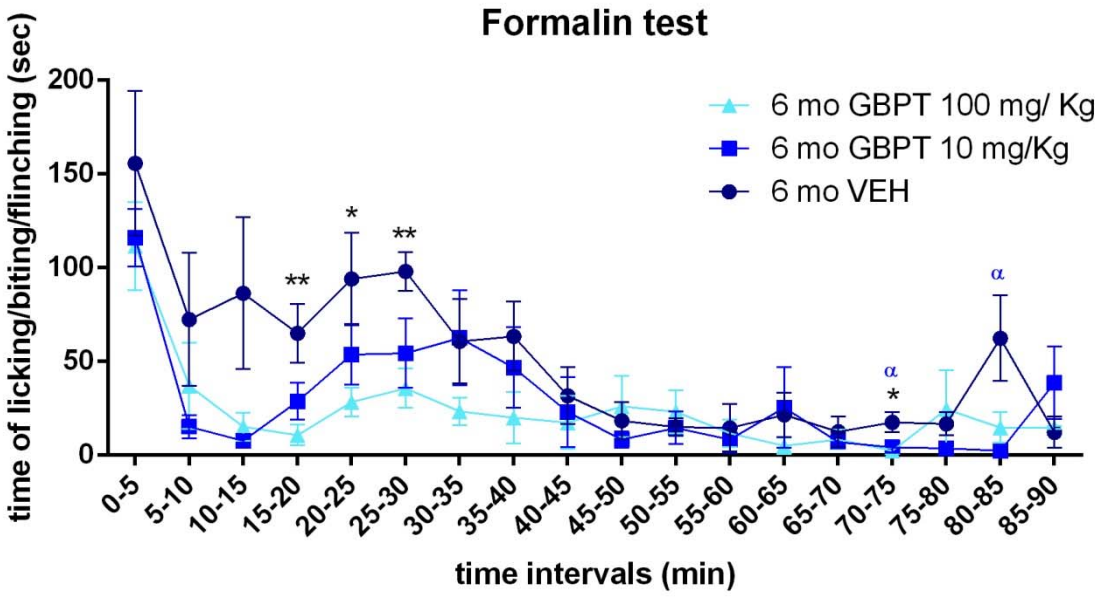


Figure 4.24. Effect of gabapentin on 2 and 6 month-old mice subjected to *formalin test.*

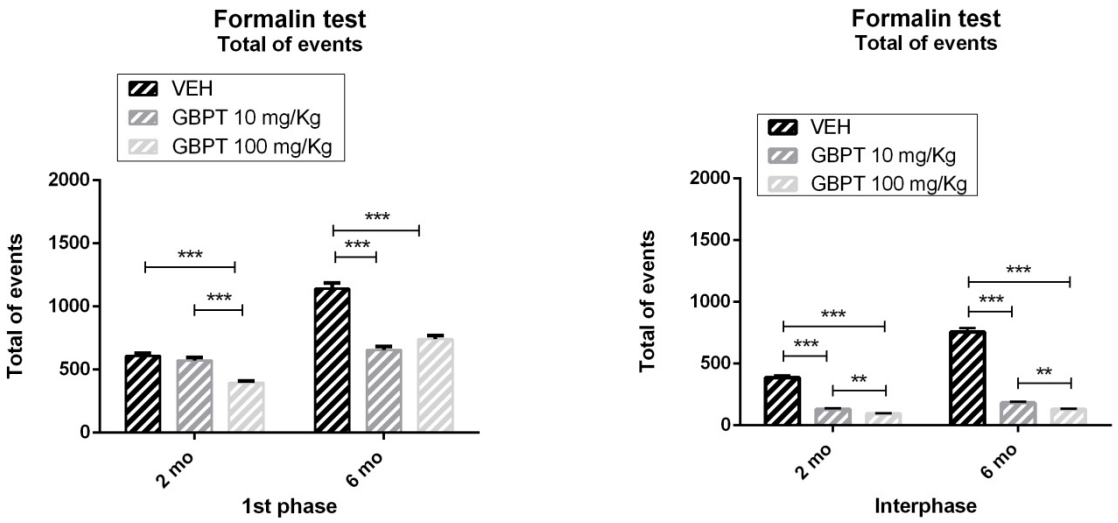
In the 2 month-old mice the 100 mg/Kg dosage only was effective in all of the phases of *formalin test* (Fig. 4.24.A), while, both the doses resulted effective in the 6 month-

old mice (Fig. 4.24.B). Data are expressed as mean ± SEM of the nociceptive reaction**.** *p* values < 0.05 were considered statistically significant. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: ***. (2 mo VEH (vehicle) n=4; 2 mo GBPT (gabapentin) 10 n=5; 2 mo GBPT 100 n=5; 6 mo VEH n=5; 6 mo GBPT 10 n=5; 6 mo GBPT 100 n=5). For the statistical analysis: *= 2 mo VEH vs 2 mo GBPT 100; **α**= 2 mo VEH vs 2 mo GBPT 10; **β**= 2 mo GBPT 10 vs 2 mo GBPT 100; *= 6 mo VEH vs 6 mo GBPT 100; **α**= 6 mo VEH vs 6 mo GBPT 10.

The graphs plotting the total of events of *licking/biting/flinching* show that, in the 6 month-old mice, the dose of gabapentin of 10 mg/Kg resulted as effective as the dose of 100 mg/Kg in the 1st phase, in the interphase and in the 3rd phase. Only in the 2nd phase, the dose of 100 mg/Kg was more effective than the 10 mg/Kg dose.

Figure 4.25

A **B**



D **E**

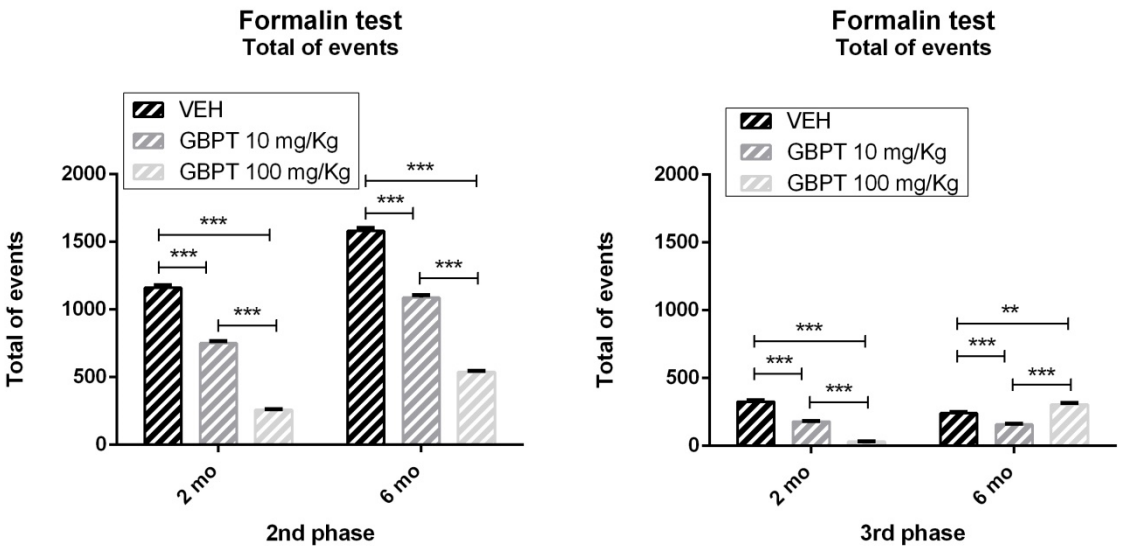


Figure 4.25. Effect of gabapentin on 2 and 6 months mice subjected to *formalin test*: total of events. In the 2 months mice the 100 mg/Kg dosage only was effective in all of the phases of *formalin test* (Fig. 4.25 A, B, C, D), while, in the 6 months mice, the gabapentin 10 mg/Kg dose resulted as effective as the dose of 100 mg/Kg in the 1st phase (Fig. 4.25. A), in the interphase (Fig.4.25. B) and in the 3rd phase (Fig. 4.25. D). In the 2nd phase (Fig. 4.25. C), the 100 mg/Kg dose was more effective than the 10 mg/Kg dose. Data are expressed as mean ± SEM of the nociceptive reaction assessed as total of events of *licking/biting/flinching*. *p* values < 0.05 were considered statistically significant. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: ***. (2 mo VEH (vehicle) n=4; 2 mo GBPT (gabapentin) 10 n=5; 2 mo GBPT 100 n=5; 6 mo VEH n=5; 6 mo GBPT 10 n=5; 6 mo GBPT 100 n=5).

