

Original Research

Metformin Alleviates Pain States by Regulating the Balance of Spinal Synaptic Transmission

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Abstract

Background: Metformin has been shown to have potent analgesic effects; however, the underlying mechanism of synaptic plasticity mediating analgesia remained ambiguous. **Methods:** In this study, animal behavioral tests, whole-cell patch-clamp recording, immunofluorescence staining, and network pharmacology techniques were applied to elucidate the mechanisms and potential targets of metformin-induced analgesia. **Results:** Single or consecutive injections of metformin significantly inhibited spinal nerve ligation (SNL)-induced neuropathic pain, and formalin-induced acute inflammatory pain. Network pharmacology analysis of metformin action targets in pain database-related targets revealed 25 targets, including five hub targets (nitric oxide synthase 1 (NOS1), NOS2, NOS3, epidermal growth factor receptor (EGFR), and plasminogen (PLG)). Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis demonstrated that metformin-induced analgesia was markedly correlated with calcium signaling and synaptic transmission. Intrathecal injection of metformin significantly reversed nerve injury-induced c-Fos (neural activity biomarker) mRNA and protein expression in neuropathic rats by regulating NOS2 expression. In addition, whole-cell recordings of isolated spinal neurons demonstrated that metformin dose-dependently inhibited the enhanced frequency and amplitude of miniature excitatory synaptic currents (mEPSCs) but did not affect those of miniature inhibitory synaptic currents (mIPSCs) in neuropathic pain. **Conclusions:** This study further demonstrated that metformin might inhibit spinal glutamatergic transmission and abnormal nociceptive circuit transduction by monitoring synaptic transmission in pain. Results of this work provide an in-depth understanding of metformin analgesia via synaptic plasticity.

Keywords: pain; metformin; mEPSCs; c-Fos; glutamatergic transmission

1. Introduction

Pain is characterized by central and peripheral inflammation [1,2]. Inflammation causes the release of inflammatory mediators, such as microglia-derived bradykinin, chemokines, nerve growth factor, pro-inflammatory cytokines, and prostaglandins, which might promote pain sensations by activating and sensitizing receptors on the terminal cell bodies of nociceptors, including G-protein-coupled receptors, ionotropic receptors, and tyrosine kinase receptors [2–4]. Inflammatory factors invade multiple ion channels in the presynaptic membrane, resulting in abnormal nociceptive circuits; animals exhibit an overreaction to external stimuli and hypersensitivity of neural circuits to nociceptive signals, leading to peripheral and central sensitization [5]. A series of calcium channel has been thoroughly investigated, including transient receptor potential cation channel subfamily V member 1 (TRPV1), T-type calcium, and transient receptor potential cation channel subfamily A member 1 (TRPA1) [6,7]. The occurrence of pain is a

consequence of neuronal adaptability in pain-coding pathways and circuits. Typically, the pain sensory balance is regulated by a delicate equilibrium between excitation and inhibition [8,9]. Alterations in the pain circuits have been linked to the severity and persistence of inflammatory and neuropathic pain [10]. Current mainstream analgesic drugs, such as morphine, exert inhibitory effects by agonizing μ opioid receptor (μ -OR), resulting in reduced neuronal excitability and the inability to switch to nociceptive signals for analgesia and sedation [11]. Notably, the noxious circuitry exhibits a dynamic increase in excitability that involves the activation of chemical mediators, such as excitatory amino acids signals. This process also recruits intracellular signaling transduction cascades and induces long-lasting changes in synaptic transmission [12]. Thus, synaptic and circuit changes are the central mechanism of pharmacological analgesia.

Metformin (biguanide) is a disease-modifying drug indicated for type 2 diabetes. Recently, pre-clinical stud-



ies have shown that metformin has antinociceptive effects and alleviates the pathological pain response via regulation of the Adenosine monophosphate-activated protein kinase (AMPK) signaling pathway in the dorsal root ganglion (DRG) of diabetic rats - mediated by the phosphorylation of nuclear factor-kappaB (NF- κ B) [13]. Moreover, metformin regulates AMPK α 2-mediated autophagy in the spinal dorsal horn and relieves sensations [14]. In addition, metformin reduces monosodium iodoacetate-induced inflammatory pathology by regulating the local inflammatory response and the autophagy-lysosomal pathway, which shows antinociceptive and cartilage-protective properties [15]. Intracellular calcium ions are essential for initiating injury and related signaling molecule transduction, and metformin has a protective effect in neurological disorders. Metformin may act through ATP-activated potassium channels, tumor necrosis factor- α (TNF- α), and NEDD4-2 to mediate inflammation and pain [16,17]. Altered biological and molecular pathways in pain contribute to hyperalgesia. Metformin treatment significantly modulates the frequency of miniature excitatory synaptic currents (mEPSCs) and enhances presynaptic glutamate release by stimulating paired-pulse ratios (PPR) in hippocampus neurons rather than gamma-aminobutyric acid (GABA) release [18]. Cyclin-dependent kinase 5 (Cdk5) is predominantly active in nociceptive neurons, and metformin reverses cellular Cdk5-potentiated calcium signaling, phosphorylation of p35, and long-term potentiation (LTP) in neuronal pathogenesis [19]. Metformin has been extensively demonstrated to be involved in synaptic transmission and circuits, but its underlying transmission target in pain states is unknown.

Therefore, the synaptic transmission of metformin may be a crucial or dominant factor for pain relief. In our study, intrathecal or intraperitoneal metformin produced potent analgesic phenotypes in rats with neuropathic- and formalin-induced pain. Network pharmacology hinted that the potential analgesic targets of metformin were related to synaptic transmission. In addition, immunofluorescence staining of the spinal cord indicated that metformin significantly reduced the expression of c-Fos following spinal nerve ligation (SNL). Thus, we found that the underlying synaptic plasticity mechanism of metformin in neuropathic pain was mediated by restoring NOS2 and c-Fos expression, subsequently inhibiting spinal glutamatergic transmission.

