1. Introduction

1.1. The Genesis of Idea: From Concept to Cure

The discovery of Bioglass® (45S5) in 1971 has led to numerous developments in tissue engineering applications (Hench, Splinter et al. 1971). Bioglass® owing to its bioactivity and osteogenic properties (i.e., osteoconduction and osteostimulation) led to its clinical use for the first time as middle ear prosthetics (MEP®) in 1985 to treat the otomastoiditis-induced hearing loss (Wilson, Clark et al. 1994). Later on, the soft connective tissue bonding of bioactive glass (BG) was first observed in 1981 (Hench 2013). Since then, numerous studies have reported the beneficial interaction of BGs with the non-osseous tissues (Tan, Romanska et al. 2003, Saravanapavan, Verrier et al. 2004, Verrier, Blaker et al. 2004, Wang, Wu et al. 2007, Dai, Yuan et al. 2009, Mao, Lin et al. 2014, Pires, Bonan et al. 2018). BGs, the bioceramic biomaterial, exhibit enormous biological functions due to leaching out of various therapeutic ions that are doped in its framework after coming in contact with the physiological fluid which induces specific intrinsic cellular responses (Perez, Singh et al. 2017). Growing evidence suggests that various trace metals like Cu^{2+}, Mg^{2+}, Zn^{2+}, and Sr^{2+} are doped in the structural framework of BGs purposefully to target specific physiological functions (Dai, Yuan et al. 2009, Zhang, Park et al. 2015, Dziadek, Zagrajczuk et al. 2018, Majumdar, Hira et al. 2021). Consequently, we have incorporated barium into the BGs (BaBG). In the previous study from our lab, it was demonstrated that the oral administration of BaBG exhibited anti-ulcer properties in various gastric ulcer models in rats because of its ability to form a protective layer in the stomach (Paliwal, Kumar et al. 2018) and it also acts like an antacid. Further, previously, fewer reports indicated that the presence of Ba^{2+} in the extracellular fluid resulted in prolonged action potential

plateaus observed in the growth cone of the regenerating axon of lamprey (Macvicar's and Llinas 1985). Barium is also reported to generate a barium spike in the growth cone of the regenerating axon (Macvicar's and Llinas 1985). Besides, the calcium channels exhibit greater selectivity for Ba^{2+} ions compared to Ca^{2+} ions (Hagiwara and Byerly 1981). In light of the aforementioned facts, we have synthesized inorganic barium-doped bioactive glass (BaBG) and performed some preliminary studies where the BaBG scaffold (Indian patent No.: 484408) had exhibited the ability to regenerate the deafferented nerve in the complete transection model of neuropathic pain (NP). So, this gave us the confidence that BaBG has the potential to be used for the treatment of NP which would probably act on the calcium channels and calcium channel blockers (CCBs) are widely used for the management of NP. Moreover, according to reports, barium is found in exoplanets, indicating that all living organisms have developed with barium in their bodies (Silva, Demangeon et al. 2022). In addition, barium is found in rocks, soil, and water (Myrvang, Gjengedal et al. 2016), meaning that every living organism is inevitably exposed to barium. Thus, our body has mechanisms to utilize as well as eliminate it, unlike the organic compounds that are foreign to the body. Therefore, we rationalized using BaBG for the treatment of NP.

1.2. The background of bioactive glass

The journey to a new era of tissue replacement and regeneration started on a bus ride in July 1967, when a U.S. army colonel projected the major agony of mankind after the loss of a limb and offered a major task of developing an artificial bone that the body does not reject. The need for the discovery was based to address the issues associated with then applicable materials for bone regeneration: a) foreign body reaction, b) lack of osseointegration, and bioactivity. This led to an arduous quest to research at the

University of Florida, leading to the discovery of 45S5 Bioglass® ($45SiO_2$–$24.5Na_2O$–$24.5CaO$–$6P_2O_5$ wt%) in November 1969 (Hench 2006).

45S5 Bioglass® synthesized by Professor Larry Hench in 1969 was the evolutionary step paving the way towards a benefic liaison of ceramic engineering and biomedical applications (Hench 2006). Being the first of a third-generation biosynthetic material that could bond to the living tissue like bone and soft tissues; 45S5 Bioglass® was soon discovered to produce *in vivo* activity through a process called osteostimulation, whereby in situ bone regeneration is controlled by the release of ionic dissolution products (IDPs) from the BG particulates leading to up-regulation of genes affecting the production of several growth factors, cytokines, osteoblast differentiation factors, etc. (Hench 2013). Ever since its discovery in 1969, the researches for clinical applications have exploded; from being used for conductive hearing loss (Bioglass Ossicular Reconstruction Prosthesis in 1985), traumatic bone repair, to arthroplasty, spine fusion, cranioplasty, the ocular implant (for fibrovascularization) to fixing oral, dental and periodontal defects to the treatment of dentinal hypersensitivity (Majumdar, Gupta et al. 2022) and metastatic colorectal carcinoma of the liver by radioactive glass TheraSphere® (by Nordion Inc. in 2018)comprising Yttrium-90 (^{90}Y) radionuclide (Baino, Hamzehlou et al. 2018, Majumdar, Gupta et al. 2022).

1.3. Role of therapeutic ions in soft tissue repair and regeneration

BGs, the bioceramic biomaterial, exhibits enormous biological functions by inducing specific intrinsic cellular response due to leaching out of various therapeutic cargoes (ions) after coming in contact with the physiological fluid (Perez, Singh et al. 2017). Various metallic ions (Ag^+, Ba^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}) are incorporated in the structural framework of BGs purposefully to target specific physiological functions (Dai, Yuan et

al. 2009, Zhang, Park et al. 2015, Dziadek, Zagrajczuk et al. 2018, Majumdar, Hira et al. 2021). Studies have reported that the ions doped in the BGs modifies the physicochemical properties of BGs and hence controls the degradation rates with subsequently better biocompatibility (Gupta, Majumdar et al. 2021). As previously reported, the trace metallic elements are toxic for the cells and tissues at a higher concentration, so to achieve desired biological actions, the leaching of ions should be in an optimum concentration in a controlled manner (Baino, Hamzehlou et al. 2018). For instance, the trace elements (Zn, Cu, Co, Sr) are reported to have anabolic effects in the hard tissue metabolism, and the doping of Co^{2+} reportedly promoted osteogenesis by activating angiogenesis-related genes (Kargozar, Lotfibakhshaiesh et al. 2016). Similarly, silicon (Si) is another important bioactive cation in biomedicine that is essential for the formation and calcification of bones and teeth. It also activates the blood clotting factor XII (Gryshchuk and Galagan 2016, Chen, Han et al. 2018) that further activate different downstream cascades of blood coagulation. Moreover, the optimum release of Ga^{3+} from the BGs matrices exhibited enhanced thrombus formation and platelet adhesion along with whole blood clotting (Pourshahrestani, Zeimaran et al. 2017). Li^{+} incorporated in the BGs activated the Wnt/β-catenin signaling pathway and enhanced the secretion of insulin-like growth factor 1, and promoted angiogenesis (Haro Durand, Vargas et al. 2017). Therefore, BGs have an excellent hemostatic property and promote vascularization of the tissue-engineered construct required for tissue regeneration.

Further, many therapeutic ions doped in the BGs act as enzyme co-factors that stimulate various biochemical pathways, essential for soft tissue repair and regeneration (Lapa, Cresswell et al. 2020). Zinc (Zn^{2+}), a co-factor of many metalloenzymes, is essential for the proliferation, differentiation, and mineralization of osteoblasts (Della Pepa and

Brandi 2016). Besides, studies have also reported that Zn^{2+} eluted from the zinc-doped BGs upregulated the expression of VEGF mRNA, angiopoietin-1, and fibroblast growth factor-2; hence have angiogenic properties required for tissue regeneration (Zhang, Park et al. 2015). Similarly, series of studies have highlighted that copper (Cu^{2+}) ions activate hypoxia-inducible factor-alpha (HIF-α), adaptive response of the human body in hypoxic conditions that aids in angiogenesis and skeletal muscle regeneration (Wan, Gilbert et al. 2008). Another important soft tissue application of the therapeutic ion (Zn^{2+}) doped in the structural framework of BGs includes peripheral nerve regeneration (Sabbatini, Boccafoschi et al. 2014). Sabbatini et al. (Sabbatini, Boccafoschi et al. 2014) explored and reported that Zn^{2+}-doped BGs favored the adhesion and proliferation of the neuronal cell line with enhanced expression of GAP-43, a protein associated with nerve growth and a major component of the growth cones of the elongating axon. Apart from this, the therapeutics ions like Ag^{+} (Pratten, Nazhat et al. 2004, Dai, Yuan et al. 2009) also have anti-bacterial properties, so they are used to coat the catheters and treat urinary tract infections. Based on this scientific evidence, it can be said that BGs have the potential to be used in soft tissue regenerative medicine due to the doped therapeutic ions. Apart from this, BGs are less risky than gene therapy and cost-effective than growth factors.