The correspondence of the time points with each phase of the *formalin test* is shown in Table 4.8.

Table 4.8.

PHASES OF THE *FORMALIN TEST*	
1st phase	0-10 min
interphase	10-20 min
2nd phase	20-40 min
3rd phase	45-60 min

Table 4.8. Phases of the *formalin test*

4.2.11. Gabapentin effectiveness on formalin-induced mechanical allodynia changes according to age

Since formalin administration induces the development of a condion of pain characterized by tactile allodynia, it was investigated whether the i.p. administration of gabapentin before the execution of formalin test could be effective in reducing this mechanical allodynia. Also in this case, gabapentin was used in the two different doses of 10 and 100 mg/Kg and compared with a vehicle treatment. Only in the 2 months C57BL/6 mice, the treatment with gabapentin resulted effective 2 hours after the execution of the *formalin test* (Figure 4.26.). On the 1st day and on the 4th day the gabapentin efficay was over: this was most probably due to the drug half life. In the 6 months group, although even a lower dose of gabapentin is sufficient to obtain a therapeutic effect (Fig. 4.24. B and Fig. 4.25.), this effect is shorter-lasting than in the 2 month-old mice, since gabapentin did not result effective anymore 2 hours after the formalin administration in these older mice (Figure 4.27.).

Figure 4.26.

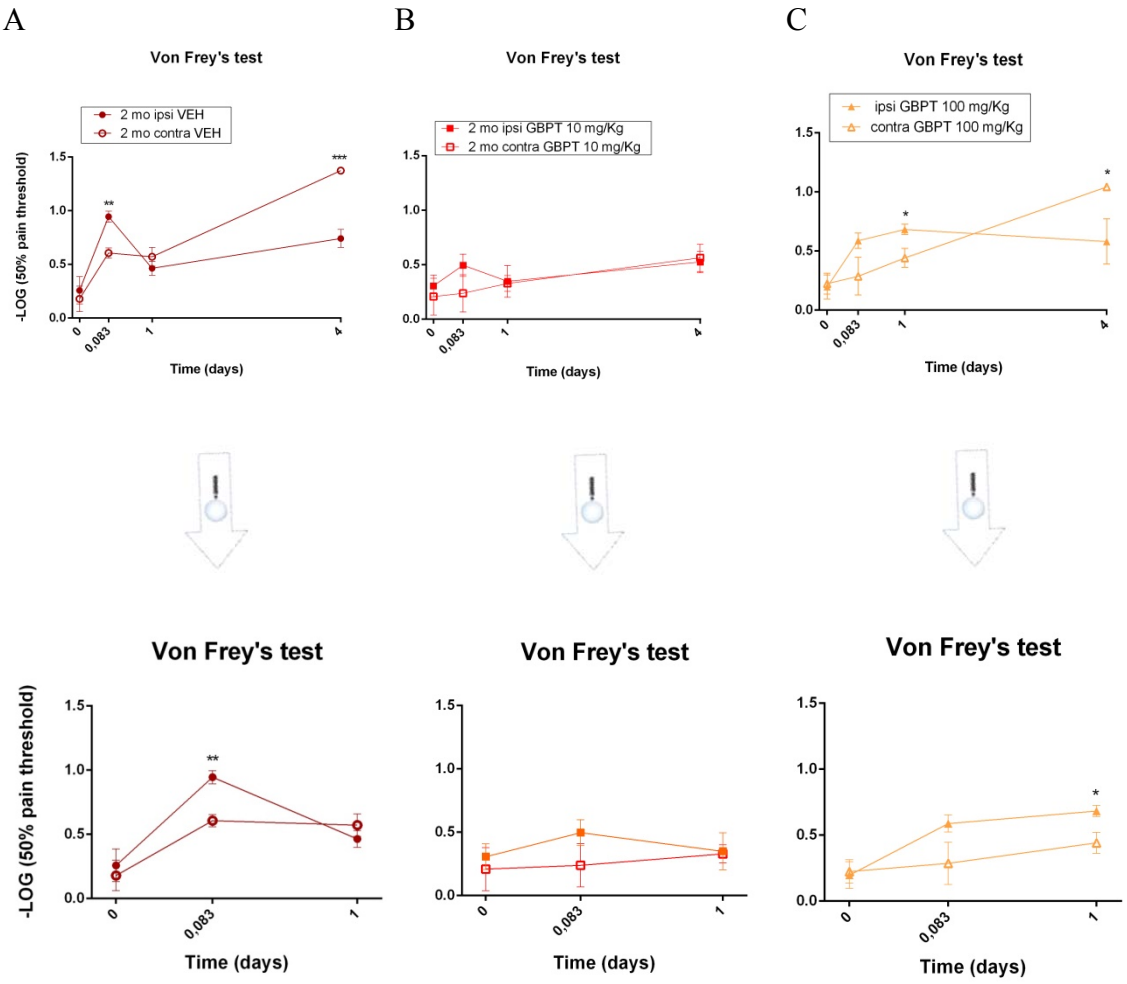


Figure 4.26. Effectiveness of gabapentin on formalin–induced mechanical allodynia in 2 month-old C57BL/6 mice. Gabapentin showed its efficacy on mechanical allodynia assessed through the *Von Frey's test,* two hours after formalin injection . Data are expressed as -LOG of the mean ± SEM of the 50% pain threshold. *p* values < 0.05 were considered statistically significant. *p* values < 0.05 were considered statistically significant. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: ***. For iconographic reasons, the time points interval between 0 and 1is enlarged compared with the real dimensions: an enlargement is shown. 0,083 day represents the time point of two hours after the *formalin test*. (2 mo VEH (vehicle) n=4; 2 mo GBPT (gabapentin) 10 n=5; 2 mo GBPT 100 n=5.)

The statistical analysis of figure 4.26. is shown in table 4.9.

Table 4.9.

STATISTIC SIGNIFICANCE			
0,083 ipsi VEH vs contra VEH 4 ipsi VEH vs contra VEH 1 ipsi GBPT 100 vs contra GBPT 100 4 ipsi GBPT 100 vs contra GBPT 100	** *** * *	0,083 ipsi VEH vs ipsi GBPT 10 0,083 ipsi VEH vs ipsi GBPT 100 1 ipsi VEH vs ipsi GBPT 100 4 contra VEH vs contra GBPT 10 4 contra VEH vs contra GBPT 100 4 contra GBPT 10 vs contra GBPT 100	** ** * *** *** **

Table 4.9. Effectiveness of gabapentin on formalin–induced mechanical allodynia in 2 month-old C57BL/6 mice: statistical analysis of all the time points. *p* values < 0.05 were considered statistically significant. 0,083 day represents the time point of two hours after the *formalin test*. Only the statistically significant points are shown. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: ***.

Figure 4.27.

