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PHARMACOLOGICAL CHARACTERIZATION OF NOVEL DRUG CANDIDATES IN STREPTOZOTOCIN-INDUCED DIABETIC NEUROPATHIC RATS

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ABSTRACT

Objective: The goal was to test the efficacy of NCE-2 and NCE-3, substituted synthesized imidazoles N-type calcium channel blockers, in a rat model of streptozotocin (STZ) induced neuropathic pain.

Method: NCE-2 and NCE-3 were evaluated for streptozotocin (STZ)- induced diabetic neuropathy for treatment of neuropathic pain. Diabetes mellitus has been induced in male Sprague Dawley rats by injecting STZ at a dose of 45 mg/kg intravenously into the tail vein. In diabetic neuropathic rats, NCE-2 and NCE-3 were dosed orally at 10 and 30 mg/kg. Mechanical allodynia and cold allodynia have been analysed using increasing weight von Frey filaments and acetone, with behaviour monitored for one minute.

Results: Overall, the findings show that the NCE-2 and the NCE-3 can be a promising option for additional studies on the neuropathic pain mitigation mechanism. With regard to both mechanical and cold allodynia, paw withdrawal threshold (PWT) and MPE % (Mechanical Allodynia), paw withdrawal latency (PWL) and % MPE (Cold Allodynia) were determined. In this Study sub-acute therapy of NCE-2 at dose 10 and 30 mg/kg exhibited 19.5% and 44.7% reversal of neuropathic pain while with treatment of NCE-3 at dose 10 and 30 mg/kg exhibited 25.7% and 61.0% reversal of mechanical allodynia a symptom of neuropathic pain. In Cold allodynia study, NCE-2 at dose 10 and 30 mg/kg exhibited 23.4 and 43.7% reversal of neuropathic pain while with treatment of NCE-3 at

dose 10 and 30 mg/kg exhibited 36.4% and 65.3% reversal neuropathic pain. NCE-2 and NCE-3 demonstrated dose dependent efficacy in reversing mechanical and cold allodynia.

Keywords: NCE-2, NCE-3, Mechanical Allodynia, Cold allodynia

INTRODUCTION

Diabetic neuropathy is characterised by a decrease of nerve conduction velocity, increased discomfort, sensory loss, and nerve fibre degradation, and is one of the most common consequences of diabetes mellitus. Neuropathic pain is a neurological condition characterised by hyperalgesia and allodynia. It is one of the most debilitating symptoms of diabetes mellitus. Toxic and non-noxious stimuli elicit spontaneous and pathologically excessive responses in neuropathic pain **[1]**. Synaptic facilitation, which contributes to secondary hypersensitivity and tactile allodynia, is one of the underlying mechanisms implicated in neuropathic pain **[2]**. Because the pathophysiology of neuropathic pain is complicated and multifaceted, existing therapies are ineffective and frequently affect glucose homeostasis. For the treatment of patients with painful diabetic neuropathy (PDN), several kinds of analgesic drugs have been utilized, and a trial-and-error strategy including a range of medications is frequently used until an acceptable regimen is developed. Antiepileptics like gabapentin and pregabalin, as well as antidepressants like duloxetine and amitriptyline, are commonly used such as first treatments for PDN. Due to the increasing probability of diabetic neuropathy as the length of the diabetic state is prolonged, changes to the etiological and pathophysiological mechanisms behind pain production which occur during disease can influence the effectivity of analgesic agents. Currently neuropathic pain pharmaceuticals are generally limited to the "off-label" use of approved medications, especially anti-convulsants and tricyclic antidepressants, in other disorders. The treatment of PDN is still hard, given to the limited efficacy and low response rate of the existing analgesic medicines, despite the existence of viable pharmacological treatments. N – the type of voltage-based calcium channels distributed on central and peripheral nerve neck endings have been shown to mediate the development and maintenance of neuronal pain sensitization processes along with pathological cerebral ischemic processes and thus to provide attractive objectives for the development of pain-dependent analgesic medicines. As a result, considerable attempts have been made thus far to produce systemically accessible small-molecule N-type calcium channel blockers as a viable contender for the next generation of neuropathic pain therapies.

