THE MECHANISM OF PAINFUL DIABETIC NEUROPATHY IN STREPTOZOTOCIN-INDUCED SPRAGUE DAWLEY RATS FOLLOWING TREATMENT WITH L-ALPHA-AMINOADIPATE OR U0126 DRUG

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by

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LIST OF SYMBOLS

α	alpha
β	beta
δ	delta
%	percentage
ºC	degree celcius
μ	mu (micro)
Δ	delta
κ	kappa

LIST OF ABBREVIATIONS

BBB	blood brain barrier
BDNF	Brain-derived neurotrophic factor
CCL1	C-X-C chemokine ligand 1
CCL2	C-C motif chemokine 2
CCR2	C-C motif chemokine receptor 2
CNS	central nervous system
CXCR2	C-X-C chemokine receptor 2
DM	Diabetes mellitus
DN	Diabetic neuropathy
DREAM	Downstream regulatory element antagonist modulator
DRG	dorsal root ganglia
ERK	extracellular signal-regulated kinase
ERK	extracellular signal-regulated kinase
FDA	Food and Drug Administration
GFAP	glial fibrillary acidic protein
H&E	Haematoxylin & Eosin (staining)
i.e.	'that is'
IHC	Immunohistochemistry (staining)
IL-1β	interleukin 1β
LAA	L-α-aminoadipate (astrocytes inhibitor)
MEK	mitogen-activated protein kinase kinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (assay)
Nav	sodium voltage-gated channel
NIH	National Institute of Health

PBS	phosphate-buffered-saline
PDN	Painful diabetic neuropathy
RM	repeated measure (ANOVA)
SEM	standard error of the mean
SNL	spinal nerve ligation
STZ	streptozotocin drug
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus

LIST OF APPENDICES

MEKANISME DIABETIS NEUROPATI YANG MENYAKITKAN ARUHAN DARIPADA STREPTOZOTOCIN TERHADAP MODEL TIKUS SPRAGUE DAWLEY DENGAN RAWATAN UBATAN L-ALPHA-AMINOADIPATE ATAU U0126

ABSTRAK

Neuropati diabetes yang menyakitkan (PDN) adalah komplikasi diabetes melitus (DM). Rawatan sakit neuropati kronik ini mencabar kerana pilihan rawatan yang terhad dan terapi yang kurang berkesan. Kajian ini bertujuan untuk meneroka mekanisme neuropati diabetes yang menyakitkan melalui penyiasatan menggunakan L-α-aminoadipate (LAA-ubatan perencat astrosit) dan U0126 (ubatan perencat Extracellular signal-regulated kinase (ERK)), dan bagaimana kedua-duanya mempengaruhi ekspresi protein dan mRNA astrosit, ERK, Downstream regulatory element antagonist modulator (DREAM) dan Brain-derived neurotrophic factor (BDNF) dalam korda spina tikus PDN yang diinduksi dari streptozotosin (STZ), khususnya DM Jenis 1 (T1DM). Seratus dua puluh enam tikus jantan *Sprague-Dawley* telah dibahagikan kepada 6 kumpulan (n=21 setiap kumpulan) yang terdiri daripada kumpulan kawalan normal (C), kumpulan kawalan neuropati diabetes yang menyakitkan (PDN), kumpulan PDN+LAA: L100 (dos rendah) dan L200 (dos tinggi) dan kumpulan PDN+U0126: U5 (dos rendah) dan U10 (dos tinggi). T1DM diinduksi dengan satu suntikan STZ (60 mg/kg). Penilaian tingkah laku kesakitan dijalankan dengan ujian *Von Frey*, ujian plat-panas dan ujian formalin untuk menentukan allodinia taktil (hari ke-0, 14 dan 22), hiperalgesia haba (hari ke-0, 14 dan 22) dan hiperalgesia bahan kimia (hari ke-23). Tempoh dua (2) minggu diberikan untuk semua tikus menunjukkan keadaan neuropati diabetes yang menyakitkan dan ditentukan

berdasarkan allodinia taktil dari ujian *Von Frey* pada hari ke-14. Kemudian, rawatan sama ada air bergaram, LAA (100 nmol/hari atau 200 nmol/hari) atau U0126 (5 µg/hari atau 10 µg/hari) diberikan secara suntikan intratekal selama tujuh hari berturut-turut. Tikus telah dikorbankan pada hari ke-26 selepas ujian formalin dan kawasan pembesaran lumbar korda spina dikumpulkan untuk pewarnaan Haematoxylin dan Eosin (H&E), imunohistokimia dan analisis PCR kuantitatif (qPCR). Keputusan menunjukkan kumpulan PDN mengalami peningkatan allodinia taktil, kedua-dua hiperalgesia haba dan bahan kimia, di mana gejala ini berkurang dalam kumpulan L100, L200, U5 dan U10 (kumpulan PDN yang dirawat) (p<0.05) pada hari ke-22 manakala pengurangan hiperalgesia bahan kimia diperhatikan dalam kumpuan L100 (fasa 1) dan U5 (fasa 2) sahaja. Sementara itu, analisis H&E mendedahkan bahawa gliosis yang ketara ditunjukkan oleh perubahan morfologi dan pengumpulan astrosit dengan penurunan skor histologi didapati pada kumpulan L100 dan U5 (p<0.05) ipsilateral dan kontralateral berbanding kumpulan PDN. Analisis immunohistokimia menunjukkan kumpulan PDN yang dirawat secara signifikan (p<0.05) menunjukkan pengurangan ekspresi protein astrosit (Glial fibrillary acidic protein (GFAP)), fosfo-ERK, DREAM dan BDNF berbanding kumpulan PDN. Dapatan ini disokong lagi oleh ekpresi mRNA qPCR yang menunjukkan ekspresi mRNA oleh GFAP, ERK1, DREAM dan BDNF juga berkurang dengan ketara dalam semua kumpulan PDN yang dirawat (p<0.05). Analisis korelasi Pearson mendedahkan korelasi positif yang signifikan (p<0.05) antara protein dan tahap ekspresi mRNA GFAP (r=0.7882), fosfo-ERK (r=0.7321), DREAM (r=0.5166) dan BDNF (r=0.5118). Konklusinya, penemuan ini mencadangkan terdapat pengurangan tingkah laku kesakitan, ekspresi astrosit, fosfo-ERK, DREAM dan BDNF tikus PDN diinduksi dari STZ dengan rawatan ubatan

LAA atau U0126 melalui modulasi perencatan laluan interaksi astrosit-ERK dalam patogenesis PDN.