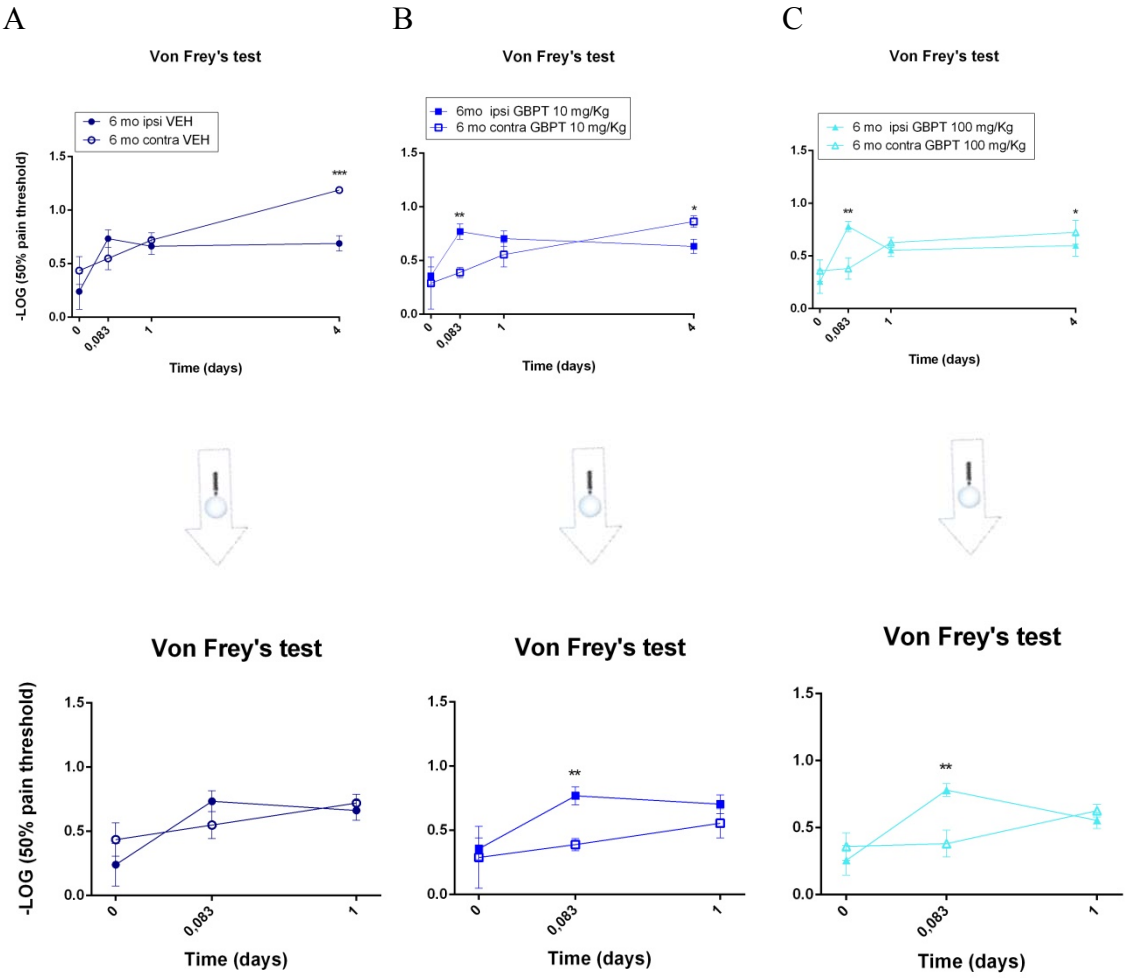


Figure 4.27. Effectiveness of gabapentin on formalin–induced mechanical allodynia in 6 month-old C57BL/6 mice. Even though a lower dose of gabapentin is effective in older mice, this effect is shorter-lasting than in the 2 month-old mice. Data are expressed as -LOG of the mean ± SEM of the 50% pain threshold. *p* values < 0.05 were considered statistically significant. *p* values < 0.05 were considered statistically significant. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: ***. For iconographic reasons, the time points interval between 0 and 1is enlarged compared with the real dimensions: an enlargement is shown. 0,083 day represents the time point of two hours after the *formalin test*. (6 mo VEH (vehicle) n=5; 6 mo GBPT (gabapentin) 10 n=5; 6 mo GBPT 100 n=5.)

.

The statistical analysis of figure 4.27. is shown in table 4.10.

Table 4.10.

STATISTIC SIGNIFICANCE			
0,083 ipsi GBPT 10 vs contra GBPT 10 0,083 ipsi GBPT 100 vs contra GBPT 100 4 ipsi GBPT 100 vs contra GBPT 100	** ** *	4 ipsi VEH vs contra VEH 4 contra VEH vs contra GBPT 100 4 contra VEH vs contra GBPT 10	*** ** ***

Table 4.10. Effectiveness of gabapentin on formalin–induced mechanical allodynia in 6 month-old C57BL/6 mice: statistical analysis of all the time points. *p* values < 0.05 were considered statistically significant. 0,083 day represents the time point of two hours after the *formalin test*. Only the statistically significant points are shown. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: ***.

4.2.12. Effects of an acute dose of gabapentin on C57BL/6 mice of different age

In order to assess the effect of an acute dose of gabapentin on C57BL/6 mice of different age, gabapentin was administered on the 4th day after formalin administration and then the mechanical allodynia was evaluated through *Von Frey's test* (Chaplan *et al*., 1994). When compared to the vehicle-treated group, gabapentin 10 mg/Kg caused a decrease of the mechanical allodynia of the contralateral hindpaw, according with the central nature of the formalin-

induced tactile allodynia also in the contralateral hindpaw. Also, the 10 mg/Kg dose resulted more effective in the 6 month-old mice than in the 2 month-old mice. The decrease of allodynia was not identifiable in the ipsilateral hindpaw and this might be due to the peripheral insult received by this paw. Two and six month-old mice treated with gabapentin 100 mg/Kg showed a decrease of mechanical allodynia both of the ipsilateral and of the contralateral hindpaw and also the 100 mg/Kg dose of gabapentin resulted more effective in the 6 month-old mice than in the 2 month-old mice (Figure 4.28.). Our results demonstrate that ageing influences the effect of gabapentin on the nocifensive response and on the mechanical allodynia induced by the *formalin test*.

Figure 4.28.

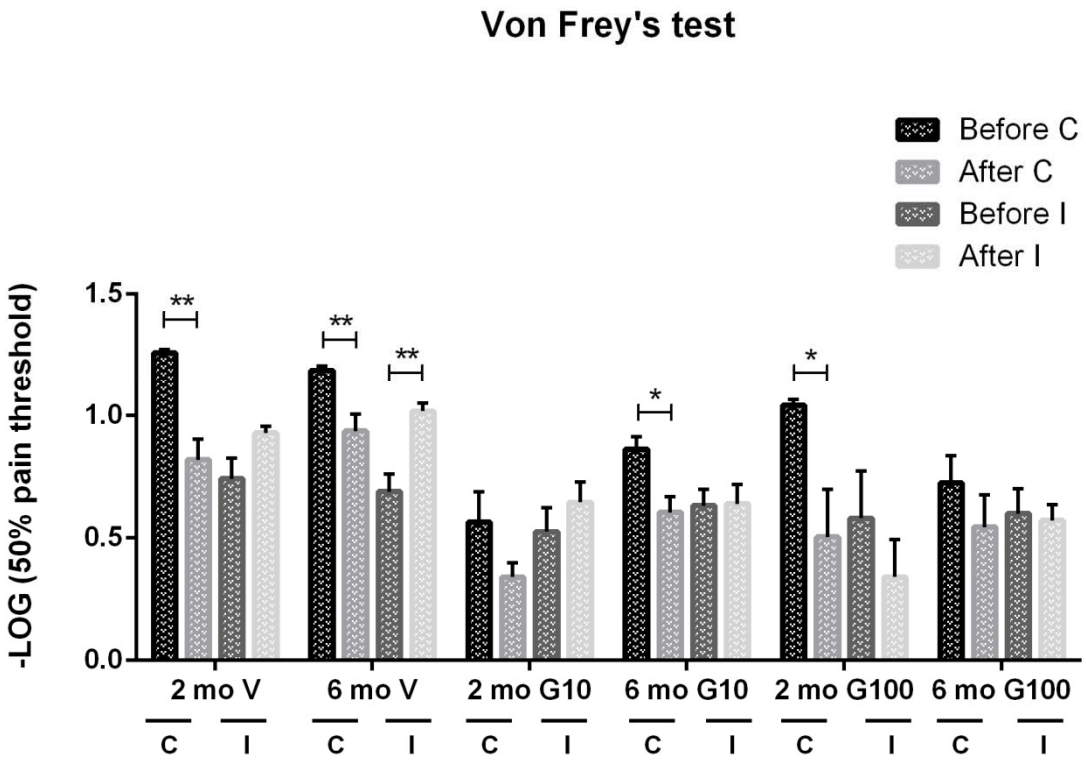


Figure 4.28. Effect of an acute dose of gabapentin on formalin–induced mechanical allodynia in 2 month-old and 6 month-old C57BL/6 mice on the 4th day after *formalin test*. **The** ageing process affects the effectiveness of gabapentin on the nocifensive response and on the mechanical allodynia induced by the *formalin test*. Data are expressed as -LOG of the mean ± SEM of the 50% pain threshold. *p* values < 0.05 were considered statistically significant. *p* values < 0.03: *; *p* values < 0.002: **; *p* values < 0.001: ***. (2 mo VEH (vehicle) n=4; 2 mo GBPT (gabapentin) 10 n=5; 2 mo GBPT 100 n=5; 6 mo VEH n=5; 6 mo GBPT 10 n=5; 6 mo GBPT 100 n=5); (C=Contra; I=Ipsi); (V=Vehicle; G=Gabapentin).