2. Materials and Methods

2.1 Animal Models and Treatments

Adult male Wistar rats, weighing 120–150 g, were provided by SPF (Beijing, China) Biotechnology Co., Ltd. All experimental procedures were approved by the National Institutes of Health and the Center for Laboratory Animals of Shanghai Mental Health Center and performed according to the guidelines of the National Research Council for the care and use of laboratory animals. The ani-

mals were kept in specific pathogen-free (SPF) conditions in a 12 h/12-h light/dark cycle at 22 °C–24 °C and 50%–60% relative humidity. All rats had free access to food and water. Every effort was made to reduce suffering and the number of rats used. Metformin (CAS:657-24-9, Mecklin, Shanghai, China) was dissolved in artificial cerebrospinal fluid (ACSF; 1.2 mM MgCl₂, 234 mM sucrose, 25 mM NaHCO₃, 12 mM glucose, 1.2 mM NaH₂PO₄, 2.5 mM CaCl₂, and 3.6 mM KCl) as a stock solution of 100 μ M. Rats were randomly divided into six groups to assign to different treatments - the sham group, sham–30 μ g of metformin group, sham–100 μ g of metformin group, SNL group, SNL–30 μ g of metformin group, or SNL–100 μ g of metformin group.

2.2 Intrathecal Catheterization and Injection in Rats

For intrathecal catheterization, polyethylene intrathecal catheters (PE-10: OD, 0.61 mm; ID, 0.28 mm, Advanced Biotechnologies Inc, South Dennis, MA, USA) were implanted into rats after inhalation of isoflurane anesthesia (R510-22, RWD, Shenzhen, Guangdong, China, 4%–5% induction, 1.5% maintenance). The catheter was inserted near the L5 spinal segmental level into the L5/L6 intervertebral subarachnoid space. Rats recovering from surgery were housed in individual cages for 14 days. Those that exhibited immediate bilateral hind-limb paralysis were chosen for the study following 10 μ L of 4% lidocaine (CAS:137-58-6, Sigma, St Louis, MO, USA) injections. For intrathecal metformin administration, 10 μ L of the metformin solution was injected slowly and then flushed with 15 μ L of ACSF.

2.3 Rat Model of Neuropathic Pain and Assessment of Mechanical Allodynia

The SNL model was established based on Kim and Chung for neuropathic rats [20]. In brief, the rat was deeply anesthetized by inhalation. The L5 and L6 spinal nerves were isolated carefully and ligatured tightly with 4-0 silk thread. The wound was sutured after complete hemostasis was confirmed. The procedure for the sham group was identical to that of the SNL group, but ligation was not performed. Rats for subsequent experimental use were screened based on obvious unilateral allodynia in response to mechanical stimuli and the absence of motor deficits.

Rats were exposed to the testing environment for at least 30 min. Rats were placed in a plexiglass box on a metal grid to assess mechanical allodynia. The behavior test was carried out by using a 2290 CE electrical von Frey hair (IITC Life Science, Woodland Hills, CA, USA), ranging from 0.1 g to 90 g. The experimenter was blinded to the different treatment groups. The paw withdrawal thresholds (PWT) were defined as the force value when the rat's contralateral or ipsilateral hind limb suddenly withdrew after being subjected to an increased upward force. The threshold was calculated by averaging the lowest force that evoked a withdrawal response over a 1 min interval [21,22].

For thermal pain hypersensitivity tests, rats were kept in the testing apparatus for 30 min on a 30 °C heated glass floor. Thermal withdrawal latency was measured by a planar test analgesic meter (II-390G, IITC Life Science, Woodland Hills, CA, USA). Each rat was tested over 3 sessions, each 5 min in length. To stimulate skin sensitization and tissue damage, the cutoff time was confined to 30 s. The average paw withdrawal latency (PWL) of the three tests was used for data analysis.

2.4 Rat Model of Formalin-Induced Pain and Assessment

Before testing, rats were placed in the plexiglass chamber for 30 min to acclimatize to the experimental environment. Rats were gently restrained and injected subcutaneously with 50 μ L of 5% formalin (221008, Yulu, Shangrao, Jiangxi, China) diluted in normal saline in the left hind paw. Rats were then immediately placed into the chamber and nociceptive behavior was observed with a mirror embedded in the rear part of the cage for better monitoring of animal behavior. Formalin-induced flinches were considered pain behavior and manually quantified by the experimenter for 1 min at 10 min intervals until 90 min; the experimenter was blind to the treatments [23]. Formalin-induced flinching was biphasic: phase I was mediated by noxious chemical stimulation immediately after formalin injection and lasted from 0–10 min, whereas phase II was mediated by a combination of peripheral inputs and formalin-induced central sensitization and began at 10 min and lasted until 90 min after formalin injection [24–26].

2.5 Ex Vivo Whole-Cell Patch-Clamp Recording

After the mechanical allodynia behavioral test, an electrophysiological test was performed on spinal cord slices obtained from rats at 14 days post surgery. In brief, the rats were deeply anesthetized via isoflurane inhalation. The lumbar enlargements of L3–L5 were quickly separated and immersed for 90 s in cold high-sucrose ACSF with a mixture of 95% O₂ and 5% CO₂ containing (in mM) 1.2 mM MgCl₂, 234 mM sucrose, 25 mM NaHCO₃, 12 mM glucose, 1.2 mM NaH₂PO₄, 2.5 mM CaCl₂, and 3.6 mM KCl. Thereafter, 300 μ m-thick slices were transversely sectioned using a vibratome (VT1200 S, Leica, Wetzlar, Germany). The spinal cord sections were incubated in ACSF containing (in mM) 1.25 mM NaH₂PO₄, 125 mM NaCl, 26 mM NaHCO₃, 3 mM KCl, 1 mM MgCl₂, 10 mM D-glucose, and 2 mM CaCl₂. The pH was adjusted to 7.3, and the fluid was filled with a mixture of 95% O₂ and 5% CO₂ for 30 min at 32 °C prior to electrophysiology recordings.