This research focuses on the most recent developments in the field of small-molecule N-Type calcium channel blockers. As a result, a study was designed to look at the issue of diabetic neuropathic pain in animal models. A rodent model of streptozotocin (STZ)-induced diabetes has been often utilized for sensory behavioral testing in experimental animal studies of diabetic neuropathy. Degeneration of sensory neurons and temporal variations in aberrant sensory input are caused by prolonged hyperglycemia, hypoinsul-inemia, and improper glucose metabolism **[3]**. Hyperalgesic sensitivity and tactile allodynia to the mechanical and thermal stimuli occur in the animal paws. Many analgesic medications, including as Mexiletine and gabapentin, have been reported in the early stage of STZ-induced diabetes **[4]** to demonstrate antiallodynic effects. This study was designed to characterize the involvement of NCE-2 and NCE-3 in against STZ induced diabetic neuropathy.

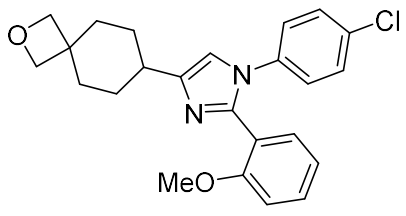
MATERIALS & METHODS

Subjects

Sprague-Dawley male adult rats (210-250 g) were housed in groups for 12:12 h dark-light cycle at controlled room temperature (25 ±2 °C) (lights on at 07:30 h-19:30 h). Water and food were accessible ad libitum. The Institutional Animal Ethics Committee (IAEC) approved all experimental treatments used in this research, and they have been performed with the permission of the Ministry of Environment and Forests, Government of India and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

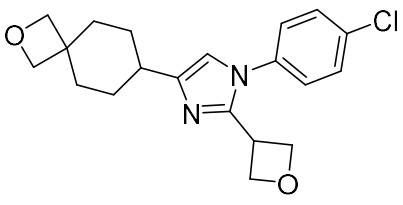
Drugs

NCE-2 and NCE-3 were synthesized at Onium Life Sciences Private Limited, Plot no. 6, KSIDC Industrial Estate, Veera Pura post Doddaballapura, Bangalore. STZ was purchased from Sigma Chemical. Gabapentin was purchased from fluorochem.



1-(4-chlorophenyl)-2-(2-methoxyphenyl)-4-(2-oxaspiro[3.5]nonan-7-yl)-1H-Imidazole

Figure 1 a: Structure of NCE-2



1-(4-chlorophenyl)-2-(2-methoxyphenyl)-4-(2-oxaspiro[3.5]nonan-7-yl)-1H-imidazole

Figure 1 b: Structure of NCE-3

Experimental design

The rats have been randomized into 7 groups, each with six individuals, with the exception of the Nave group, which had three animals. Group 1: Normal control animals given vehicle (0.5 percent Methyl cellulose); Group 2: Diabetic control animals given STZ dissolved in pH 4.5 citrate buffer; Group 3: Diabetic animals given NCE-2 (10 mg/kg, p.o); Group 4: Diabetic animals given NCE-2 (30 mg/kg, p.o); Group 5: Diabetic animals given NCE-3 (10 mg/kg, p.o); Group 6: Diabetic animals were treated with NCE-3 (30 mg/kg, p.o.) and Group 7: diabetic animals were treated with Gabapentin (100 mg/kg, i.p.). Day 0 was designated as the day of diabetes induction. From Day 28 of STZ induction, NCE-2 and NCE-3 sub-acute treatment was administered as a single dose, and pain parameters were assessed. For seven days, sub-acute treatment was administered.