DISCUSSION AND CONCLUSIONS

5. DISCUSSION AND CONCLUSIONS

The results obtained during this PhD research project characterize the *Formalin test* (Dubuisson & Dennis, 1977) as a valid chronic pain model and suggest that the ageing process affects the nociceptive threshold to the stimuli both of mechanical and of thermal nature and the nocifensive behaviour in this experimental model of chronic pain. Actually, thanks to the progresses of modern medicine and to an improved life style (Scully, 2012), the elderly stand for the most represented and ever-increasing segment of our society. This increased longevity favours the development of long-term conditions (Abdulla *et al*., 2013), such as chronic pain. Ageing is a predisposing factor to chronic and wasting diseases characterized by pain (Dziechciaż *et al*., 2013). Chronic pain states in the elderly are often undertreated and misunderstood (Bruckenthal & D'Arcy, 2007) and represent a particular challenge beacuse of age-related pharmacokinetic and pharmacodynamic issues, comorbities, polypharmacological treatments (Sawynok, 2014) and so on. Therefore, one of the purposes of this PhD project was to study and characterize a model of pain that can mirror a chronic pain condition comparable to the painful states experienced by an increasing number of old patients, including in itself both the inflammatory features of joint pain, often resistant to treatment (Scaglione *et al*., 2014), and the central aspects of neuropathic pain conditions. We focused our attention on the *Formalin test* (Dubuisson & Dennis, 1977). This choice was driven by several strenght points of this experimental pain model. The *formalin test,* characterized by a first and a second phase, is a reliable model of nociception and it is also sensitive for various classes of analgesic drugs (Hunskaar & Hole, 1987). It was suggested that the first phase originates from the activation of Aδ- and C-fibers (Heapy *et al*., 1987). It was demonstrated that the intraplantar formalin injection causes the activation of the TRPA1 (Transient Receptor Potential), a cation channel that plays an

important role in inflammatory pain (McNamara *et al*., 2007). At molecular level, it was recently highlighted that the injection of 5% formalin produces an internalization of the neurokinin 1 (NK1) receptor in the ipsilateral superficial dorsal horn (Takasusuki & Yaksh, 2011). On the contrary, the mechanisms underlying the second phase have not been completely understood yet. It is now common thought that this phase is correlated with a central sensitization (Yamamoto & Yaksh, 1992). Indeed, it was demonstrated that the central sensitization of the dorsal horn neurons, paralleled by the cortical somatosensory evoked potentials observed after formalin injection (Lebrun *et al*., 2000). is involved in the development of the late phase of the formalin test (Abram & Yaksh, 1994; Ji *et al*., 1999). Interestingly, two hours after formalin administration, hyperalgesia, enhanced 1 to 3 days after injection and lasting for 3 – 4 weeks, was observed (Fu *et al*., 2001). Based on literature (Lin *et al*., 2007; Guida *et al*., 2012), the *formalin test* induces tactile allodynia on the 3rd and 7th day after the administration of formalin up to 14 days. Seen this long-term sensitization, the *formalin test* was studied in 2 month-old C57BL/6 mice, not only as an acute inflammatory pain model anymore, but also as a chronic pain model. Apart from examinating the formalin-induced *licking/biting/flinching* nocifensive behaviour, the mechanical sensitivity of the mice after formalin administration was examined through the *Von Frey's test* (Chaplan *et al.*, 1994). The *Von Frey's test* was performed two hours after formalin injection and on the 1st, 4th, 7th, 9th, 11th and 14th day. Our results demonstrated that the onset of mechanical allodynia occurred already two hours after formalin administration in the hindpaw ipsilateral to the insult and further increased on the 1st day. The allodynic peak was reached on the 4th day following formalin administration and it affected also the contralateral hindpaw. The recovery from this mechanical allodynia occurs around the 14th day after the *formalin test*. Based on literature (Burke *et al*., 2010), the oedema of the hindpaw administered

with formalin was measured using a caliper and, 2 hours after formalin administration, the oedem size resulted marked. This phenomenon kept to enhance till the 4th day and the recovery was shown around the 11th-14th day. Gabapentin is one of the current therapeutic treatments of chronic pain states such as painful diabetic neuropathy and postherpetic neuralgia (Backonja *et al*., 1998; Rowbotham *et al*., 1998). Thus, it was decided to pre-treat the C57BL/6 2 month-old mice with gabapentin 15 min before injecting to them 5% intraplantar formalin. It resulted very effective on the second phase. This confirmed the existance of a central sensitization at the root of this phase. The slight efficay of gabapentin on the first phase could be the result of a different mechanism of action of gabapentin. Even if it is known that it binds the α2δ-1 calcium channel subunit, the exact mechanism of action of gabapentin is unknown (Shannon *et al*., 2005). Because of the multiple functions of the α2δ-1 subunit, it was hypothesized that gabapentin can increase synaptic transmission (Hoppa *et al*., 2012), modulate trafficking processes (Hendrich *et al*., 2008; Tran-Van-Minh & Dolphin., 2010). Furthermore, this subunit was recently demonstrated to be a neuronal receptor for the thrombospondin involved in the central synaptogenesis (Eroglu *et al*., 2009), so gabapentin could inhibit synaptogenesis (Eroglu *et al*., 2009). Later, it was investigated whether gabapentin worked well on formalin-induced mechanical allodynia. Two hours after the formalin administration, gabapentin resulted effective on formalin-induced mechanical allodynia in C57BL/6 mice. Therefore, gabapentin resulted as active on formalin-induced allodynia as it is in the allodynic states induced by other experimental pain models such as SNL or Spared Nerve Injury (SNI) (Decosterd & Woolf, 2000). All the obtained results show that the *formalin test* can be used as a chronic pain model with some noteworthy advantages: it is not very invasive, it provides valid information more rapidly, it is sensitive to most of the common use drugs. Furthermore, since the nociception modifications induced by ageing have not

been well understood yet (Taguchi *et al*., 2010), this PhD project aimed at understanding the effects of the ageing process on basal sensitivity first, and, later, on the development and maintainance of a condition of pain that can be assimilated to a clinically relevant state in C57BL/6 mice. First, mechanical and thermal sensitivity were characterised during ageing. For this, behavioural tests able to assess basal mechanical and thermal (to heat and cold) sensitivity (*Von Frey's test* (Chaplan *et al*., 1994), *Pin-prick* test (Chan *et al.,* 1992), *Hargreaves'test* (Hargreaves *et al*., 1988), acetone test (Choi *et al*., 1994)), were performed on C57BL/6 mice belonging within an interval of ages that covers a remarkable segment of the whole human life from young to old adult. Both the mechanical and thermal sensitivity seemed to increase with age. The increase of mechanical sensitivity resulted more evident than the one of thermal sensitivity. Moreover, basal sensitivity increased progressively among 2 month-old mice and 6, 12 and 18 month-old mice, but the shown differences were much more marked between 2 and 6 or 12 month-old mice than between 12 and 18 month-old mice. Indeed, the basal sensitivity, and in particular the mechanical one, seemed to be characterized by a *plateau* around the age of 12 months. The first significant changes of sensitivity could be seen at 6 months of age, mostly in terms of cold sensitivity. Longitudinal studies performed on young (1 month-old), young adult (6 month-old) and adult (12 month-old) mice confirmed that groups of young and young adult mice that get older present an increase in mechanical and thermal sensitivity as older mice. These results find their validation in literature since there are several hypothesis and a lot of evidences showing that, during ageing, the excitatory processes are more active than the inhibitory ones (Lautenbacher, 2012) and that the sensation and reaction to pain changes with age (Dziechciaż *et al*., 2013). Since nociception resulted to be modified in the older animals, it was decided to examine the effect of age on the nocifensive response to the *formalin test* (Dubuisson & Dennis., 1977).