1.4. Diverse Applications of Bioactive Glass: Innovations in Medicine and Beyond

1.4.1. Bioactive glasses exhibit wound healing property

Wound healing is the dynamic and well-orchestrated physiological process involving the overlapping of four different mechanisms: hemostasis, inflammation, proliferation, and tissue remodeling. It requires an optimal environment to heal, but the extreme loss of blood increases the chance of hypothermia, acidosis, infections and causes multiple

organ failure (Heckbert, Vedder et al. 1998). The existing treatment strategies mainly involve palliative wound dressings, which otherwise are incompetent; thus, there is a need for safe and biocompatible biomaterials to stop arterial and venous bleeding. Current research has paved the way for BGs as an effective hemostatic agent due to the release of ions simulating various processes involved in different stages of wound healing (Naseri, Lepry et al. 2017, Majumdar, Gupta et al. 2022). A study has investigated the silver exchanged mesoporous silica sphere (AgMSS) for its antibacterial properties for uncontrollable hemorrhage control (Dai, Yuan et al. 2009). The thromboelastographic analysis reported the thrombotic property of AgMSS. The time for the fibrin formation in the silver-doped mesoporous silica sphere (2.9 ± 0.2 min) was significantly lower than the control (9.1 ± 2.5 min). The prothrombin (PT) and activated partial thromboplastin time (aPTT) are critical parameters for predicting the hemostatic properties of a biomaterial. AgMSS treatment reduced PT, and aPTT, supporting the fascinating role of BG in hemostasis. Similarly, silver-doped nanoporous BG (n-BGS) prepared using the sol-gel process possessed significantly greater hemostatic and antibacterial properties compared to the BG without nanopores (BGS) (Hu, Xiao et al. 2012). Further, due to the high surface area of the nonporous n-BGS, complete *in vivo* hemostasis in substantially less time, i.e., 27 ± 2.0 seconds, than BGS and control (86 ± 3 and193 ± 8 sec, respectively) was observed.

More recently, BGs were explored in diseases like diabetes, where the wound healing ability is primarily compromised and hence causes a greater risk of infection. Diabetic foot ulcer (DFU), one of the severe complications of diabetes mellitus, was treated using the rubidium-doped BG nanospheres (Rb-BGNs) (He, Ding et al. 2019). The *in vitro* study reported that the ionic dissolution products (IDPs) of Rb-BGMs induced the

proliferation of fibroblasts (FBs) and human immortal keratinocytes (HaCaTs) along with cell migration in the scratch assay, gold standard for repair of the injured tissues. The consolidated findings support the role of Rb-BGMs in wound healing as FBs and HaCaTs are primarily associated with the re-epithelialization and collagen deposition, vital steps of wound healing. Besides, Rb-BGMs increased the expression of various growth factors essential for wound healing (i.e., VEGF (vascular endothelial growth factor), EGF (epidermal growth factor), and PDGF (platelet-derived growth factor)). Based on the effectiveness of growth factor on wound closure, EF was loaded into Rb-BGM and produced a synergistic effect with improved hemostasis and collagen deposition compared to unloaded Rb-BGMs. A further study by Cong et al. (Mao, Lin et al. 2014) established the potential of BGs in accelerating the healing of full-thickness diabetes wounds in rodents. Bioglass® ointment significantly improved the fibroblast proliferation and formation of the granulation tissue compared to the saline-treated diabetic rats. Hence, BGs can heal wounds by incorporating various other ions to elicit several different cellular responses.

The US-FDA has approved a melt-derived borate-based Bioglass® matrix as a wound dressing (MIRRAGEN™, ETS Wound Care LLC, USA) to treat acute and chronic deep wounds (http://etissuesolutions.com/ 2017). The preclinical study data reported MIRRAGEN® advanced wound matrix to exhibit the slightest inflammation and healed deep wounds in less time. There was significantly more granulation tissue than synthetic polymer fiber and silver-doped collagen fiber (http://etissuesolutions.com/ 2017). Following these seminal results, BGs can serve as an advanced material system for faster wound healing. However, more preclinical and clinical research is required to translate BGs efficiently.

1.4.2. Bioactive glasses facilitate angiogenesis

The beneficial role of 45S5 in promoting endothelial cell proliferation and tubule formation suggestive of angiogenesis was first evidenced in 2004 (Day 2005). The study reported the stimulatory role of IDPs of 45S5 on de novo release and expression of VEGF and bFGF in human intestinal fibroblasts (CCD-18Co) (Day 2005). The same research group (Day, Boccaccini et al. 2004) also reported that composite scaffolds of polyglycolic acid (PGA)/45S5 after 28 and 42 days of subcutaneous (s.c.) implantation in Lewis rats promoted neovascularization without any inflammatory response. In another study, sintered 45S5 promoted angiogenesis using a model of an arteriovenous loop formed between the contralateral sides of the femoral artery and vein in the medial thigh of the Lewis rats (Arkudas, Balzer et al. 2013). The loop placed in a Teflon isolation chamber and embedded in a sintered 45S5 granular matrix filled with fibrin gel showed axial vascularization and dense sprouting angiogenesis three weeks post-operation. The authors suggested that the intrinsic type of vascularization with an equal number of blood vessels in the arterial and venous parts could allow transplantation of the entire construct using the AVL pedicle to confer neovascularization in larger soft tissues.

Moreover, the IDP of BGs stimulates fibroblasts to secrete angiogenic growth factors, which activates endothelial cells to form a vascular network on the scaffolds, thereby enhancing *ex vivo* pre-vascularization of the scaffolds (Gorustovich, Roether et al. 2010, Yu, Peng et al. 2016, Balasubramanian, Hupa et al. 2017, Qazi, Berkmann et al. 2018). Furthermore, Co^{2+} ions also produce chemical hypoxia and induce HIF-1α with further activation of the downstream hypoxic pathway. In a study (Wu, Zhou et al. 2012), sustained release of Co^{2+} ions from a Co-doped BG scaffold has been shown to

upregulate the gene expression of VEGF and HIF-1α, leading to vascularized tissue regeneration

1.4.3. Bioactive glasses in gastrointestinal tissue regeneration

Peptic Ulcer Disease (PUD) is said to have a worldwide prevalence of 4% and have a high recurrence rate despite long-term chronic drug treatment and its associated adverse effects (Lanas and Chan 2017, Paliwal, Kumar et al. 2018). Moosvi et al. (Moosvi and Day 2009) reported that 45S5 promotes healing of injured superficial intestinal mucosa through a mechanism involving epithelial restitution, which involves epithelial cell migration adjacent to the denuded basal lamina. 45S5 affected paracrine mucosal signaling, promoting rapid epithelial repair modulating intestinal epithelial cell growth and migration without causing cell proliferation in a dose-dependent manner.

1.4.4. Bioactive glasses in myocardial tissue engineering

The most versatile application of BGs lies in the possibility of regenerating tissues with inferior regenerative potentials like that of the heart and the lungs. Chen et al. (Chen, Jin et al. 2010) developed a cardiac patch using elastomeric poly(glycerol sebacate) (PGS) with nanoparticles of the melt-derived 45S5 to provide mechanical support to the damaged tissues. However, PGS has limited applicability due to the cytotoxicity of the acidic degradation products, which the alkaline BGs neutralize. In an *in vitro* study, PGS-BG exhibited reduced cytotoxicity to the hESC-CM compared to PGS and even promoted cell proliferation. BG incorporated hydrogel has also been investigated in myocardial TE (Li and Guan 2011). Barabadi et al. (Barabadi, Azami et al. 2016) fabricated gelatin-COL (G/C) hydrogel containing sol-gel-derived BG (G/C/BG). The G/C/BG scaffold-treated cells showed significantly higher viability than the G/C

hydrogel after 72 h of incubation. In addition, cell attachment assay showed more significant infiltration of cells through the pores of the scaffold (G/C/BG) along with increased expression of cardiac tissue-specific proteins like Desmin and α-Actin.

1.4.5. Bioactive glass aids in the peripheral nerve regeneration

Traumatic injury, surgery, chemotherapy, and diseases like diabetes, hypothyroidism, and rheumatoid arthritis lead to motor and sensory deprivation of the affected area due to peripheral nerve damage. Treatment of damaged nerves involves allografts or autografts, with the implicit drawbacks of finding the matching donor and blunted complete functional recovery (Grinsell and Keating 2014). Thus, the other possible alternatives preferred nowadays to bridge the gaps between the damaged peripheral nerves include the nerve guide conduits (NGC). Based on the importance of directional guidance to the regenerating axons, Kim et al. (Kim, Lee et al. 2012) designed a biocompatible and biodegradable scaffold filled with phosphate glass fibers (PGf). The *in vitro* study reported that the length of the neurites of the DRG (dorsal root ganglion) grew to 1220.8 ±168.4 μm length that was significantly larger compared to the control group (875.1±185.1 μm) after incubating for three days. Further, in the *in vivo* experimental model, the sciatic nerve was transected, and the gap was bridged using the conduits. Interestingly, the *in vivo* study results were similar to the *in vitro* data regarding neurite length and number. Similarly, in a very recent study, PGf formulated into gelatin methacrylate (GeIMA) hydrogel (GeIMA-PGf) exhibited significantly more aligned directional growth and spreading of C6 cells over its surface after 14 days incubation compared to GeIMA (Keskin-Erdogan, Patel et al. 2021). This observed growth of C6 cells might be due to the biophysical cues provided by the PGf for neural

growth. Hence, BGs can be considered an essential biomaterial for nerve conduits, guiding regenerated fibers and supporting further nerve regeneration.