Induction of experimental diabetes

Diabetes mellitus was produced in a dose of 45 mg/kg and a dose volume of 2 mL/kg by a single intravenous STZ injector into the dorsal tail vein (Day-0). Blood glucose was estimated by using a glucometer (Accu-check, Roche). Diabetes was considered and included in the study animals with blood glucose levels over 250 mg/dL. Animals were allowed to develop neuropathic pain for 21 days after induction of diabetes mellitus. On 22nd day, the symptoms of mechanical allodynia and cold allodynia were evaluated for animal development. When mechanical allodynia (i.e. paw withdrawal or a flinching behaviour in response to a bending force less than 4 g) and cold Allodynia exhibited in the same case (i.e., paw withdrawal latency response was observed at a paw withdrawal or flinching behaviour of less than or equal to 70 g), animals were regarded as neuropathic. Post evaluation of symptoms animals were randomized into different treatment groups.

Evaluation of mechanical and cold allodynia

Before beginning the tests, rats were kept in a wire grid floor acrylic cages and left to sit 20 to 30 minutes in a quiet environment. The animals were quiet prior to paw stimulation, but not lying on their paws, and had stopped exploring their cages or marking them by defecating or urinating. Von Frey filament (Bioseb), which consisted of series of von Frey filaments (0.4, 0.7, 0.16, 0.40, 0.60, 1.0, 1.4, 2.0, 4.0, 6.0, 8.0, 10, 15, 26 and 60 g) used to determine the lowest mechanical threshold, which evokes a brisk paw withdrawal reflex in the rat hindpaws. Commencing with 2g filament for the rat, the filament will be applied to the plantar surface of the hindpaw, until the filament buckles slightly. Absence of withdrawal response,

upto 3 seconds after application of the filaments, will be considered unresponsive, and will prompt use of next filament of increasing force. Conversely, a hindpaw withdrawal response within 3 sec will prompt use of next filament of decreasing force. In rats, a score of 15 g will be given to the animals that do not respond to any of the Vonfrey filament (0.4 – 15 g). During the selection phase, the rats are considered to be allodynic, if their PWT ≤ 4 g.

Statistical analysis

The results have been presented as a mean ± standard deviation. The PWT (both mechanical and cold allodynia) differences were determined by a two-way variance analysis and the post-hoc test Bonferroni. Statistically significant were the differences in *p<0.05, **p<0.01, ***p<0.001]

RESULT

Time course for development of mechanical allodynia, plasma glucose levels, and body weights in STZ-induced diabetic rats

The rapid increase in the mean plasma glucose levels to >500mg/dL induced by intravenous injections of 45 mg/kg STZ, which lasted up to four weeks; **(Figure 2A)**. During observation, the body weight of rats treated with STZ remained nearly steady, with non-STZ control rats increasing dramatically in body weights **(Figure 2B)**.

Effect of sub-acute treatment of NCE-2 and NCE-3 on Mechanical allodynia in STZ induced diabetic rats:

The cut-off PWT for all animals was 15 g. PWT was not significantly altered in normal control (Nave) rats. PWT decreased from 15 g (naive) to 2 g in diabetic control rats (pre-dose). One-way ANOVA followed by post hoc Dunnett's test revealed that oral administration N-Type calcium channel blockers, NCE-2 [F (4, 29) =132, P<0.001] as well as NCE-3 [F (4, 29) =117, P<0.001] produced significant improvement in neuropathic pain as evidenced by increase in paw withdrawal threshold as compared to diabetic control. In NCE-2 (10 mg/kg) treatment group, naive and diabetic control post-dose PWT were found to be 4.36 ± 0.81, 14.2 ± 0.75 and 1.99 ± 0.59 respectively. In NCE-2 (30 mg/kg) treatment group, naive and diabetic control post-dose PWT were found to be 7.43 ± 1.22, 14.2 ± 0.75 and 1.99 ± 0.59 respectively. Gabapentin (100 mg/kg) as a standard control group post-dose PWT were found to be 12.0 ± 1.69. Whereas, In NCE-3 (10 mg/kg) treatment group, naive and diabetic control PWT post-dose were found to be 5.12 ± 0.91, 14.2 ± 0.75 and 1.99 ± 0.59 respectively. In NCE-3 (30 mg/kg) treatment group, naive and diabetic control post-dose PWT were found to be 9.42 ± 1.33, 14.2 ± 0.75 and 1.99 ± 0.59 respectively. The effect