To this aim, C57BL/6 mice of four different age groups (2, 6, 12, 18 months) were subjected to the *formalin test* and the nociceptive response was monitored and expressed in seconds of *licking/biting/flinching* and as total of events of *licking/biting/flinching*. The aged mice showed a different trend of the classical behaviour presented by the 2 month-old mice, with no more only 2 but 3 peak phases, a shift in time and a varied amplitude, thus suggesting the existance of possible modifications in the mechanisms of central sensitization. The six month-old mice nocifensive behaviour resulted quite intermediate.The central sensitization and the plastic modifications occurring at the level of the dorsal horn (Tjølsen *et al*., 1992) that seem to be responsible for the second phase of formalin test and possibly for the late and long-term mechanical allodynia (Guida *et al*., 2012) that, according to our previous results (chapter 1) make of it a chronic pain model could undergo modifications with the increased age of the animal. The mechanical allodynia induced by *formalin test* (Dubuisson & Dennis., 1977) developed the day after formalin administration but showed different features according to the age of the animal: the level of mechanical allodynia shown by the older mice resulted higher, compared to the 2 months mice and, while in the mice of two months of age the recovery of the basal sensitivity level was beginning, the mechanical allodynia still kept to remain stable at the peak. Therefore, the mechanical allodynia presented by the 6, 12 and 18 months mice resulted much higher than the one of the 2 month-old mice up to 14 days and even the recovery resulted much slower than in the 2 months mice. These results likely are due to the different basal threshold of these mice according to their age. Interestingly, some days following formalin administration, the ipsilateral hindpaw showed a lesion that had different features according to the age of the animal, thus demonstrating that even the healing process is subjected to modifications with ageing. The three groups of older mice developed an earlier and more extensive lesion than the 2 months mice and even the

formalin-induced oedema persisted longer. Indeed, the degree of oedema of the hindpaw subcutaneously injected with formalin was evaluated on the same time points in which the assessment of mechanical allodynia was performed. Since we observed that ageing causes an increase in the nociceptive threshold, it was decided to investigate the molecular basis of this phenomenon and, in particular, whether the autophagic machinery and the levels of one of the main chronic pain markers, the Ca^{2+} channel subunit α2δ-1, were modificated according to the age of C57BL/6 naïve mice. While the spinal levels of LC3-I, LC3-II and p62 were not changed, Beclin 1 resulted reduced suggesting that the autophagic pathway is modulated and that the autophagic flux is possibly reduced in spinal dorsal horn during ageing. Further studies will try to clarify whether the different mechanical and thermal sensitivity during ageing might be related to these molecular changes at the spinal cord. Also α2δ-1 expression, which resulted almost absent in 2 month-old naïve mice, was markedly up-regulated following a bell-shaped trend in 6 and 12 months mice. The up-regulation of α2δ-1 could be at the root of the increased sensitivity of 6 month-old mice and, in the 12 month-old mice, also other mechanisms could be involved.These results find correspondence in the evidence that the cutaneous mechanical nociceptive threshold, measured through the *Von Frey's test* (Chaplan *et al*., 1994), and the mechanical withdrawal threshold measured by the paw pressure test were decreased in the aged Lou/C/Jall rats (Jourdan *et al*., 2000). Interestingly, a novel low molecular weight band, which we named α2δ-1*, resulted overexpressed in the older mice, as the classic α2δ-1. According to its low molecular weight (25-17 KDa), α2δ-1* could represent a cleavage product or an abnormal variant of the known subunit recognised by the antibody used, present in aged mice. It remains to establish, in future experiments, whether the α2δ-1* contributes to the decreased nociceptive threshold presented by aged mice. In a second set of experiments, it was tested whether the effects of gabapentin,

one of the most used painkillers in clinic together with the antidepressants, (Moore *et al*., 2009, 2011) could be influenced by ageing. Two and six month-old C57BL/6 mice subjected to the *formalin test* were pre-treated with 10 and 100 mg/Kg gabapentin and the effectiveness of these dosages was affected by ageing. Indeed, the obtained results demonstrated that, in the 2 month-old mice subjected to the *formalin test*, only the 100 mg/Kg dosage was effective in all of the phases, while, both the higher and the lower dosages resulted effective in the 6 month-old mice. In the older mice, moreover, the dose of gabapentin of 10 mg/Kg presented the same efficacy as the dose of 100 mg/Kg on the 1st phase, on the interphase and on the 3rd phase; the 100 mg/Kg dose resulted more effective than the 10 mg/Kg one just on the 2nd phase. The finding that even a low dose, normally ineffective (Dixit *et al*., 1999), resulted effective in older mice could be explained by the increased basal sensitivity presented by these mice, likely due to the up-regulation of the subunit α2δ-1 and, maybe, to a contribution of the α2δ-1*. Even the effect of gabapentin on formalin-induced mechanical allodynia varies according to age in C57BL/6 mice. In the 2 month-old mice, the treatment with gabapentin reduced mechanical allodynia 2 hours after the formalin injection. Because of the drug half life, on the 1st day and on the 4th day its effect resulted used up. In the 6 months mice, it was not possible to underline any significant changes between the mechanical threshold of the vehicle-treated and of the drug-treated mice. These results suggest that, in older mice, even a lower dose of gabapentin results therapeutic but the therapeutic effect is shorter-lasting than in the 2 month-old mice. There could be several explanations for this phenomenon characteristic of the aged animals: the occurrence of drug-resistance mechanisms, the α2δ-1* competes with the α2δ-1 for the drug-binding. The most likely explanation is given by the pathologic up-regulation of α2δ-1 per membrane unit, highlighted in the older mice already in basal conditions. This α2δ-1 overexpression,

responsible for the increased sensitivity of the aged mice, could make the older mice more sensitive to gabapentin efficacy, thus even the lower 10 mg/Kg dose results active in the *formalin test*. For the same reason, the presence of more receptors could cause a rapid complete binding of all the drug and so the therapeutic effect has a shorter duration. This would suggest that a single therapeutic dose is longer-lasting in the young than in the older mice and so that more administrations would be needed in the aged population. This observation could be very useful for the clinical management of chronic pain in the elderly. Furthermore, this hypothesis is confirmed by the finding that, an acute administration of gabapentin resulted more effective on formalin-induced mechanical allodynia in the 6 than in the 2 months mice. Indeed, finally, the eventual influence of the ageing process on the acute treatment condition was reproduced administering gabapentin (10 and 100 mg/kg) on the 4^{th} day after formalin test and evaluating the mechanical allodynia in 2 and 6 month-old age C57BL/6 mice. Our results demonstrated that ageing affects the effectiveness of gabapentin on the nocifensive response to the *formalin test* and on the formalin-mechanical allodynia. In the future experiments, the effect of gabapentin on 12 and 18 month-old mice will be examined to get other indications about all the four age groups investigated. The collected data are endowed with a remarkable translational value as they can provide relevant information for the study and the treatment of chronic pain in the in the continuously growing elderly population. .

REFERENCES

6. REFERENCES

Abbadie, C., Taylor, B.K., Peterson, M.A., Basbaum, A.I., 1997. Differential contribution of the two phases of the formalin test to the pattern of c-fos expression in the rat spinal cord: studies with remifentanil and lidocaine. Pain, 69(1-2), 101-110.

Abdulla, A., Adams, N., Bone, M., Elliott, A.M., Gaffin, J., Jones, D., Knaggs, R., Martin, D., Sampson, L., Schofield, P; British Geriatric Society. Guidance on the management of pain in older people. Age Ageing, 42 Suppl 1, i1-57.

Abram, S.E., & Yaksh, T.L., 1994. Systemic lidocaine blocks nerve injury-induced hyperalgesia and nociceptor-driven spinal sensitization in the rat. Anesthesiology, 80, 383-391.

AGS Panel on Persistent Pain in Older Persons., 2002. The management of persistent pain in older persons. J. Am. Geriatr. Soc., 50: S205-S224.

Allen, R.R., 1998. Neuropathic pain in the cancer patient. Neurol. Clin., 16(4), 869-888.

Arneric, S.P., Laird, J.M., Chappell, A.S., Kennedy, J.D., 2013. Tailoring chronic pain treatments for the elderly: are we prepared for the challenge? Drug Discov. Today, S1359-6446(13)00284-00285.

Backonja, M., Beydoun, A., Edwards, K.R., Schwartz, S.L., Fonseca, V., Hes, M., LaMoreaux, L., Garofalo, E., 1998. Gabapentin for the symptomatic treatment of painful neuropathy in patients with diabetes mellitus: a randomized controlled trial. J. Am. Med. Assoc., 280, 1831-1836.

Backonja, M.M., Attal, N., Baron, R., Bouhassira, D., Drangholt, M., Dyck, P.J., Edwards, R.R., Freeman, R., Gracely, R., Haanpaa, M.H., Hansson, P.,

Hatem, S.M,, Krumova, E.K., Jensen, T.S., Maier, C., Mick, G., Rice, A.S., Rolke, R., Treede, R.D., Serra, J., Toelle, T., Tugnoli, V., Walk, D., Walalce, M.S., Ware, M., Yarnitsky, D., Ziegler, D., 2013. Value of quantitative sensory testing in neurological and pain disorders: NeuPSIG consensus. Pain, 154(9), 1807-1819.

Barron, K.D., 1995. The microglial cell, a historical review, J. Neurol. Sci., 134, 57-68.

Berliocchi, L., Russo, R., Maiarù, M., Levato, A., Bagetta, G., Corasaniti, M.T., 2011. Autophagy impairment in a mouse model of neuropathic pain. Mol. Pain, 7, 83.