1.5. Innovation in Biomaterials: The Development of Barium-Doped Bioactive Glass (BaBG)

Barium (Ba^{2+}) is a trace alkaline earth metal present in the human body (22 mg in a 70 kg adult) (Schroeder, Tipton et al. 1972), mainly in the bones and also in muscle, skin, connective tissue, and the lungs and elicits various physiological functions (Satoh, Kubota et al. 1987, Majumdar, Gupta et al. 2021, Majumdar, Hira et al. 2021). The enrichment of barium in the bone of rodents is also reported and is essential for their calcification (Moore Jr 1964). There are various reports on the use of barium as radiocontrast agents as well as in radio-osteometric analysis (Madanat, Moritz et al. 2009). Barium causes concentration-dependent contraction of the smooth muscle (Satoh, Kubota et al. 1987) but there is a paucity of information on the pharmacological potential of barium. According to reports, barium is found in exoplanets, indicating that all living organisms have developed with barium in their bodies (Silva, Demangeon et al. 2022). In addition, barium is found in rocks, soil, and water (Myrvang, Gjengedal et al. 2016), meaning that every living organism is inevitably exposed to barium. Thus, our body has mechanisms to utilize as well as eliminate it, unlike the organic compounds that are foreign to the body. In light of the aforementioned facts, we had incorporated in the BGs to elicit specific physiological functions. In our laboratory, we have reported that barium-doped BGs possess anti-ulcer properties (Paliwal, Kumar et al. 2018). Additionally, we have reported that Ba^{2+} released from BaBG possesses anti-inflammatory properties (Majumdar, Hira et al. 2021). Previously, fewer reports indicated that the presence of Ba^{2+} ions in the extracellular fluid resulted in prolonged

action potential plateaus observed in the growth cone of regenerating axon of lamprey (Macvicar's and Llinas 1985). The calcium channels also exhibit greater selectivity for Ba^{2+} ions compared to Ca^{2+} ions (Hagiwara and Byerly 1981). Therefore, we presumed that BaBG has the potential to be used as a therapeutic strategy for the treatment of neuropathic pain (NP) by acting on the calcium channels.

1.6. Understanding Neuropathic Pain: An Introduction

Neuropathic pain (NP) is a persistent pain condition that occurs due to injury or lesion to the central or peripheral nervous system (Baron, Maier et al. 2017). The International Association for the Study of Pain (IASP) has defined pain as "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" (Raja, Carr et al. 2020). It mostly arises as a direct consequence of a lesion or diseases affecting the somatosensory system (Gierthmühlen and Baron 2016). The somatosensory nerves mostly originate in the skin, muscles, joints, and fascia and comprise thermoreceptors, mechanoreceptors, chemoreceptors, and nociceptors (Colloca, Ludman et al. 2017). These nerves transmit the sensory signals from the periphery to the spinal cord (SC) and subsequently to the brain for perception of "sensations" such as touch, pain, pressure, vibrations, and temperature. Therefore, any lesions or disorders affecting the somatosensory nervous system can cause abnormal and disrupted transmission of sensory information to the spinal and supraspinal regions which results in the emergence of sensory anomalies (Colloca, Ludman et al. 2017). The identification of distinct sensory abnormalities is essential for accurately diagnosing NP and differentiating it from other types of pain. The damage to bodily tissues leads to the occurrence of acute pain which serves as a warning signal for the body and plays a crucial role in safeguarding its well-being. Nevertheless, acute pain has the potential to progress into a chronic state in the presence

of a disease, as a result of maladaptive changes in the neural pathways within the SC and regions above it. The International Classification of Disease (ICD) given by World Health Organization (WHO) defines chronic pain as the pain that persists for more than three months of duration (Nicholas, Vlaeyen et al. 2019). The emergence of chronic NP has a significant impact on an individual's physical capabilities, psychological state, and interpersonal engagements and imposes a huge burden on the healthcare system. Further, the global epidemiological studies suggested that NP affects around 7-10 % of people worldwide (Colloca, Ludman et al. 2017, Bannister, Sachau et al. 2020) with greater rates documented in individuals with metabolic diseases like diabetes mellitus (up to 26 %) or in case of spinal cord injury (around 40%) (Widerström-Noga 2017, Smith, Hébert et al. 2020). Besides, it is anticipated that the number of patients suffering from NP will rise in the years to come. Therefore, NP is a serious clinical illness that necessitates further investigation to comprehend its pathogenesis and to devise effective treatment of persistent pain.

1.7. Symptoms of Neuropathic pain

The peripheral nerve injury normally induces the NP which may either be spontaneous pain or the stimulus evoked pain (Van Hecke, Austin et al. 2014) as represented in **Table 1.1**. The spontaneous pain is stimulus independent which can be either shooting electrical attacks (paroxysmal pain) or the painful ongoing burning sensation (superficial pain) occurring due to spontaneous ectopic activity in the nociceptive C-fibers thus causing central sensitization of the dorsal horn neurons (Baron, Maier et al. 2017). The second category of the NP is the stimulus evoked pain which may either be allodynia or hyperalgesia (Loeser and Treede 2008). According to the International Association for the Study of Pain (IASP), allodynia refers to the experience of pain in response to a stimulus that that is normally non painful and is caused due to the action

of the Aβ fibers on the CNS. Similarly, hyperalgesia is increased response to a stimulus that is normally painful due to decrease in the threshold of the nociceptive nonmyelinated C-fibers (Buonocore, Demartini et al. 2016). Further, the evoked pain can easily be stimulated by touch, vibration, hot, cold and pin prick (Loeser and Treede 2008).

Table 1.1: Definition of the negative and positive symptoms observed clinically in patients suffering from neuropathic pain

Negative symptoms and signs	
Hypoaesthesia	Reduced sensation to non-painful stimuli
Pall-hypoaesthesia	Reduced sensation to vibration
Hypoalgesia	Reduced sensation to painful stimuli
Thermal hypoaesthesia	Reduced sensation to cold or warm stimuli
Spontaneous sensations or pain	
Paraesthesia	Non-painful ongoing sensation (skin crawling sensation)
Paroxysmal pain	Shooting electrical attacks for seconds
Superficial pain	Painful ongoing sensation, often a burning sensation
Evoked pain	
Mechanical dynamic allodynia	Pain from normally non-painful light moving stimuli on skin
Cold hyperalgesia	Pain from normally non-painful cold stimuli
Heat hyperalgesia	Pain from normally non-painful heat stimuli
Mechanical punctate, pin-prick hyperalgesia	Pain from normally stinging but non-painful stimuli

1.8. Epidemiology of Neuropathic pain

The prevalence rates of persistent pain range from 11 % to 40 % worldwide, and according to a study conducted by the US Centers for Disease Control and Prevention (CDC), 20.4 % of people suffers from chronic pain in United States (Dahlhamer 2018, Cohen, Vase et al. 2021). Similarly, a comprehensive analysis of research conducted in

the UK found that the overall prevalence rate of chronic pain was 43.5 %, with the incidence of moderate-to-severe debilitating pain ranging from 10.4 % to 14.3 % (Fayaz, Croft et al. 2016). In Asia, the prevalence rate of chronic pain ranges from 13 to 51 % (Bhattarai, Pokhrel et al. 2007, Sá, Moreira et al. 2019). Specifically, as per the meta-analysis study performed, the data collected from India revealed that around 19.3 % of people are experiencing NP (Bhattarai, Pokhrel et al. 2007, Dureja, Jain et al. 2014, Saxena, Jain et al. 2018). Additionally, it was more prevalent in the older population i.e., beyond the age of 65 (Saxena, Jain et al. 2018). In the older population, the prevalence of NP was reported to be 23.5 % compared to 15.5 % in the younger population in India (Saxena, Jain et al. 2018). The Global Burden of Disease Study 2019 has confirmed once again that pain and pain-related disorders are the primary cause of disability and disease burden worldwide (Mills, Nicolson et al. 2019, Shin, Shin et al. 2022). Hence, the substantial worldwide burden of chronic pain necessitates addressing the origin and consequences of NP, with a focus on both individual and population-based interventions.

1.9. The physiology of pain perception

The primary afferent fibers of the somatosensory nervous system are comprised of the nociceptive non-myelinated C-fibers that generally terminate at the spino-thalamic projection neuron in the upper laminae as depicted in **Figure 1.1** (highlighted in red). In addition, there are non-nociceptive myelinated A-fibres that are projected into the deeper laminae of the spinal cord (indicated in blue color). Aβ fibers are responsible for perceiving delicate sensations such as touch, while the C and Aδ fibers detect more intense stimuli including mechanical and thermal sensations. (Meyer, Ringkamp et al. 2005). Further, there is second order projection neuron of wide dynamic range (WDR)

type (marked in orange in **Figure 1.1**) that directly receives input from the nociceptive C-fiber terminals. However, there is multisynaptic input from the A-fiber. The WDR neuron also receives inhibitory input from the GABAergic interneurons (colored green). Injury to the sensory peripheral fibres (A and C fibers) causes activation of the nociceptors in response to the external stimulus like temperature, pressure, touch, vibration, etc. which are relayed to the dorsal horn of SC via the generation of action potential (Peirs and Seal 2016). After a peripheral nerve lesion, the nociceptive neurons become abnormally sensitive and develop pathological spontaneous activity (Colloca, Ludman et al. 2017). The hyperactivity of nociceptors causes secondary neuroplastic changes in the spinothalamic tract and modulates the synaptic transmission at the spinal level leading to central sensitization (D'Mello and Dickenson 2008).

The spinal projections then carry the nociceptive information via the spinothalamic and spinoreticulothalamic tract to the supraspinal areas including the periaqueductal grey (PAG) and thalamus (TH) that acts as a central relay station for sensory information (Tracey and Mantyh 2007, Peirs and Seal 2016). Projections to the posterolateral thalamus convey specific information about the stimulus and originate from the endpoints of the column/medial lemniscus route. On the other hand, the medial thalamic nuclei get information related to the emotional aspect of pain (Peirs and Seal 2016). Subsequently, the signals reaches the complex “pain neuromatrix” located in the cortical regions i.e., somatosensory cortex (SC), prefrontal cortex (PFC), insular (INS), and anterior cingulated cortex (ACC) (Tracey and Mantyh 2007). SC is reported to encode the sensory-discriminative aspects of pain while INS, ACC, and PFC is implicated to regulate the affective (emotional) and interoceptive aspects of pain along

with the pain-related decision making by PFC (Apkarian, Bushnell et al. 2005, Tracey and Mantyh 2007) as shown in **Figure 1.2**.

