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Probing the putative role of imprinted Begain in the modulation of neuropathic pain

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Abstract

Neuropathic pain is a complex pain condition that generally results from a somatosensory system lesion or disease. Changes in pain signaling, second-order nociceptive neurons, and inhibitory modulation are only a few of the pathophysiology's components that contribute to its establishment and chronicity. For instance, peripheral nerve injury-induced chronic pain appears impact on NMDA receptor association with the postsynaptic density (PSD) complexes. Begain is a protein highly expressed in the nervous system and thought to interact with the NMDA receptor through the PSD-95/SAP90 protein and to be recruited to synapses dependent on NMDAR activity. Begain has already been shown to be upregulated in mice with peripheral nerve injury, and its absence in a knockout mouse model resulted in a significantly lower pain level. The Begain gene is nevertheless susceptible to epigenetic control since it uses alternate first exons to produce two isoforms with unique N-termini, one of which is, termed *Begain* 1B, is imprinted. Throughout embryonic development and in adult life, epigenetic mechanisms regulate gene expression, offering a highly adaptable response mechanism to environmental influences. It is generally accepted that epigenetic mechanisms may be involved in persistent pathological pathologies such as chronic neuropathic pain. The aim of this project was to investigate whether the imprinted *Begain* isoform modulates neuropathic pain. To that effect, we carried out molecular and nociceptive behavioral studies on Begain isoform 1B-specific knock-out mice. Heterozygous mutant mice of either parental transmission, null mice, and wild type littermates were all subjected to peripheral nerve injury-induced neuropathic pain (or sham surgery) and analyzed using the von Frey test to assess mechanical allodynia on days 7, 14 and 21 after surgery. The nociceptive analysis showed that allodynia induced by SNI is not significantly affected by the silencing of imprinted Begain 1B.
Samples of spinal cord (SC), dorsal root ganglia (DRG) and rostral ventromedial medulla (RVM) were collected to probe for potential changes in steady-state mRNA levels induced by the neuropathic pain condition. Using RT-qPCR, expression analyses of Begain isoforms 1A and 1B, and the Dlk1 gene (an imprinted neighbor), as well as relevant NMDA receptor subunits, were conducted at day 7 after surgery. The expression of the Begain 1B was unaffected by the SNI model. Nevertheless, the

expected genotype effect was evident, showing full silencing in KO mice and a significant downregulation in Het Pat animals as compared to Het Mat or WT mice (since this imprinted isoform is mostly paternally expressed and maternally repressed). Similarly, the transcript levels of Begain 1A and Dlk1 remained unaltered under the neuropathic pain condition, and, unlike Begain 1B, did not show altered expression in mutant animals. The expression analyses of four NMDAR subunits, on the other hand, revealed interesting results in the DRG. Despite remaining unaltered in the SC and RVM, the expression of the NR1 and NR2D subunits showed significant changed in the DRG. In comparison to their sham counterparts, the null mice subjected to SNI revealed considerably lower NR1 expression. Instead, the NR2D displayed significantly decreased expression levels in KO and heterozygotes with maternal inheritance of the silence allele (Het Mat) relative to their WT littermates. Heterozygotes with paternal inheritance (Het Pat) also had their transcript levels reduced, but not statistically significantly. To analyze the parental-specific expression (imprinting) of Begain 1A, 1B, and Dlk1, qualitative Sanger sequencing of cDNA from hybrids of reciprocal crosses was carried out. Begain 1B and Dlk1 exhibited paternal expression in SC and RVM consistent with their “canonical” parental expression. Interestingly, Begain 1B revealed a pattern of biallelic expression in the DRG, in contrast to the behavior of DLK1, which showed obvious and nearly exclusively paternal expression. Conversely, Begain 1A showed the expected biallelic expression in WT animals in all tissues, but in Het Pat the expression in SC and RVM came preferentially from the paternal allele in mice subjected to sham surgery, although under the SNI condition the expression “reverted” to biallelic. The same was seen for Het Mat animais but in SC only, whereby in sham animals the expression was preferentially maternal and then reverted to biallelic in SNI-subjected animals. This was not seen in DRG for Het Pat mice, and DRG and RVM for Het Mat mutants. The lack of a pain phenotype (as assayed herein) in the Begain 1B mutant can be attributed to several factors. Namely, in spite of this isoform being more expressed than the 1A isoform, its silencing may be compensated by the presence of the latter. Also, the model of neuropathic pain used in this work may not be the most adequate to reveal the impact of the silencing of the imprinted isoform. Another possible explanation would be that Begain may not be an essential player in the molecular

pathways underlying the neural paradigms associated with neuropathic pain. Molecular studies focusing on day 21 post-surgery and are still to be completed, as is the analysis of additional potential interactors that may respond to Begain 1B silencing. Extensive research however is required to better understand the role of the Begain 1B isoform in the latter stages of pain chronification.

Resumo

A dor neuropática é uma complexa condição de dor que geralmente resulta de uma lesão ou doença do sistema somatossensorial. Alterações na sinalização da dor, neurónios nociceptivos de segunda ordem, e modulação inibitória são apenas alguns dos componentes da fisiopatologia que contribuem para o seu estabelecimento e cronicidade. Por exemplo, a cronificação da dor induzida por lesão nervosa periférica parece ter impacto nas subunidades do recetor de NMDA e levar à formação de complexos da densidade pós-sináptica (PSD). Begain é uma proteína altamente expressa no sistema nervoso que se pensa interagir com o recetor NMDA através da proteína PSD-95/SAP90, sendo recrutada para as sinapses de forma dependente da atividade dos recetores de NMDA. O Begain já demonstrou estar super-regulado em murganhos com lesão nervosa periférica, e a sua ausência num modelo de murganho knock-out resultou num nível de dor significativamente mais baixo. O gene Begain é, no entanto, suscetível ao controlo epigenético, uma vez que utiliza exões alternativos para produzir duas isoformas com N-termini únicos, uma das quais está paternalmente *imprinted*. Desde a fase embrionária e durante o decorrer da vida, os mecanismos epigenéticos regulam fisiologicamente a expressão genética e do desenvolvimento, oferecendo uma resposta altamente adaptável às influências ambientais. É presumível que alguns mecanismos epigenéticos, que inclusive já se verificaram ter sido desencadeados em resposta a doenças, possam estar envolvidos em perturbações patológicas persistentes, tais como a dor crónica neuropática. O objectivo deste projecto foi investigar de que forma a isoforma *imprinted* do begain modula a dor neuropática. Para isto, realizámos estudos moleculares e comportamentais e adotamos uma estratégia experimental utilizando um *knock-out* específico para a isoforma 1B do Begain. Murganhos heterozigóticos mutantes provenientes de transmissão parental alternativa, murganho mutantes *knock-out*, e *wild-type* foram sujeitos a dor neuropática induzida por lesão nervosas periférica e o teste de Von Frey utilizado para avaliar a alodinia mecânica. Uma vez terminadas as análises comportamentais, foram recolhidas amostras de *SC*, *DRG*, e *RVM* para analisar potenciais alterações nos níveis de mRNA induzidas pela condição de dor neuropática. Usando RT-qPCR, foram feitas análises de expressão das isoformas Begain 1A e 1B, gene Dlk1 e das subunidades recetoras de NMDA.

Além disso, foram realizados ensaios de *imprinting* baseadas na sequenciação de Sanger para analisar a expressão parental específica de Begain 1A, 1B e Dlk1. A análise comportamental demonstrou que a alodinia induzida pelo SNI não é significativamente afetada pela mutação na forma *imprinted* do Begain. Em termos da análise da expressão genética, este projeto centrou na avaliação no sétimo dia após a cirurgia, sendo que representa uma fase inicial no processo de cronificação da dor. A expressão da isoforma Begain1B não foi afetada pelo modelo SNI. No entanto, o efeito genótipo esperado era evidente, mostrando um silenciamento total em murganhos KO e uma desregulação significativa em animais Het Pat em comparação com ratos Het Mat ou WT. Relativamente ao imprinting, Begain 1B e Dlk1 exibiram expressão paterna em SC e RVM consistente com a sua expressão parental classicamente descrita. Begain 1B, curiosamente, revelou um padrão de expressão bialélica no DRG, em contraste com o comportamento do Dlk1, que mostrou uma clara e quase exclusivamente paterna. Esta é a primeira vez que Begain 1B é referido como tendencialmente bialélico em vez de impresso. As descobertas preliminares do modelo Begain^{tm1bMLS} sugerem que este será uma ferramenta útil para integrar o controlo dos genes impressos por mecanismos epigenéticos em contextos patológicos. Contudo, é necessária uma investigação extensiva para compreender melhor o papel da isoforma Begain 1B nas últimas fases da cronificação da dor.

Table of contents

List of Abbreviations

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

BDNF - brain-derived neurotrophic factor

Begain - brain-enriched guanylate kinase associated protein

bp – base pairs

CAMKII - Ca2+ calmodulin-dependent protein kinase II

CNS - central nervous system

CRISPR - clustered regularly interspaced short palindromic repeats

Cas – CRISPR-associated

Dio3 - type III iodothyronine deiodinase

Dlk1 - delta-like homolog 1

DMR - differentially methylated region

DRG - dorsal root ganglion

GABA - γ-aminobutyric acid

gDMR - germline differentially methylated region

IASP - International Association for the Study of Pain

ICR - imprinting control region

lncRNA - long non-coding RNA

mRNA - messenger RNA

ncRNA - non-coding RNA

NMDA - N-methyl-D-aspartate

NMDAR - N-methyl-D-aspartate receptor

PCR - polymerase chain reaction

PSD - post synaptic density

SAP - synapse-associated protein

SNI - spared nerve injury

ssDNA - single-strand DNA

Introduction

Introduction

Neuropathic Pain

Neuropathic pain is a complex pain state usually in consequence of a lesion or disease of the somatosensory system (Merskey and Bogduk, 1994). Common causes for neural damage and subsequent pain are injury, ischemia or metabolic diseases (IASP, 2005). After a pathological change, peripheral nerves become more sensitive and develop spontaneous excitability to otherwise innocuous chemical, thermal and mechanical stimuli (Merskey and Bogduk, 1994; Colloca et al., 2017). The neuropathic pain pathophysiology comprises several dimensions that contribute for its establishment and chronicity. Such dimensions include alterations in pain signaling and ion channels, second-order nociceptive neuron and inhibitory modulation changes (Colloca et al., 2017). Chronification of pain, which is defined by a prolonged, chronic pathologic occurrence that is spontaneous and without a physiologically evident origin, like when tissue injury occurs, and pain persists for some time after the primary noxious stimulus has disappeared (Tracey and Bushnell, 2009). Between 6.9% and 10% of people globally are estimated to experience neuropathic pain (Van Hecke et al., 2014).

Pain signaling molecular and circuit changes

In physiological conditions nociceptive inputs travel from the primary afferents C and Aδ fibers predominantly to the lamina I-II of spinal dorsal horn neurons and then to the deeper laminae IIi-IV (Christensen and Perl, 1970; Woolf and Fitzgerald, 1986). In a pathological state, we first come across a disruption, notably in these laminae. Sensory signal transmission is altered as a result of decreased interneuron synaptic effectiveness. So, noxious stimuli directly engage the projection cells and, via a circuit that is dorsally directed, provide the projection cells more feedforward excitatory drive (Braz et al., 2014). As a result, the pain circuitry that originates in the spinal dorsal horn functions abnormally, disrupting inhibitory control or facilitating excitatory control (Braz et al., 2014; Duan et al., 2015; Peirs et al., 2015). Changes in ion channels (sodium, calcium, and potassium) inside the injured nerves, which can include all types of afferent fibers, leads to neuropathy, which directly effects sensory

signaling in the spinal cord and the brain (Yang et al., 2004). There will be sensory loss if an afferent fiber is severed from the periphery as a result of trauma or a lesion. However, the damaged fiber remnants may still produce ectopic activity, which causes pain to spread from the injured location (Tesfaye et al., 2013). The irritable nociceptors that were still intact became hyperexcitable (Fields et al., 1998). Higher expression of calcium channel activity in the nerve terminal and altered inputs to the spinal cord lead to increased neurotransmitter release and enhanced excitatory synaptic transmission in the nociceptive circuit (IASP, 2005). Increased sensory responses result from increased spinal neuron excitability, which also enables afferent fibers to stimulate second-order nociceptive neurons. Consequently, a given stimulus excites more second-order nociceptive neurons, creating the phenomenon known as central sensitization (Woolf, 2011; Baron et al., 2013). Second-order nociceptive neurons undergo postsynaptic alterations as a result of continuous peripheral afferent fiber firing and concomitant release of excitatory molecules; this excess of signaling is caused in part by phosphorylation of NMDA and AMPA receptors. These alterations are reflected by increased sensory thalamic neural activity, which may explains mechanical allodynia (Patel and Dickenson, 2016; Peyron, 2016). Scaffold proteins and NMDA receptors, components of PSD, are involved in pain and abnormal pain transmission through activation of signalling cascade proteins in the spinal dorsal horns and brain (Craven and Bredt, 1998; Garner et al., 2000; Luo et al., 2014). The balance between pathological and physiological conditions is determined by reversible changes of PSD complex components in the spinal dorsal horn (Abe et al., 2005). NMDA receptors interact with scaffold proteins such as postsynaptic density (PSD)-95 and synapse-associated protein (SAP) 90 though the GluN2B component of these receptors (Katano et al., 2008). After peripheral nerve injury, the interactions between these scaffold proteins is augmented (Peng et al., 2013). Disruption of PSD components PSD-95/SAP90 and GluN2B has been shown to attenuate neuropathic and inflammatory pain (Katano et al., 2011). Neuropathic pain caused by peripheral nerve injury seems to be crucially dependent of GluN2B phosphorylation at Tyr1472 (Y1472) and subsequent formation of PSD complexes at spinal dorsal horn neurons (Nakazawa et al., 2006; Katano et al., 2008, 2011; Matsumura et al., 2010).