Brickley, K., Campbell, V., Berrow, N., Leach, R., Norman, R.I., Wray, D., Dolphin, A.C., Baldwin, S.A., 1995. Use of site-directed antibodies to probe the topography of the α2 subunit of voltage-gated Ca^{2+} channels. FEBS Lett., 364, 129–133.

Bruckenthal, P., D'Arcy, Y.M., 2007. Assessment and management of pain in older adults: a review of the basics. Topics in Advanced Practice Nursing eJournal, 7(1).

Burke, N.N., Hayes, E., Calpin, P., Kerr, D.M., Moriarty, O., Finn, D.P., Roche, M., 2010. Enhanced nociceptive responding in two rat models of depression is associated with alterations in monoamine levels in discrete brain regions. Neuroscience, 171(4), 1300-1313.

Campbell, J.N., Basbaum, A.I., Dray, A., Dubner, R., Dworkin, R.H., Sang, C.H., eds., 2006. Emerging Strategies for the Treatment of Neuropathic Pain. Seattle, WA: IASP Press.

Chaplan, S.R., Bach, F.W., Pogrel, J.W., Chung, J.M., Yaksh, T.L., 1994. Quantitative assessment of tactile allodynia in the rat paw. J. Neurosci. Methods, 53, 55-63.

Chapman, C.R., Gavrin, J., 1999. Suffering: the contributions of persistent pain. Lancet, 353: 2233–2237.

Chan, A.W., MacFarlane, I.A., Bowsher, D., Campbell, J.A., 1992. Weighted needle pinprick sensory thresholds: a simple test of sensory function in diabetic peripheral neuropathy. J. Neurol. Neurosurg. Psychiatry., 55, 56-59.

Choi, Y., Yoon, Y.W., Na, H.S., Kim, S.H., Chung, J.M., 1994. Behavioral signs of ongoing pain and cold allodynia in a rat model of neuropathic pain. Pain., 59, 369-376.

De Jongh, K.S., Warner, C., Catterall, W.A., 1990. Subunits of purified calcium channels. J. Biol. Chem., 265, 14738–14741.

Decosterd, I., Woolf, C.J., 2000. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. Pain, 87(2), 149-158.

Dixit, R., Bhargava, V.K., Kaur, N., 1999. Antinociceptive effect of gabapentin in the formalin test. Methods, Find Exp. Clin. Pharmacol., 21(7), 481-482.

Dixon, W.J., 1980. Efficient analysis of experimental observations. Annu. Rev. Pharmacol. Toxicol., 20, 441-462.

Dolly, J.O., O'Connell, M.A., 2012. Neurotherapeutics to inhibit exocytosis from sensory neurons for the control of chronic pain. Curr. Opin. Pharmacol., 12(1), 100-108.

Dolphin, A.C., 2012. Calcium channel auxiliary α(2)δ and β subunits: trafficking and one step beyond. Nat. Rev. Neurosci., 13, 542–555.

Dray, A., 1995. Inflammatory mediators of pain. Br. J. Anaesth., 75, 125-131.

Dray, A., 2008. Neuropathic pain: emerging treatments. Br. J. Anaesth., 101, 48-58.

Dubuisson, D., Dennis, S.G., 1977. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain, 4(2), 161-174.

Dziechciaż, M., Balicka-Adamik, L., Filip, R., 2013. The problem of pain in old age. Ann. Agric. Environ. Med., Spec. no. 1, 35-38.

Eroglu, C., Allen, N.J., Susman, M.W., O'Rourke, N.A., Park, C.Y., Ozkan, E., Chakraborty, C., Mulinyawe, S.B., Annis, D.S., Huberman, A.D., Green, E.M., Lawler, J., Dolmetsch, R., Garcia, K.C., Smith, S.J., Luo, Z.D., Rosenthal, A., Mosher, D.F., Barres, B.A., 2009. Gabapentin receptor alpha2delta-1 is a neuronal thrombospondin receptor responsible for excitatory CNS synaptogenesis. Cell, 139(2), 380-392.

Field, M.J., Cox, P.J., Stott, E., Melrose, H., Offord, J., Su, T.Z., Bramwell, S., Corradini, L., England, S., Winks, J., Kinloch, R.A., Hendrich, J., Dolphin, A.C., Webb, T., Williams, D., 2006. Identification of the α2δ-1 subunit of voltage-dependent calcium channels as a novel molecular target for pain mediating the analgesic actions of pregabalin. Proc. Natl. Acad. Sci. U. S. A., 103, 17537–17542.

Fu, K.Y., Light, A.R., Maixner, W., 2001. Long-lasting inflammation and long-term hyperalgesia after subcutaneous formalin injection into the rat hindpaw. J. Pain, 2(1), 2-11.

Gagliese, L., 2009. Pain and aging: the emergence of a new subfield of pain research. J. Pain. 10, 343-353.

Garrison, S.R., Stucky, C.L., 2014. Contribution of transient receptor potential ankyrin 1 to chronic pain in aged mice with complete Freund's adjuvant-induced arthritis. Arthritis Rheumatol., 66(9), 2380-2390.

Grayson, M., 2012. Ageing. Nature, 492(7427), S1.

Guida, F., Luongo, L., Aviello, G., Palazzo, E., De Chiaro, M., Gatta, L., Boccella, S., Marabese, I., Zjawiony, J.K., Capasso, R., Izzo, A.A., de Novellis, V., Maione, S., 2012. Salvinorin A reduces mechanical allodynia and spinal neuronal hyperexcitability induced by peripheral formalin injection. Mol. Pain, 8, 60.

Gustafsson, H., Flood, K., Berge, O.G., Brodin, E., Olgart, L., Stiller, C.O., 2003. Gabapentin reverses mechanical allodynia induced by sciatic nerve ischemia and formalin-induced nociception in mice. Exp. Neurol., 182(2), 427-434.

Hanks-Bell, M., Halvey, K., Paice, J.A., 2004. Pain assessment and management in aging. Online J Issues Nurs., 9(3).

Hargreaves, K., Dubner, R., Brown, F., Flores, C., Joris, J., 1988. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain., 32, 77-88.

Haroun, O.M., Hietaharju, A., Bizuneh, E., Tesfaye, F., Brandsma, J.W., Haanpää, M., Rice, A.S., Lockwood, D.N., 2012. Investigation of neuropathic pain in treated leprosy patients in Ethiopia: a cross-sectional study. Pain, 153(8), 1620-1624.

Heapy, C.G., Jamieson, A., Russel, N.J.W., 1987. Afferent C-fiber and A-delta activity in models of inflammation. British Journal of Pharmacology, 90, 146.

Helme, R.D., & Gibson, S.J., 2001. The epidemiology of pain in elderly people. Clinics in Geriatric Medicine, 17, 417-31.

Hendrich, J., Van Minh, A.T., Heblich, F., Nieto-Rostro, M., Watschinger, K., Striessnig, J., Wratten, J., Davies, A., Dolphin, A.C., 2008. Pharmacological disruption of calcium channel trafficking by the α2δ ligand gabapentin. Proc. Natl. Acad. Sci. U S A., 105, 3628–3633.

Hewitt, D.J., McDonald, M., Portenoy, R.K., Rosenfeld, B., Passik, S., Breitbart, W., 1997. Pain syndromes and etiologies in ambulatory AIDS patients. Pain, 70(2-3), 117-123.

Hoppa, M.B., Lana, B., Margas, W., Dolphin, A.C., Ryan, T.A., 2012. α2δ couples calcium channels to neurotransmitter release sites to control release probability. Nature, 486, 122–125.

Hunskaar S., & Hole K., 1987. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain, 30(1), 103-114.

IASP Task Force on Taxonomy, "Part III: Pain Terms, A Current List with Definitions and Notes on Usage" In Classification of Chronic Pain, Second Edition, 1994. Merskey, H., & Bogduk, N., eds., IASP Press, Seattle.

Institute of Medicine (US) Committee on Advancing Pain Research, Care, and Education (IOM)., 2011. Relieving Pain in America: A Blueprint for Transforming Prevention, Care, Education, and Research. Washington (DC): National Academies Press (US).

International Association for the Study of Pain. Classification of chronic pain. 1986. Pain, suppl 3, S1-S226.

Jakobsson, U., Klevsgard, R., Westergren, A., & Rahm Hallberg, I., 2003. Old people in pain: A comparative study. Journal of Pain and Symptom Management, 26, 625-636.

Ji, R.R., Baba, H., Brenner, G.J., Woolf, C.J.. 1999. Nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity. Nat. Neurosci., 2, 1114-1119.