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ABSTRACT

Painful diabetic neuropathy (PDN) is a complication of diabetes mellitus (DM). This chronic neuropathic pain treatment is challenging due to limited options and less effective therapeutics. This study aimed to explore the mechanism of PDN by investigating how L-α-aminoadipate (LAA-astrocytes inhibitor drug) and U0126 (ERK inhibitor drug) affect the expression of protein and mRNA of astrocytes, extracellular signal-regulated kinase (ERK), Downstream regulatory element antagonist modulator (DREAM) and Brain-derived neurotrophic factor (BDNF) in the spinal cord of streptozotocin-induced PDN rats, specifically Type 1 DM (T1DM). One hundred twenty-six Sprague-Dawley male rats were assigned to 6 groups (n=21 per group) consisting of normal control group (C), PDN-diabetic control group (PDN), PDN+LAA treated groups: L100 (low dose) and L200 (high dose) and PDN+U0126 treated groups: U5 (low dose) and U10 (high dose). T1DM was induced with a single intraperitoneal injection of streptozotocin at 60 mg/kg. The pain behavioural assessment was performed by Von Frey test, hot plate test and formalin test to determine tactile allodynia (Day 0, 14 and 22), thermal hyperalgesia (Day 0, 14 and 22) and chemical hyperalgesia (Day 23). Two (2) weeks period was allowed for all DM rats to develop PDN condition and was determined based on tactile allodynia by Von Frey test results on Day 14. Then, treatment of either saline, LAA (100 nmol/day or 200 nmol/day) or U0126 (5 µg/day or 10 µg/day) was administered intrathecally for seven consecutive days. The rats were sacrificed on Day 26 after the formalin test and

the lumbar enlargement region of the spinal cord was collected for Haematoxylin and Eosin (H&E) staining, immunohistochemistry and qPCR analyses. The results showed that PDN group developed tactile allodynia and thermal hyperalgesia by which these symptoms were attenuated in L100, L200, U5 and U10 groups (PDN-treated groups) (p<0.05) on Day 22 whilst attenuation of chemical hyperalgesia was observed in L100 group (Phase 1) and U5 group (Phase 2) only. Meanwhile, H&E analysis revealed a marked gliosis as shown by the morphological changes and accumulation of astrocytes with a decreased histological score of L100 and U5 groups (p<0.05) ipsilaterally and contralaterally when compared with the PDN group. Immunohistochemistry analysis showed that PDN-treated groups significantly (p<0.05) attenuated the total number of astrocytes (Glial fibrillary acidic protein (GFAP)), phospho-ERK (phospho-ERK), DREAM and BDNF protein levels when compared to the PDN group. This finding is further supported by qPCR mRNA expression, which demonstrated that GFAP, ERK1, DREAM and BDNF mRNA expression were significantly reduced in all PDN-treated groups (p<0.05). Pearson correlation analysis revealed that there was a significant positive correlation (p<0.05) between protein and mRNA expression level of GFAP (r=0.7882), phosho-ERK (r=0.7321), DREAM (r=0.5166) and BDNF (r=0.5118). In conclusion, the findings suggested that all PDN-treated groups showed attenuation in tactile allodynia, thermal and chemical hyperalgesia, down-regulation of astrocytes, phospho-ERK, DREAM and BDNF of STZ-induced PDN rats following treatment with LAA or U0126 drug through modulating the inhibition of astrocytes-ERK interaction pathways in the pathogenesis of PDN.

CHAPTER 1

INTRODUCTION

1.1 Painful diabetic neuropathy

Pain, a complex and unpleasant phenomenon with physical, emotional, and cognitive dimensions, has become a significant concern in healthcare nowadays. In Malaysia, the Ministry of Health launched the Pain as 5th Vital Sign (P5VS) initiative in 2008, followed by the Pain Free Program in 2011, and continued progress until now (National Pain Free Program, 2023). This initiative, a cornerstone in pain management, instills in-depth attention to holistic pain management through a multidisciplinary approach including pharmacological, modern surgical techniques and non-pharmacological (physiotherapy, occupational therapy or traditional and complementary medicine). The approach is concerned with providing a pain-free experience for patients suffering.

Pathological pain is a severe medical condition that severely impairs individuals, resulting from nervous system damage or abnormality (Reed, 2022). It may be brought on by a long-healed injury, disease-related conditions or from unknown cause. Even while therapies exist, they frequently have unbearable adverse effects or are ineffective. More significantly, when treatments are stopped, the symptoms frequently recur since they do not reverse the modifications in the nervous system that mediate pathological pain (Price et al., 2016).

Neuropathic pain is caused by abnormal neuronal responses along the pain pathway and serves as pathological pain. It is often characterized by lancinating sensation or burning pain, as well as abnormal sensory symptoms such as allodynia and hyperalgesia (Bridges et al., 2001). Allodynia, which refers to pain caused by a

normally non-noxious stimulation, and hyperalgesia, which is an enhanced sensitivity to pain from a typically painful stimulus, are notable symptoms observed in individuals suffering from neuropathic pain (Ji et al., 2019).

Diabetic neuropathy (DN) is one of the neuropathic pain syndromes that affects diabetic individuals. Numerous difficulties accompany it, which lower the quality of life. The primary factor contributing to the pathophysiology of DN is hyperglycemia which contributes to inflammation and neurotoxicity (Block et al., 2007). In addition, hyperglycemia in diabetic patients has been reported to activate astrocytes and microglia, leading to the release of reactive oxygen species (ROS) that serve as hallmarks of inflammation (Fang et al., 2022).