Begain gene and its role in functional pain mechanisms

Begain (brain-enriched guanylate kinase-associated protein) is a PSD-95/SAP90 binding protein highly expressed in the brain and thought to sustain the structure of the PSD (Naisbitt et al., 1997; Deguchi et al., 1998). In neurons, Begain is localized at synapses and in the nucleus. The Begain gene encodes two isoforms termed Begain 1A and Begain 1B (Soares et al., 2018), which result from the usage of alternative first exons, thus having different N-termini. While the N-terminus of the Begain 1B isoform only has one nuclear localization signal, that of the Begain 1A isoform has two. Both neuronal and non-neuronal cells can detect these. Specifically, the N-terminus amino acid composition of the isoforms is MWTGGRRPGRLRRA and MGSHQQSSQ for Begain 1A and Begain 1B respectively; the remainder 601 amino acids that make up the proteins being fully shared among the two. The N-terminal region is involved in nuclear localization but also mediates the recruitment from the nucleus to dendrites, and from dendrites to synapses in an NMDA receptor activity-dependent manner (Katano et al., 2016). Begain is only found in the nucleus of non-neuronal cells, such as epithelial cells, although the protein is also found at synapses in neurons (Yao et al., 2002). In fact, Yao and collegues (2002) propose that the recruitment of Begain to synapses may take place in two steps: first, there may be neuron-specific components that bind Begain's N-terminal and either transport it from the nucleus to the dendrites or sequester it there; and second, Begain is transported from dendrites to synapses by PSD-95/SAP90 binding in an NMDAR activity-dependent manner. Strikingly, whereas the 1A isoform is biallelic expressed, the Begain 1B isoform is imprinted (paternally expressed), as previously demonstrated in sheep (Smit et al., 2005) and in the mouse (Tierling et al., 2009; Soares et al., 2018). The precise role, however, played by each isoform in pathological pain is still unknown. It is significant that imprinting, a crucial epigenetic regulatory process, has not yet been implicated in the role or relative contribution of imprinted Begain 1B in neuropathic pain.

Epigenetics and imprinting

Genomic imprinting is an epigenetic process that results in the monoallelic expression of genes in a parent-of-origin-specific manner. In other words, a normal

gene regulation is dependent of only one of the two parental chromosomes being expressed, some imprinted genes are maternal and other paternal. The specific imprinting occurs during the gamete formation, and indicates that the maternal and paternal genomes are not functionally equivalent (Barlow and Bartolomei, 2006). In fact, epigenetic changes control gene expression without changing the DNA's physical sequence. Stress, tissue injury, or diseases that impact physiological and pathological processes can acutely elicit epigenetic processes such as DNA methylation, histone changes, and the expression of non-coding RNA (ncRNA) (Smith and Meissner, 2013; Bai et al., 2015; Descalzi et al., 2015) . By converting the chromatin state between euchromatin and heterochromatin, DNA methylation and histone tail post-translation changes affect DNA access by permitting or blocking the physical binding of transcriptional factor proteins and controlling gene transcription (Jenuwein and Allis, 2001; Birney et al., 2007; Ruthenburg et al., 2007). However, ncRNAs suppress mRNA degradation and/or act as translation inhibitors (Grishok et al., 2001; Bartel, 2004; He and Hannon, 2004). This process is dynamic and is brought about by the selective epigenetic marking of genomic regions, termed imprinting control regions (ICRs) that coordinately control the allelic expression of several genes in clusters. One of the most prevalent epigenetic mechanisms that significantly affects gene expression is DNA methylation. Various tissues acquire different patterns of methylation throughout prenatal development, which confers cell-type differentiation by influencing gene activity (Razin and Szyf, 1984). Studies involving monozygotic twins showed that although having identical genetic makeup, their methylation profiles differed a little (Ziller et al., 2013). Disruption of imprinting is associated with human syndromes and cancer (Paulsen and Ferguson-Smith, 2001). Numerous imprinted genes are crucial for human development, neonatal growth, and neural plasticity. If their expression is disrupted, it may increase the chance of developing diseases like cancer, transitory neonatal diabetes mellitus, and Prader-Willi syndrome, among others (Peters, 2014). The influence of epigenetic mechanisms regulating the expression of specific genes after damages or disorders in the somatosensory system is now well established. These pathways can retain long-lasting effects on gene expression and are inherited (Penas and Navarro, 2018). The majority of cell types, imprinted genes frequently exhibit monoallelic expression.

However, certain genes only exhibit monoallelic expression at a particular developmental stage or tissue, and in some instances, this methylation or monoallelic expression can vary between individuals (Rougeulle et al., 1997; Vu and Hoffman, 1997; Travers et al., 2013; Hanna et al., 2016; Sanchez-Delgado et al., 2016; Zink et al., 2018). Located at the 5' upstream boundary, Begain is part of the Dlk1-Dio3 imprinted domain, which defines a ~1 Mb genomic region of paternally expressed protein-coding genes and maternally expressed non-coding regulatory RNA transcripts important for pre- and post-natal development (Rocha et al., 2008; Charalambous et al., 2012). In this cluster, imprinting is regulated by a germline-derived differentially methylated region (DMR) - termed IG-DMR - located between Dlk1 and the non-coding RNA Gtl2, which is fully methylated in sperm but remains unmethylated in the oocyte (Takada et al., 2002).

Recently, Begain has been implicated in the maintenance of neuropathic pain following peripheral nerve injury. In wild type mice, Begain was upregulated in the spinal dorsal horn upon spared nerve injury (SNI). Furthermore, in Begain knock-out mice, neuropathic pain was significantly attenuated after SNI revealing that Begain was involved in pathological pain transmission through activation of NMDA receptors in the spinal dorsal horn. However, these functional studies were conducted following the simultaneous knock-out of Begain 1A and 1B since the gene was targeted through the removal of exons 4 and 5, which are common to both isoforms. Thus, the specific involvement of each isoform in pathological pain remains elusive. Importantly, the role and/or relative contribution of imprinted Begain 1B in neuropathic pain is yet to be demonstrated, begging the question of whether imprinting (a crucial epigenetic regulatory process) is in anyway associated with abnormal neural plasticity in the context of pathological pain. The epigenetic regulation of the mammalian genome constitutes a fundamental mechanism that modulates several biological processes from embryonic development to plasticity in the adult nervous system. Notably, it acts as a mediator interface between genes and the environment.

Aims

Aims

This project aims to explore the role of the imprinted begain isoform in the modulation of neuropathic pain. For thus, we implemented an experimental approach using the Begain isoform 1B-specific knock-out model, by conducting molecular and behavioral studies. To that end, peripheral nerve injury-induced neuropathic pain was induced in heterozygous mutant mice of alternative parental transmission, mutant knock-out, and wild type littermates.

Thus, we carried out:

1) Nociceptive behavioral analyses, using the Von Frey test to assess mechanical allodynia on three distinct timepoints.

2) Characterization of the expression levels of Begain 1A, Begain 1B and Dlk1 in spinal cord (SC), dorsal root ganglia (DRG) and rostral ventromedial medulla (RVM).

3) Analysis of NMDA receptor sub-units expression levels in SC, DRG and RVM.

4) Imprinting analysis by Sanger sequencing.

In summary, our strategy will contribute to deepen the understanding of the interplay between the genome, epigenetic control, and pain physiology, which will contribute to the development of new therapeutic strategies for the treatment of chronic pain.

Material and Methods

Material and Methodology

1. Animals and Ethical Statement

All the experiments were performed in adult mice (in the weight range 25-35g) housed at the animal facility in the *Centro de Investigação Médica (CIM)* of the *Faculdade de Medicina da Universidade do Porto (FMUP).* All procedures were conducted according to *Direção Geral de Alimentação e Veterinária* licensing and regulations, as well as the guidelines set forth by the institution's Animal Welfare and Ethics Body (*Órgão Responsável pelo Bem-estar e Ética Animal*, ORBEA) on the use of animals for research purposes based on the 2010/63/EU directive. Furthermore, all experimental methods were carried out in compliance with the International Association for the Study of Pain's (IASP) ethical criteria for the study of experimental pain in conscious animals. The animals were maintained in a 12-hour light/dark cycle with controlled temperature (21±2ºC) and humidity (50±5%). Behavioural habituation and testing sessions were performed around the same period during the light cycle. The mice were collectively maintained in conventional type II cages, in groups of 2 to 4 animals. During all the experiments, mice had water and food *ad libitum*. At the beginning of each set, the animals were given a period for habituation to the experimenter handling, laboratory room environment and Von Frey apparatus. Experimental animals resulted from crosses between C57BL/6J and Begain^{tm1bMLS} mutant males (C57BL/6J background), ensuring transmission of the inversion of 1B exon. The experimental groups were composed by Begain1B mutants, both heterozygous and homozygous, and wild type littermates.

2. Begain^{tm1bMLS} model

A method that involved inverting the genomic region containing exon 1B was developed to target the Begain 1B isoform. This disrupted the transcript while preserving the genomic region unaffected. High specificity (minimum off-targets) and predicted efficiency were features of the CRISPR site sequences 5'-ACTCCAGCACTGCCTTTCGC **GGG**-3' and 5'-CCTGAAGGATGATTCGTAGA **AGG**-3' (PAM sequences in bold). A long single-strand DNA (ssDNA) fragment was

designed with arms of homology to the endogenous locus, flanking the inverted target sequence, to be introduced by homology directed repair upon Cas9-mediated cleavage and removal of the endogenous target sequence since we intended to replace the sequence delimited by the CRISPR-Cas target sites rather than just discard it.

3. Surgical Procedure - Spared Nerve Injury

The spared nerve injury (SNI) model was induced as described by the authors (Decosterd and Woolf, 2000). A SNI or a sham lesion intervention (control group) with an identical level of skin incision and muscle dissection was applied to each mouse. Deep general anesthesia induced by 1.5–2.5% isoflurane was used during the procedure. For this, the mice were placed in a container to induce anesthesia with 3.0% isoflurane, which was subsequently maintained under 1.5% isoflurane using a head mask throughout the entire procedure. The left hindlimb was lifted, extended laterally, and immobilized. The mid-thigh incision was made using the femur and the knee joint as reference points. In order to avoid extensive lesion of the muscle, the *biceps femoris* muscle was exposed, and the artery *genus descendens* was used to guide the incision in accordance with the orientation of the muscle fibers. Following the muscle incision, the three branches of the sciatic nerve (the sural, common peroneal, and tibial nerves) were carefully exposed without stretching nervous structures. A 5.0 silk suture was tightly wrapped around the peroneal and tibial nerves to tied them together and a 1-2 mm wide section was then severed. The sural nerve was carefully monitored during the procedure to prevent straining of the nerve by avoiding contact with the surgical tools. After the nerve was severed, the muscle and skin were stitched together in two separate layers, and the head mask was taken off to reverse the anesthetic effect. An identical surgical procedure was carried out on the sham group, however without inflicting the nerve lesion.

4. Behavioural Procedures - Von Frey Filaments Test

Von Frey monofilaments were used as a tool for nociceptive behavioral assessment following SNI-induced neuropathic pain. The animals were habituated to the environment, equipment, and experimenter handling for a week. Each mouse was individually placed on the elevated platform with a wire mesh flooring prior to the testing session and given 15 to 30 minutes to acclimate. The mice were then tested in the mid-plantar area of the paw. The paw was stimulated by perpendicular contact with a von Frey filament until it bent slightly. Each stimulus should be present for maximum of 5 seconds, or until a response was elicited.