observed with NCE-2 and NCE-3 in reversing mechanical allodynia values were statistically significant ($p < 0.001$) post-dose. The effect was dose dependent, with Gabapentin (100 mg/kg, i.p.) showing a significant ($p < 0.001$) reversal of PWT post-treatment as compared to Diabetic control PWT. Similarly, the %MPE (log) for mechanical allodynia was 39.3 ± 8.3 and 65.3 ± 7.8 at 10 and 30 mg/kg of NCE-2 respectively, whereas for NCE-3 %MPE (log) for mechanical allodynia was 47.1 ± 8.6 and 77.0 ± 6.9 at 10 and 30 mg/kg **(Figure 3a, 3b)**.

Effect of sub-acute treatment of NCE-2 and NCE-3 on Cold allodynia in STZ induced diabetic rats:

PWL cutoff was 60 seconds in all animals. PWL was not significantly affected in normal control (Nave) rats. PWL decreased from 60 g (naive) to 7 sec in diabetic control rats (pre-dose. One-way ANOVA followed by post hoc Dunnett's test revealed that oral administration N-Type calcium channel blockers, NCE-2 [F (4, 29) =183, P<0.001] as well as NCE-3 [F (4, 29) =117, P<0.001] produced significant improvement in neuropathic pain as evidenced by increase in paw withdrawal latency as compared to diabetic control. In NCE-2 (10 mg/kg) treatment group, naive and diabetic control post-dose PWL were found to be 19.3 ± 3.39, 57.1 ± 4.74 and 7.77 ± 2.03 respectively. In NCE-2 (30 mg/kg) treatment group, naive and diabetic control post-dose PWL were found to be 29.3 ± 3.88, 57.1 ± 4.74 and 7.77 ± 2.03 respectively. Gabapentin as a standard control group post-dose PWL were found to be 51.9 ± 4.42. Whereas, In NCE-3 (10 mg/kg) treatment group, naive and diabetic control post-dose PWL were found to be 19.3 ± 3.4, 57.1 ± 4.74 and 7.77 ± 2.03 respectively. In NCE-3 (30 mg/kg) treatment group, naive and diabetic control post-dose PWL were found to be 40.0 ± 7.10, 57.1 ± 4.74 and 7.77 ± 2.03 respectively. The effect observed with NCE-2 and NCE-3 in reversing Cold allodynia values were statistically significant ($p < 0.001$) post-dose. The effect was dose dependent, with Gabapentin (100 mg/kg, i.p.) showing a significant (p<0.001) reversal of PWL post-treatment when compared to diabetic control PWL. Similarly, the %MPE (log) for cold allodynia was 44.8 ± 8.3 and 65.2 ± 6.5 at 10 and 30 mg/kg of NCE-2, respectively whereas %MPE (log) for mechanical allodynia was 57.6 ± 14.4 and 79.8 ± 9.2 at 10 and 30 mg/kg of NCE-3 **(Figure 4a, 4b)**.