Painful diabetic neuropathy (PDN) refers to the painful sensation resulting from injury to the peripheral somatosensory system due to diabetes (Jensen et al., 2021). PDN is not a rare condition, but a significant health issue that affects a substantial portion of diabetic individuals in general; hence it is important to note the variability in PDN incidence among diabetic individuals. Data shows that one-third of them experience PDN (Bouhassira et al., 2013; Javed et al., 2015), while the incidence of PDN among all individuals with DM ranges from 10 to 50% (Truini et al., 2018). It is estimated that 50% of the patients develop evidence of nerve damage after over 25 years of having DM (Block et al., 2007). A recent survey conducted by Viatris, an American multinational pharmaceutical and healthcare firm, revealed that 68% of patients in Malaysia who have diabetic neuropathy were aware of the link between pain sensations and diabetes before being diagnosed (The Star, 2023). Unfortunately, no recommended medication is approved worldwide that specifically blocks the pathological processes leading to excruciating DN. This high prevalence highlights the urgent need for effective treatments.

Clinical research has demonstrated the effectiveness of anticonvulsant and antidepressant treatments in the treatment of PDN-related pain (Coelho et al., 2024). The FDA has currently approved duloxetine, pregabalin, tapentadol extended release, and capsaicin (for foot PDN only) as treatments for PDN. Other regulatory organizations have advised the following medications such as sodium valproate, venlafaxine, gabapentin, amitriptyline, dextromethorphan, tramadol, and 5% lidocaine patch (Qureshi et al., 2022). Generally, anticonvulsants such as pregabalin, gabapentin and antidepressants (duloxetine) are often used as first-line treatments. Gabapentin, the anticonvulsant drug, has been demonstrated to reduce the manifestation of both allodynia and hyperalgesia symptoms in multiple animal models (Bannister et al., 2017; Chen et al., 2001; Zhang et al., 2013). Nonetheless, these medications only provide pain relief and are partially effective (less than 50% of patients) with sub-optimal side effects (Bridges et al., 2001). Oral opiate use is also less effective in neuropathic pain compared with inflammatory pain and can cause people to feel more pain than usual. Prescription opiate abuse has also been highlighted, which leads to potentially life-threatening conditions or adverse effects, such as significant residual disability or disfigurement (Green et al., 2017). Because of this, their use is not recommended.

Converging interests have been shown on neuromodulation treatments such as spinal cord stimulation, dorsal root ganglion stimulation, and medicine delivery through the intrathecal space that is becoming more critical in treating PDN (Staudt et al., 2022). However, many chronic pain problems do not react well to conventional painkillers; therefore more clinical trials for both novel and conventional medicines are needed to develop a more effective treatment for PDN with fewer side effects, as

treating painful symptoms in diabetes patients can be frustrating for both patients and healthcare professionals.

Recent research has shed light on the potential role of glial cells in the development and maintenance of PDN. Neuroglial cells such as microglia and astrocytes, are reported to be possible players in PDN through activation of various mitogen-activated protein kinase (MAPK), especially extracellular signal-regulated kinase (ERK). MAPK is critical in signalling and gene expression in the inflammatory response. MAPKS family consists of three major members, extracellular signal-regulated kinase (ERK), p38, and cjun N-terminal kinase (JNK), representing three different signalling cascades (Johnson and Lapadat, 2002). In the pain model, p38 has been activated in microglia while JNK is primarily activated in astrocytes, but ERK is activated in microglia and astrocytes (Zhuang et al., 2006; Ma and Quirion, 2002). A study has found that intravenous injection of L-aminoadipate (an astrocyte-specific inhibitor) reduced allodynia in the T2DM mouse model (Liao et al., 2011). This could suggest that astrocyte activation and the ERK signalling pathway play a key role in the PDN process. However, the signalling mechanism of astrocyte-ERK interaction in PDN remains unknown and requires additional research. The findings could open up new avenues for understanding and potentially treating PDN while sparking curiosity and interest in the medical research community.

There are growing shreds of evidence reported that the downstream regulatory element antagonist modulator (DREAM) protein and/or gene is involved in pain modulation by acting as putative transcriptional repressor for the protein involved in pain processing such as prodynorphin and c-fos (Fang et al., 2022; Ying Zhang et al., 2007). Knocking down the DREAM gene increases the generation of dynorphin peptide in the spinal cord area and lowers generalized pain response in various pain

models (Cheng and Penninger, 2002). DREAM posits a comparable role in a few neurological disorders such as stroke, Alzheimer's and Huntington's diseases, amyotrophic lateral sclerosis, and neuropathic pain (Molinaro et al., 2023). Several studies have reported the involvement of DREAM protein in pain processes (Cheng et al., 2002; Cheng and Penninger, 2002; Long et al., 2011). In addition, DREAM can suppress the transcription of certain genes that are important for protecting the nervous system - the neuroprotective genes, such as sodium/calcium exchanger isoform 3 (NCX3), BDNF, pro-dynorphin, and c-fos (Molinaro et al., 2023).

Meanwhile, Brain-derived neurotrophic factor (BDNF), a type of neurotrophin, has also been found to play a significant role in pain modulation. In animal research, BDNF was shown to influence rapid excitatory (glutamatergic) and inhibitory (GABAergic/glycinergic) signals, as well as slow peptidergic neurotransmission in the spinal cord (Merighi et al., 2008). A study demonstrated that the administration of an inhibitor of astrocytic activation, through a spinal injection, was able to reverse mechanical allodynia in the maintenance phase of pain generated by the spinal nerve ligation (SNL) pain model, whereas the microglial inhibitor did not produce the same effect (Zhang et al., 2012). The study also suggested that the impact of the astrocyte inhibitor was associated with a reduction in BDNF expression in the dorsal horn. Ismail et al., (2020), also demonstrated that DREAM and BDNF were significantly modulated in an STZ-induced PDN rat model, by co-interaction with microglia activation, suggesting future avenues in future research.