If the response was inconclusive, the stimulus was presented again. Paw withdrawal was considered a defensive and thus a positive response. Starting with the filament with the lowest force (in this case 0,02 grams-force), stimulation was repeated for filaments with progressively higher forces. According to the *ascending stimulus method* (Deuis et al., 2017), each filament was tested ten times, and the force necessary to elicit at least two out of ten positive responses was used to estimate the sensory threshold for mechanical stimulation. The *percent response method* (Deuis et al., 2017) was also applied for assessing frequency of withdrawal curves. In this instance, the ten filaments were applied ten times in ascending sequence with forces ranging from 0,02 to 4g, and the results of each trial were reported.

5. Tissue collection

On the seventh day following surgery, the brain, spinal cord, and dorsal root ganglia were dissected from adult animals in order to examine the levels of gene expression in the Begain^{tm1bMLS} model mutant mice and wildtype littermates. Adult mice were euthanised by placing them on a chamber with 5% isoflurane followed by decapitation on the guillotine. Their tissues were quickly collected, placed into 1.5 ml or 5 ml tubes, snap frozen on liquid nitrogen, and then kept at -80 °C until use. The adult spinal cord was taken from the area between the L3 and L6 lumbar segments, which is where most of the inputs from the paw fibers are received. The spinal cord sample was further partitioned into the ipsilateral and contralateral halves before

freezing. DRG were bilaterally extracted and pooled from the same L3 to L6 area. Before the RVM area was isolated, the whole brain was collected and frozen.

6. Molecular Assays

a. Genotyping

Ear punch samples of over 12 days-old mice were collected. The ear fragments were boiled in a 100ul aqueous boiling genotyping solution for 20 minutes (25mM NaOH and 0.2mM EDTA). Afterward, 100 ml of a neutralizing solution containing 40 mM Tris-HCl (pH=8.0) was added, and one ml of the recovered suspended DNA was used for amplification. The DNA was amplified in a 15 ml reaction using Kapa Ready Mix (Sigma), 3% DMSO, and the specific primer pair for each desired amplicon. The PCR cycling conditions were the following: 95 °C initial denaturing for 5 min; 36 cycles of 95 °C denaturing for 30 sec; 60 °C annealing for 30 sec; 72 °C extension for 30 sec; and final extension at 72 °C for 5 min. The presence or absence of the amplicon allowed us to assign the genotype to each animal after the PCR results were run on a 2% agarose gel electrophoresis.

Transcript primer	Primer sequence	Size (bp)	Annealing temperature
Bg1b _5Pgenot_Fw	ACCCACTAGCCCCTGTTAAC	400	60ºC
Bg1b _5Pgenot_Rv	AACTGAGGCTCGGGGAGA		
Bg1b _5Pgenot_Fw	ACCCACTAGCCCCTGTTAAC	242	60ºC
Bg1b _5Pgenot_Inv_Rv	CCCTCCCCGCACACTTTAAT		
Bg1b_Native_Genomic_Fw	AGACCTGGGAAGACTGATGG	249 (WT) 171 (Mut)	60ºC
Bg1b_Native_Genomic_Rv	GCCTTCCCTCCTCCAGATTT		

b. RNA extraction

Total RNA was extracted from adult mice RVM, DRG and spinal cord, dissected according to established procedures. RNA was isolated using the NZYTech Total RNA Isolation Kit and quantified using the Nanodrop 2000 by spectrophotometry (Thermofisher). In a short, tissue samples were placed in RNase-free tubes with magnetic beads with 1 mL of QIAzol Lysis reagent (Qiagen) for homogenizing in a MagNA Lyser instrument (Roche). The top aqueous phase was transferred into a 1.5 mL tube, to which 1 volume of 70% ethanol was added, and mixed by pipetting up and down. Phase separation was then achieved by centrifugation (15 min at 12000 g) after the addition of 200 ul of chloroform per 1 mL of QIAzol. According to the manufacturer's instructions, the mixture was pipetted into a NZYSpin Binding column for RNA extraction (NZYtech, Portugal). After RNA binding, DNase treatment, and a series of washing steps, the RNA was measured after being eluted with 40–60 ul RNase-free water. Through the use of the 2100 Bioanalyzer Instrument, RNA integrity was determined (Agilent, USA). All RNA samples were kept at -80°C until analysis.

c. cDNA Synthesis

Reverse transcription was carried out with 0.5 to 1 µg of total RNA using the NZY First-Strand cDNA Synthesis Kit (NZYtech, Portugal). To enhance sensitivity, oligo(dT)18 primers and random hexamers were used in the reaction mix. Following synthesis, RNase was applied on all cDNA samples. For later processing, the cDNA was diluted 1:20, aliquoted, and kept at 4 °C. To ensure that there was no genomic DNA contamination in the qPCR analysis that followed, negative control reactions - without reverse transcriptase - were also set up for a number of samples.

d. Real Time Quantitative PCR

Expression studies were conducted by RT-qPCR for biallelic Begain 1A, paternally expressed Begain 1B and Dlk1. Total RNA was isolated using TRIzol (Invitrogen) and silica-based spin columns according to manufacturer’s instructions. One microgram of total RNA was reverse transcribed using a cDNA synthesis kit with random primers. The resulting cDNA was diluted 1:20, aliquoted and stored at -20ºC for

subsequent quantification. Real-time quantitative PCR with SYBR Green was performed to measure the levels of gene expression. Target gene expression was normalised to the expression of ActinB. Thermocycling was carried out with 3µl of cDNA synthesized and diluted as described above, in triplicate. A standard curve made up of doubling dilutions of pooled cDNA from the samples being assayed will be run on each plate, and quantification performed relative to the standard curve.

Transcript primer	Primer sequence	Annealing
Begain 1A_Fw	GTAGTGGCGCGTGGAGTC	68ºC
Begain 1A_Rv	GCAGAGCCGGGGCCATGT	
Begain1B_Fw	GTAGTGGCGCGTGGAGTC	67ºC
Begain1B_Rv	ATGGGCAGCCATCAGTCTT	
Dlk1_Rv	GAAAGGACTGCCAGCACAAG	60ºC
Dlk1_Rv	CACAGAAGTTGCCTGAGAAGC	

7. Imprinting analysis

This task aims to examine the allelic expression (imprinting status) of Begain and Dlk1 in DRG, SC, and RVM in mice that have undergone sham surgery or SNI-induced neuropathic pain. Imprinting analyses were carried out on the seventh postoperative day. From reciprocal crosses between heterozygous mutants (C57BL/6 background) and Cast/EiJ mice, F1 hybrid offspring with expressed polymorphisms at Chr.12 was produced (Dlk1: rs253217941, alleles T and C; Begain: rs36806417, alleles G and A respectively). The imprinting assays were based on Sanger sequencing of PCR amplified cDNA to analyse parental-specific expression of Begain 1A, 1B and Dlk1.

8. Data Analysis

The GraphPad Prism program was used to perform statistical analysis (version 6.01). To determine whether the data was normal, the Shapiro Wilk test was performed, and parametric or nonparametric tests were utilized accordingly. A two-way ANOVA followed by a Dunn's multiple comparison test was used to analyse the variation of

genotypes' frequency in response to the filaments. The Kruskal-Wallis test was used to analyse expression studies and withdrawal threshold levels, also followed by the Dunn’s post-hoc test. Each figure's legend lists the statistical tests that were used and their corresponding significance levels. 5% was established as the significance level. Results are presented as the mean ± standard error of the mean (SEM).

Results

Results

1. Nociceptive behavioral analysis

a) Nociceptive behavior of Begaintm1bMLS KO, Pat and Mat mutants and WT littermates at baseline

Following an initial period of habituation to the experimental handling conditions, laboratory environment and Von Frey apparatus, a baseline measurement was established. The mice were tested a few days prior to surgery in a randomized and blind way to the experimenter. Two-way ANOVA was performed to analyze the effect of increasingly higher force von Frey filaments on nociception levels among the four distinct genotype groups at baseline. The test revealed a similar behavior amongst genotypes: there was not a statistically significant interaction between the force of the applied filaments and the animal genotype (p=0.764) (Fig. 1a). Similarly, when analyzing the paw withdrawal thresholds, no statistically significant differences were observed among the genotype groups (p=0.165) (Fig 1b).

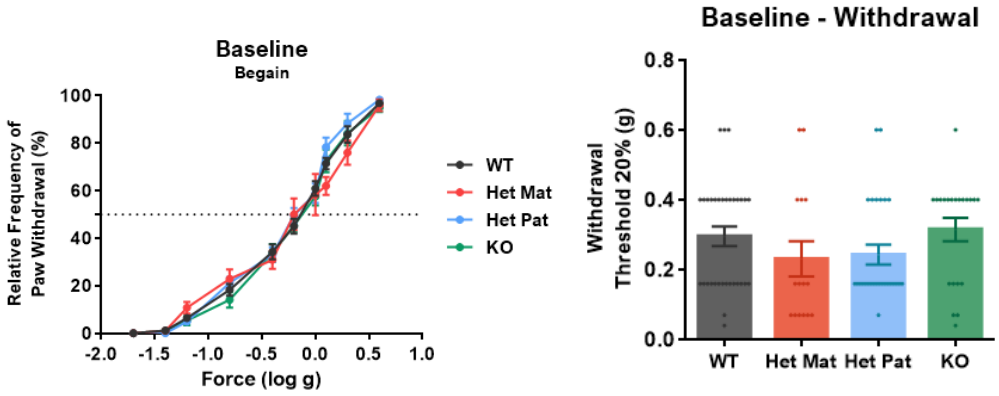


Figure 1. Naïve mice exhibit the same nociceptive behavior at baseline regardless of genotype.

Relative frequency of paw withdrawal (a) and paw withdrawal threshold (b) of Begain^{tm1bMLS} KO, Pat and Mat mutants and WT littermates at baseline.

Baseline measurements prior to sham or SNI surgery showed no statistical differences in nociceptive perception among groups. WT: n=33, Het Mat: n=15, Het Pat: n=27, KO: n=21. Values presented as mean ± SEM. Analyses were carried out using two-way ANOVA with repeated measures followed by Tukey's Multiple Comparisons post-hoc

test for frequency of paw withdrawal, and Kruskal–Wallis test followed by Dunn's post hoc test for withdrawal thresholds.

b) The SNI model of neuropathic pain was successfully induced in all genotype groups

Mechanical allodynia was assessed 7, 14 and 21 days after nerve lesion or sham surgery. The plantar surface of each subject's paw was stimulated with a series of monofilaments of ascending force. Frequency of paw withdrawal was determined for each filament and the threshold was taken as the lowest force that evoked two withdrawal responses in ten replicated stimuli. As expected, a significant decrease in withdrawal threshold was observed in wild-type animals at each time point, indicating that the SNI model of pain had been properly induced. The same was seen for the Begain 1B mutants, regardless of genotype class (Fig.2).

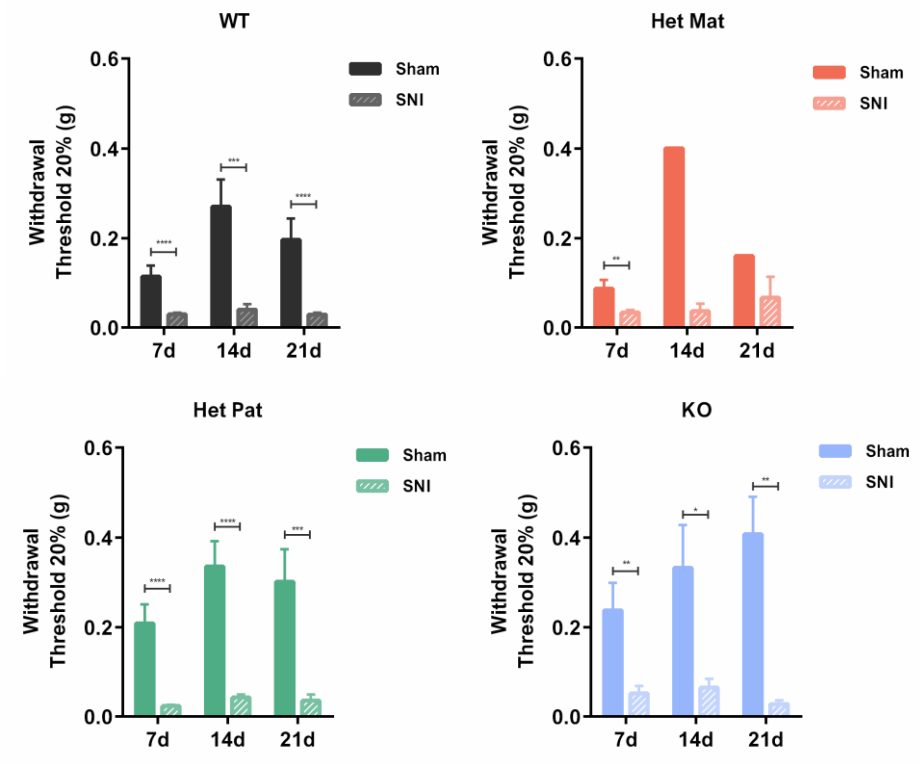


Figure 2. SNI induces mechanical allodynia regardless of genotype.