Normally, the descending efferent pathways from the PAG and rostroventral medulla (RVM) runs downward to the dorsal horn of the SC through the dorsal section of the lateral funiculus (Peirs and Seal 2016) which regulate the pain signal and effectively block its facilitation (Wood 2020). However, in case of chronic pain like NP, here is loss of descending analgesic pathway (Baron, Maier et al. 2017). Additionally, there is impairment of the inhibitory input from the GABAergic interneurons into the SC, hence causes hyper excitation of nociceptors that enhances the pain sensation.

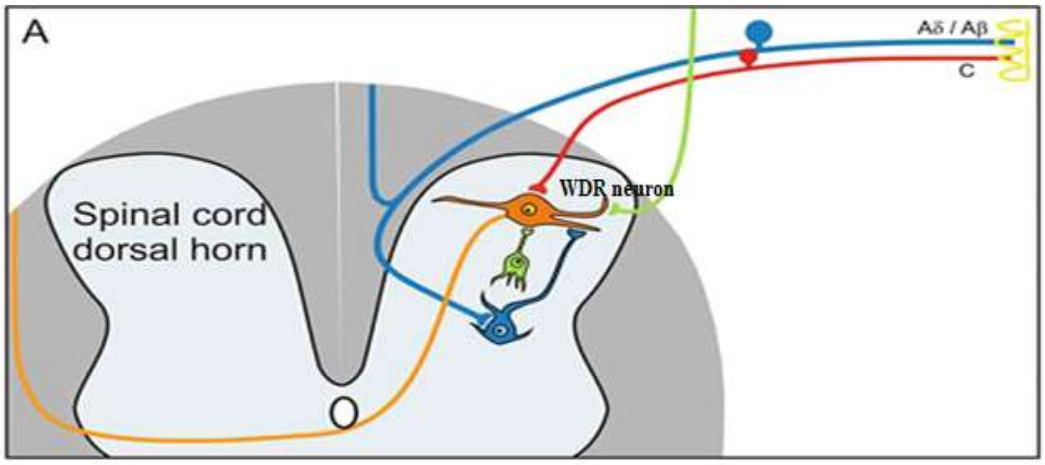

Figure 1.1. The primary afferent pathways and its connections in the dorsal horn of spinal cord. Reprinted with permission from Elsevier (Baron, Binder et al. 2010).

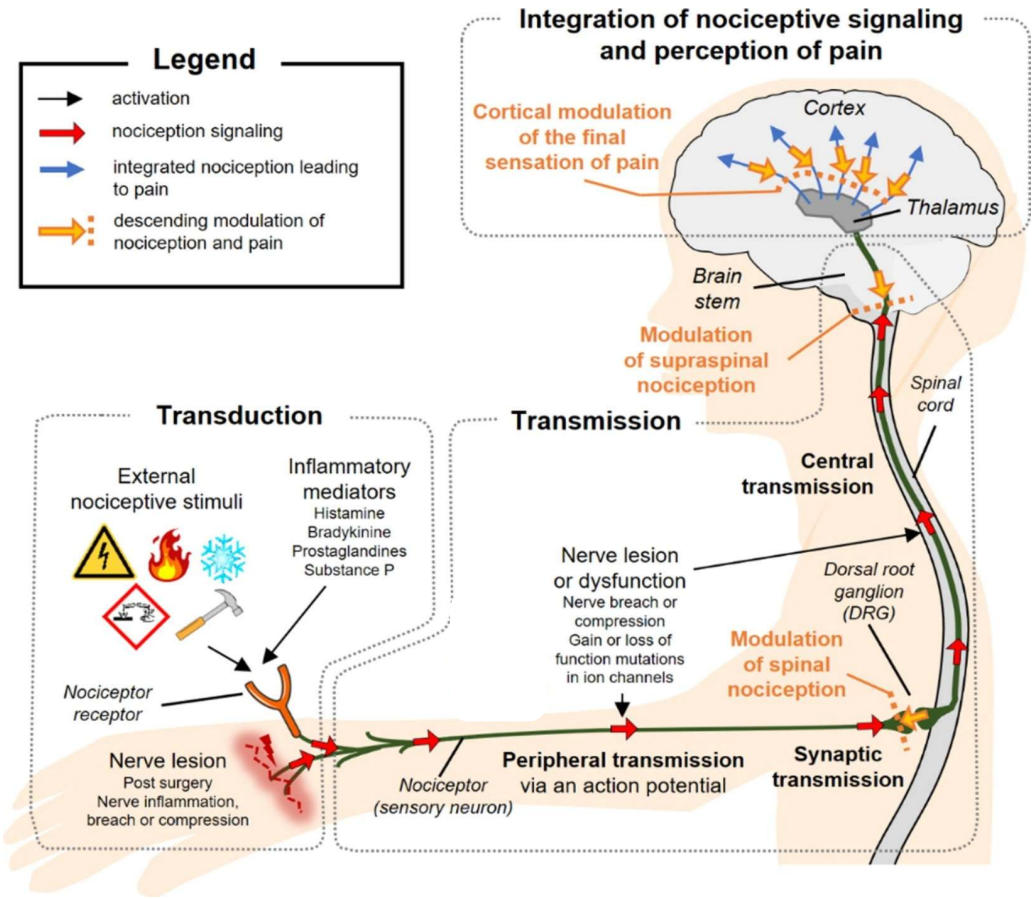

Figure 1.2. The ascending pathway of the pain perception. Reprinted with permission from Elsevier (da Silva, Lepetre-Mouelhi et al. 2022).

1.10. Pathophysiology and molecular mechanism of Neuropathic pain

1.10.1. Peripheral sensitization:

Peripheral sensitization is the ectopic activity developed in the primary afferent nociceptors after partial peripheral nerve lesion or damage (Baron 2006). Typically, following an injury, certain axons get damaged and disintegrate, while others stay intact and connected to the organ, as depicted in as depicted in **Figure 1.3**. This leads to the activation of voltage-gated sodium channels ($Na_v1.7$, $Na_v1.8$ & $Na_v1.9$) on both the damaged and intact sections of the nociceptive C fiber axon (Woolf and Mannion 1999, Waxman, Merkies et al. 2014)**,** which results in a decrease in the threshold for action

potential generation in the injured nerve and consequently leads to increased nerve activity and excruciating pain sensations (Lai, Hunter et al. 2003). Additionally, research has indicated a mutation in the SCN9A gene, responsible for encoding the sodium channel $Na_V1.7$. This mutation results in a change in the gating properties of the channel, causing a shift in its activation towards hyper polarization. As a result, the channel becomes easier to activate, leading to a rapid generation of action potential (Yang, Wang et al. 2004).

Additionally, the peripheral nerve injury induces an upregulation of TRPV 1 expression on the intact A-fibers and C-fibers, which leads to thermal sensitivity (Hudson, Bevan et al. 2001, Hong, Morrow et al. 2004). Out of all the members of the TRP family, the receptor TRPV1 has been extensively researched and is responsible for detecting heat beyond 42°C, which is near the threshold where pain is sensed (Julius 2013). Further, other temperature-sensitive ion channels, such as TRPV2, become active at temperatures exceeding 52°C and TRPV3 and TRPV4 are activated at temperatures ranging from 25°C to 35°C (Julius 2013). In addition, the TRPV2 and TRPA1 receptors are involved in the perception of mechanosensation, indicating their ability to detect multiple types of stimuli (Baron, Maier et al. 2017). Even after peripheral nerve loss or lesion, there is evidence of over expression of the TRPM 8 receptor, which sensitizes cold-sensitive C-fibers and causes cold hyperalgesia (Wasner, Schattschneider et al. 2004).

Moreover, several signaling pathways are involved in the development of peripheral sensitization in NP conditions. Calcitonin gene related peptide and substance P are examples of inflammatory mediators that are released from nociceptive terminals following injury. These mediators enhance the vascular permeability, which in turn

causes localized edema and allows the byproducts of injury, including as prostaglandins, bradykinin (Wang, Ehnert et al. 2006), growth factors, and cytokines, to escape (Cohen and Mao 2014). These mediators stimulate the ion channels that are present on the nociceptive terminal by either direct processes (phosphorylation) or indirect mechanisms (the prostaglandin pathway) which have the ability to sensitize them, which can lead to decreased firing thresholds and more discharges (Fornasari 2012).

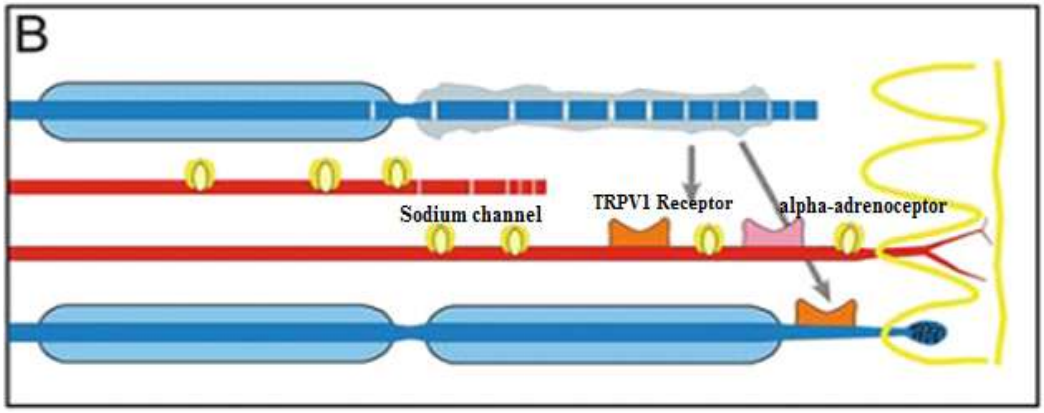

Figure 1.3. The peripheral changes in the primary afferent neuron after the peripheral nerve injury. Reprinted with permission from Elsevier (Baron, Binder et al. 2010).