To record glutamatergic transmission, a bath application of 0.5 μ M tetrodotoxin (TTX; Aladdin, Shanghai, China), 1 μ M strychnine (Sigma, St Louis, MO, USA), and 100 μ M PTX (Sigma, St Louis, MO, USA) was used to inhibit sodium channels, glycine, and GABA receptors. The recording was conducted using a borosilicate glass recording electrode (BF150-86-10, Sutter, Sacramento,

CA, USA), which was filled with a K⁺-based internal solution (composition in mM: 10 HEPES, 130 K-gluconate, 2 NaCl, 2 MgCl₂, 5 KCl, 1.1 EGTA, 5 QX314, 10 sodium phosphocreatine, 4 Na₂-ATP, 1 MgSO₄, 0.4 Na-GTP; the pH was adjusted to pH 7.2 with KOH). To record the inhibitory transmission, the electrode was filled with a high-Cl solution (Formula: 10 mM HEPES, 1.1 mM EGTA, 2 mM MgCl₂, 140 mM CsCl, 0.3 mM Tris-guanosine triphosphate and 3 mM MgATP; the pH was adjusted to pH 7.4 with CsOH), and was clamped at –70 mV during bath application of TTX (0.5 μ M), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Sigma, St Louis, MO, USA) (20 μ M), and D-AP5 (50 μ M) to block kainate, N-methyl-D-aspartate receptor (NMDAR) activity, and amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPA), respectively.

2.6 Immunofluorescence Staining

Isoflurane inhalation was used to induce deep anesthesia. About 100 mL of normal saline was perfused intracardially, followed by 100 mL of 4% paraformaldehyde (PFA) (w/v) (BL539A, Biosharp, Hefei, Anhui, China) in phosphate-buffered saline (PBS, pH 7.4). Spinal lumbar enlargements were immediately removed and post-fixed for 12 h in the same fixative at 4 °C before being dehydrated in sucrose solutions dissolved in PBS, with concentrations ranging from 10% to 30%. Tissue was mounted in an optimal cutting temperature (OCT) compound and sliced into 30 μ m frozen sections using a Leica Microsystems (CM1950, Leica, Wetzlar, Germany). The frozen sections were incubated in 10% goat serum (v/v) (Sigma, St Louis, MO, USA) and 0.5% Triton X-100 (v/v) (CAS: 9002-93-1, Sangon, Shanghai, China) in PBS for 1 h at room temperature before being incubated with the rabbit anti-c-Fos (1:1000; 226008, SYSY, Gottingen, Germany) and mouse anti-NeuN antibodies (1:300; ab128886, Abcam, Boston, MA, USA) for an additional 24 hours at 4 °C. The sections were washed three times in PBST for 5 min each and then incubated with the appropriate secondary antibodies (conjugated with Alexa Fluor 488 or 594; 1:1000; A-10631, A-21205, Invitrogen, Carlsbad, CA, USA) for 2 h at room temperature. Finally, the sections were mounted on slides before being dried, cleared, and cover slipped. Immunofluorescence images were captured with a confocal scanning laser microscope (Fluo View FV1000, Olympus, Tokyo, Japan).

2.7 Western Blot

Spinal cord tissue was extracted and homogenized with a Radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) to determine the expression level of pain-related proteins. Protein concentrations were measured with a BCA protein detection kit (Beyotime, Shanghai, China). The proteins were separated on 8–20% Precast-Gels (Beyotime, Shanghai, China), followed by electro-transfer to polyvinylidene difluoride

membrane (PVDF) (Sigma, St Louis, MO, USA) membranes. The membranes were then blocked with 5% skim milk for 1 h at room temperature. After being blocked, the membranes were incubated with primary antibodies for c-Fos (1:1000, ab190289, Abcam, Cambridge, UK) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1000, Cat No. 60004-1-Ig, Proteintech, Chicago, IL, USA) at 4 °C overnight. Subsequently, they were incubated with corresponding HRP-conjugated secondary antibody (1:1000, 31402, ThermoFisher, Waltham, MA, USA) for 1 h at room temperature. Proteins were detected and quantified using the iBright FL1000 Imaging System (Invitrogen, Carlsbad, CA, USA).

2.8 RNA Isolation and qPCR

The lumbar enlargements of the spinal dorsal were quickly separated for RNA extraction after rats were sacrificed by decapitation. Total RNA was extracted with Trizol reagent (9108, Takara, Shiga, Japan) and converted into cDNA using the PrimeScript RT reagent kit (RR036A, Takara, Shiga, Japan). The specific primer sequences used for cDNA amplification were as follows: NOS1, forward 5'-ATGGAAGAGAACACGTTTGGGGTTC-3' and reverse 5'-CTCCCACTTTGCGTTTGAAGA-3', NOS2, forward 5'-ATGGCTTGCCCCTGGAAGTTTCTCT-3', reverse 5'-CTCCACGTTGTTGTTAATGTCCT-3', c-Fos, forward 5'-AGGCAGAGCTGGAGTCGGAGAT-3', reverse 5'-GCCGAGGACTTGAACCTCACTCG-3', GAPDH, forward 5'-CCAAGGTCATCCATGACGAC-3', reverse 5'-TCCACAGTCTTCTGAGTGGC-3'. qPCR analysis was performed by SYBR-Green Supermix and a LightCycler 480 system (04887352001, Roche Molecular Diagnostics, Alameda, CA, USA). The ratio of mRNA expression relative to GAPDH was determined by the $2^{-\Delta\Delta CT}$ method.

2.9 Network Pharmacology Analysis

First, we searched for potential targets of metformin. The PubChem database (<https://pubchem.ncbi.nlm.nih.gov>) contains chemical information such as molecular formula, chemical structure, biological activities, and other identifiers. The SwissTargetPrediction database (<http://www.swisstargetprediction.ch>) was employed to predict the most possible protein targets of small molecules. We uploaded the structure of metformin from PubChem to acquire its putative targets. Second, we searched the potential pathogenic targets on the GeneCards database (<https://www.genecards.org>), Online Mendelian Inheritance in Man (OMIM) database (<https://www.omim.org>), DisGeNET database (<https://www.disgenet.org>), and Therapeutic Target Database (TTD) database (<https://db.idrblab.net/ttd>) using “pain” as the keyword. A pain potential target databank was created after deleting duplicate targets. The targets of metformin were compared with the pain databank to obtain the intersection targets. These intersection tar-

gets were the potential targets of metformin for pain treatment. Third, the overlapped targets were displayed as a Venn figure (Venny 2.1.0, CNB, Madrid, Spain), and the protein-protein interaction (PPI) network of targets shared by metformin and pain was acquired from the STRING database (<https://string-db.org/>, version 11.5). The PPI network was uploaded to Cytoscape 3.9.1 (<https://cytoscape.org/>) to search hub targets according to the topological features “Betweenness centrality”, “closeness centrality”, and “degree” [27,28]. Finally, to clarify the biological function of shared targets between metformin and pain, an enrichment analysis of pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG) was performed with “clusterProfiler” package in the RStudio 4.2.2 (<https://rstudio.com>) [29].