A mechanical allodynia behavior was developed following SNI surgery, as demonstrated by the significant decrease in withdrawal threshold. Values presented as mean±SEM. WT (7, 14, 21d, respectively): sham, n=15, 10, 10; SNI, n=18, 12, 12; Het Mat (7, 14, 21d,

respectively): sham, n=7, 2, 2; SNI, n=8, 3, 3; Het Pat (7, 14, 21d, respectively): sham, n=12, 8, 8; SNI, n=15, 10, 10; KO (7, 14, 21d, respectively): sham, n=10, 6, 6; SNI, n=11, 6, 6. The effect of treatment was analyzed using the Mann-Whitney test. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.

Indeed, the filament force required to elicit 20% of positive responses was significantly lower in SNI-subjected mice, as shown by the Mann-Whitney non-parametric test (WT time-point 7d: p<0,0001; 14d: p=0,0001; 21d: p<0,0001; Het Pat time-point 7d: p<0,0001; 14d: p<0,0001; 21d: p=0,0002; KO time-point 7d: p=0,0016; 14d: p=0,0152; 21d: p=0,0043). The Het Mat group displayed the same behavior at day 7 (p=0,0191), but despite the clear decreases also seen on days 14 and 21, the sample size (n=2-3) was insufficient for statistical analysis.

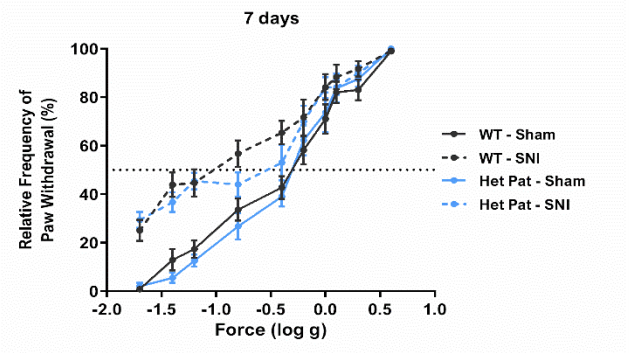
c) The null mutation on imprinted Begain 1B has no significant effect on SNI-elicited mechanical allodynia.

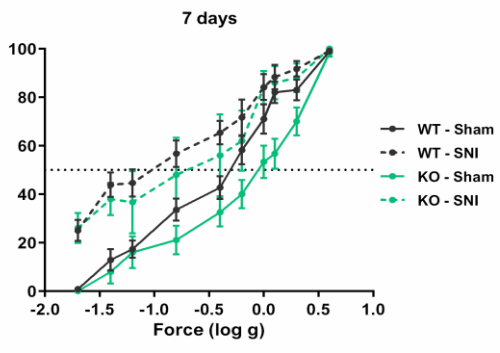
Following SNI and sham surgery, mechanical allodynia was assessed in all experimental groups and evaluated at time points 7, 14 and 21 days post-operation. Due to the small sample size of heterozygotes with maternal transmission of the silent allele (Het Mat) and the characteristic inter-individual variability of the model, we focus henceforth on the KO, Het Pat and WT genotypes (Fig. 3).

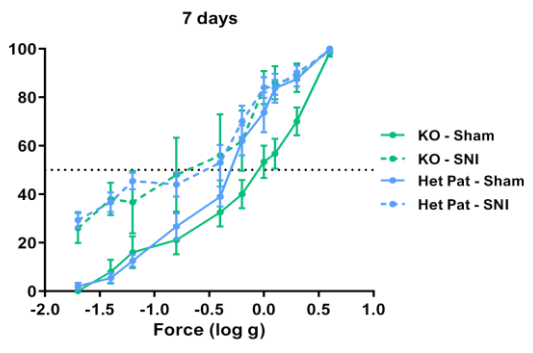
After 7 days of surgery, both sham and SNI-treated KO mice showed a tendency to display a lower frequency of withdrawal than the WT littermates (Fig 3, panel B); however, Kruskal-Wallis analysis did not confirm that these differences were statistically significant. Conversely, the Het Pat group exhibited a somewhat similar response to that of the WT animals for both treatments. Since the behavioral analyses were carried out in 3 distinct sets of animals (all genotypes being equally represented in each working set), preliminary analyses indicated that the KO mice had a significantly lower pain threshold on the first set of day 7 (data not shown). However, the differences faded thereafter with the inclusion of the results from sets 2 and 3.

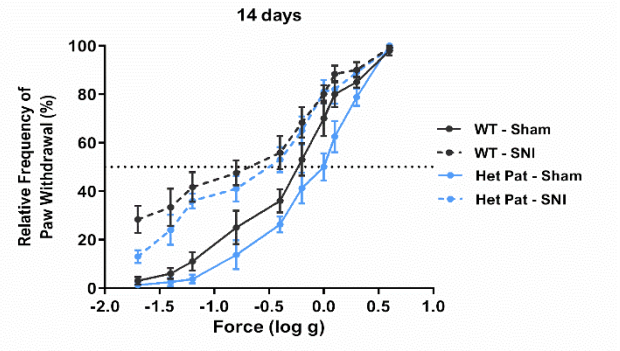
On day 14, a small reduction of the withdrawal frequency was still apparent in the Het Pat animals (both for SNI and sham surgery), and in KO animals subjected to

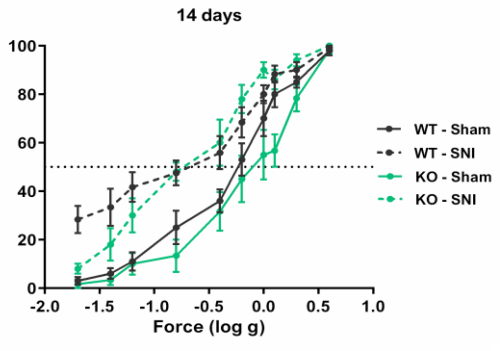
sham surgery. Again, these small differences did not reach statistical significance. Likewise, the KO group seemed to stand out on the 21st day after surgery, showing a lower frequency of withdrawal than WT and Het Pat animals upon sham treatment, though not statistically different.

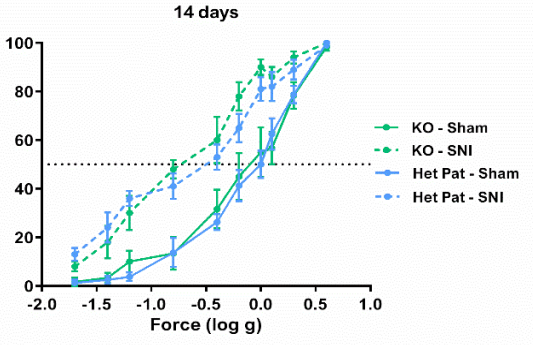

7 days
Relative Frequency of Paw Withdrawal (%)
Force (log g)
WT - Sham
WT - SNI
Het Pat - Sham
Het Pat - SNI

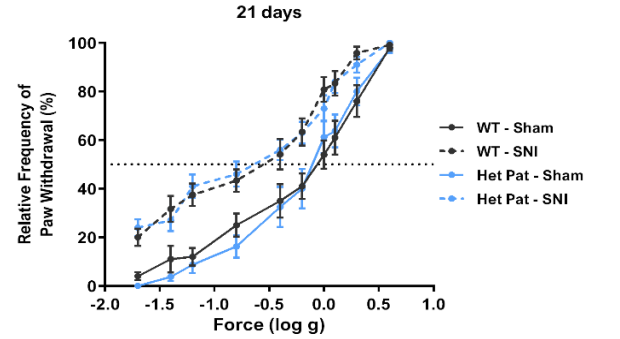

7 days
Force (log g)
WT - Sham
WT - SNI
KO - Sham
KO - SNI

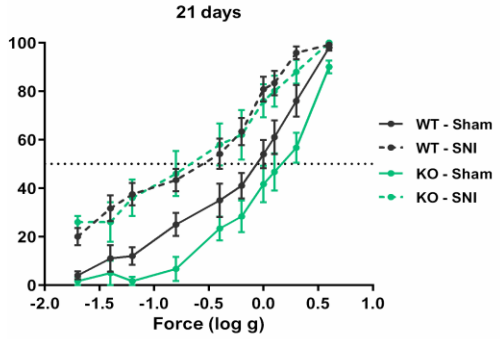

7 days
Force (log g)
KO - Sham
KO - SNI
Het Pat - Sham
Het Pat - SNI


14 days
Relative Frequency of Paw Withdrawal (%)
Force (log g)
WT - Sham
WT - SNI
Het Pat - Sham
Het Pat - SNI


14 days
Force (log g)
WT - Sham
WT - SNI
KO - Sham
KO - SNI


14 days
Force (log g)
KO - Sham
KO - SNI
Het Pat - Sham
Het Pat - SNI


21 days
Relative Frequency of Paw Withdrawal (%)
Force (log g)
WT - Sham
WT - SNI
Het Pat - Sham
Het Pat - SNI


21 days
Force (log g)
WT - Sham
WT - SNI
KO - Sham
KO - SNI

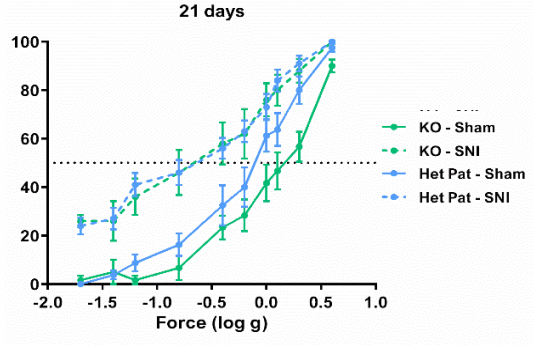

21 days
Force (log g)
KO - Sham
KO - SNI
Het Pat - Sham
Het Pat - SNI

Figure 3. SNI-induced nociception is not significantly impacted by the knockout of the Begain 1B isoform.

Relative frequency of paw withdrawal of Begain^{tm1bMLS} KO and Pat mutants and WT littermates at days 7, 14 and 21 after surgery.

The frequency of paw withdrawal for each Von Frey filament of Het Pat vs. WT littermates (panel A), KO vs. WT littermates (panel B), and Het Pat vs. KO animals (panel C) indicate a similar level of mechanical allodynia amongst genotypes. The effect of each filament between groups was analyzed using the Kruskal–Wallis test followed by post hoc Dunn's test. Values are presented as mean±SEM. WT (7, 14 and 21d, respectively): Sham, n = 15, 10, 10; SNI, n = 18, 12, 12; Het Mat (7, 14 and 21d, respectively): Sham, n=7, 2, 2; SNI, n=8, 3, 3; Het Pat (7, 14 and 21d, respectively): Sham, n=12, 8, 8; SNI, n=15, 10, 10; KO (7, 14 and 21d, respectively): Sham, n=10, 6, 6; SNI, n=11, 6, 6. Statistical differences referring to treatment (sham vs. SNI) are not plotted.

In addition to the frequency of withdrawal, we studied the withdrawal threshold in an attempt to clarify the impact of Begain 1B silencing on the pain phenotype (Fig.4). The effect of genotype did not produce significant differences among the thresholds displayed by sham- and SNI-treated animals. The only exception being at day 14, in the SNI model, where the threshold of withdrawal was higher in null mutants than in WT littermates ($p=0.0191$). The Mann-Whitney test comparing the withdrawal thresholds confirmed the already established significant differences between treatments seen in both genotypes, throughout the time points analyzed ($p<0.0001$ to $p<0.05$).

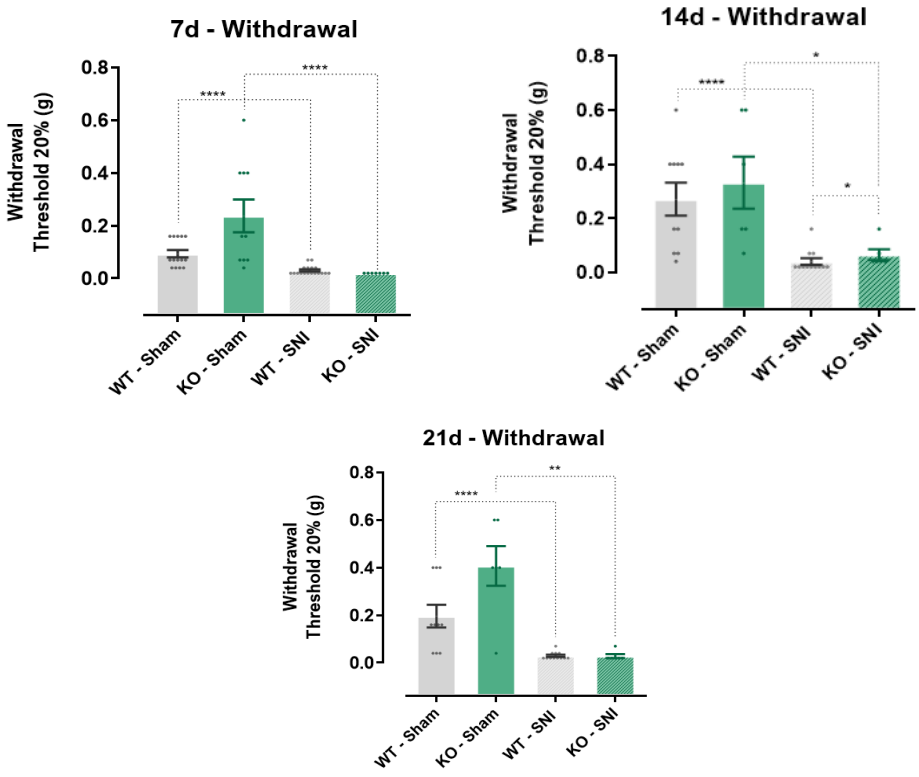


Figure 4. Paw withdrawal threshold did not vary significantly within treatment.