1.10.2. Central sensitization:

Peripheral sensitization of the afferent C nociceptive fibers causes neuroplastic changes in the spinothalamic tract, which enhances the synaptic excitability of neurons in the SC, brainstem, and brain This ultimately leads to central sensitization (Baron, Maier et al. 2017). Spinal cord dorsal horn neurons are sensitized by primary afferent sensory fibers through the release of glutamate. The glutamate then acts on post-synaptic N-methyl-D-aspartate (NMDA) receptors, which play an integral part in the development and maintenance of central sensitization (Baron 2006). In addition, there is an abnormal rise in the upregulation of calcium channels, leading to an enhanced inflow of calcium

ions (Cui, Wu et al. 2021). The influx of calcium ions removes the Mg^{2+} block from the NMDA receptors, resulting in the further entry of calcium. These events trigger different intracellular cascade pathways, including MAPK, PKA, PKC, extracellular signal-related kinase (ERK), and Src. This contributes to an increased sensitivity of the central nervous system, causing normal inputs to elicit exaggerated responses (Woolf 2007). In addition to all of these various cytokines, prostaglandin, BDNF, and substance P also stimulate NMDA receptors and contribute to increased sensitivity to heat and mechanical stimuli (Baron 2006). All of these physiological alterations contribute to, or uphold, the presence of persistent pain as represented in **Figure 1.4**.

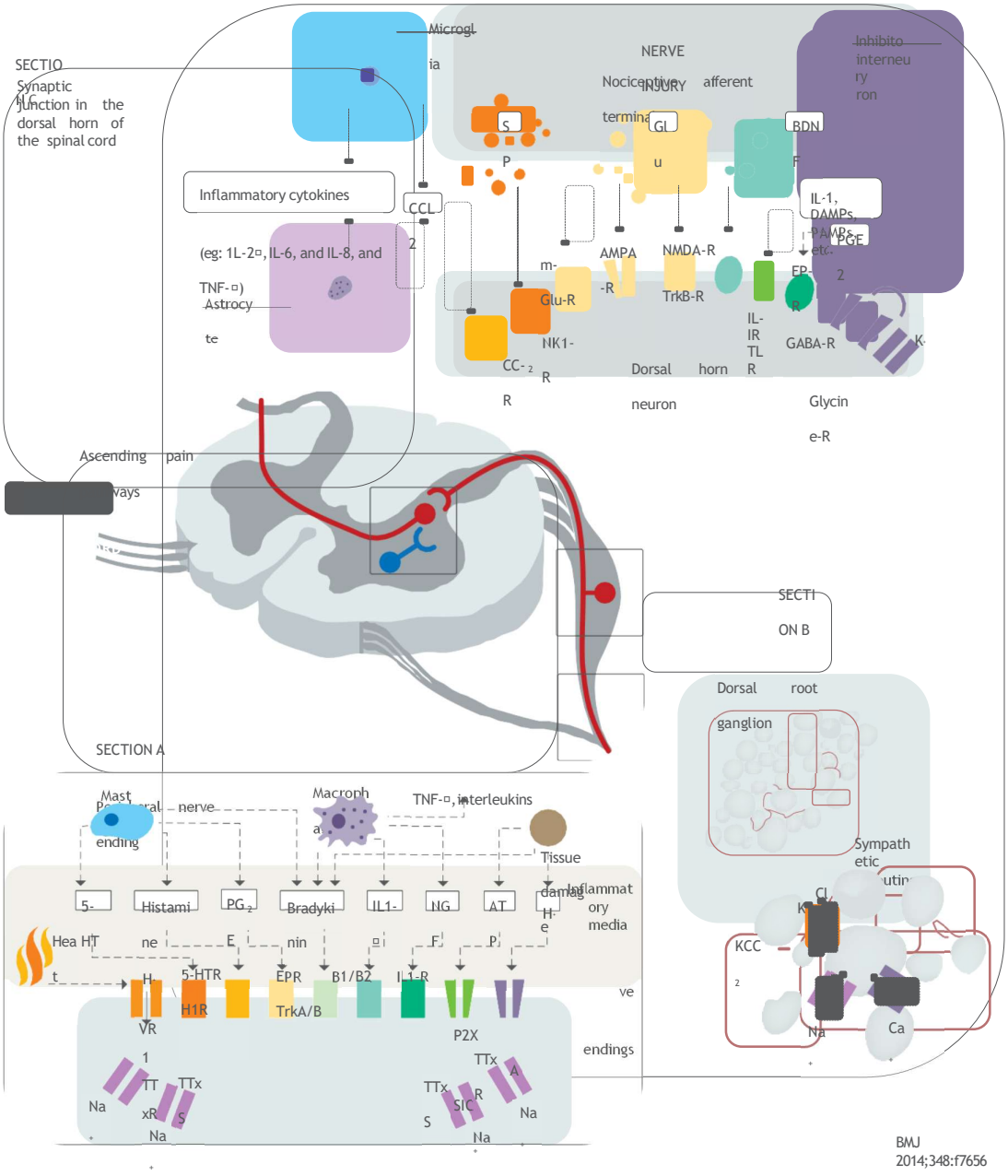

Figure 1.4. The mechanisms of central sensitization of WDR neurons. Diagram showing the various mechanisms involved in neuropathic pain at different sites in the nociceptive pathway. AMPA=α-amino-3-hydroxy-5-methyl- 4-isoxazolepropionic acid; ASIC=acid sensing ion channel; B1/B2=bradykinin receptor 1/2; BDNF=brain derived neurotrophic factor; CCL=chemokine (C-C motif) ligand; CC-R2=CC-chemokine receptor; DAMPs=danger associated molecular patterns; EPR=prostaglandin E2 sensitive receptor; GABA: γ-aminobutyric acid; Glu=glutamate; H1R=histamine receptor; 5-HT=5-hydroxytryptamine; IL=interleukin; KCC=potassium-chloride cotransporter; m-Glu=metabatropic glutamate; NGF=nerve

growth factor; NK=neurokinin; NMDA=N-methyl-D-aspartate; PAMPs: pathogen associated molecular patterns; PG=prostaglandin; P2X=purinergic receptor channel; -R=receptor; SP=substance P; TLR=toll-like receptor; TNF=tumor necrosis factor; Trk=tyrosine kinase; TTxR=tetrodotoxin resistant sodium channel; TTxS=tetrodotoxin sensitive sodium channel; VR=vanilloid receptor (transient receptor potential cation channel subfamily V member 1 TRPV-1). Reprinted with permission from BMJ Publishing Group Ltd. (Cohen and Mao 2014).

1.11. Clinical pharmacotherapy for the treatment of Neuropathic pain

The management of neuropathic pain mostly involves addressing symptoms, and only in certain pathological conditions, the underlying reasons can be treated to alleviate pain. Treating neuropathic pain entails using a range of drugs with the goal of alleviating pain and enhancing overall quality of life. However, around 35% of NP patients do not respond to classical analgesics and even exhibit substantial side-effects (Dworkin, O'connor et al. 2007, Kamerman, Wadley et al. 2015). This is mostly owing to the fact that there is complex overlaying of the molecular and cellular mechanisms contributing to NP which are not entirely comprehended.

1.11.1. Calcium channel blockers/ anticonvulsants

Anticonvulsants are used for the treatment of a wide range of chronic pain conditions, particularly those that are of a neuropathic origin. Within the context of neuropathic pain, activation of many ion channels takes place, including the voltage-gated calcium channel, which is responsible for modulating neuronal firing (Zamponi, Lewis et al. 2009, Alles and Smith 2021). Carbamazepine, oxcarbazepine, topiramate, gabapentin, pregabalin, and lamotrigine are the anticonvulsants that are most frequently used in the management of chronic pain, which includes the treatment of medical conditions such as diabetic neuropathy, trigeminal neuralgia, and other similar conditions (Guy, Mehta et al. 2014). Gabapentin and pregabalin are currently the first-line treatment for the

management of chronic pain (Saxena, Jain et al. 2018, Cavalli, Mammana et al. 2019). Both of these medications have the ability to control the levels of intracellular Ca^{2+}, and they also have active effects on substance P, involved in sensitization of the pain pathways (Gilron and Flatters 2006). Gabapentinoids are reported to bind to the auxiliary unit of calcium channel i.e., α2δ and regulates the calcium influx and aberrant synaptic transmission (Patel and Dickenson 2016). However, the clinical drawbacks of them includes increased risk of atrial fibrillation (Afib) along with somnolence, dizziness, and peripheral edema (10 % to 15 %) (Park, Hunter et al. 2023). Further, the withdrawal rate for pregabalin is 19 % which is mainly due to dizziness and somnolence (Moore, Straube et al. 2009).

1.11.2. Nonsteroidal anti-inflammatory drugs (NSAIDs)

NSAIDs are the analgesics that are used the most frequently by people of varying ages, with the elderly being the most likely to use them for treating acute painful conditions (Vo, Rice et al. 2009). They are usually used frequently for conditions like sprains, headaches, menstrual cramps, surgical pain, and so forth. Additionally, these medications are utilized to treat chronic pain conditions, such as rheumatoid arthritis and osteoarthritis due to their ability to diminish inflammation and other mediators of peripheral sensitization (Crofford 2013). Cyclooxygenase (COX) inhibition is the primary mechanism of action for this class of medicines, which also inhibits the production of prostaglandins that are involved in the progression of NP. The use of these medications is being restricted in clinics, despite the fact that they are effective. This is because there have been worries raised about the contraindications and side effects that they may cause (Paglia, Silva et al. 2021). Acetaminophen, a strong and safer nonsteroidal anti-inflammatory drug (NSAID) analgesic, works by inhibiting COX-3

in the acute therapy paradigm. Acetaminophen poses the dangers of liver damage and nephropathy. Therefore, patients with a history of alcohol and tobacco use are administered lower dosages of this medication (Gloor, Schvartz et al. 2019).