1.2 Problem statement

Currently, there is no effective treatment aimed at inhibiting or reversing the progression of the disease since available medications mostly relieve pain symptoms and are unable to reverse the PDN mechanism. These medications only provide pain relief (Bridges et al., 2001) or may lead to severe, life-threatening illnesses or adverse effects that result in considerable residual disability or disfigurement (Green et al., 2017). For instance, the prescription opiate abuse manifests in deliberate actions, such as crushing a tablet for faster pain relief, or unintentional ones, such as splitting extended-release tablets to reduce the dose or crushing them to facilitate easier intake by mixing with food. This underscores the urgent need for more effective treatments that address the underlying causes of PDN. Due to the insufficient understanding of the intricate pathophysiology of nociception and pain perception in health and illness, the therapy of PDN continues to be inadequate.

The exact mechanism of PDN is still not clearly understood. We still do not fully understand why certain neuropathic patients can have painful symptoms. In addition, the mechanism by which our central nervous system processes nociception produced in the peripheral nervous system is also not fully understood. Diabetic patients with PDN, a condition that affects their nervous system, will experience neuropathic pain, a chronic pain condition and become one of the major late complications, which significantly limits the life expectancy of affected diabetic patients while impacting their quality of life.

Therefore, one of the approaches is to focus on inflammatory pathways such as ERK pathway that could interact with the glial cells (neuron-supporting cells in our nervous system), particularly astrocytes. Other contributing factors such as the

involvement of signalling neuromodulators, i.e, DREAM and BDNF, may play a comparable role in the interaction of the astrocytes-ERK pathway.

1.3 Rationale of study

PDN is a multifaceted disease influenced by various substantial factors such as inflammation, metabolic and vascular abnormalities, and poor glycaemic control (Themistocleous and Backonja, 2023). Although the etiology of PDN is complex and has multiple forms of complications, prolonged hyperglycemia leading to neurotoxicity may be recognized as a key factor in its development, which is more pronounced in T1DM (Courteix et al., 1993; Edwards et al., 2008). In T2DM, this factor is less significant since hyperlipidemia, insulin resistance, and other variables might also influence the development of PDN (Yorek, 2022). Current treatment only alleviates the pain symptoms, does not reverse the mechanism and also produces serious side effects such as permanent impairment or disfigurement (Green et al., 2017). Due to the insufficient understanding of the pathophysiology and PDN mechanism, the therapy of PDN continues to be inadequate.

This present study aims to achieve a greater understanding of the pain behaviour and the molecular mechanism behind the development of PDN, which would enable early-stage diagnosis and treatments. It is essential to identify the plausible molecular processes behind a particular disease to develop preventative treatments and improve progression prediction. In particular, findings from this study may elucidate the exact role of astrocytes, phospho-ERK, DREAM and BDNF protein and mRNA expression levels that may eventually offer analgesic potential for PDN treatments.

Non-neuronal cells such as astrocytes, cancer cells and stem cells play an important role in pain modulation by interacting with the nociceptive neurons (Ji et al., 2016). As reported by Dauch et al. (2012), the investigation on the sensory neuron and regional astrocyte activation of the T2DM mouse model suggested that the neuron-astrocyte interaction posits a significant factor in multiple chronic pain conditions, together with the involvement of ERK signalling, presenting a promising approach in treating PDN. However, the previous study did not report on T1DM rats and other signalling neuromodulators, such as DREAM and BDNF involvement. This knowledge gap suggests that understanding the neuron-astrocyte network could inspire more research and lead to a better understanding and improved treatment of PDN. Accordingly, it could be highlighted that the use of the T1DM STZ-induced PDN model is also crucial to be investigated, thus potentially providing analgesic strategies and transforming our way of addressing PDN.

1.4 Hypothesis

The central hypothesis guiding this present study is a novel approach, suggesting that spinal astrocytes activation, ERK phosphorylation, and up-regulation of DREAM and BDNF protein and mRNA are involved in the mechanism of PDN in the STZ-induced rat model. If this hypothesis is substantiated by inhibiting astrocyte activation (LAA drug) and ERK signalling cascades (U0126 drug), this study could offer a new perspective on the mechanism of PDN. In brief, this study hypothesizes that STZ-induced PDN rats exhibit allodynia and hyperalgesia whilst increasing the GFAP, ERK, DREAM and BDNF protein and mRNA expression. Further, the LAA and U0126 drug treatment significantly improves the PDN rats by attenuating the allodynia and hyperalgesia, reducing the gliosis and down-regulating the protein and mRNA expression of astrocyte activation (GFAP marker), ERK, DREAM and BDNF. In addition, it is hypothesised that there is a positive correlation between GFAP, ERK, DREAM and BDNF protein and mRNA expression with the tactile allodynia, thermal hyperalgesia and chemical hyperalgesia.

1.5 Objectives of the study

1.5.1 Main objectives

The study aimed to determine the mechanism of PDN in the STZ-induced T1DM Sprague-Dawley rats following treatment with L-α-aminoadipate or U0126 drug.

1.5.2 Specific objectives

1. To determine the effects of L-α-aminoadipate (LAA; astrocytes inhibitor) or 1,4-diamino-2-3-dicyano-1,4-bis (2-aminophenylthio) butadiene (U0126;

ERK inhibitor) administration intrathecally on tactile allodynia, thermal hyperalgesia and formalin-induced pain response in the STZ-induced PDN rat.

2. To determine the histopathological changes and histological score after intrathecal administration of LAA or U0126 drugs by H&E analysis in the STZ-induced PDN rat spinal cord.

3. To determine the protein expression changes of GFAP, phospho-ERK, DREAM and BDNF after intrathecally administration of LAA or U0126 drugs by immunohistochemistry analysis in the of STZ-induced PDN rat spinal cord.

4. To determine GFAP, ERK, DREAM and BDNF mRNA expression after intrathecally administration of LAA or U0126 drugs by qPCR analysis in the STZ-induced PDN rat spinal cord.