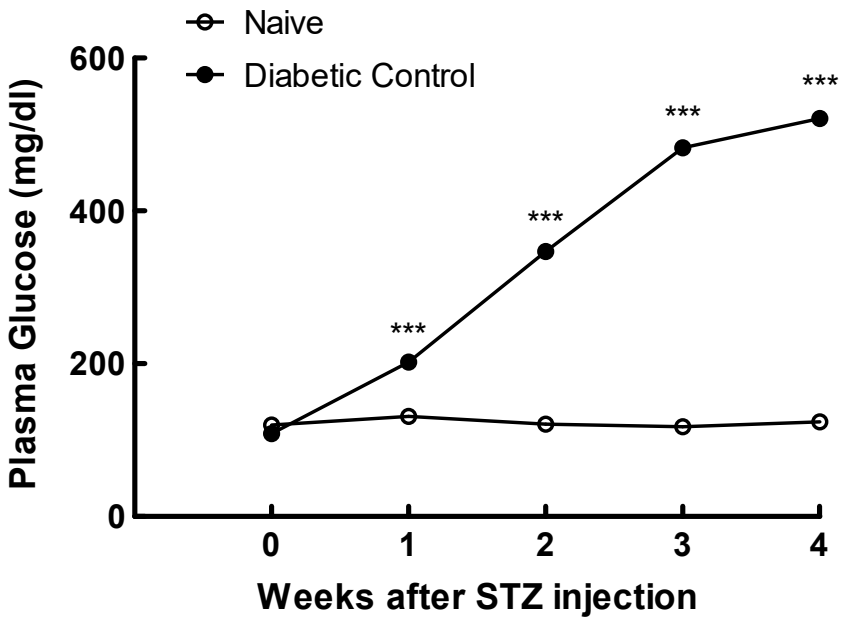


Figure 2a: Time course of Plasma Glucose Level Changes After STZ Injection

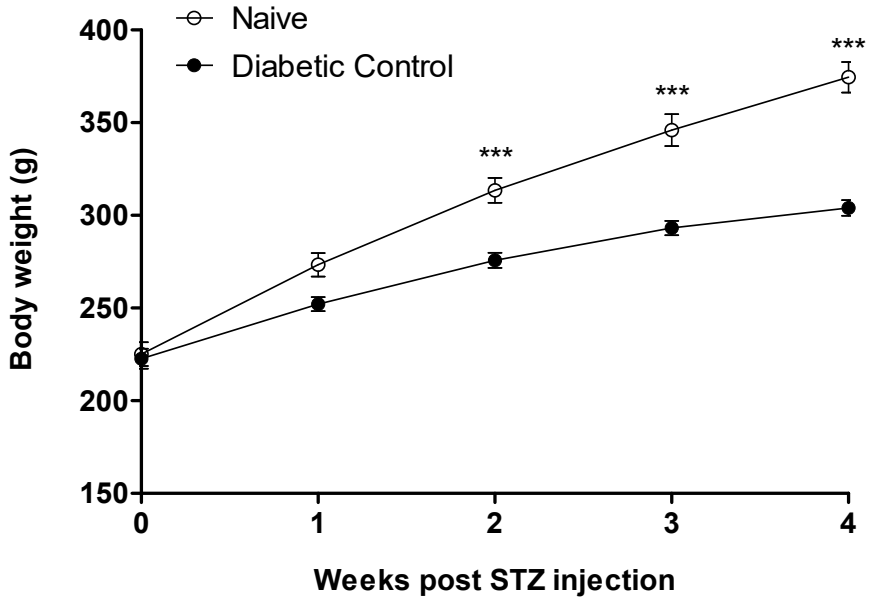


Figure 2b: Time course of Body Weight Changes After STZ Injection

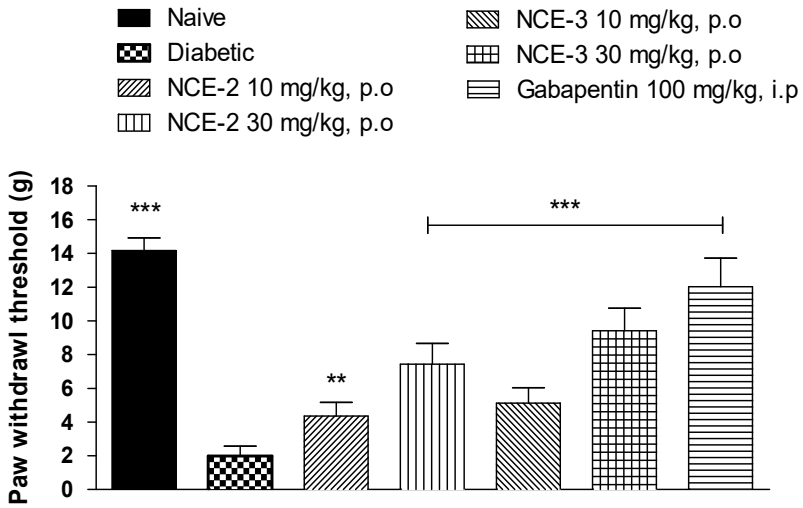


Figure 3a: Effect of sub-acute treatment of NCE-2 and NCE-3 on Mechanical allodynia in STZ induced Neuropathic Rats