Table 1.2. Clinical pharmacomanagement of neuropathic pain

	Class	Drugs	Dose
First-line therapy	Calcium channel α2-δ ligands	Gabapentin	100–1800 mg/day
		Pregabalin	50–450 mg/day
	Tricyclic antidepressants (TCAs)	Amitriptyline	10–150mg/day
	Serotonin–norepinephrine reuptake inhibitors (SNRI)	Duloxetine	20–120mg/day
		Venlafaxine	37.5–225mg/day
Second-line therapy	Opioids	Tramadol	25–100mg/day
	Topical treatment	Lidocaine	5% patches or gel
		Capsaicin	8% patches
Third-line therapy	Strong opioids	Morphine	10–120mg/day
		Oxycodone	10–120mg/day
	Neurotoxin	Botulinum toxin	25–300 U BTX-A 0.9% saline

(Saxena, Jain et al. 2018, Cavalli, Mammana et al. 2019)

1.11.3. Antidepressants

The analgesic action of tricyclic antidepressants (TCA) is a result of their ability to prevent the reuptake of norepinephrine at the synapses in the spinal dorsal region. The analgesic effect of TCA is attributed to their ability to prevent the reuptake of norepinephrine at the synapses in the spinal dorsal region (Zilliox 2017). Additionally, they also exhibit secondary activity via affecting sodium channels. Amitriptyline has a moderate level of inhibition in both norepinephrine and serotonin reuptake. On the other hand, nortriptyline, which is equally effective but has lesser side effects, demonstrates a higher level of inhibition specifically in norepinephrine reuptake. TCA are efficacious

in treating several forms of neuropathic pain, including severe diabetic neuropathy and postherpetic neuralgia (Park and Moon 2010). TCA are deemed efficacious in the treatment of central pain; nonetheless, there is a scarcity of data about their effectiveness in particular central pain causes. The primary issue associated with the utilization of tricyclic antidepressants is the occurrence of adverse effects. Typical adverse reactions consist of drowsiness and anticholinergic symptoms, such as dry mouth, impaired vision, constipation, urine retention, and postural hypotension. These medications should be taken carefully in individuals who have a history of heart illness. It is advisable to do a screening electrocardiogram (ECG) to check for any abnormalities in the heart's conduction before starting treatment. Additionally, attention should be exercised in patients with glaucoma, urinary retention, or autonomic neuropathy (Park and Moon 2010). Similarly, serotonin norepinephrine reuptake inhibitors (SNRIs) such as venlafaxine, duloxetine, milnacipran are also prescribed for pain management but they carry serious side effects including suicidal tendencies, liver damage, weight gain, withdrawal symptoms, etc. (Park and Moon 2010).

1.11.4. Opioids

Opioids, which are highly strong and effective pain relievers, have recently been relegated to a third-line treatment option for chronic pain in medical clinics, despite their well-established therapeutic benefits (Park and Moon 2010). Morphine, methadone, tramadol, tapentadol, and buprenorphine are frequently used for pain management purposes (Nadeau, Wu et al. 2021). Opioids tolerance is the primary element that restricts the effectiveness of treatment, resulting in the need for higher doses, the occurrence of severe adverse effects, and in rare cases, even death of patients (Nadeau, Wu et al. 2021). Tramadol exhibits a dual mode of action, functioning both as

a central opioid agonist at the mu receptor and as an inhibitor of norepinephrine and serotonin reuptake (da Rocha, Mizzaci et al. 2020). It has demonstrated efficacy in several neuropathic pain situations, such as severe diabetic neuropathy and mixed neuropathic pain syndromes. Despite being regarded as a safer option compared to other narcotic painkillers, this medication is classified as a schedule IV prohibited substance and is experiencing a growing problem of misuse and abuse. The negative consequences of tramadol usage encompass dizziness, nausea, constipation, somnolence, and orthostatic hypotension. Further, codeine, morphine, oxycodone, and fentanyl are examples of potent opioids commonly used for chronic pain management. Their efficacy in managing various neuropathic pain syndromes is a subject of debate, and the utilization of opioids for the treatment of persistent noncancerous pain is a matter of public health concern due to the increasing number of fatalities associated with prescription opioids in the United States (Dowell 2022).

1.11.5. Additional pharmacotherapy

Additional approaches for managing chronic pain include the use of low dose naltrexone, topical medications such as diclofenac, lidocaine, and capsaicin, skeletal muscle relaxants like baclofen, tizanidine, and cyclobenzaprine, as well as botulinum toxin A and cannabinoids (Dowell 2022). However, further research is needed to determine the most effective therapeutic approach, such as combination therapy. Divalproex sodium has also demonstrated success in the treatment of painful diabetic neuropathy and trigeminal neuralgia. However, it does not effectively reduce overall pain or enhance quality of life (Muresanu, Verisezan Rosu et al. 2021).

1.12. Role of ion channels in the development and progression of Neuropathic pain

Modifications in the expression, trafficking, and functioning of ion channels in primary sensory neurons can elicit neuronal hyperexcitability and spontaneous activity, thus leading to the development and maintenance of NP (Aurilio, Pota et al. 2008, Bouali-Benazzouz, Landry et al. 2021). As previously stated, the expression and function of ion channels have been reportedly altered in the primary afferent of animals with neuropathic pain (Tibbs, Posson et al. 2016). Besides, remodeling caused by alterations to the membrane-bound proteins comprising ion channels, might also alter the electrical characteristics of the damaged neuron. Preclinical research on NP indicate that membrane modification leads to increased neuronal excitability due to decrease in the threshold of action potential generation because of disruption in the ionic homeostasis (Bouali-Benazzouz, Landry et al. 2021). The hyper excitability of the nociceptors is observed both at the specific location of nerve damage as well as farther away in the dorsal root ganglia and dorsal horn of the SC. These patterns of anomalous and excessive discharge are considered to be the main reasons for the positive symptoms (paresthesia, dysesthesia, hyperalgesia, and allodynia) experienced by people with NP (Markman and Dworkin 2006). Hence, ion channels have a crucial function in the development and maintenance of NP by regulating the neuronal excitability, the release of neurotransmitters, and the activation of glial cells. The intricate pathophysiology of NP involves voltage-gated sodium and calcium channels, transient receptor potential (TRP) channels, potassium channels, hyperpolarization-activated cyclic nucleotide (HCN) channels, and acid-sensing ion channels (ASICs) (Tibbs, Posson et al. 2016).

1.12.1. Voltage-Gated Sodium Channels

Neuronal action potentials depend on voltage-gated sodium channels (Na_v). $Na_v1.3$, $Na_v1.7$, $Na_v1.8$, and $Na_v1.9$ subcategories of Na_v channels have been implicated of causing NP (Tibbs, Posson et al. 2016). $Na_v1.3$ is reportedly upregulated in sensory neurons after nerve injury. This particular subtype is typically not present in the fully developed sensory neurons, but it reemerges after nerve injury leading to heightened activity and spontaneous firing of the neurons, which ultimately contributes to the perception of pain (Liu, Zhong et al. 2020). Further, hereditary pain syndromes have also been linked to $Na_v1.7$ gene mutations (Niu, Liu et al. 2021). This mutation results in a change in the gating properties of the channel, causing a shift in its activation towards hyper polarization. As a result, the channel becomes easier to activate, leading to a rapid generation of action potential (Yang, Wang et al. 2004). $Na_v1.7$ is highly expressed in PNS sensory neurons and regulates pain sensitivity in erythromelalgia and paroxysmal severe pain condition (Niu, Liu et al. 2021). Similarly, $Na_v1.8$ and $Na_v1.9$ channels are mostly found in nociceptive neurons and plays a role in producing action potentials when the body is exposed to painful stimuli leading to allodynia (Niu, Liu et al. 2021).

1.12.2. Transient receptor potential (TRP) channels

The lesion to the peripheral nerve causes an increase in the expression of TRPV 1 on the A-fibers and C-fibers that are still intact, which ultimately results in a heightened sensitivity to heat (Hudson, Bevan et al. 2001, Hong, Morrow et al. 2004). Among all the members of the TRP family, the receptor TRPV1 has been the subject of substantial investigation. It is the receptor that is responsible for detecting heat that is higher than

42 °C, which is near the threshold where pain is perceived (Julius 2013). Further, other temperature-sensitive ion channels that are reportedly upregulated aberrantly and are involved in the pathophysiology of NP includes TRPV2 which become active at temperatures exceeding 52°C, and TRPV3 and TRPV4 that are activated at temperatures ranging from 25°C to 35°C (Julius 2013). In addition, the TRPV2 and TRPA1 receptors are also reported to be involved in the perception of mechanosensation, indicating their ability to detect multiple types of stimuli (Baron, Maier et al. 2017). TRPA1 is expressed in nociceptive neurons and has been implicated in the development of mechanical allodynia and hyperalgesia following nerve injury (Souza Monteiro de Araujo, Nassini et al. 2020). Even after peripheral nerve loss or lesion, there is evidence of over expression of the TRPM 8 receptor, which sensitizes cold-sensitive C-fibers and causes cold hyperalgesia clinically and preclinically (Wasner, Schattschneider et al. 2004). TRP family receptors are calcium-permeable receptors and their upregulation following peripheral nerve injury leads to an increase in intracellular calcium levels, which in turn promotes neuronal hyperexcitability (Liu, Miao et al. 2023). These mechanisms synergistically amplify synaptic transmission and increase sensitivity, resulting in persistent pain.