2.10 Statistical Analysis

The data were expressed as means \pm standard error of the mean (SEM). Two-tailed and unpaired Student *t*-test, and one-way or repeated measures two-way analysis of variance (ANOVA) were used to generate the statistical significance values using GraphPad Prism 6 (version 6.0, GraphPad Software Inc., San Diego, CA, USA). The post-hoc Student-Newman-Keuls test was performed when the effect of the drug dose was statistically significant. Probability values were two-tailed, and the statistical significance criterion *p* value was 0.05.

3. Results

3.1 Metformin Suppressed Both SNL- and Formalin-Induced Pain

To assess the analgesic effects following metformin treatments, we applied an SNL and formalin-induced hypersensitivity model. An intrathecal injection of 30 or 100 μ g (dissolved in 10 μ L of ACSF) of metformin was administered to two groups of neuropathic pain rats. The PWT to mechanical or thermal stimuli was measured before and after injections at 1, 2, and 4 h. Intrathecal injections of 30 or 100 μ g of metformin and ACSF did not change contralateral hind paws in sham rats over 4 h. In contrast, metformin via intrathecal delivery prolonged the mechanical and thermal response in ipsilateral hind paws in a dose- and time-dependent manner (Fig. 1A, mechanical allodynia $F_{(5,96)} = 509.4$, $p < 0.0001$; two-way repeated measures analysis of variance (ANOVA) followed by Sidak's post-tests, Fig. 1B, thermal hyperalgesia: $F_{(5,96)} = 118.1$, $p < 0.0001$; two-way repeated measures ANOVA followed by Sidak's post-tests).

Furthermore, we assessed the antinociceptive effects of metformin in formalin-induced acute inflammatory pain. The rats displayed flinching behaviors following 5% formalin injection into the left hind paws. These responses were separated into phase I and phase II, and counted with the frequencies of flinching behaviors (Fig. 1C, $F_{(2,120)} = 164.9$, $p < 0.0001$). The results showed that metformin did not significantly affect the number of flinching behaviors

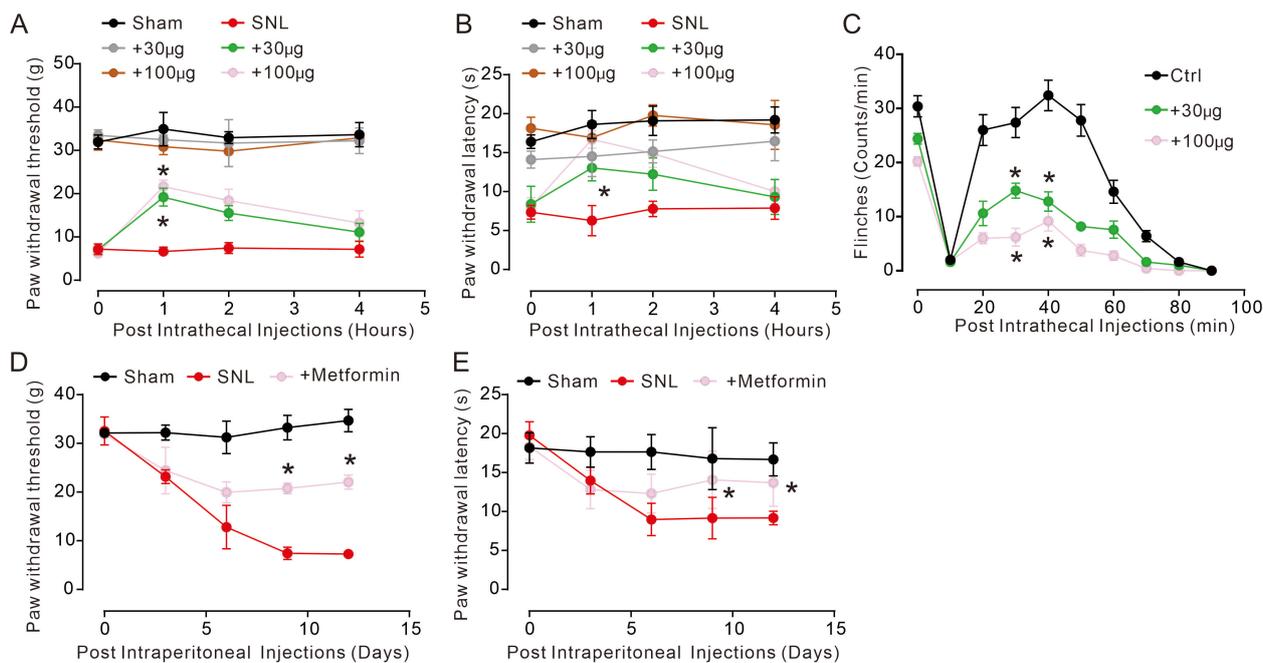


Fig. 1. Intrathecal administration of metformin attenuated neuropathic- and formalin-induced pain. (A,B) Intrathecal delivery of metformin exhibited mechanically antiallodynic and thermal hyperalgesia effects in SNL-induced neuropathic pain rats. (C) Intrathecal delivery of metformin significantly reduced flinch counts and exhibited analgesic effects in formalin-induced inflammatory pain rats ($n = 5-6$ per group). $*p < 0.05$, by two-tailed Student t -test or repeated measures two-way ANOVA followed by Sidak's post-tests. (D,E) Consecutive intraperitoneal metformin suppressed mechanically antiallodynic and thermal hyperalgesia effects, mechanical allodynia: $F_{(2,60)} = 273.1, p < 0.0001$; two-way repeated measures ANOVA followed by Sidak's post-tests, thermal hyperalgesia: $F_{(2,60)} = 28.74, p < 0.0001$; $*p < 0.05$, two-way repeated measures ANOVA followed by Sidak's post-tests, $n = 5$. Data were presented as means \pm SEM. SNL, spinal nerve ligation; ANOVA, analysis of variance; SEM, standard error of the mean.

in phase I, but intrathecal injection of 30 or 100 μg of metformin inhibited the formalin-induced flinching response in phase II and peaked at around 30–40 min. Therefore, metformin produced analgesic effects in SNL and formalin-induced hypersensitivity. Consecutive intraperitoneal injections of 100 mg of metformin also suppressed nerve injury-associated hypersensitivity in rats (Fig. 1D, mechanical allodynia $F_{(2,60)} = 273.1, p < 0.0001$; two-way repeated measures ANOVA followed by Sidak's post-tests, Fig. 1E, thermal hyperalgesia: $F_{(2,60)} = 28.74, p < 0.0001$; two-way repeated measures ANOVA followed by Sidak's post-tests).