Paw withdrawal threshold of Begain^{tm1bMLS} KO mutants and WT littermates at days 7, 14 and 21 after surgery.

The minimum force to elicit paw withdrawal during filament stimulation was not significantly different between KO and WT animals, with the exception of SNI-subjected mice on day 14 and corresponding to a putative increase in KO mutants relative to WT (p=0,0191). Values presented as mean ± SEM. WT (7, 14, 21d, respectively): sham, n=15, 10, 10; SNI, n=18, 12, 12, and KO (7, 14, 21d, respectively): sham, n=10, 6, 6; SNI, n=11, 6, 6. Threshold differences were analysed using the Mann-Whitney test. ****p<0.0001, **p<0.01, *p<0.05.

2. Gene expression studies

Expression studies of Begain isoforms 1A and 1B, the paternally expressed neighboring Dlk1 gene, and interacting molecules deemed relevant to the biology of pain transmission, were conducted by RT-qPCR.

All studies were performed in L3-L6 sectioned spinal cord (ipsilateral half), L3-L6 DRG, and RVM in Begain^{tm1bMLS} mutants and WT littermates subjected to SNI and sham surgery. Upon completion of the behavioural analyses, animals were sacrificed and tissues were collected to evaluate possible changes in steady-state mRNA levels elicited by the neuropathic pain condition.

Two time points were chosen for expression analyses: day 7 after surgery, as an early time point along the pain chronification process, and day 21 after surgery, at which point chronic neuropathic pain is considered to be fully established. In this thesis, we focus on the analyses of gene expression at the former time point only, which emerged as a good candidate for molecular studies based on the earliest indications of the behavioral analyses, namely the results arising from the first set of studied animals.

a) The Begain 1B isoform is more abundant than the Begain1A isoform in SC, DRG and RVM

Analysis of Begain isoform levels of WT animals subjected to sham surgery revealed that the Begain 1B isoform was the most abundant, with expression levels 7.7x, 3.3x and 16.7x higher than those of isoform 1A in SC, DGR and RVM, respectively. The Dlk1 gene displayed higher expression levels, 2.8x, 4.0x and 8.7x stronger than Begain 1B in SC, DGR and RVM, respectively (Fig. 5).

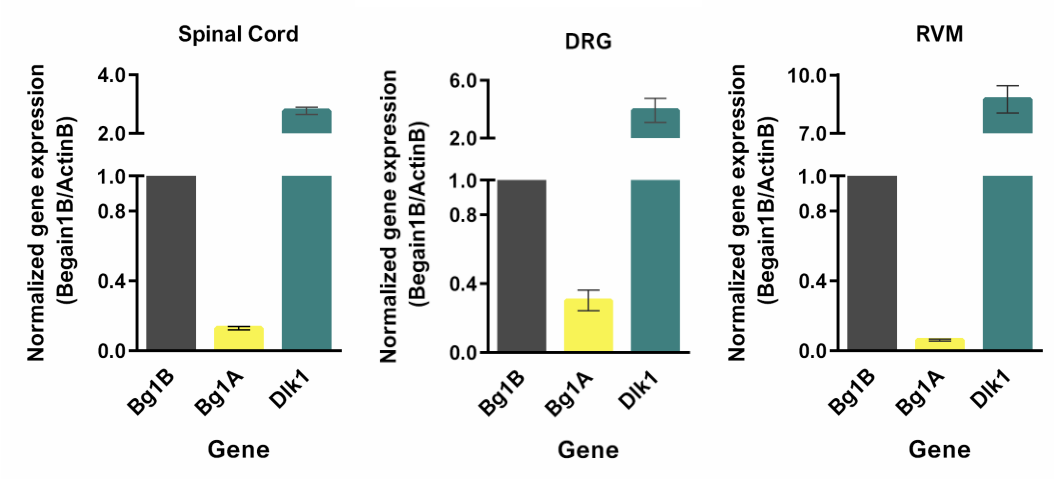


Figure 5. Relative levels of Begain 1A, Begain 1B, and Dlk1 transcripts

Relative gene expression of Begain 1A, 1B and Dlk1 in SC, DRG and RVM of wild type mice. Target gene expression was normalized to Actinβ expression.

The expression of the Begain isoform 1B is higher than that of isoform 1A in the three tissues. Values presented as mean ± SEM. Bg1B: n=5; Bg1A: n=5; and Dlk1: n=5.

b) Begain 1B expression in the SC and RVM is abolished in KO mice, severely depressed in paternal heterozygotes and slightly diminished in maternal heterozygotes.

The validity of the Begain^{tm1bMLS} mutant model was confirmed by assessing the expression levels of Begain isoform 1B in null animals (carrying the silencing mutation on both alleles). An absence of expression was seen in the three tissues under study (Fig. 6). In accordance to the expected behavior of an imprinted transcript (of paternal expression), the transcript levels in heterozygotes inheriting the mutation on the paternal allele (Het Pat) were severely reduced; whereas those in heterozygotes inheriting the mutation on the maternal allele (Het Mat) showed a small reduction only. This pattern was seen in SC and RVM, whereas in the DRG the levels of expression in both Het Pat and Het Mat animals were reduced to a similar extent, corresponding to about half of the expression displayed by the WT littermates (Fig. 6). These results indicate that Begain isoform

1B may be (predominantly) biallelic expressed (i.e. not imprinted) in the DRG.

c) Begain 1A, 1B and Dlk1 expression in the SC, DRG and RVM remains unaltered under the neuropathic pain condition

No statistically significant differences were observed regarding the expression levels of the three transcripts in the analyzed tissues when comparing animals subjected to SNI and sham surgeries (Figs. 6 and 7). Whereas all two-way ANOVA statistics revealed highly significant differences in expression of Begain 1B between genotypes within each treatment group, no differences were seen between treatments for any of the transcripts, nor in within-treatment (genotype) groups for Begain 1A and Dlk1. The spinal cord showed consistent expression of Begain 1A and Dlk1 among sham- and SNI-treated animals, revealing no differences evoked by treatment (Bg1A: p=0.8926; Dlk1: p=0.8196). Similarly, the DRG and RVM did not display significant changes of Bg1A (DRG: p=0,1609; RVM: p =0,4166) and Dlk1 (DRG: p=0.8082; RVM: p=0.6484) transcript levels induced by the spared nerve injury paradigm. Therefore, the silencing or downregulation of Begain 1B did not elicit a response at the level of Begain's other isoform (1A) expression, either in a "basal" state (sham) or in the context of neuropathic pain (SNI). Likewise, the "perturbation" of expression of Begain's imprinted isoform 1B had no impact nor echo on that of imprinted (and possible co-regulated at the level of genomic imprinting) neighbor Dlk1, again either in the absence (sham) or presence of neuropathic pain (SNI).

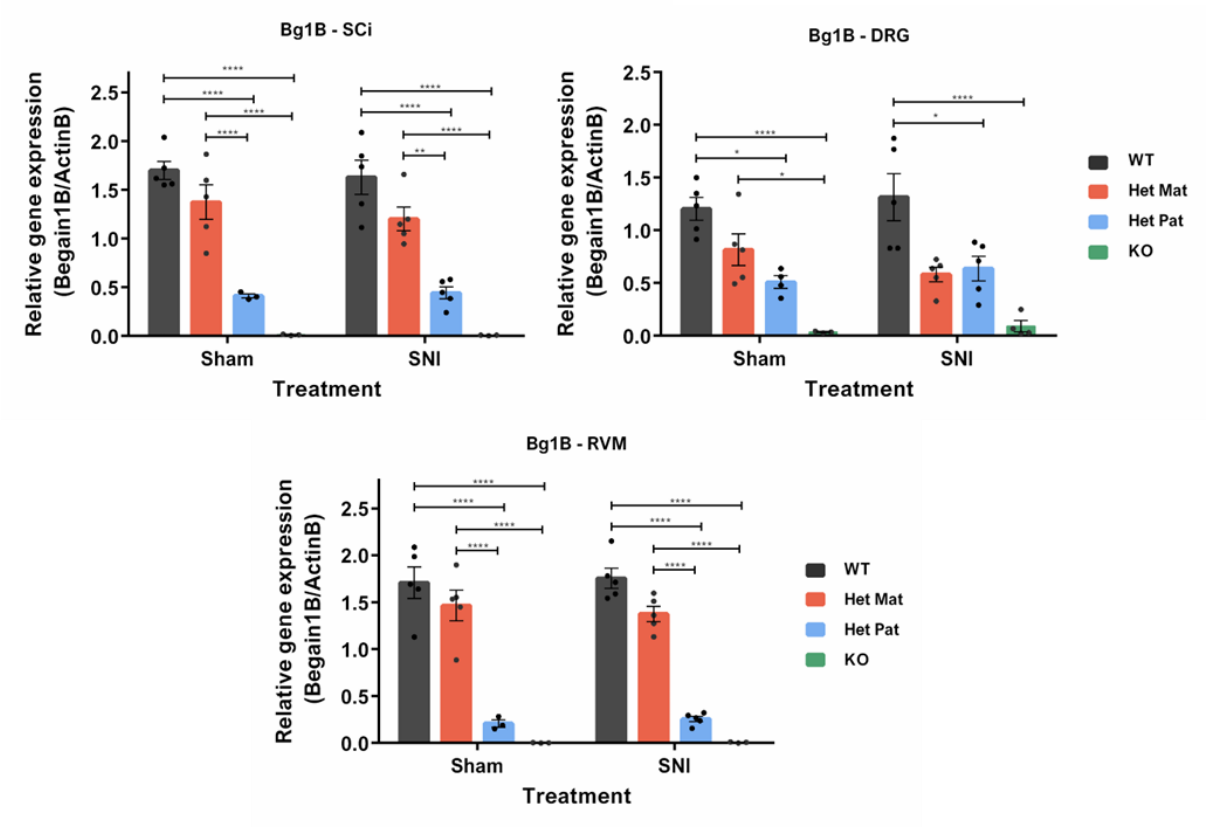


Figure 6. Expression of Begain 1B is differentially compromised depending on the pattern of inheritance of the null alleles, but is unaffected by the neuropathic pain condition.