1.12.3. Calcium channels and their role in the development of Neuropathic pain

Voltage-gated calcium channels (VGCC) open when the membrane depolarizes and are involved in both generating the action potential and triggering a variety of downstream physiological cytoplasmic cascades. However, under pain-inducing pathological circumstances there is abnormal upregulation of the VGCC which probably enhances the synaptic vesicle release of pain-inducing transmitters like glutamate, substance P, and calcitonin gene-related peptide, thus altering the sensory excitability and leading to

heightened pain sensations. (Altier and Zamponi 2004). Alteration in the voltage-gated calcium channel (VGCC) expression, leads to an elevation in the inflow of Ca^{2+}, which in turn mediates a variety of neuronal processes, including membrane excitability, neurotransmitter release, synaptic plasticity along with the release of pro-inflammatory cytokines, resulting in an exacerbation of pain sensation following sciatic nerve damage (Berridge, Lipp et al. 2000, Yaksh 2006). Further, The N-type VDCC α1β subunit immunoreactivity expression has been reported to rise in the dorsal root ganglion (DRG) cells and lamina II of the spinal cord in 5 to 20 days after nerve CCI (Yaksh 2006) that contribute to neuronal firing and development of NP (Nowycky, Fox et al. 1985, Bourinet, Francois et al. 2016, Hoppanova and Lacinova 2022). Moreover, the CCI model demonstrated that the density of T-type current increases in response to injury of the SN (Jagodic, Pathirathna et al. 2008). This finding was further supported by Yue and colleagues, who observed an increase in both T-current density and amplitude following spinal nerve ligation (Watanabe, Ueda et al. 2015). Ca_V3 channels not only have a role in neuropathic pain caused by nerve injury, but also contribute to the development of painful diabetic neuropathy (Todorovic and Jevtovic-Todorovic 2014). Therefore, it is worth mentioning that CCBs are the first-line therapeutic strategies for the treatment of NP.

1.13. Role of calcium ions in the pathophysiology of Neuropathic pain

According to studies performed on the pathophysiology of NP, one of the most important factors that lead to the development and maintenance of neuropathy is the dysregulation of calcium homeostasis that occurs after neurons have been injured (Siau and Bennett 2006, Cui, Wu et al. 2021). There are reports that demonstrated an alteration in the voltage-gated calcium channel (VGCC) expression, leading to an

elevation in the inflow of Ca^{2+} (Berridge, Lipp et al. 2000, Yaksh 2006). This, in turn, mediates a variety of neuronal processes, such as membrane excitability, neurotransmitter release, and synaptic plasticity along with the release of pro-inflammatory cytokines, which ultimately leads to an intensification of the pain sensation following sciatic nerve (SN) damage (Kawamata and Omote 1996, Berridge, Lipp et al. 2000, Yaksh 2006, Tilley, Cedeño et al. 2022). In addition, the activation of calcium-permeable receptors, such as TRPV1, also results in an increase in the levels of (Ca^{2+})i, which in turn promotes neuronal hyperexcitability (Liu, Miao et al. 2023). The increase in the $(Ca^{2+})_i$ has been reported to further facilitate the increase in the influx of calcium through various ion channels leading to neuroplastic changes in the primary afferent fibres and glial cell-induced neuroinflammation that exacerbates pain (Kawamata and Omote 1996). These mechanisms synergistically amplify synaptic transmission and increase sensitivity, resulting in persistent pain. Moreover, the elevated $(Ca^{2+})_i$ also stimulate many calcium-binding proteins that activate glial cells and trigger the production of pro-inflammatory cytokines and chemokines, which further heighten the sensitivity of pain pathways (Cho and Huh 2020, Jager, Goodwin et al. 2024). Additionally, the sustained elevation of intracellular calcium levels can activate various calcium-dependent enzymes, such as proteases and kinases, which modify neuronal function and enhance pain signaling. As $(Ca^{2+})_i$ ions contribute to membrane depolarization and also act as a second messenger, therefore an aberrant increase in the calcium ion post-injury triggers a wide range of cellular processes including the release of neurotransmitters such as glutamate, substance P, and calcitonin gene-related peptides and increasing the chronicity of pain (Smith, Cabot et al. 2002, Takasusuki and Yaksh 2011). Therefore, calcium ions play a pivotal role in the

development and persistence of NP and clinically CCBs are considered as the first-line therapy for the management of NP conditions.

1.14. Significance of glial cells in Neuropathic pain

Glia encompasses a wide range of specialized cell types that exist in both the peripheral nervous system (including Schwann cells, satellite glia, and perineural glia) and the central nervous system (including astrocytes, oligodendrocytes, microglia, and perivascular glia). These cells make up 70% of the total cell population in brain and spinal cord. When activated, both astrocytes and microglia emit several signaling molecules that can have either protective or harmful effects (Jha, Jeon et al. 2012). Glia have lately been recognized as significant contributors to the mechanisms of chronic pain (Mika, Zychowska et al. 2013), and are now being considered as a promising target for therapeutic research (Pocock and Kettenmann 2007). After the peripheral nervy injury, studies have reported an increase in the number of activated microglia (DH) on the ipsilateral side of the dorsal horn of SC (Jergová and Čížková 2007), leading to systemic release of cytokines that mediate pain hyper sensitization (McGinnis and Ji 2023). Further, studies have also indicated microglia to have an pivotal role in the initial stages of NP, while astrocytes are responsible for maintaining NP phenotypes (Zhuo, Wu et al. 2011). Similarly, following neuronal damage, interleukins was found to be elevated in various NP models that induces allodynia and hyperalgesia in rats (Khan, Noboru et al. 2018). The role of glial cells were further confirmed using fluorocitrate, an anti-metabolic drug that disrupts the Krebs cycle via the inhibition of glia-specific aconitase which mitigated NP phenotypes in NP model (Clark, Gentry et al. 2007). Moreover, in a recent study, elevated levels of S100b was observed that reflect the activation of glial cells, which in turn stimulates inflammatory responses, hence

contributing to the chronic nature of NP (Michetti, Di Sante et al. 2021). In addition, prolonged activation of astroglia following neuronal injury is reported to be associated with the maintenance of a prolonged state of chronic neuroinflammation, contributing to the persistence of NP (Fan, Zhang et al. 2023). The aforementioned compiled evidence underscores the significance of glial cell activation as a pivotal element in the progression and endurance of NP. In the light of this, we have elucidated the temporal changes in the glial-cell associated neuroinflammation following the CCI of SN in rats.

1.14.1. Calcium -binding protein: S100 proteins

S100 proteins are a group of small, acidic calcium-binding proteins that are involved in both intracellular and extracellular functions (Kligman and Hilt 1988). Initially, these proteins were isolated from a sub cellular fraction of a bovine brain and the name S100 denotes its 100% solubility in saturated neutral ammonium sulfate solution (Moore 1965). The S100 protein family is one of the largest Ca^{2+} binding protein subfamilies having an EF-hand motif, which was originally grown from two members (S100A1 and S100b) and is expressed only in vertebrates (Moore 1965). A distinguishing feature of these proteins is that the individual members are confined to particular cellular compartments and can migrate on Ca^{2+} activation, and Ca^{2+} signal transduction in spatial as well as temporal manner by interacting with target-specific S100 proteins (Heizmann 2022). Further, the S100 are cytosolic proteins carrying a wide range of functions by regulations of calcium balance, cell growth, cell proliferation, apoptosis, inflammation, and energy metabolism (Marenholz, Heizmann et al. 2004, Xia, Braunstein et al. 2018). While the intracellular function comprises membrane protein transportation, recruitment, intracellular receptor interaction, transcriptional regulation, its integration with nucleic acids or enzymes, and DNA repair. When S100 binds to

calcium they trigger a cascade of downstream pathways by binding to various cellular proteins (Leclerc, Fritz et al. 2009). The receptor for advanced glycation end products (RAGE) or the cytokine-activated Toll-like receptor (TLR) mediated innate immunity pathways are triggered depending upon the type of S100 protein released by monocytes and neutrophils, which further elevates the levels of interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumor necrosis factor (TNF-α) (Leclerc, Fritz et al. 2009). The increased levels of these inflammatory cytokines further starts a vicious inflammatory cycle (Sims, Rowe et al. 2009). Moreover, the activated S100 proteins tends to modulate glial cells activation, triggering inflammatory responses due to the release pro-inflammatory cytokines and chemokines via NF- κB or calcium-dependent pathway, contributing to pathogenesis of inflammatory diseases (Michetti, Di Sante et al. 2021).

1.14.2. S100 proteins: A key player in the pathophysiology of various diseases

S100 proteins have been observed to serve as a reliable indicator of neuroinflammation or glial activation in several neurodegenerative disorders, such as Parkinson's disease (PD). The notion that S100b may have a role in the development of PD arose from the findings of preclinical investigations, which revealed increased levels of S100B expression in the glial cells of mice exposed to MPTP (Muramatsu, Kurosaki et al. 2003). Further, the clinical findings showed a correlation between neuroinflammation and degeneration, and higher expression of S100b in the substantia nigra of the six patients with PD (Sathe, Maetzler et al. 2012). Studies have also reported a direct relationship exists between the levels of serum S100 and the severity of motor dysfunctions in PD patients (Schaf, Tort et al. 2005). Moreover, five years post-diagnosis, the level of serum auto antibodies against the S100B proteins was found to be almost four times higher in PD patients as compared to the controls (Gruden, Sewell

et al. 2011). Similarly, there is scientific evidence from preclinical studies that demonstrates an elevated level of S100b protein being produced and released in a culture of C6 rat glioma cells that were exposed to MPTP. The elevation in S100 release is associated with heightened glial activation, resulting in decreased neuronal viability (Iuvone, Esposito et al. 2007).