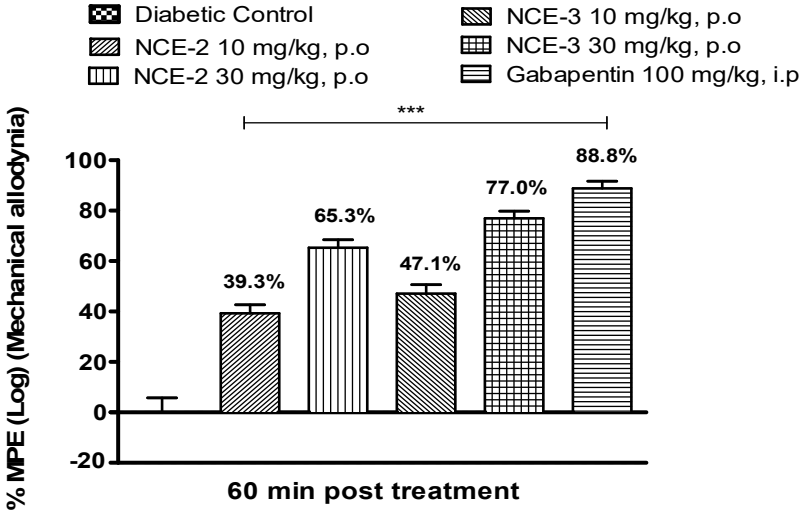


Figure 3b: Effect of sub-acute treatment of NCE-2 and NCE-3 on Mechanical allodynia in STZ induced Neuropathic Rats

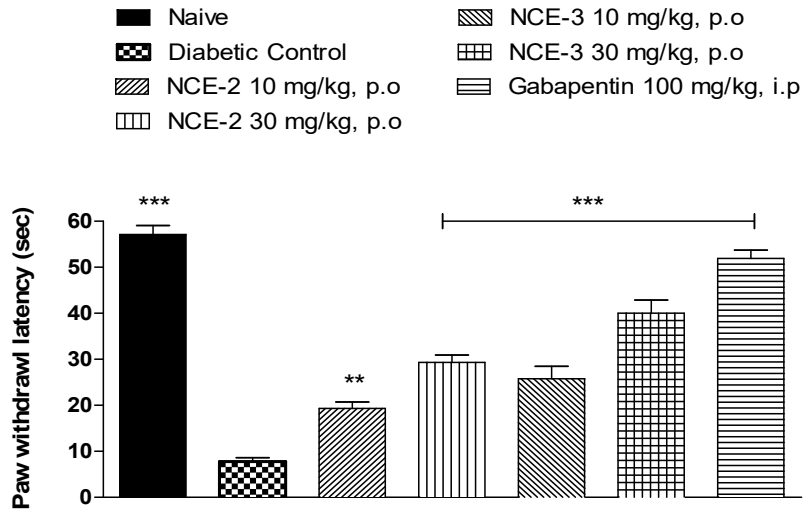


Figure 4a: Effect of sub-acute treatment of NCE-2 and NCE-3 on Cold allodynia in STZ induced diabetic rats