*Relative expression levels of Begain 1B in SC, DRG and RVM of Begaintm1bMLS mutants and wild type littermates subjected to SNI or sham surgery. Target gene expression was normalized to Actinβ expression. The SNI model did not elicit changes in expression of the Begain1B isoform. The expected genotype effect was, nonetheless, clear, with a complete silencing in KO mice and a strong downregulation in Het Pat animals comparing to Het Mat or WT mice. Values presented as mean ± SEM. WT: sham, n=5; SNI: n=5. Het Mat: sham, n=5; SNI, n=5. Het Pat: sham, n=4; SNI, n=5. KO: sham, n=3; SNI, n=3. ****p<0.0001, **p<0.01, *p<0.05.*

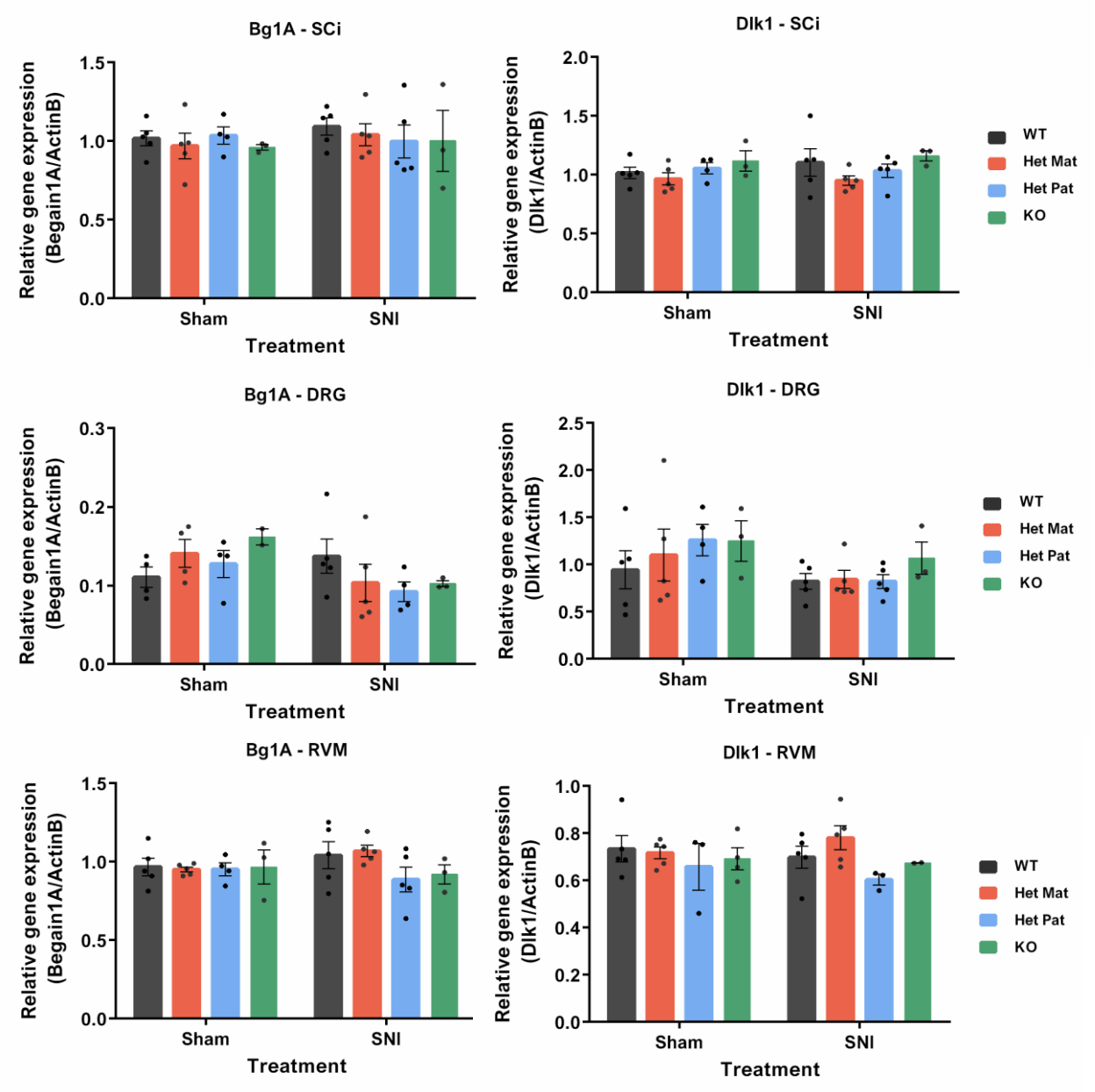


Figure 7. Expression of Begain isoform 1A and Dlk1 is unaffected by the Begain^{tm1bMLS} mutation and by the neuropathic pain condition

Relative expression levels of Begain 1A and Dlk1 in SC, DRG and RVM of Begain^{tm1bMLS} mutants and wild type littermates subjected to SNI or sham surgery. Target gene expression was normalized to Actinβ expression.

The SNI model did not elicit changes in expression of the Begain1A isoform and Dlk1 gene. Values presented as mean ± SEM. WT: sham, n=5; SNI: n=5. Het Mat: sham, n=5; SNI, n=5. Het Pat: sham, n=4; SNI, n=5. KO:

sham, n=3; SNI, n=3. Two-Way ANOVA and Tukey's multiple comparison test, no significant differences identified.

3. The relative abundance of NMDA receptor subunits varies among tissues

Since Begain is believed to interact with the NMDA receptor via the PSD-95/SAP90 protein, and to be recruited to the synapses in a NMDAR activity-dependent manner (Yao et al., 2002), we have elected to study the expression levels of relevant NMDA receptor subunits: NR1, encoded by *Grin1*; and NR2A, 2B, 2C and 2D, encoded by *Grin2a*, *Grin2b*, *Grin2c* and *Grin2d*, respectively (Fig.8). Expression of the NR2C sub-unit transcript was exceptionally low in the three tissues, thus quantification was not possible.

In the spinal cord of wild type animals, the NMDAR subunits displayed a somewhat similar degree of expression, whereas in DRG and RVM there were substantial differences in transcript levels. In the DRG, for example, the NR1 and NR2D subunits are dominant, the former with an expression 43x higher than that of NR2A and NR2B (and 3,5x higher than that of NR2D), and the latter with an expression 9x stronger than that of the two lest expressed subunits. Conversely, the NR2B subunit shows the highest expression levels in the RVM, with approximately 4x more expression than the average of the reminder subunits.

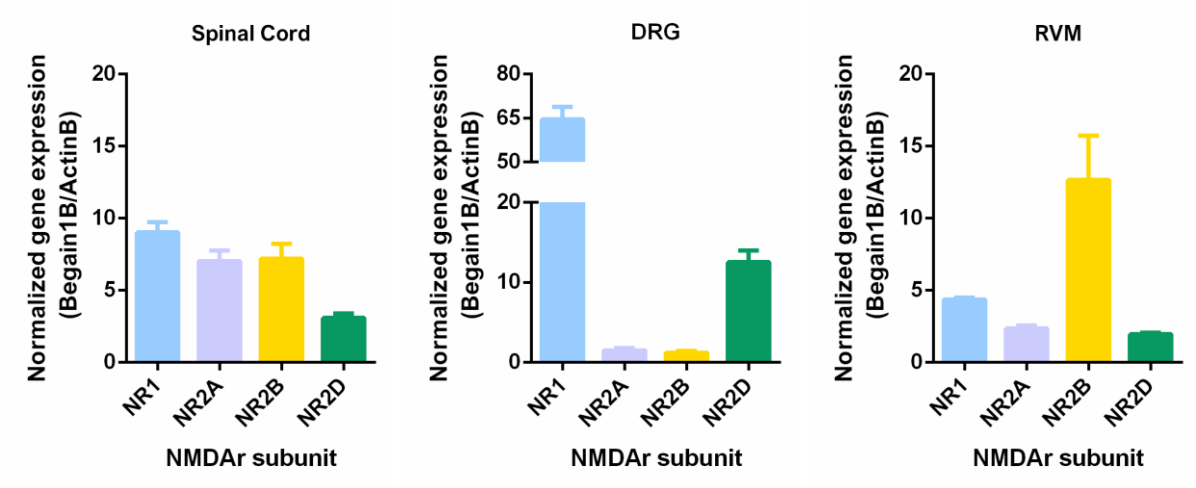


Figure 8 – Relative levels of expression of relevant NMDA receptor subunits

Relative gene expression of NR1, NR2A, NR2B and NR2D in SC, DRG and RVM of wild type mice. Target gene expression was normalized to Actinβ expression.

The relative level of the transcripts is variable and tissue-specific. Values presented as mean ± SEM. NR1: n=5, NR2A: n=5, NR2B: n=5 and NR2D: n=5.

a) Expression of NMDA receptor subunits NR1 and NR2D is affected in the DRG

To probe for a possible the impact of the Begain^{tm1bMLS} mutation on NMDA receptor expression, we assessed the transcript levels of the four subunits in knockout animals, heterozygotes of paternal and maternal transmission, and WT littermates under the neuropathic pain model.

Whereas the expression of subunits NR1 and NR2D was unchanged in SC and RVM, whether between genotype or treatment groups, we observed statistically significant changes in the DRG (Fig.9), where their expression is highest. In this tissue, there was a statistically significant decrease of 31% in expression of NR1 in null mice subjected to SNI relative to null mice subjected to sham surgery. In Het Pat animals, a smaller decrease (17%) was also observed, but it did not reach statistical significance . The latter tendency was also seen in WT and Het Mat animals (16% and 12% expression reduction under SNI, respectively), but again

short of statistical significance (Fig.9, panel A). Regarding the NR2D subunit, no differences were observed between treatments. Rather, there were statistically significant reductions in expression in KO and Het Mat mutants (and close to significance in Het Pat mutants; 25%) relative to the levels of the WT littermates, but only in the SNI-elicited neuropathic pain condition (Fig. 9, panel B).

Expression of NR2A and NR2B subunits was not altered in either SC, DRG or RVM (Fig.10). All genotypes displayed similar levels, and the subunits' expression was not affected by the SNI surgery.

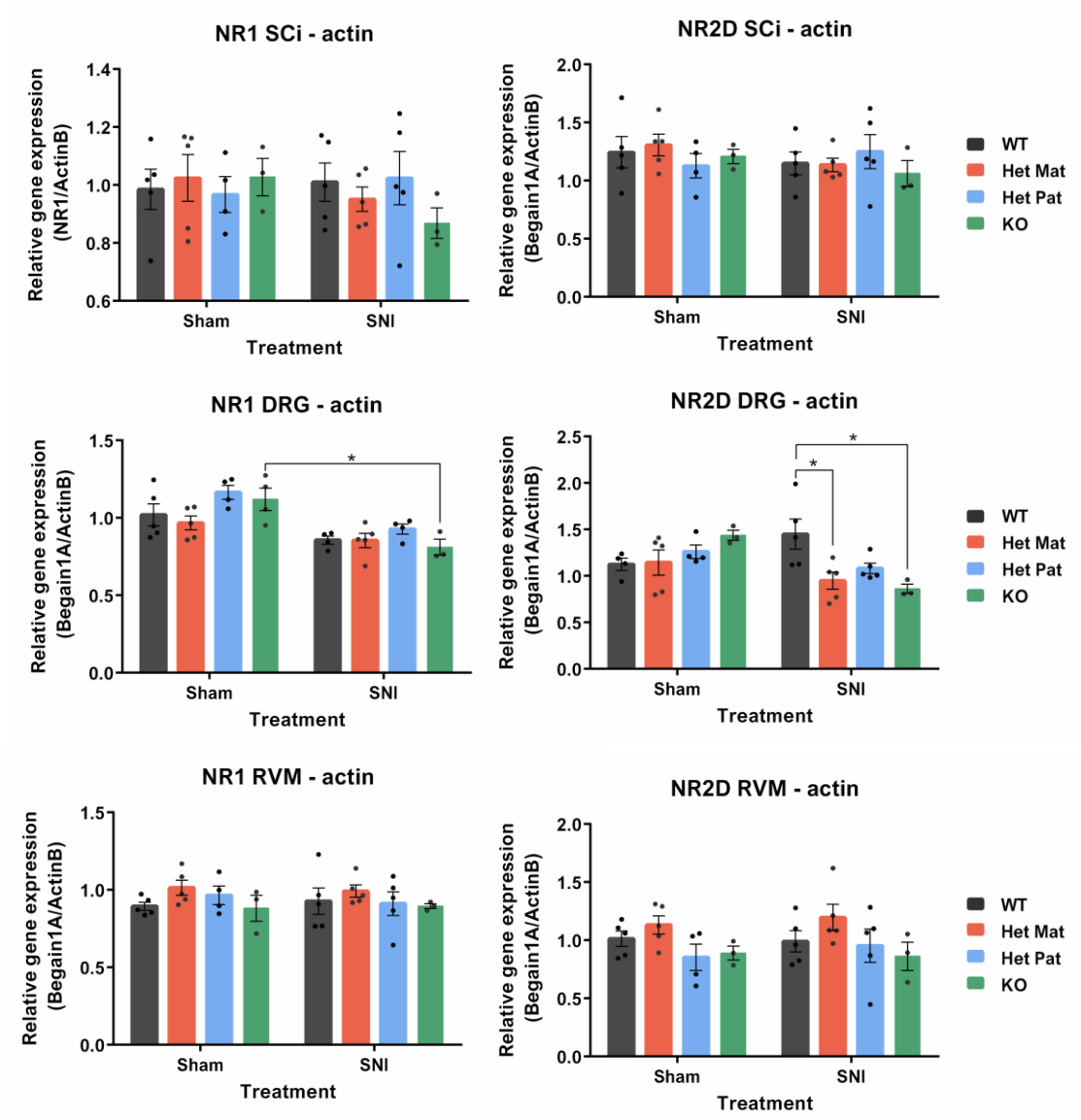


Figure 9. The DRG displays genotype-specific altered expression of NR1 and NR2D elicited by treatment

The KO animals subjected to SNI showed a statistically significant reduction in NR1 transcript levels relative to those of the KO mice subjected to sham surgery in the DRG. Also in DRG, the levels of NR2D are significantly reduced in Het Mat and KO mutants (and Het Pat mutants, although not statistically so) when compared to the levels of the WT littermates within the SNI-treated group. Values presented as mean ± SEM. WT: sham, n=5; SNI, n=5; Het Mat: sham, n = 5; SNI, n=5. Het Pat: sham, n=4; SNI, n=5. KO: sham, n=3; SNI, n=3. Kruskal–Wallis test followed by post hoc Dunn's test , *p < 0.05.