5. To examine whether the expression level of GFAP, phospho-ERK, DREAM and BDNF of protein and mRNA expression in the spinal cord correlates with the tactile allodynia, thermal hyperalgesia and formalin-induced pain response in STZ-induced PDN rat by correlation analysis.

1.6 Analytical framework

This study aims to determine the mechanism of PDN, specifically the astrocytes, phospho-ERK, DREAM, BDNF protein and mRNA expression of STZ-induced T1DM rats following treatment with LAA or U0126 drug. The summary of the analytical framework is shown in Table 1.1.

Table 1.1 Analytical framework of the present study.

Research question	**Research objective**	**Strategy of inquiry (methodology)**
Do LAA (astrocytes inhibitor) and U0126 (ERK inhibitor) administration affect tactile allodynia, thermal hyperalgesia and formalin-induced pain behavioural stimulus in STZ-induced PDN rat?	To determine the effects of LAA and U0126 administration intrathecally on tactile allodynia, thermal hyperalgesia and formalin-induced pain response in STZ-induced PDN rat.	Behavioural pain assessment: 1. Von Frey test – tactile allodynia 2. Hot plate test – thermal hyperalgesia 3. Formalin test – chemical hyperalgesia
Are there any changes in the histopathology of astrocytes in spinal cord of STZ-induced PDN rat after intrathecal administration of LAA and U0126 drugs?	To determine the histopathological changes and histological score of astrocytes after intrathecal administration of LAA or U0126 in the spinal cord of STZ-induced PDN rat.	Haematoxylin & Eosin (H&E)

Table 1.1 Continued

Research question	Research objective	Strategy of inquiry (methodology)
Are GFAP, phospho-ERK, BDNF and DREAM protein involved in the underlying mechanism of STZ-induced PDN rat after intrathecally administration of LAA and U0126 drugs?	To determine GFAP, ERK, DREAM and BDNF protein expression after intrathecal administration of LAA or U0126 in the spinal cord of STZ-induced PDN rat.	Immunohistochemistry (IHC) - protein expression
Are GFAP, ERK, BDNF and DREAM mRNAs involved in the underlying mechanism of STZ-induced PDN rat after intrathecally administration of LAA and U0126 drugs?	To determine GFAP, ERK, DREAM and BDNF mRNA expression after intrathecal administration of LAA or U0126 in the spinal cord of STZ-induced PDN rat.	qPCR - mRNA expression
Is there any correlation between GFAP, ERK, DREAM and BDNF protein and mRNA expression with the tactile allodynia, thermal hyperalgesia and formalin-induced pain behaviour in STZ-induced PDN rat?	To examine whether the expression level of GFAP, ERK, DREAM and BDNF of protein and mRNA expression in the spinal cord correlates with the tactile allodynia, thermal hyperalgesia and formalin-induced pain response in STZ-induced PDN rat.	Correlation analysis

CHAPTER 2

LITERATURE REVIEW

This chapter aims to review the relevant works of literature related to astrocytes, phospho-ERK, DREAM and BDNF proteins in the STZ-induced PDN rat model. The chapter begins by defining a few pain terminologies, followed by pain classification and pain pathways. The mechanism and symptoms pertaining to neuropathic pain – an exclusive type of pain, are also discussed. This is followed by an introduction to PDN – prevalent symptoms of neuropathic pain due to complications of DM, the pathophysiological aspects and molecular mechanisms related to PDN. Furthermore, the STZ-induced PDN rat model is also reviewed, followed by behavioural pain assessment related to this model. Next, this chapter also discusses the role of astrocytes, phospho-ERK, DREAM and BDNF proteins in the mechanism of PDN which are the parameters highlighted in this present study. Finally, a brief overview of interaction involving all the parameters is also summarized as a conceptual framework at the end of this chapter. To better understand this current study, a few important terminologies are listed in Table 2.1.

Table 2.1 Terms and definitions from the literature related to the present study.

Term	Definition	References
Pain	Unpleasant sensory and emotional experiences associated with actual or potential tissue damage	(Raja et al., 2020)
Somatosensory nervous system	Specialized sub-systems of the somatic nervous system detect distinct stimuli like temperature and touch.	(Le Pichon and Chesler, 2014)
Neuropathic pain	Pain that arises from a lesion or disease of the somatosensory nervous system	(J. Cheng, 2018)
Painful diabetic neuropathy	Leading source of neuropathic pain and a consequence of diabetes with painful sensation.	(Aslam et al., 2014)
Nociception	The sensory perception that enables organisms to detect and avoid tissue-damaging stimuli (noxious stimuli)	(Tracey, 2017)
Nociceptors	Primary afferent of peripheral nerve ending specifically tuned to perceive stimuli arising either from actual or potential tissue damage	(Nikolenko et al., 2022)
Peripheral sensitisation	Increased sensitivity and excitability of nociceptors at the site of injury	(Vardeh and Naranjo, 2017)
Central sensitisation	Forms of neuronal and/or synaptic plasticity characterized by increased responsiveness of nociceptive neurons in the CNS to their normal input	(Ji et al., 2019)