Furthermore, the levels of S100B are elevated in CSF as well as sera, in both chronic active and active demyelinating multiple sclerosis plaques (Petzold, Eikelenboom et al. 2002). Previous studies have demonstrated that when demyelination occurs in *ex vivo* demyelinating models, there is a significant rise of astrocytic S100B; however, when S100B action is inhibited, demyelination is reduced and the expression of inflammatory markers is downregulated (Barateiro, Afonso et al. 2016). A recent study has shown that Pentamidine, an approved antiprotozoal drug, inhibits S100b activity. This leads to improvements in biomolecular and neuropathological parameters, as well as the clinical disease score, in the relapsing-remitting experimental autoimmune encephalomyelitis SJL/J mouse model of MS (Di Sante, Amadio et al. 2020).

Traumatic brain injury is a pathological condition caused by an external force or physical impulse that disrupts brain function and physiology (Timofeev, Santarius et al. 2012). Clinically, the cohort study demonstrated the association between the neurological outcome post-severe head injury and the serum S100b levels (Netto, Conte et al. 2006). Moreover, the sudden increase in the serum S100b followed by brain injury has been reported to be due to the disintegrated blood-brain barrier or the systematic inflammatory reaction in the brain (Netto, Conte et al. 2006). Similarly, rheumatoid arthritis is a complex systemic autoimmune pathology characterized by chronic

inflammation and the inflammation associated with it is believed to be linked with the S100 proteins (Croia, Bursi et al. 2019).

Compelling clinical evidences have highlighted increased levels of calcium-binding proteins i.e., S100 in the cerebrospinal fluid of patients suffering from sciatica pain (Brisby, Olmarker et al. 1999, Skouen, Brisby et al. 1999). In parallel, a recent study also found that S100b was elevated in individuals suffering from trigeminal neuralgia, representing all of the symptoms observed in people suffering from NP (Ito, Seki et al. 2023). Similarly, preclinically, there are reports on enhanced expression of S100b in the SC post-spinal nerve transection with the rodents displaying behavioral hypersensitivity (Tanga, Raghavendra et al. 2006, Chen, Huang et al. 2020). Besides, S100b induces the aberrant release of pro-inflammatory cytokines that are involved in the development and progression of NP phenotypes (Tanga, Raghavendra et al. 2006, Stefani, Leite et al. 2019). Based on the above clinical and preclinical consolidated evidences, S100b is recognized as a pathophysiological marker for persistent pain caused by nervous system damage. However, there is no information on the time-dependent changes in S100b in NP conditions. Hence, the aim of the present study was to evaluate the temporal changes in calcium-binding protein (S100b) level in rats with CCI-induced NP. This analysis will provide valuable insights into the dynamics of disease pathophysiological progression and also will assist in identifying the optimal timing for interventions. In addition, prior research has shown that the presence of Ba^{2+} inhibits the release of S100b from primary astrocyte cultures (Vizuete, Hansen et al. 2019). Consequently, we believe that barium leached from the BaBG has the potential to mitigate the S100-induced neuroinflammation, a crucial factor involved in the persistence of NP.

1.15. Hypothesis

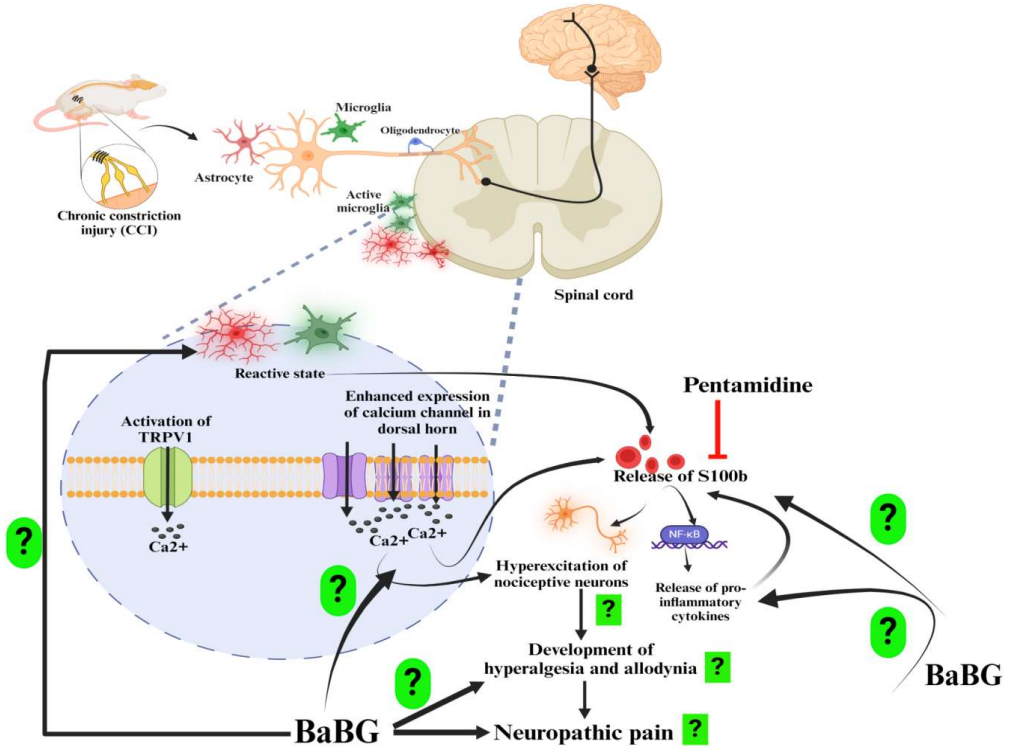

Figure 1.5: The proposed hypothesis of pharmacological evaluation of BaBG in the experimental model of neuropathic pain. Red and Green arrow denotes inhibition and unknown.

The injury to the sciatic nerve (SN) leads to the development of central sensitization due to the upregulation in the expression of calcium channels ($Ca_v2.2$) in the dorsal horn of spinal cord (SC). Besides, there are also enhanced expression of heat-sensing TRPV1 channels in the SN and SC following neuronal injury that leads to the development of thermal hyperalgesia. Upregulation in the expression of these channel results in an increase in the influx of calcium ions leading to hyper excitation of the nociceptive neurons and development of core neuropathic pain (NP) phenotypes i.e., hyperalgesia and allodynia (Kawamata and Omote 1996, Tilley, Cedeño et al. 2022). In addition, the resident immune cells of the central nervous system i.e., glial cells (astrocytes and microglia) get activated post-CCI (chronic constriction injury) leading

to increase in the release of calcium-binding proteins i.e., S100b. Further, S100b gets activated in presence of calcium ions causing release of pro-inflammatory cytokines that further exaggerates the NP conditions (Tanga, Raghavendra et al. 2006, Chen, Huang et al. 2020). However, there is lack of comprehensive knowledge on the temporal changes in the ionic concentration post-injury that causes the development of NP. Therefore, our study's major aim was to evaluate the temporal changes in the intracellular calcium and S100b protein level in the CCI model of NP which will help to identify the pharmacological window of opportunity. CCI model holds clinical relevance and presents face validity as the animal exhibits similar painful and abnormal peripheral neuropathies as observed clinically like the spontaneous pain, hyperalgesia to heat and mechanical stimuli along with the development of allodynia (Bennett and Xie 1988). Further, in terms of the predictive validity, CCI model is preferred widely for drug discovery as the pain symptoms persists for a longer time (Whiteside, Adedoyin et al. 2008). Further, to validate the role of S100b in the pathogenesis and progression of NP in this model, our next objective involved the use of specific S100b inhibitor i.e., pentamidine to access its effect on NP phenotypes. This analysis will offer vital insights into the dynamics of disease pathophysiological progression and also will assist in identifying the optimal timing for interventions.

Inorganic biomaterials like the barium-doped bioactive glass (BaBG) has the tendency to leach the pharmacologiaclly active ions from its framework into the physiological milieu, which impart various biological effects (Majumdar, Gupta et al. 2021). Since, barium has calcium-modulating effects and calcium channels also exhibit greater selectivity for Ba^{2+} ions compared to Ca^{2+} ions (Hagiwara and Byerly 1981). Therefore, we have hypothesized that BaBG has the potential to be used for the treatment of NP that would probably act on the calcium channels and calcium channel blockers (CCBs)

are widely used clinically for the management of NP. Barium released from BaBG may also prevent the activation of calcium-binding protein i.e., S100b, reduce neuroinflammation, and concurrently cause axonal repair and remodeling; hence may have disease-modifying effects. Therefore, the use of BaBG in CCI model of NP would provide new insight into pathophysiology and pharmacotherapy of NP. Hence, our specific objectives are to evaluate the pharmacological effects of BaBG in alleviating the sensory and motor deficits observed in NP condition.

Furthermore, the clinical translation of BaBG necessitates investigating the release kinetic profile and biodistribution of the dopants leached from BaBG. Besides, the understanding of the fate of dopants released after the oral administration is highly important for optimizing the dose regimens therefore; another aim of our study was to determine the *in vivo* pharmacokinetic parameters of the leached ions from BaBG and its biodistribution and excretion profile.

1.16. Objectives:

Objective 1: Synthesis and characterization of barium-doped bioactive glass (BaBG) and its in vitro regenerative potential.

Objective 2: Evaluation of the *in vivo* pharmacokinetics, biodistribution and excretion of dopants released from BaBG after the oral administration.

Objective 3: Acute and sub-acute oral toxicity study of BaBG and 45S5 according to the OECD guidelines.

Objective 4: To evaluate the temporal changes in the intracellular calcium and S100b protein levels in the pathophysiology of the CCI-induced neuropathic pain in rats.

Objective 5: Pharmacological effect of BaBG in the treatment of Neuropathic pain.