3.2 GO and KEGG Analyses Inferred that Metformin Regulated Synaptic Transmission in Pain

Network pharmacology was used to determine the underlying mechanism mediated by metformin treatment to further assess the underlying target and signaling pathways. Through the online Venn diagram tool, we found 25 shared targets for metformin to treat pain (Fig. 2A). The targets were imported into the STRING database to construct and identify the PPI network and then visualized by Cytoscape. Subsequently, we collected the hub targets, including nitric oxide synthase 1 (NOS1), NOS2, NOS3, epidermal growth factor receptor (EGFR), and plasminogen (PLG),

by the Centiscape 2.2 (<https://apps.cytoscape.org/apps/centiscape>) plugin in Cytoscape based on three topological features: degree, betweenness centrality, and closeness centrality (Fig. 2B). Gene Ontology (GO) and KEGG pathway enrichment analyses of the 25 shared targets were performed using RStudio. The top 10 remarkably enriched biological processes (BP), molecular functions (MF), cellular components (CC), and KEGG signaling pathways involved in the shared targets were identified. The BP of the targets included postsynaptic signal transduction, regulation of hemostasis, and biosynthetic processes (Fig. 2C). The MF comprised neurotransmitter receptor activity and binding, mitogen-activated protein (MAP) kinase kinase activity, and calcium-dependent protein binding (Fig. 2D). The CC consisted of synaptic cleft, neuronal cell body and spine, dendritic spine, and membrane raft (Fig. 2E). The underlying target genes of metformin in pain were specifically and predominantly enriched in the Relaxin signaling pathway, the cyclic guanosine monophosphate (cGMP)-protein kinase G (PKG) signaling pathway, neuroactive ligand-receptor interaction, calcium signaling pathways, neuroactive ligand-receptor interaction, and cholinergic synapses (Fig. 2F). These findings suggest that the main bioactive components of metformin had different action targets in synaptic transmission pathways.

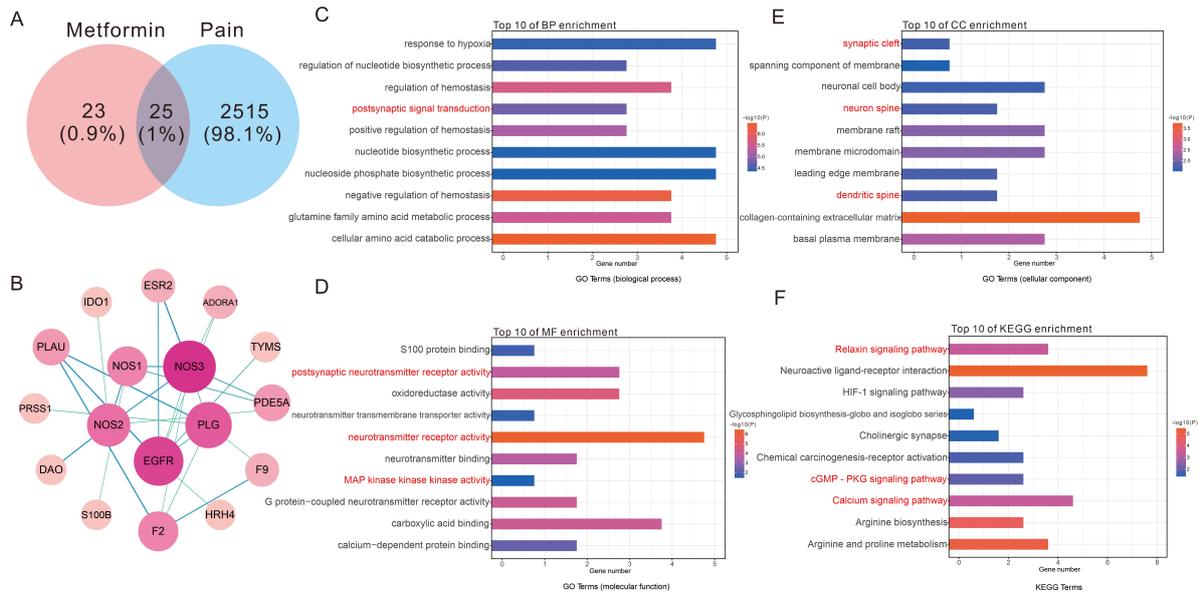


Fig. 2. GO and KEGG enrichment analyses on metformin. (A) Venn diagram of targets for metformin to treat pain. (B) A PPI network of metformin to treat pain. (C) The top 10 significantly enriched terms in biological process (BP). (D) The top 10 significantly enriched terms in molecular function (MF). (E) The top 10 significantly enriched terms in cellular component (CC). (F) The top 10 significantly enriched terms in KEGG. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein-protein interaction; BP, biological process; MF, molecular function; CC, cellular component.

3.3 Metformin Reduced Neuronal Activity of the Spinal Cord in Neuropathic Rats

Network pharmacology highlighted that metformin significantly remodeled the painful information mediated by complex circuits associated with excitatory and inhibitory networks. The protooncogene c-Fos is a gene expression-regulated nuclear protein with elevated expression in neurons following neuronal activation. The increase in c-Fos protein was stimulated by neurotransmitters on membrane receptors under physiological stimuli, especially in pain states in response to mechanical stimulus. To further illustrate neuronal activity after metformin treatment in neuropathic rats, we used double immunofluorescence staining to identify c-Fos expression levels (Fig. 3A–C). The results showed that c-Fos expression significantly increased in neuropathic rats, and intrathecal injections of metformin abolished c-Fos expression in the spinal cord (Fig. 3A–D, immunofluorescence staining: $F_{(2,17)} = 25.67, p < 0.0001$, one-way ANOVA followed by Sidak's post-tests; Fig. 3E,F, protein: $F_{(2,10)} = 4.387, p = 0.0429$, one-way ANOVA followed by Sidak's post-tests). In addition, a network pharmacology analysis inferred that NOS may be involved in metformin treatments. We observed that metformin significantly restored NOS2 mRNA without affecting NOS1 in neuropathic pain (Fig. 3G,H, NOS1: $F_{(2,9)} = 0.2736, p = 0.7667$, NOS2: $F_{(2,9)} = 30.95, p < 0.0001, *p < 0.05, n = 4$, one-way ANOVA followed by Sidak's post-tests). Therefore, our study demonstrated that metformin produced anticipative effects by inhibiting spinal neuronal activity in neuropathic pain.