2.1 Pain

2.1.1 Overview of pain

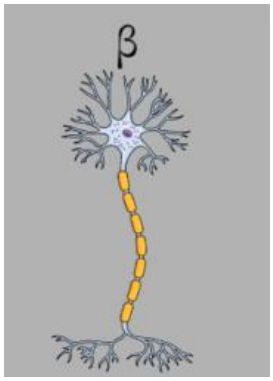
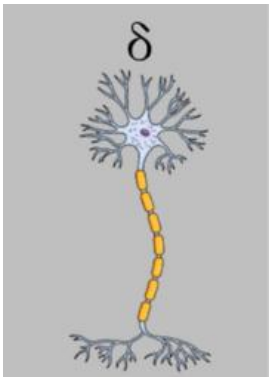
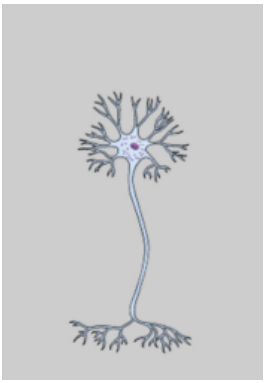
Pain is a complex sensory and subjective perception based on an individual's emotional state. It is an interpretation of our brain on sensory information that begins at nociceptors and is perceived at the central or peripheral somatosensory of the nervous system (Silverthorn, 2019). The nociceptors are the primary sensory neurons located mainly in our skin that detect or respond to noxious (harmful) stimuli (Patapoutian et al., 2009) such as chemical, mechanical or thermal that potentially can cause tissue damage. Generally, the pain sensation is controlled by the nociception system in our brain (Walton, 2019). In 1979, the International Association for the Study of Pain (IASP) council defined pain as “An unpleasant sensory and emotional experience that is associated with actual or potential tissue damage or [is] described in terms of such damage’. However, the definition of pain has been debated since some researchers suggested broadening the term to include both physical and psychological aspects (Biro, 2011). In 2020, the IASP reassessed the definition and recommended their retention of modification. It was revised to “An unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage” (Raja et al., 2020). This IASP definition has remained unchanged since then and has been used by many researchers.

Afferent signals from sensory receptors called nociceptors are the primary neurons with free nerve endings that are carried to the central nervous system (CNS). Nociceptors are the sensory neurons that are primarily responsible for detecting and responding to stimuli that can cause or indicate tissue damage (Nikolenko et al., 2022). There are two classes of primary somatosensory nerve fibres (Gonzalez-Hermosillo et al., 2023). Firstly, A fibres (Aβ-beta, large myelinated neurons and another is Aδ-delta,

small myelinated neurons). Secondly, C fibres (unmyelinated neurons) as depicted in Table 2.2. The sensation carried by this nociceptor pathway is perceived as pain and most of them respond to only one type or several types of stimulation. Activation of the nociceptor pathways mainly initiates adaptive and protective responses (Silverthorn, 2019). In general, the pain conduction may occur very fast, moderately fast or slow transmission, with most myelinated fibres such as the Aβ-fibres being associated with very fast pain transmission. Meanwhile, the Aδ-fibre mainly receives and transmits the pain sensation to the CNS quickly, associated with thermoreceptors and mechanoreceptors. In contrast, polymodal receptors are carried on C fibres and are mainly related to slow pain (Gonzalez-Hermosillo et al., 2023).

Pain consists of two related conditions, either physiological or pathological circumstances. The physiological type plays a pivotal protective role as a warning to avoid noxious stimuli such as heat, chemical irritants or a cold state (Lu and Gao, 2023) and to facilitate healing by making our body sensitive to movement that could impede the healing process (Finnerup, 2019). Meanwhile, pathological pain is a medical condition that results from tissue or nerve damage and is usually associated with neuropathic pain which no longer serves an adaptive purpose (Silverthorn, 2019).

Table 2.2 Primary afferent nerve fibres type related to pain transmission.
The sensory nerve fibres related to pain sensation comprise of two classes. Type A are the myelinated fibres whilst Type C is the unmyelinated fibres (adapted from Gonzalez-Hermosillo et al., 2023; Silverthorn, 2019).

Nerve Type	**Type A**		**Type C**
	β	δ	
			
Fibre characteristic	Large, myelinated Diameter 6-12 µm	Small, myelinated Diameter 1-5 µm	Small, unmyelinated Diameter 0.02-1.5 µm
Speed of conduction	30-70 meters/sec	12-30 meters/sec	0.5-2 meters/sec
Function association	Touch and pressure from skin -very fast pain	Mechanical, thermal and touch from skin – fast pain	Polymodal receptor, mechanical, thermal and chemical – slow pain

2.1.2 Types of pain

Pain can be classified based on several factors such as duration, causal mechanism, severity and pathophysiology (Dissanayake and Dissanayake, 2016). Generally, three types of pain are commonly used based on mechanism: nociceptive, neuropathic and nociplastic (Patapoutian et al., 2009; Srishty Sharma et al., 2023; Woolf, 2004) as shown in Table 2.3. Nociceptive pain originates from sensory receptors (nociceptors) and is stimulated due to injury or inflammation which can also overlap with neuropathic pain that arises from nerve tissue damage (Patapoutian et al., 2009). Notably, DN falls under the neuropathic pain. Meanwhile, nociplastic pain refers to a type of pain for which there is not enough data to distinguish whether it is nociceptive or neuropathic (Yoo and Kim, 2024). It has been suggested to involve the central nervous system across multiple locations and is more extensive than inflammation and nerve damage (Srishty Sharma et al., 2023). Although nociceptive and neuropathic pain were thoroughly defined in the 2011 International Association for the Study of Pain (IASP) taxonomy, a significant proportion of patients remained whose pain that could not be placed in any of the two groups. Consequently, the nociplastic pain was proposed in 2016 as a third way to describe the pain (Atta et al., 2023; Lu and Gao, 2023).

Interestingly, there is also another type of pain - the psychogenic pain which is associated with psychiatric disorder (Woolf, 2004) and perceived with absent evidence of trauma, inflammation of illness such as headache (Srishty Sharma et al., 2023).

Pain can also be classified in terms of duration. Pain is originally acute however if it lasts more than 3 months, it is suggested to be chronic pain (Zolezzi et al., 2022). The chronic pain is classified as primary or secondary chronic pain. The primary chronic pain is evident without any chronic condition such as fibromyalgia

(generalized pain) and irritable bowel syndrome (visceral pain). Meanwhile, secondary chronic pain is linked to other chronic diseases such as neuropathic pain (Alves et al., 2022).

Table 2.3 Pain classification (adapted from Patapoutian et al., 2009).