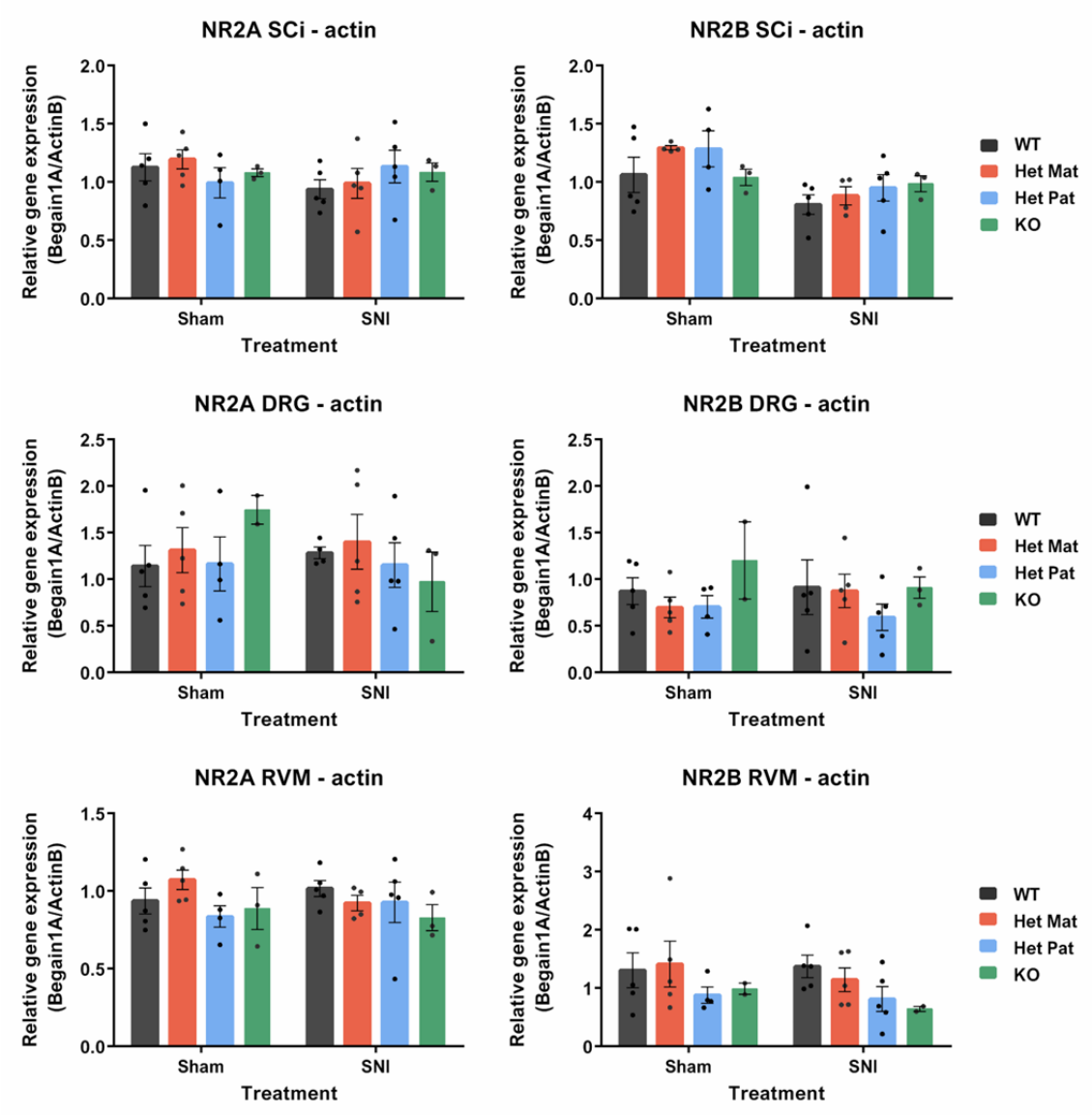


Figure 10. The expression of NMDA receptors NR2A and NR2B is not affected by the Begain 1 B isoform silencing or the neuropathic pain model.

The transcript levels of NR2A and NR2B NMDAR subunits did not change with genotype or induction of the neuropathic pain. Values presented as mean ± SEM. WT- sham: n = 5, SNI: n = 5; Het Mat - sham: n = 5, SNI: n = 5; Het Pat - sham: n = 4, SNI: n = 5; KO - sham: n = 3, SNI: n = 3. Kruskal–Wallis test followed by post hoc Dunn's test, no significant differences identified.

4. Imprinting studies

We aimed to ascertain the allele-specific expression of Begain isoforms 1A, 1B and Dlk1 in SC, DRG and RVM of wild type and mutant animals subjected to SNI or sham surgery, in order to 1) assess the imprinting status of the transcripts in a condition of neuropathic pain, an 2) to probe if the silencing of the Begain 1B isoform could influence the allelic expression pattern of the transcripts in the absence or presence of the pain paradigm. To that end, we studied expressed strain-specific polymorphisms in hybrid animals obtained from reciprocal crosses between Begain^{tm1bMLS} heterozygous mutants (C57BL/6J background) and Cast/EiJ mice, by Sanger sequencing. The qualitative analysis of the expressed polymorphisms in Chr. 12 (Dlk1: rs253217941, alleles T and C; Begain: rs36806417, alleles G and A, from C57BL/6 and Cast/EiJ, respectively) enabled us to determine the parental-specific expression of the transcripts (Figs.11 and 12).

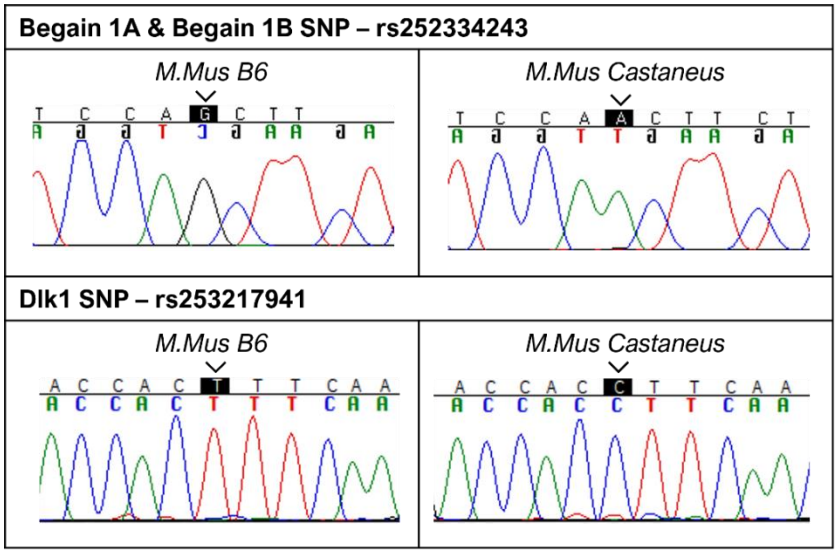


Figure 11 – Chr. 12 polymorphisms in Begain and Dlk1

Sequencing electropherograms of genomic DNA encompassing the Begain and Dlk1 expressed single-nucleotide polymorphisms (rs252334243 and rs253217941, respectively). Arrow depicts the SNP (highlighted in black).

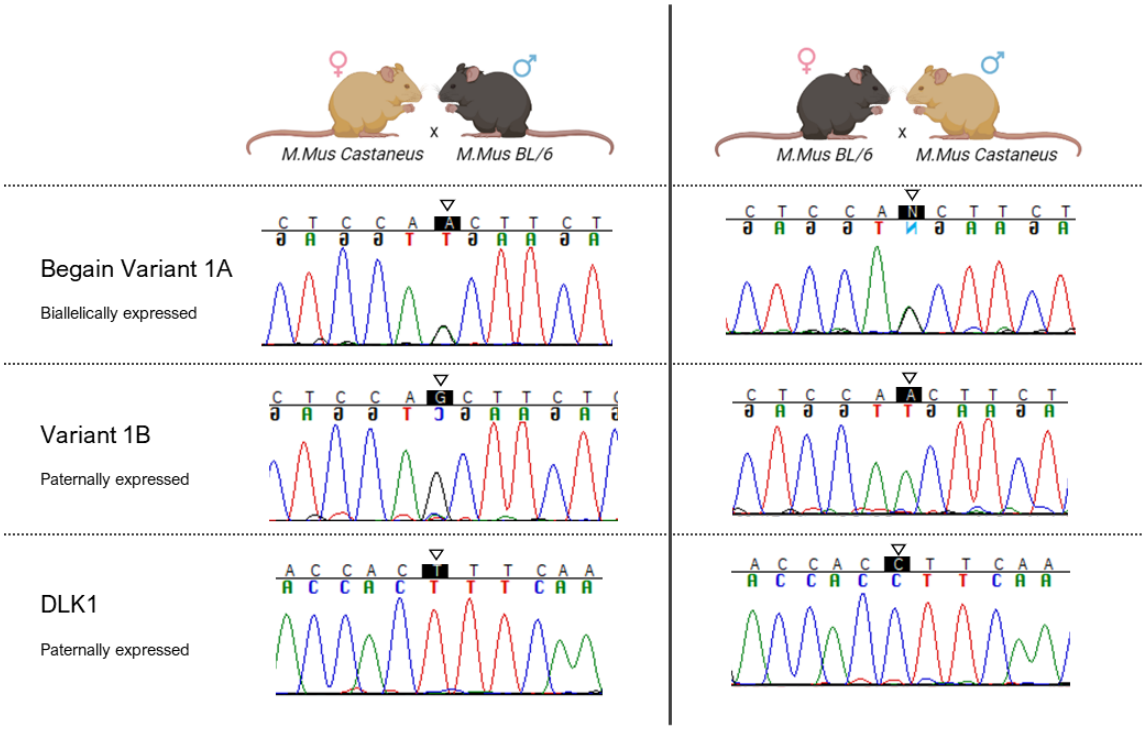


Figure 12 – Schematics of reciprocal crosses and respective electropherograms of cDNA sequencing showing the "canonical" parental expression of the three transcripts.

Example of biallelic expression of Bg1A and monoallelic (paternal) expression of Begain1B and Dlk1. Arrow depicts the SNP, highlighted in black.

a) Begain 1B is paternally expressed in SC and RVM but tendentially biallelic in the DRG.

In accordance to their "canonical" parental expression (as determined is other tissues elsewhere), Begain 1B and Dlk1 showed paternal expression in the SC and RVM of WT animals. The electropherograms exhibited a clear peak corresponding to the allele inherited from the father, although for Begain 1B, expression was not exclusive of the paternal allele: a small peak corresponding to the maternal allele could also be seen, indicating that the repression of this allele is not absolute (Fig. 13). Accordingly, in mutants inheriting the Begain 1B silent allele on the paternal chromosome (Het Pat), the sequencing traces revealed that "residual" expression from the maternal allele, the only instance of the imprinting studies where Begain 1B was seen as "maternally" expressed (Fig.13, Table 2). In DRG, however, Begain 1B displayed a pattern of biallelic expression, unlike that seen for DLK1, which - as in SC and RVM - showed a clear and almost exclusive paternal expression in WT animals (Fig.14). This is the first instance in which Begain 1B is described as not imprinted, but tendentially biallelic. The term "tendentially" is used to acknowledge exceptions observed in a few WT animals that displayed either almost exclusive paternal expression or, unexpectedly, maternal expression. In mutants, when the mutation was on the maternal chromosome, Begain 1B expression came exclusively from the paternal allele, whereas when the mutation was borne on the paternal chromosome, Begain 1B expression came exclusively from the paternal allele, as expected.

SNI or sham surgery, whether in a wild type or mutant context, did not elicit a change of the allelic expression of either gene.

b) The silencing of Begain 1B and SNI-induced pain seem to influence the allelic expression of Begain 1A

Analysis of the allelic expression of Begain 1A in WT animals showed the "canonical" biallelic expression of the transcript in the three tissues. Expression in DRG was, nonetheless, classified as tendentially biallelic since, once again, there were instances where a bias towards paternal or maternal expression was seen. Furthermore, its pattern of expression was not affected by SNI-induced neuropathic pain. However, when the Begain^{tm1bMLS} mutation was borne on the paternal allele (Het Pat mutants), Begain 1A expression in the SC and RVM seems to have changed to a preferentially paternal (imprinted) expression in sham animals (Table 2). On the other hand, SNI-subjected paternal heterozygotes displayed the "original" biallelic expression in SC, and a mixture of biallelic and paternal expression in the RVM, suggesting that neuropathic pain has had an effect on the allelic expression of Begain 1A in Het Pat mice. In the DRG of mutant mice (either Het Mat or Het Pat), Begain 1A retained the biallelic expression as seen in WT littermates, but under the SNI pain model the allelic expression turned to a mixture of biallelic and paternal expression in both genotypes.