3.4 Metformin Significantly Alleviated SNL-Induced Glutamatergic Transmission.

We demonstrated that metformin inhibited nerve injury-induced neuronal activity, but the underlying plastic mechanism remains unclear. Electrophysiological recording is the most dynamic method used to study neural plasticity [30]. Behavioral tests suggested that metformin may significantly lower nociceptive behaviors in the ipsilateral side after SNL (Fig. 1A). Thus, the mechanism for neural plasticity was assessed after nerve injury. Both the frequency and amplitude of mEPSCs in the spinal lamina II significantly increased during neuropathic pain, and this finding was consistent with our prior studies [31]. Frequency reflected the release of presynaptic glutamate, whereas amplitude was associated with postsynaptic receptors. Bath application of 10–100 μM metformin ameliorated the increase in mEPSCs amplitude and frequency in a dosage-dependent manner (Fig. 4A–E, frequency: $F_{(5,62)} = 71.41, p < 0.0001$; amplitude: $F_{(5,62)} = 24.23, p < 0.0001$, one-way ANOVA followed by Tukey's post-tests). Inhibitory transmission was also associated with neuropathic pain. We found that 100 μM metformin treatment did not significantly inhibit the frequency and amplitude of mIPSCs after the application of D-AP5, CNQX, and TTX in both SNL and sham groups (Fig. 4F–J, frequency: $F_{(3,38)} = 19.28, p < 0.0001$; amplitude, $F_{(3,38)} = 2.017, p = 0.1279$, one-way ANOVA followed by Tukey's post-tests). These findings demonstrated that metformin alleviated spinal glutamatergic transmission in neuropathic rats.

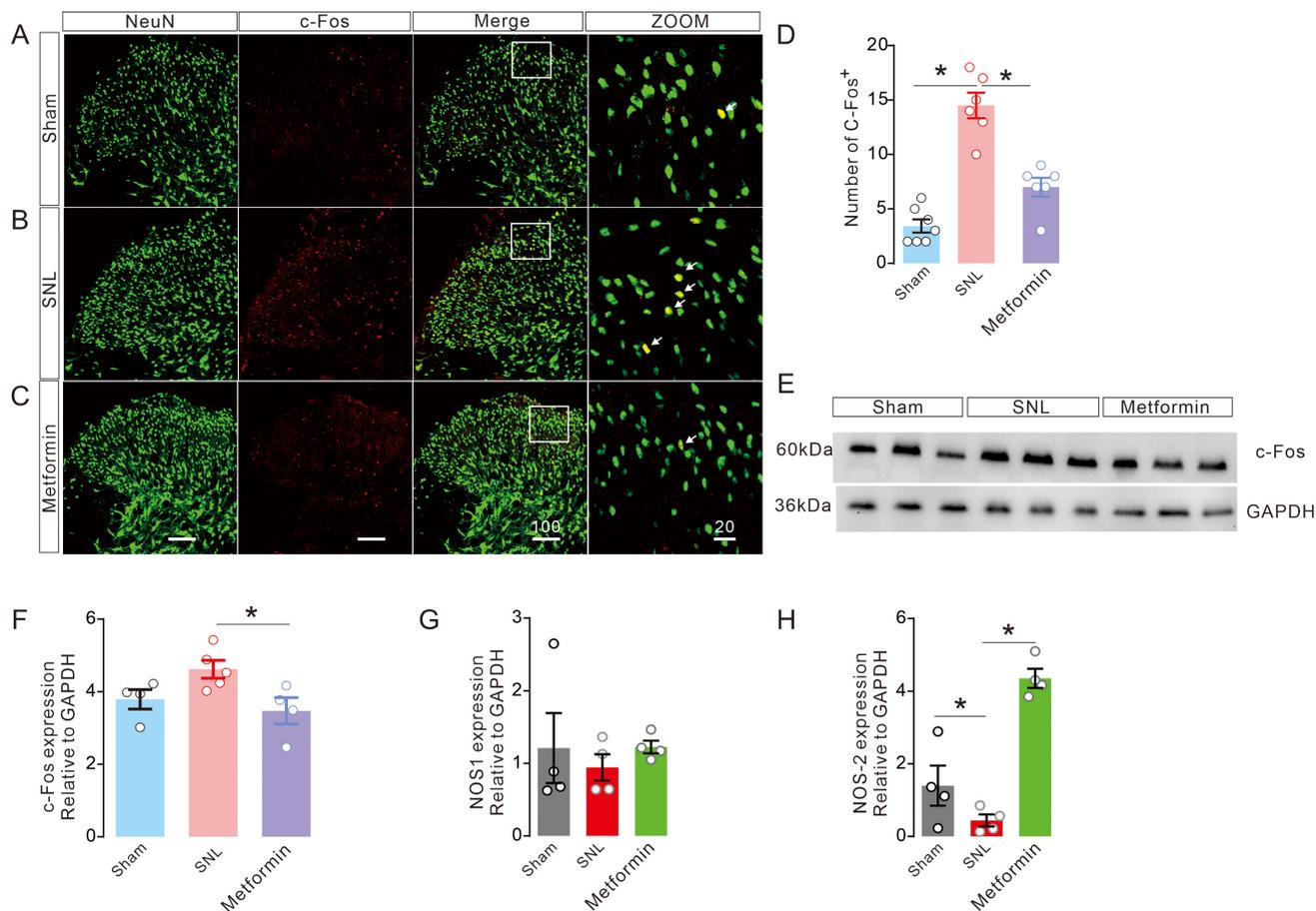


Fig. 3. Dual fluorescence labeling of c-Fos with NeuN among three different groups. (A) c-Fos with NeuN co-labeling in the sham group. (B) c-Fos with NeuN co-labeling in the SNL group. (C) c-Fos with NeuN co-labeling in the metformin-treated group. Images were captured in laminae I–III of spinal cord (n = 6–8 animals). Scale bar: 100 μ m ($\times 10$), 20 μ m ($\times 20$). (D) Statistics on number of c-Fos positive cells. (E,F) c-Fos expression. (G,H) mRNA expression of nitric oxide synthase 1 (NOS1) and NOS2. NOS1: $F_{(2,9)} = 0.2736$, $p = 0.7667$, NOS2: $F_{(2,9)} = 30.95$, $p < 0.0001$, $*p < 0.05$, n = 4. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