Type of pain	**Nociceptive/ Inflammatory**	**Neuropathic**	**Nociplastic/ central sensitisation**
Stimulus	Injury or inflammation	Neural damage, pinching, irritation	Central nervous system dysfunction
Neurons	Nociceptor and non-nociceptor	Nociceptor and non-nociceptor	Non-nociceptor
Site	Peripheral and central nervous system	Peripheral and central nervous system	Central nervous system
Clinical setting	Acute trauma, post-operative, arthritis	Nerve lesions, diabetic neuropathy, shingles, carpal tunnel	Fibromyalgia and a variety of other pain disorders
Function	Protective, healing/ repair, pathological	Pathological	Pathological
Pain sensitivity	High or low threshold	Low threshold	Low threshold

2.1.3 Pain pathways

Pain pathways are important aspects of therapeutic application in that they provide targets for pain management and explain how pain is perceived in our brain. These pathways are constituted of three basic nerve fibres as described in the previous section 2.1.1, based on the degree of myelination which can be myelinated, poorly myelinated or unmyelinated.

Three groups of neurons constitute in pain pathways as suggested by Trigo Blanco et al., (2018): primary sensory neuron, secondary sensory neuron and tertiary neuron (Figure 2.1). The pathways involve three main structures: the peripheral sensory system, the thalamus and the cortical area in the brain that corresponds to the respective neuron groups (Table 2.4).

In general, the pain pathway involves signal transduction, conduction, modulation and perception (Alves et al., 2022). Pain transduction involves the synapse between nociceptors and afferent sensory neurons in response to stimuli. Meanwhile, pain conduction is described between the peripheral and cranial nerves, where the primary sensory neurons synapse onto secondary neurons and eventually onto tertiary neurons in the somatosensory cortex. The signal transmission happens along the pathways (Silverthorn, 2019).

The cell bodies of primary sensory afferent neurons are found within the dorsal root ganglion of the spinal cord, except for certain neurons supplying the viscera (which are found in the cranial ganglia and nodose ganglia) and those providing sensory innervation to the face (which are found in the trigeminal ganglion). Nociceptive fibres are activated upon depolarization of axonal nerve terminals (Bridges et al., 2001).

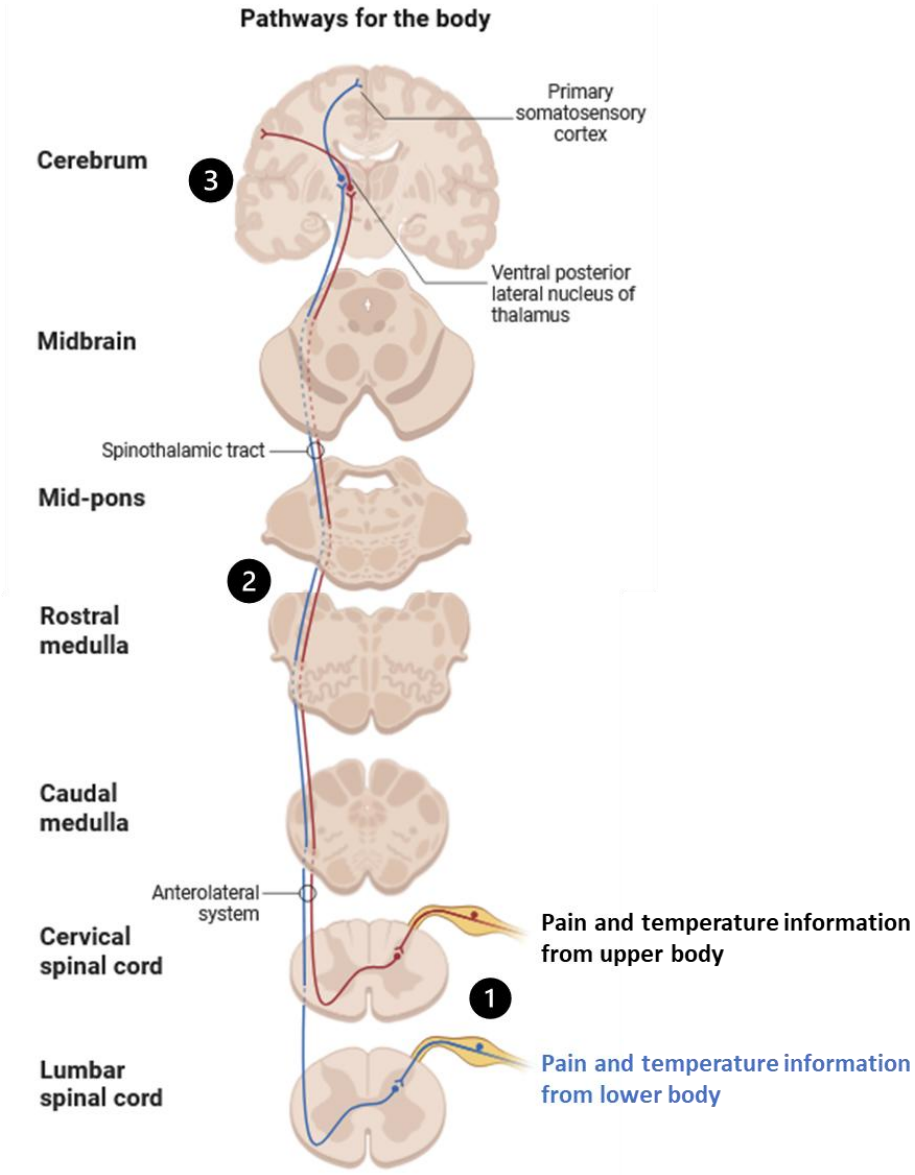


Figure 2.1 Somatosensory pathways (ascending pathways) in pain conduction. 1) Primary sensory neuron that transmits sensory inputs from nociceptors to the spinal cord 2) Secondary sensory neuron that transmits the nociceptive signal from the spinal cord to the thalamus 3) Tertiary neuron that propagates the nociceptive signal from the thalamus onto the somatosensory cortex in the brain. All secondary neurons cross the midline of the body at some point and process the signals contralaterally in our brain. The utilization of neuroimaging techniques has been essential in furthering our understanding of pain pathways, specifically in pinpointing the brain regions responsible for pain modulation and perception (adapted from Trigo Blanco et al., 2018).

Table 2.4 Detailed structures related to pain pathway of the nervous tract (adapted from Gonzalez-Hermosillo et al., 2023).