Jourdan, D., Boghossian, S., Alloui, A., Veyrat-Durebex, C., Coudore, M.A., Eschalier, A., Alliot, J., 2000. Age-related changes in nociception and effect of morphine in the Lou rat. Eur J Pain, 4, 291–300.

Kim, S.H., Chung, J.M., 1992. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain, 50(3), 355-363.

Kim, S.J., Thomas, K.S., Calejesan, A.A., Zhuo, M., 1998. Macromolecular synthesis contributes to nociceptive response to subcutaneous formalin injection in mice. Neuropharmacology, 37(8), 1091-1093.

Kim, C., Jun, K., Lee, T., *et al.*, 2001. Altered nociceptive responses in mice deficient in the alpha1d subunit of the voltage dependent calcium channel. Mol. Cell. Neurosci., 18, 235–245.

Klugbauer, N., Lacinova,´ L., Marais, E., Hobom, M., Hofmann, F., 1999. Molecular diversity of the calcium channel α2δ subunit. J. Neurosci., 19, 684–691.

Lautenbacher, S., 2012. Experimental approaches in the study of pain in the elderly. Pain Med., 13 Suppl., 2, S44-50.

Lebrun, P., Manil, J., Colin, F., 2000. Formalin-induced central sensitization in the rat: somatosensory evoked potential data. Neurosci. Lett., 283(2), 113-6.

Lee, I.O., Crosby, G., 1999. Halothane effect on formalin-induced paw edema and flinching in rat. J. Korean Med. Sci., 14(1), 34-38.

Li, X., Sahbaie, P., Zheng, M., Ritchie, J., Peltz, G., Mogil, J.S., Clark, J.D., 2010. Expression genetics identifies spinal mechanisms supporting formalin late phase behaviors. Mol. Pain, 11, 6-11.

Lin, C.S., Tsaur, M.L., Chen, C.C., Wang, T.Y., Lin, C.F., Lai, Y.L., Hsu, T.C., Pan, Y.Y., Yang, C.H., Cheng, J.K., 2007. Chronic intrathecal infusion of minocycline prevents the development of spinal-nerve ligation-induced pain in rats. Reg. Anesth. Pain Med., 32(3), 209-216.

Luo, Z.D., Chaplan, S.R., Higuera, E.S., Sorkin, L.S., Stauderman, K.A., Williams, M.E., Yaksh, T.L., 2001. Upregulation of dorsal root ganglion alpha2delta calcium channel subunit and its correlation with allodynia in spinal nerve injured rats. J. Neurosci., 21, 1868–1875.

Luo, Z.D., Calcutt, N.A., Higuera, E.S,, *et al*., 2002. Injury type-specific calcium channel alpha (2) delta-1 subunit up-regulation in rat neuropathic pain models correlates with antiallodynic effects of gabapentin. J. Pharmacol. Exp. Ther., 303, 1199–1205.

Marais, E., Klugbauer, N., Hofmann, F., 2001. Calcium channel alpha(2)delta subunits-structure and Gabapentin binding. Mol. Pharmacol., 59(5), 1243-1248.

Mathieu-Kia, A.M., Fan, L.Q., Kreek, M.J., Simon, E.J., Hiller, J.M., 2001. Mu-, delta- and kappa-opioid receptor populations are differentially altered in distinct areas of postmortem brains of Alzheimer's disease patients. Brain Res., 893(1-2), 121-134.

Matthews, F.E., Dening, T., UK Medical Research Council Cognitive Function and Ageing Study., 2002. Prevalence of dementia in institutional care. Lancet, 360(9328), 225-226.

McNamara, C.R., Mandel-Brehm, J., Bautista, D.M., Siemens, J., Deranian, K.L., Zhao, M., Hayward, N.J., Chong, J.A., Julius, D., Moran, M.M., Fanger, C.M., 2007. TRPA1 mediates formalin-induced pain.. Proc. Natl. Acad. Sci. U S A., 104(33), 13525-13530.

Merskey, H., Bogduk, N., 1994. Classification of Chronic Pain. Seattle, WA: IASP Press.

Miranda HF, Noriega V, Zanetta P, Prieto JC.

Mogil, J.S., Wilson, S.G., Bon, K., Lee, S.E., Chung, K., Raber, P., Pieper, J.O., Hain, H.S., Belknap, J.K., Hubert, L., Elmer, G.I., Chung, J.M., Devor, M., 1999. Heritability of nociception I: responses of 11 inbred mouse strains on 12 measures of nociception. Pain 80, 67-82.

Moore, R.A., Straube, S., Wiffen, P.J., Derry, S., McQuay, H.J., 2009. Pregabalin for acute and chronic pain in adults. Cochrane Database Syst. Rev., 3, CD007076.

Moore, R.A., Wiffen, P.J., Derry, S., McQuay, H.J., 2011. Gabapentin for chronic neuropathic pain and fibromyalgia in adults. Cochrane Database Syst. Rev., 3, CD007938.

Morimoto, R.I., Cuervo, A.M., 2014. Proteostasis and the aging proteome in health and disease. J. Gerontol. A Biol. Sci. Med. Sci., 69 Suppl 1, S33-S38.

Newton, R.A., Binghamb, S., Casec, P.C., Sangerb, G.J., Lawson, S.N., 2001. Dorsal root ganglion neurons show increased expression of the calcium channel alpha-2 delta-1 subunit following partial sciatic nerve injury. Mol. Brain Res., 95, 1–8.

Owen, R.T., 2007. Pregabalin its efficacy safety and tolerability profile in fibromyalgia syndrome. Drugs Today, 43, 857–863

Paudel, K.R., Bhattacharya, S., Rauniar, G., Das, B., 2011. Comparison of antinociceptive effect of the antiepileptic drug gabapentin to that of various dosage combinations of gabapentin with lamotrigine and topiramate in mice and rats. J. Neurosci. Rural Pract., 2(2), 130-136.

Purves D, Augustine GJ, Fitzpatrick D, et al., eds. Sunderland (MA): Sinauer Associates; 2001.

Raut, A., Ratka, A., 2009. Oxidative damage and sensitivity to nociceptive stimulus and opioids in aging rats. Neurobiol. Aging, 30(6), 910-909.

Risser, D., You, Z.B., Cairns, N., Herrera-Marschitz, M., Seidl, R., Schneider, C., Terenius, L., Lubec, G., 1996. Endogenous opioids in frontal cortex of patients with Down syndrome. Neurosci. Lett., 203(2), 111-114.

Rode, F., Broløs, T., Blackburn-Munro, G., Bjerrum, O.J., 2006. Venlafaxine compromises the antinociceptive actions of gabapentin in rat models of neuropathic and persistent pain. Psychopharmacology (Berl)., 187(3), 364-375.

Sawynok, J., 2014. Topical Analgesics for Neuropathic Pain in the Elderly: Current and Future Prospects. Drugs Aging, Epub ahead of print.

Scaglione, M., Fabbri, L., Dell'Omo, D., Gambini, F., Di Rollo, F., Guido, G., 2014. A case of bilateral stress fractures in an old woman: three years of pain. Clin. Cases Miner. Bone Metab., 11(2), 149-152.

Scully, T., 2012. Demography: To the limit. Nature, 492(7427), S2-3.

Shannon, H.E., Eberle, E.L., Peters, S.C., 2005. Comparison of the effects of anticonvulsant drugs with diverse mechanisms of action in the formalin test in rats. Neuropharmacology, 48(7), 1012-1020.

Staaf, S., Oerther, S., Lucas, G., Mattsson, J.P., Ernfors, P., 2009. Differential regulation of TRP channels in a rat model of neuropathic pain. Pain, 144 (1-2), 187-199.

Stratakis, C.A., Chrousos, G.P., 1995. Neuroendocrinology and pathophysiology of the stress system. Ann. N. Y. Acad. Sci., 771, 1 – 1 8 .

Stuesse, S.L., Cruce, W.L., Lovell, J.A., McBurney, D.L., Crisp, T., 2000. Microglial proliferation in the spinal cord of aged rats with a sciatic nerve injury. Neurosci, Lett., 287(2), 121-124.

Su, L., Wang, C., Yu, Y.H., Ren, Y.Y., Xie, K.L., Wang, G.L., 2011. Role of TRPM8 in dorsal root ganglion in nerve injury-induced chronic pain. BMC Neurosci., 12, 120.

Taguchi, T., Ota, H., Matsuda, T., Murase, S., Mizumura, K., 2010. Cutaneous C-fiber nociceptor responses and nociceptive behaviors in aged Sprague-Dawley rats. Pain, 151(3), 771-782.