4. Discussion

In the present study, we observed that intrathecal injection of metformin exhibited remarkable analgesic effects in a dose-dependent manner, which significantly inhibited SNL-induced chronic pain and formalin-induced acute pain. Using whole-cell recording and immunofluorescence, we demonstrated that metformin significantly inhibited spinal nociceptive transduction by alleviating glutamatergic transmission, thereby attenuating chronic and acute pain in rats. Through network pharmacology, we discovered the analgesic targets of metformin by overlapping its predicted targets with pain targets and revealed the signal transduction mechanism of metformin analgesia through calcium signaling and cGMP-PKG signaling. Intracellular calcium ions are essential for initiating nerve injury and related signaling molecule transduction. Our results further demonstrated that metformin significantly reversed AMPK-mediated nerve injury. Thus, metformin exerted an important neuroprotective effect by regulating intracellular calcium ion levels and reversing neuropathic pain caused by

spinal nerve ligation. We demonstrated that metformin produced analgesic behaviors by inhibiting spinal glutamatergic transmission, calcium signaling, and cGMP-PKG signaling in nociceptive hypersensitivity.

Neural activity is a critical mechanism in the nervous system for maintaining functions and behavioral performance. Metformin significantly inhibited excitatory synaptic transmission, but did not affect inhibitory synaptic transmission. Our previous study demonstrated that analgesia could be achieved by simply interfering with excitatory synaptic transmission [31]. Glutamate transmission is central to changes in the nociceptive circuit, leading to neuronal excitotoxicity and loss via a trans-synaptic mechanism [32]. In addition, intrathecal injection of metformin was insufficient to reverse hypersensitivity in neuropathic rats, as shown in Fig. 1A. In brief, the immunohistochemical and electrophysiological whole-cell recording results showed that metformin had crucial neuroprotective effects.

Network pharmacology is currently the most critical and mature method for drug target research. In our study,

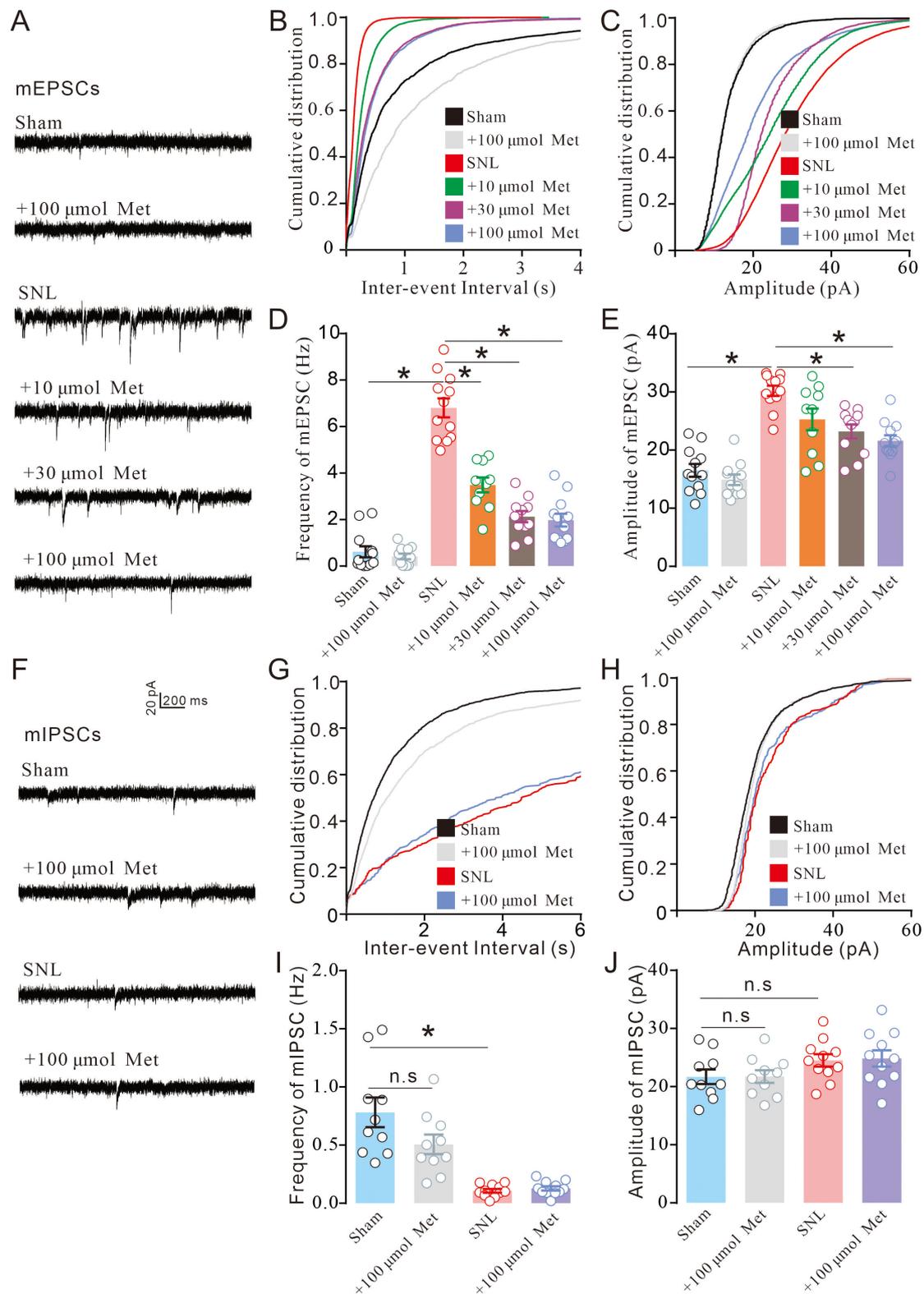


Fig. 4. Metformin attenuated glutamatergic transmission in the spinal dorsal horn of the ipsilateral hind paws in pain states.