When the Begain^{tm1bMLS} mutation was borne on the maternal allele (Het Mat mutants), Begain 1A expression in the SC was preferentially maternal in sham animals, as opposed to the biallelic expression seen in the DRG and RVM (and displayed by WT animals in either tissue). Upon a condition of neuropathic pain, in SNI-subjected animals, the expression "reverted" to biallelic, the apparently "canonical" situation found in WT animals (Table 2).

		ALLELIC EXPRESSION								
		Bg1A			Bg1B			Dlk1		
		SCi	DRG	RVM	SCi	DRG	RVM	SCi	DRG	RVM
WT	Sham	B	B	B	P	TB	P	P	P	P
	SNI	B	TB	B	P	TB	P	P	P	P
Het Pat	Sham	PP	B	PP	M	M	M	P	P	P
	SNI	B	V	V	M	M	M	P	P	P
Het Mat	Sham	PM	B	B	P	P	P	P	P	P
	SNI	B	V	B	P	P	P	P	P	P

Table 2. Summary of the allelic expression of Begain isoforms 1A and 1B and Dlk1 in the probed tissues.

B: biallelic expression; P: paternal expression; M: maternal expression; TB: tendentially biallelic expression; PP: preferentially paternal expression; PM: preferentially maternal expression; V: biallelic and paternal expression.

Discussion and Conclusions

Discussion

The present work contributed to elucidate how neuropathic pain impacts the Begain imprinted isoform genetic expression and epigenetic regulation. Literature about begain gene is still scarce, however, has been recently implicated in pathological pain transmission following peripheral nerve injury in mice (Katano et al., 2016). To that effect, we have used an Begain 1B-specific knockout mouse model produced on our lab (Vasconcelos, 2019). The obtained results showed that (1) imprinted Begain 1B has no significant effect on SNI-elicited mechanical allodynia; (2) genetic expression of Begain 1B is abolished in KO mice, significantly depressed in paternal and slightly diminished in maternal heterozygotes; (3) Begain1A, 1B and Dlk1 expression is not altered under the neuropathic pain condition; (4) NR1 and NR2D expression is affected in the DRG of SNI mice; (5) Begain 1B is paternally expressed in SC and RVM, but not in the DRG; (6) Allelic expression of Begain 1A seems to be influenced by silencing of Begain 1B and neuropathic pain.

1. Pain Behavior

Neuropathic pain is thought to be the result of abnormal neuronal plasticity caused by damage, dysfunction or injury of the somatosensory system. In pathological conditions, peripheral nerves become more sensitive and develop spontaneous excitability to otherwise innocuous chemical, thermal and mechanical stimuli (Colloca et al., 2017). Katano and colleagues, showed for the first time the involvement of Begain in a pathological side of pain. By inducing a peripheral neuropathic pain model, they saw a significantly attenuated nociceptive behaviour in the complete Begain KO, in the end concluding was involved in pathological pain transmission through N-methyl-d-aspartate receptor activation after the phosphorylation of GluN2B (Katano et al., 2016). The Begain gene however encodes two isoforms termed Begain 1A and Begain 1B (Soares et al., 2018), which

result from the usage of alternative first exons. Strikingly, whereas the 1A isoform is biallelic expressed, the Begain 1B isoform is imprinted (paternally expressed), as previously demonstrated in sheep (Smit et al., 2005) and in the mouse (Tierling et al., 2009; Soares et al., 2018).

Along with this notion, and the main focused of comprehending the epigenetic influence in sustaining or inhibit a pathophysiologic pain state, a specific mutant for only 1B isoform was the best suited model. Our group focused therefore on developing this model. An approach was developed that involved inverting the genomic region containing exon 1B in order to target the Begain 1B isoform (Vasconcelos, 2019). As a result, the transcript would be disrupted whereas the genetic area would mostly remain unaltered. Exon 1B's surrounding target CRISPR sites were chosen to be adequate. We designed a long single-strand DNA (ssDNA) fragment with arms of homology to the endogenous locus, flanking the inverted target sequence, to be introduced by homology directed repair after Cas9-mediated cleavage and removal of the endogenous target sequence since we needed to replace the sequence delimited by the CRISPR-Cas target sites rather than just remove it. Once the genotype of the founder male was successfully confirmed, crosses between Begaintm1bMLS (C57BL/6J background) and C57BL/6J mice generated this new colony. All experimental animals resulted from crosses that originated heterozygotes with paternal inheritance of the KO allele (Het Pat), heterozygotes with maternal inheritance of the KO allele (Het Mat) and homozygous mutants (KO) in addition to their wild type littermates. The spared nerve injury (SNI) model, a well-established and widely used neuropathic pain (Decosterd and Woolf, 2000) was the elected for studying the interplay between begain and pain pathophysiology. Following SNI and sham surgery, mechanical allodynia was assessed in all experimental groups and evaluated at time points 7, 14 and 21 days post-operation (Bourquin et al., 2006). Both sham and SNI-treated KO mice after 7 days of surgery tended to exhibit a lower frequency of withdrawal than the WT littermates. In contrast, the WT animals' response to both treatments was nearly identical

in the Het Pat group. Preliminary results revealed that the KO mice had a considerably reduced pain threshold on the first set of day 7 animals. Since the behavioral analyses were conducted in 3 different sets of animals with equal representation of all genotypes (data not shown). When the results from sets 2 and 3 were included, the differences eventually diminished. It's possible that the loss of 1B is compensated for by the 1A isoform because Begain isoforms 1A and 1B only differ at the N-terminal region. The N-terminal region is involved in nuclear localization and, in an NMDA receptor activity-dependent manner, mediates recruitment from the nucleus to dendrites and from dendrites to synapses (Yao et al., 2002). PSD components scaffold proteins and NMDA receptors are implicated in pain and abnormal pain transmission through activation of signalling cascade proteins in the brain and spinal dorsal horns (Craven and Bredt, 1998; Garner et al., 2000; Luo et al., 2014). The balance between pathological and physiological conditions is determined by reversible changes of PSD complex components in the spinal dorsal horn (Abe et al., 2005). Primary afferent C and A fibers convey nociceptive inputs to the spinal dorsal horn (predominantly lamina I-II) (Christensen and Perl, 1970; Woolf and Fitzgerald, 1986) and deeper laminae (Todd, 2010; Braz et al., 2014). An abnormal functioning of the pain circuitry in the spinal dorsal horn results in innocuous stimuli-triggered nociception. These changes in the efficacy of synaptic interneurons in laminae IIi–IV include disruption of inhibitory control or facilitation of excitatory control (Braz et al., 2014; Duan et al., 2015; Peirs et al., 2015). Begain, is undoubtedly a natural candidate, as is a PSD-95/SAP90 binding protein highly expressed in the brain and thought to sustain the structure of the PSD (Naisbitt et al., 1997; Deguchi et al., 1998). Therefore, despite the strong functional evidence linking Begin to neuronal activity, little is known about its potential pathological or structural role in the nervous system.

2. Expression studies

Gene expression studies were conducted to investigate the dynamics of Begain and Dlk1 expression in the SC, DRG and RVM of adult KO, paternal and maternal heterozygous, and wildtype mice subjected to SNI. Thus, these studies were carried out at a single timepoint at seven days after SNI (or sham surgery). In accordance with the expected behavior of an imprinted transcript (of paternal expression), the transcript levels in heterozygotes inheriting the mutation on the paternal allele (Het Pat) were severely reduced; whereas those in heterozygotes inheriting the mutation on the maternal allele (Het Mat) showed a small reduction only. This pattern was seen in SC and RVM, whereas in the DRG the levels of expression in both Het Pat and Het Mat animals were reduced to a similar extent, corresponding to about half of the expression displayed by the WT littermates. A previously created mutant line known as RepL1del exhibits a substantial downregulation of imprinted Begain 1B in embryonic brain yet preserving expression levels of the biallelically expressed Begain 1A isoform (Soares et al., 2018). The mutation, which involves the deletion of a 260 kb L1-rich array of repetitive sequence between Begain and Dlk1, also impacts the latter gene's expression in the brain, both in embryos and adults, where it has been shown to be downregulated. Latter studies revealed that imprinted Begain 1B was substantially downregulated in the SC and DRG of RepL1del mutants, but that Begain 1A expression was unaffected (Vasconcelos, 2019). These findings are in accordance with those of Soares and colleagues (2018), who found that the imprinted isoform of Begain was the only form of Begain affected by the RepL1del mutation. To clarify the interplay between the imprinted begain isoform 1B and pain, we also analyzed on the 7th day after SNI. Begain 1A, 1B and Dlk1 expression in the SC, DRG and RVM remained unaltered under the neuropathic pain condition. Therefore, in a "basal" condition (sham) or in the condition of neuropathic pain, the silencing or downregulation of Begain 1B did not elicit a response in the level of Begain's other isoform

(1A) expression (SNI). Similar to Begain's imprinted isoform 1B, Dlk1's imprinted (and maybe co-regulated at the level of genomic imprinting) neighbor had neither an effect nor an echo on its expression, whether in the absence (sham) or presence of neuropathic pain (SNI). Since Begain is believed to interact with the NMDA receptor via the PSD-95/SAP90 protein, and to be recruited to the synapses in a NMDAR activity-dependent manner (Yao et al., 2002), we have elected to study the expression levels of relevant NMDA receptor subunits: NR1, encoded by Grin1; and NR2A, 2B, 2C and 2D, encoded by Grin2a, Grin2b, Grin2c and Grin2d, respectively. In contrast to how genotype or treatment groups had no effect on the expression of NR1 and NR2D subunits in SC and RVM, we found statistically significant changes in the DRG, where their expression is highest. When compared to null mice who underwent sham surgery, there was a significant decrease in the expression of NR1 in this tissue in null mice who underwent SNI. There were no treatment differences seen in terms of the NR2D subunit. Statistically substantial decreases in expression were seen in KO. In the SC, DRG, or RVM, the expression of NR2A and NR2B subunits was unaltered.

3. Imprinting

Genomic imprinting is an epigenetic process that leads to the monoallelic expression of genes in a way that is specific to the parent of origin. Imprinting disruption is linked to cancer and human diseases (Tierling et al., 2009). Given that bg1b is imprinted and given our interest in the regulation of genomic imprinting in neuropathic pain, we went to verify the imprinting status of the Begain 1A, Begain 1B and Dlk1. We aimed to scrutinize possible epigenotype switches or de-repression of the repressed allele. This data was obtained by analysis of electropherograms results of Sanger sequencing. A quantitative approach, namely pyrosequencing, would be more precise to determine the contribution of each parental allele and possibly draw more meaningful conclusions. Begain is a component of the Dlk1-Dio3 imprinted domain, which is situated at the 5' upstream

boundary and defines a 1 Mb genomic region of paternally expressed protein-coding genes and maternally expressed non-coding regulatory RNA transcripts crucial for pre- and post-natal development (Rocha et al., 2008; Charalambous et al., 2012). In this cluster, the non-coding RNA Gtl2, which is fully methylated in sperm but is unmethylated in the oocyte, is positioned between Dlk1 and the germline-derived differentially methylated region (IG-DMR), which controls imprinting. Our results showed that, in accordance with their canonical parental expression Begain 1B and Dlk1 showed paternal expression in the SC and RVM of WT animals. However, Begain 1B revealed a pattern of biallelic expression in DRG, in contrast to Dlk1, which, like SC and RVM, demonstrated a distinct and largely paternal expression in WT mice. This is the first time Begain 1B is described as being tendentially biallelic rather than imprinted. Whether in a wild type or mutant setting, SNI or sham surgery did not cause a change in the allelic expression of either gene.

Conclusion

The work that we propose to develop during this past year constituted an original approach to investigate a potential relationship between epigenetic regulation of Begain and neuropathic pain. This work represents the first steps of characterizing a novel mutant mouse model (Begain^{tm1bMLS}) and several research pathways. So far, we have shown that the KO of Bg1B alone does not result in a pain phenotype, as determined by the SNI paradigm. However, it may do so, under a different paradigm of pain. The KO bg1b, under condition of different parental transmission appears to influence the expression of NR1 and NR2D on the DRG, the consequences of which need to be properly dissected. Disruption of Bg1B does not elicit a change in the imprinting status of the Dlk1 gene or the isoform itself. Bg1A however, not always shows a clear biallelic characteristics, instead we also observe preferential paternal expression. Considering that, though the isoform 1B is the most abundant in the three studied components of the pain system it must have an important role in synaptic transmission.

However, the resulting phenotype of its disruption is not revealed by the SNI model so we can not precipitate into excluding this isoform role in the mechanisms underlying pain's (patho-) physiology. Nevertheless, it's conceivable that even in this model, it will be possible to identify significant changes in gene expression and imprinting in the later timepoint (21st day) currently underway.

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