Takasusuki, T., Yaksh, T.L., 2011. The effects of intrathecal and systemic gabapentin on spinal substance P release. Anesth. Analg., 112(4), 971-976.

Taneja, A., Troconiz, I.F., Danhof, M., Della Pasqua, O., Neuropathic Pain Project of the PKPD Modelling Platform., 2014. Semi-mechanistic modelling of the analgesic effect of gabapentin in the formalin-induced rat model of experimental pain. Pharm., Res., 31(3), 593-606. Epub 2013 Oct 5.

Tjølsen, A., Berge, O.G., Hunskaar, S., Rosland, J.H., Hole, K., 1992. The formalin test: an evaluation of the method. Pain, 51(1), 5-17.

Tran-Van-Minh, A., Dolphin, A.C., 2010. Gabapentin inhibits the Rab11-dependent recycling of the calcium channel subunit α2δ-2. J. Neurosci., 130, 12856–12867.

U.S. Census Bureau., 2004. U.S. Interim Projections by Age, Sex, Race, and Hispanic Origins. Retrieved July 26, 2004 form the world wide web at: www.census.gov/ipc/www/usinterimproj/

Veves, A., Backonja, M., Malik, R.A., 2008. Painful diabetic neuropathy: epidemiology, natural history, early diagnosis, and treatment options. Pain Med., 9(6), 660-674.

Vierck, C.J., Yezierski, R.P., Light, A.R., 2008. Long-lasting hyperalgesia and sympathetic dysregulation after formalin injection into the rat hind paw. Neuroscience. 153(2), 501-506.

Vos, T., Flaxman, A.D., Naghavi, M., Lozano, R., Michaud, C., *et al*., 2012. Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and

injuries 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet, 380 (9859), 2163-2196.

Wang, H., Dwyer-Lindgren, L., Lofgren, K.T., Rajaratnam, J.K., Marcus, J.R., Levin-Rector, A., Levitz, C.E., Lopez, A.D., Murray, C.J., 2012. Age-specific and sex-specific mortality in 187 countries, 1970-2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet, 380 (9859), 2071-2094.

Yakovleva, T., Marinova, Z., Kuzmin, A., Seidah, N.G., Haroutunian, V., Terenius, L., Bakalkin, G., 2007. Dysregulation of dynorphins in Alzheimer disease. Neurobiol. Aging, 28(11), 1700-1708.

Yamamoto, T., Yaksh, T.L., 1992. Comparison of the antinociceptive effects of pre- and post treatment with intrathecal morphine and MK801, an NMDA antagonist, on the formalin test in the rat. Anesthesiology, 77, 757-763.

Yoon, M.H., Yaksh, T.L., 1999. The effect of intrathecal gabapentin on pain behavior and hemodynamics on the formalin test in the rat. Anesth. Analg., 89(2), 434-439.

www.ncbi.nlm.nih.gov/pccompound/

PUBLICATIONS AND CONTRIBUTIONS

7. PUBLICATIONS AND CONTRIBUTIONS

Publications

Berliocchi, L., Maiarù, M., **Scuteri, D.,** Corasaniti, M.T., Bagetta, G., 2012. New trends in pain research: from basic research to clinical translation. Funct. Neurol., 27(4), 253-255.

Contributions

Characterization of alpha2-delta1 expression and response to chronic treatment with gabapentin in a mouse model of neuropathic pain. new trends in pain research.

Damiana Scuteri, Michelangelo Certo, Laura Berliocchi, Luigi A. Morrone, Cristina Tassorelli, Giacinto Bagetta, Maria Tiziana Corasaniti. From Basic Research to Clinical Translation. Parghelia (VV), Calabria, Italy. 13th-15th September, 2012. **Oral Communication.**

Current opportunities for drug repurposing in pain management: is there a role for autophagy modulation?

Laura Berliocchi, Maria Maiarù, Rossella Russo, Damiana Scuteri, Giuseppe Varano, Carlotta Chiappini, Cristina Tassorelli, Giacinto Bagetta, Maria Tiziana Corasaniti. Drug Repurposing And Beyond: The Fundamental Role

Of Pharmacology. Sala stampa, Aula Magna, University of Calabria (CS), Arcavacata di Rende (CS), Calabria, Italia. 13-14 Giugno 2014

Spinal expression of autophagic markers following intraplantar formalin injection

Federica Cavaliere, Maria Maiarù, Damiana Scuteri, Rossella Russo, Laura Berliocchi, Luigi A. Morrone, Giacinto Bagetta and Maria Tiziana Corasaniti. From Basic Research to Clinical Translation. Parghelia (VV), Calabria, Italy. 13th-15th September, 2012.

Gender differences in pain response to peripheral injury in mice.

Giuseppe Varano, Maria Maiarù, Damiana Scuteri, Laura Berliocchi, Luigi Antonio Morrone, Cristina Tassorelli, Giorgio Sandrini, Maria Tiziana Corasaniti. From Basic Research to Clinical Translation. Parghelia (VV), Calabria, Italy. 13th-15th September, 2012.

NEW TRENDS IN PAIN RESEARCH

From Basic Research to Clinical Translation

FINAL PROGRAMME AND ABSTRACT BOOK

(Eds. Laura Berliocchi, Maria Maiarù, Damiana Scuteri and Luigi A. Morrone)

AWARDS

SINS (Società Italiana di Neuroscienze) scholarhip for the congress “From Basic Research to Clinical Translation. Parghelia (VV), Calabria, Italy. 13th-15th September, 2012.”

PARTICIPATION TO CONGRESSES AND SEMINARS

8. PARTICIPATION TO CONGRESSES AND SEMINARS

DRUG REPURPOSING AND BEYOND: THE FUNDAMENTAL ROLE OF PHARMACOLOGY

Sala stampa, Aula Magna, University of Calabria (CS), Arcavacata di Rende (CS), Calabria, Italia. 13-14 Giugno 2014

NUTRIGENOMICA: NUOVE TENDENZE SCIENTIFICHE – Prof. Giovanni Scapagnini; Dr. Vincenzo Capilupi.

Università della Calabria, Arcavacata di Rende (CS), Calabria, Italia. 27 Maggio 2014

DOTTORATO DI RICERCA IN BIOCHIMICA CELLULARE ED ATTIVITA' DEI FARMACI IN ONCOLOGIA XXVI CICLO – SEDUTA DI DOTTORATO

Università della Calabria, Arcavacata di Rende (CS), Calabria, Italia. Dicembre 2013

THE R&D PROCESS FOR ANTI-INFLAMMATORY MEDICINES – Prof. Richard G. Knowles. SEMINARIO

Università della Calabria, Arcavacata di Rende (CS), Calabria, Italia. 2 Dicembre 2013

IL RUOLO DEI VIRUS ONCOGENICI E DEI FATTORI AMBIENTALI NELLA CARCINOGENESI UMANA – Dr. Massimo Tommasino. SEMINARIO

Università della Calabria, Arcavacata di Rende (CS), Calabria, Italia. 23 Maggio 2013

CERIMONIA DI CONFERIMENTO DELLA LAUREA MAGISTRALE *HONORIS CAUSA* IN SCIENZA DELLA NUTRIZIONE A VANDANA SHIVA

Università della Calabria, Arcavacata di Rende (CS), Calabria, Italia. 9 Aprile 2013

FARMACI E PRODOTTI PER LA SALUTE: NUOVE PROSPETTIVE NELLA RICERCA TRASLAZIONALE

Università della Calabria, Arcavacata di Rende (CS), Calabria, Italia. 8 Aprile 2013

DOTTORATO DI RICERCA IN BIOCHIMICA CELLULARE ED ATTIVITA' DEI FARMACI IN ONCOLOGIA XXV CICLO – SEDUTA DI DOTTORATO

Università della Calabria, Arcavacata di Rende (CS), Calabria, Italia. 20 Dicembre 2012

DEPRESSIONE ED ANTIDEPRESSIVI: QUALE RUOLO PER LA FARMACOGENETICA? – Prof. Tullio Giraldi. SEMINARIO

Università della Calabria, Arcavacata di Rende (CS), Calabria, Italia. 27 Novembre 2012

NEW TRENDS IN PAIN RESEARCH – From Basic Research to Clinical Translation. INTERNATIONAL MEETING

Parghelia (VV), Calabria, Italia. 13-15 Settembre 2012.

METABOTROPIC $GABA_B$ RECEPTORS AND THEIR POTENTIAL AS THERAPEUTIC TARGETS – Prof. N. Bowery. SEMINARIO

Università della Calabria, Arcavacata di Rende (CS), Calabria, Italia. 13 Marzo 2012

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