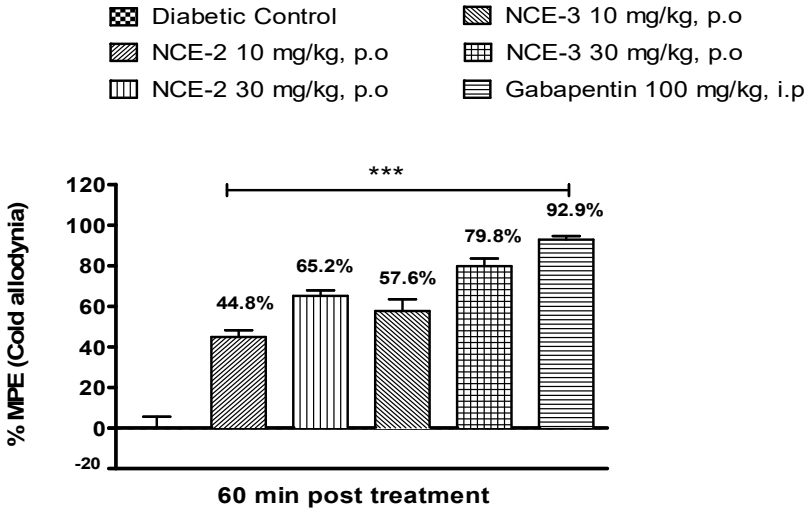


Figure 4b: Effect of sub-acute treatment of NCE-2 and NCE-3 on Cold allodynia in STZ induced diabetic rats

DISCUSSION

Several processes, including metabolic alterations and neurovascular dysfunction, are thought to be implicated in the aetiology of diabetic neuropathy **[5]**. However, the precise mechanism by which these metabolic and vascular aberrations induce nerve damage is still unknown **[6]**. Disrupted neuronal (Ca2 +) homeostasis was proposed to be involved in diabetic neuropathy aetiology. Abnormal [Ca2+] regulation and calcium channel activity have been discovered in primary and secondary sensory neurons, and have been connected to the pathogenesis of diabetic neuropathic impairments **[7]**. Diabetic mice and rats' dorsal root ganglion and dorsal horn neurons have been shown to have

enhanced voltage-dependent calcium currents via N-type channels **[8]**. We confirm that Ca-channels are essential cellular targets for drug development in PDN in this work. We have shown that pharmacological blockage of Ca-channels has considerable therapeutic benefits on the most crucial and difficult-to-treat indications of PDN – mechanical and cold sensitivity – using our NCES. allodynia. Our compounds NCE-2 and NCE-3 potently inhibited activation of Ca-type channels showing IC50 values 17.1 and 14.7 nM, with good plasma exposure AUC (0–24 h) 7471 ng·h/mL and AUC (0–24 h) 12062 ng·h/mL in the in vivo pharmacokinetic study. Chronic treatment is effective the normalization of impaired calcium homeostasis and showed the comparable analgesic responses to gabapentin for neuropathic pain in STZ model **[9]**, implicating that NCE-2 and NCE-3 potent calcium channel inhibitors of the N type calcium channel can be developed as attractive options for treating neuropathic pain diabetes-induced cold allodynia and mechanical allodynia to noxious stimuli responses **[10]**. Our research results clearly indicate that Ca channels in sensory neurons are an important target for NCE-2 and NCE-3 potent analgesic effects. However, the possibility of other ion channels contributing to its analgesic effects cannot be ruled out. Experiments and clinical trials may also lay the foundation for the pharmacological development of new and more targeted medicines that target ion channels in peripheral nociceptors. This method could help diabetic neuropathy patients manage their discomfort while limiting unwanted effects.

CONCLUSION

These finding suggest that NCE-2 and NCE-3 block N-type calcium channels and decrease depolarization-induced rise of intracellular Ca2 +concentration in a dose-dependent manner with a reasonable in vivo pharmacokinetic profile. However, more research into the beneficial usage of new drugs in animal models of pain and neurodegeneration.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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