(A) Sample traces of mEPSCs. (B,C) Cumulative distribution of frequency and amplitude in mEPSCs. (D,E) Statistical analysis of frequency and amplitude in mEPSCs. (F) Sample traces of mIPSCs. (G,H) Cumulative distribution of frequency and amplitude in mIPSCs. (I,J) Statistical analysis of frequency and amplitude in mIPSCs. Data were presented as the means \pm SEM ($n = 5-6$ animals). n.s., not significant. $*p < 0.05$, by one-way or repeated-measures two-way ANOVA followed by Sidak's post-tests. mEPSCs, miniature excitatory synaptic currents.

we discovered that EGFR and NOS may be the targets of metformin analgesia. The study demonstrated that altered tetrahydrobiopterin (BH4) production following sensory neuron injury contributes to pain sensitivity and duration, regulating Gch1 expression in sensory neurons. A molecular link exists between BH4 and pain perception via EGFR/Kirsten ras sarcoma virus (KRAS), and the pharmacological modulation of BH4 has significant analgesic effects via EGFR/KRAS [33]. Clinical trials have revealed that EGFR inhibitors have positive analgesic effects in patients suffering from neuropathic pain [34]. NO activates soluble guanylate cyclase activity in response to the production of cGMP. cGMP then activates cGMP-dependent protein kinase (PKG), which causes multiple targets to be activated, including the ATP-sensitive K⁺ channels, thereby promoting synaptic processing and transmitter release in response to pain.

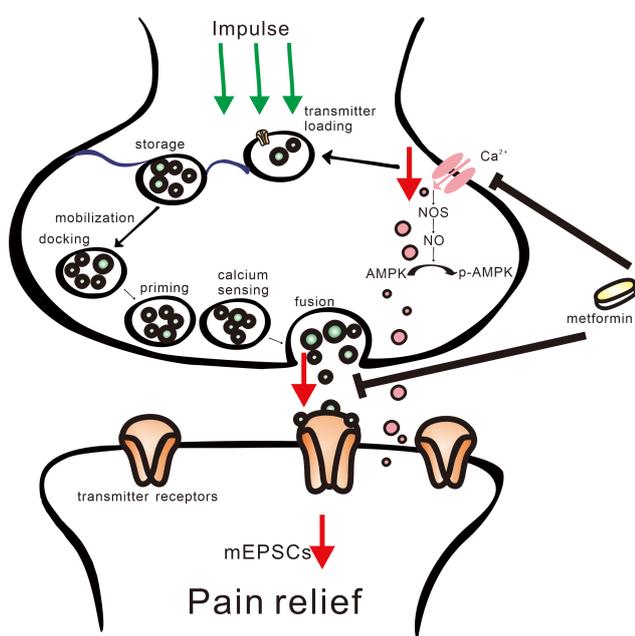


Fig. 5. Schematic showing the role of metformin in the spinal excitatory synaptic transmission and pain hypersensitivity in neuropathic pain.

The activation of NO/cGMP signaling in pain states clearly causes a series of intracellular molecular activities, as well as reactive astrogliosis processes and microglial polarization, both of which contribute to chronic pain sensations. The natriuretic peptide commonly binds to membrane guanylyl cyclase in DRG neurons - generating and further activating the cGMP/PKG pathway, the release of microglial-mediated inflammatory factors, and the development of chronic pain [35]. Inflammatory factors significantly promote glial cell-induced alteration in spinal synaptic plasticity in the early stages of nerve injury. Microglia have the potential to induce signal trans-

duction and synaptic plasticity in neural circuits by modifying synaptic proteins via the complement ligand pathway [36]. In whole-cell recording mode, perfusion of TNF- α and IL-6 significantly alters the strength of neuronal transmission in spinal cord neurons and enhances synaptic connections, which are essential for nociceptive circuitry [37]. In chronic pain models, inhibiting inflammatory factor expression and blocking TNF- α -mediated activation of the downstream Fbxo-45-dependent signaling pathway can significantly inhibit the ubiquitination of Munc13-1, resulting in potent analgesic effects [38]. Moreover, other inflammatory mediators that diffuse intracellularly after nerve injury invade the primary integration centers in DRG through similar mechanisms. When using knockout animals and inhibiting inflammatory mediators-related receptors, whole-cell recordings revealed that knockout animals and pharmacology significantly inhibit the activation of signaling pathways such as the inflammatory signaling pathway p38 and guanosine triphosphate (GTP)-RhoA/Rho-associated coiled-coil-forming protein serine/threonine kinase 2 (ROCK2)-mediated nociceptive hypersensitivity behavior after nerve injury. Those fundamental alterations form the basis of synaptic plasticity and nociceptive transmission in pain states. Interestingly, our data illustrated that the increased dosage of metformin exhibited pharmacologically analgesic effects on glutamatergic transmission (Fig. 4), and this result was consistent with GO and KEGG in network pharmacology (Fig. 2).

5. Conclusions

Spinal nerve ligation caused changes in neural circuits, whereby depolarization led to the opening of calcium channels. Large amounts of calcium ions diffused into the presynaptic neuron, allowing synaptic vesicles to enter circulation. These vesicles carried neurotransmitters to merge with the synaptic membrane, which released into the synaptic cleft and finally diffused into the postsynaptic neuron (Fig. 3). Metformin significantly reversed calcium ion efflux, intracellular second messenger-mediated AMPK phosphorylation levels, and NOS2 gene expression. This led to inhibited fusion of presynaptic glutamatergic vesicles with presynaptic membranes in the dorsal horn of the spinal cord, preventing glutamatergic neurotransmission and mechanically stimulated c-Fos expression. Therefore, metformin may regulate the synaptic vesicle cycle by inhibiting calcium channels and glutamatergic synaptic transmission, providing analgesia (Fig. 5).

Availability of Data and Materials

All data supporting the conclusion of the article are included in this article.

Author Contributions

YM, YMM and LM designed the research study. DXD, XJW and UA performed the research. DW analyzed the data. XL and RML provided help and advice on visualization. DXD wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All animal experimental protocols and procedures in this study were reviewed and approved by the Animal Care and Welfare Committee of Shanghai Mental Health Center (A2018081).

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Conflict of Interest

The authors declare no conflict of interest.

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