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Main structures in pain pathway	**Details structures**
First-order neurons (peripheral sensory system)	Spinothalamic tracts Spinal cord Dorsal root ganglia Sensory receptors
Second-order neurons (Thalamus)	Amygdala Hypothalamus Basal ganglia Periaqueductal gray matter
Third-order neurons (cortical areas)	Somatosensory cortex Insula Orbitofrontal cortex Dorsolateral prefrontal cortex Cingulate cortex

The nervous tract (anterolateral system) can be divided into three divisions: spinothalamic tract (the direct pathway that mediates the pain and temperature sensitivity such as location and intensity of stimuli), spinoreticular tract (the indirect pathway that carries emotional sensation of pain) and spinomesencephalic tract (involve in central modulation of pain) (Gonzalez-Hermosillo et al., 2023).

In nociceptive projection neurons, nociceptive (responsive to noxious stimuli) and non-nociceptive (responsive to innocuous stimuli) impulses are combined to form a control gate theory (Wall, 1978) on the signal input mechanism to the brain (Figure 2.2). The gate control model of pain modulation proposed that non-painful stimuli (low intensity of pain) can diminish the pain signal, in which the absence of input from C fibres suppresses the pain pathway. On the other hand, with strong pain, the C fibres which act as inhibitory interneuron, stops the inhibition of the pathway, allowing a strong signal to be sent to our brain. The pain pathway which involves the somatosensory cortex, is part of the brain that recognizes the pain signal tracts that

originate where the pain is then perceived by our brain (Gonzalez-Hermosillo et al., 2023; Silverthorn, 2019). Millions of people worldwide are being impacted by the serious problem of inadequate pain management options for chronic pain, the variability of how people perceive pain and react to pain management techniques is another issue that should be highlighted.

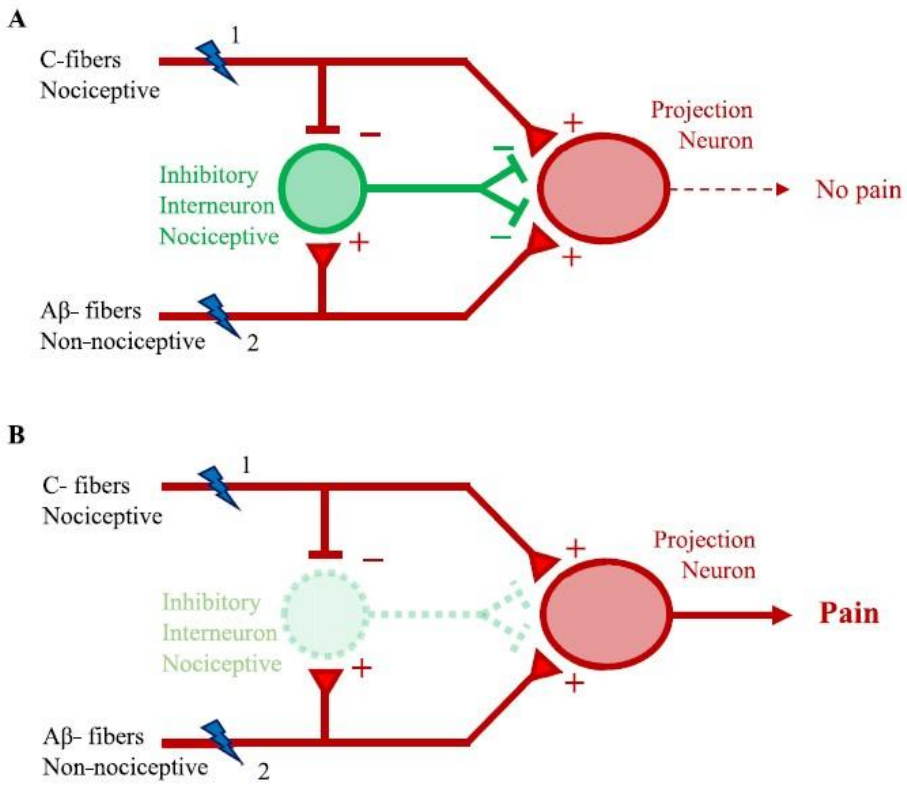

Figure 2.2 Forward inhibition in pain modulation at the spinal cord level.
(A) Normal condition, simultaneous pain activation=(1) or touch activation=(2) receptors do not lead to pain sensation**. (B)** Inactivation of inhibitory interneuron (disinhibition) where the tactile stimuli alone=(2) also leads to pain sensation (adapted from Breitinger and Breitinger, 2023).

2.2 Neuropathic pain

2.2.1 Introduction to neuropathic pain

Neuropathic pain is defined as pain derived from a lesion or disease of the somatosensory nervous system (Cheng, 2018; Treede et al., 2008). Nervous injury or damage in the peripheral or central nervous system may cause neuropathic pain. This pain can be triggered by the excessive stimulation of the nerve system, such as mechanical damage, lesion, illness, trauma, inflammation, neurological diseases or the result of long-term metabolic disorders such as diabetes that affects the somatosensory system without any noxious stimuli leading to allodynia (Archvadze et al., 2018; Breitinger and Breitinger, 2023).

Neuropathic pain is a form of chronic pain caused by the impairment of the peripheral or central somatosensory nervous system (Fang et al., 2022). Neuropathic pain is a unique form of pain that is distinguished from other chronic pain such as inflammatory pain by its specific pathophysiological mechanisms and clinical manifestations of the pain. Neuropathic pain and inflammatory pain may coexist in the same individual (Bouhassira, 2008). Hence, it is crucial to identify neuropathic pain, regardless of whether it is isolated or associated with other chronic pain, in order to provide appropriate treatment. The most frequently accepted neuropathic pain models of chronic pain typically include traumatic nerve injury (e.g. spared nerve injury, SNI), drug-induced neuropathy (e.g. chemotherapy-induced painful neuropathy, CIPN), or metabolic illness (e.g. diabetic neuropathy) (Price and Ray, 2019).