Journal of Integrative Neuroscience

J. Integr. Neurosci. **2022**; 21(4): 121
https://doi.org/10.31083/j.jin2104121

Original Research

Endogenous Cannabinoid Receptors Modulate Plasticity at Immature Synapses

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Academic Editor: Rafael Franco
Submitted: 23 November 2021 Revised: 1 March 2022 Accepted: 10 March 2022 Published: 28 June 2022

Abstract

Background: To explore the mechanism of endocannabinoid cannabinoid receptor 1 (CB1) receptor pathway that regulates synaptic plasticity in the dorsal horn of the spinal cord of rats with neuropathic pain at different ages. **Methods**: Neonatal, juvenile, and adult male sprague dawley (SD) rats were divided into the spinal nerve preservation injury (SNI), SNI + Anandamide (AEA), SNI + D-AP5, SNI + CNQX, SNI + D-AP5 + AEA, SNI + CNQX + AEA, sham SNI, sham SNI + AEA, sham SNI + D-AP5, sham SNI + CNQX, sham SNI + D-AP5 + AEA, and sham SNI + CNQX + AEA groups, respectively. Paw withdrawal threshold (PWT) and long-term potentiation (LTP) of the spinal dorsal horn PS (field potential) were assessed to judge the spinal cord's functional state. Immunohistochemical staining and Western blot were conducted to detect CB1 protein levels in the spinal dorsal horn. **Results**: The LTP response in the spinal cord was alleviated in the SNI + AEA group. After treatment with the N-methyl-D-aspartate (NMDA) receptor blocker D-AP5, the LTP of neonatal A nerve was relieved further. After treatment with the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor blocker CNQX, LTP change in the A nerve was not obvious. The LTP of the A and C nerves were relieved after D-AP5 or CNQX treatment in young and adult animals; however, the blocking effect of CNQX was obvious. The altered levels of PWT and CB1 support these results. **Conclusions**: The CB1 receptor activation produces analgesia in neonatal rats through NMDA receptor formation for PS inhibitory activity. In juvenile and adult rats, this phenomenon was effectuated through NMDA and AMPA receptors. This difference could be attributed to the varied number of NMDA and/or AMPA receptors activated during development and changes in the NMDA/AMPA receptor ratio.

Keywords: cannabinoid signaling pathway; NMDA receptor; AMPA receptor; neonatal rat; juvenile rat

1. Introduction

Trauma, inflammation, and direct nerve injury may first cause nerve fiber damage, resulting in a large amount of ectopic electrical activity. The long-term effects of this ectopic electrical activity lead to long-term potentiation (LTP) of synaptic transmission of nociception in the dorsal horn of the spinal cord, ultimately causing hyperexcitability and central sensitization of spinal nociceptive sensory neurons and neuropathic pain. The long-term plasticity of synaptic transmission in the dorsal horn of the spinal cord plays a major role in the pathogenesis of neuropathic pain. Similar changes were detected in synaptic transmission plasticity during the occurrence, development, and maintenance of neuropathic pain [1]. A previous study constructed an adult rat model of neuropathic pain, he spinal nerve preservation injury model (SNI model), which readily induces synaptic transmission of LTP in the dorsal horn of the spinal cord [2]. The decrease in LTP induction thresholds for A and C fiber evoked potentials in the dorsal horn of the spinal cord in SNI model rats suggested that the plasticity of synaptic transmission efficiency in the spinal dorsal horn may play an important role in the development of neuropathological pain. In addition, ectopic electrical activity of nerves in the dorsal horn of the spinal cord after spinal nerve ligation, disruption of the central descending inhibitory system of the spinal cord, phenotypes of injured nerve fibers, and changes in neurochemical factors have been associated with neuropathic pain [3].

The endogenous cannabinoid receptor agonist Anandamide (AEA) acts through cannabinoid receptors (CB1 and CB2) and other targets, such as G protein-coupled receptor 55 (GPR55) and transient receptor potential cation channel, subfamily V, member 1 (TRPV1) receptors, thereby promoting LTP. The CB1 receptors are mainly distributed in the brain, spinal cord, and peripheral nervous system, as well as in the pain transmission pathways, such as the posterior horn of the spinal cord. Some studies on the dorsal horn of the adult rat spinal cord have shown that presynaptic CB1 receptors are activated, thereby inhibiting glutamate release and C nociceptive sensory nerve fibers, decreasing spinal excitatory transmission, and effectuating analgesia in the spinal cord [4]. In the present study, we analyzed PS in the neonatal and juvenile rat SNI models. This confirmed the LTP changes in synaptic plasticity-generating field potentials (FPs) in the dorsal horn of the spinal cord via modulation of glutamatergic α-amino-3-

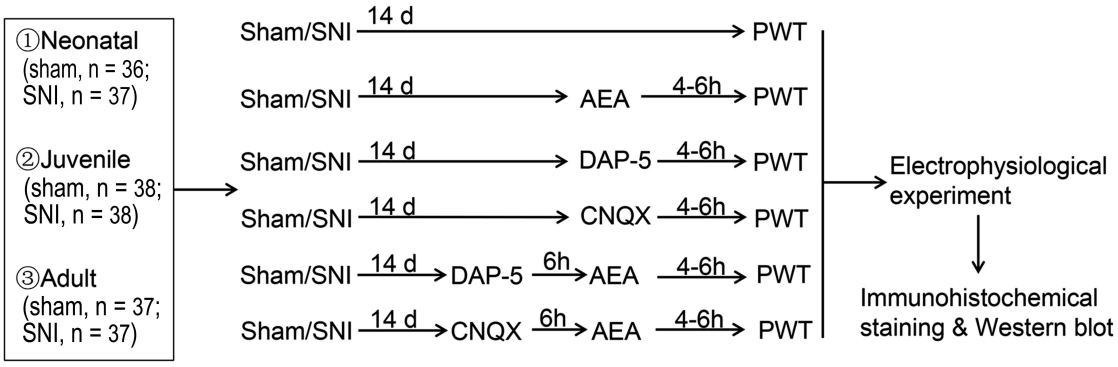

Fig. 1. Experimental design. SNI, spared nerve injury; d, days; h, hour; PWT, 50% paw withdrawal threshold; DAP-5, DL-2-amino-5-phosphonopentanoic acid; CNQX, 6-cyano-7 nitroquinoxaline-2, 3-dione.

hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors by cannabinoid CB1 receptor in neonatal and juvenile rats.

Recording the PS of synaptic transmission in the dorsal horn of the spine is a crucial aspect of LTP and interference with respect to the onset and development of neuropathic pain. LTP is a sustained enhancement of neuronal signaling, and its formation is associated with glutamate AMPA and NMDA receptors, postsynaptic calcium release, and activation of signaling pathways [5]. Previous studies have shown that the modulation of AMPA and NMDA receptors affects LTP formation in the FPs in the dorsal horn of the spine. However, the formation of LTP in the FPs of immature SNI rats is yet to be clarified. In the neonatal period, the spinal dorsal horn develops central sensitization in young individuals [6,7]. Repeated stressful stimuli (pain signals) redistribute the neurons in the sensory system of the spinal cord with simultaneous appearance of C and A fibers. These phenomena lead to the retention of a large number of synapses or the formation of abnormal connections, causing painful changes that cause chronic pain syndrome, physical discomfort, growth retardation, and other future diseases in children. Therefore, the detection and pharmacological intervention of LTP in the dorsal horn of the spinal cord is essential in neonatal and adolescent individuals. CB1 receptors are widely distributed in the central nervous system and are targets for central analgesia. Therefore, the present study aimed to investigate the mechanisms of endogenous cannabinoid CB1 receptor pathway in regulating synaptic plasticity in the dorsal horn of the spinal cord in rats with neuropathic pain at different ages.

2. Materials and Methods

2.1. SNI Model Preparation

A total of 273 specific pathogen-free (SPF) rats, including neonatal (20 days after birth; P20), juvenile (P40), and adult (P60) (6–8 rats per group, 36 groups; Fig. 1 and Table 1), were purchased from Unilever (Beijing, China), and all the animal experiments were conducted at the Laboratory A III of Center for Animal Experiment of Wuhan University. After one week of early adaptation or feeding acclimatization (newborn rats were fed by their mothers), SNI or sham SNI surgery was performed on the animals [8,9]. According to the method by Bennett and Xie [10], the surgery involved the blunt separation of the sciatic nerve and its three branches, ligation of the tibial peroneal and tibial nerves and preservation of the small sural nerve, silk ligation and shear of the tibial and common peroneal nerves, and preservation of the small sural nerve to establish the SNI model. Local and intramuscular injections of penicillin were adopted to promote wound healing in rats. Sham operation was also performed in the neonatal, juvenile, and adult rat groups. The skin was incised and the nerve separated but not ligated before suturing. After the operation, the rats were kept in a single cage for 14 days.

2.2 Animal Treatment

The animals were randomized into the SNI, SNI + AEA, SNI + D-AP5, SNI + CNQX, SNI + D-AP5 + AEA, SNI + CNQX + AEA, sham SNI, sham SNI + AEA, sham SNI + D-AP5, sham SNI + CNQX, sham SNI + D-AP5 + AEA, and sham SNI + CNQX + AEA groups. A volume of 0.5 mL of 1 mmol/L [11] AEA (Asc-429, Xiamen Huijia Biotechnology Company, Xiamen, China) or 10 mmol/L [12] D-AP5 (Asc-003, Xiamen Huijia Biotechnology Company, Xiamen, China) or 10 mmol/L [13] CNQX (MCE, HY-15066, NJ, USA) was injected into the caudal vein at 14 days after SNI (sham operation) in SNI/sham + AEA, SNI/sham + D-AP5, and SNI/sham + CNQX groups. After 6 h post-D-AP5 or -CNQX administration, 0.5 mL of 1 mmol/L anandamide (AEA) [11] was injected into the caudal vein in the SNI/sham + DAP-5 + AEA or SNI/sham + CNQX + AEA group. After 4–6 h, mechanical stimulation was performed to shrink the right palm and assess the leg shrinkage threshold (50% paw withdrawal threshold (PWT)). Then, mechanical stimulation and electrophysiological experiments were carried out in the SNI group, following which, the second and third spinal cord segments were collected.

Table 1. 50% PWT before and after SNI in rats of different ages.

Neonatal group (P20)									
	Before operation	3 days after operation	7 days after operation	14 days after operation		Before operation	3 days after operation	7 days after operation	14 days after operation
SNI	6.78 ± 1.26	5.17 ± 1.21	3.72 ± 1.13$^{\#▲}$	2.1 ± 0.48$^{\#▲}$	Sham SNI	6.37 ± 1.43	6.01 ± 1.28	5.87 ± 1.39	5.92 ± 1.14
SNI + AEA	6.72 ± 1.62	5.8 ± 1.65	3.99 ± 1.51$^{\#▲}$	4.27 ± 1.16$^{\#▲}$	Sham SNI + AEA	6.82 ± 1.54	6.09 ± 1.41	5.92 ± 1.49	6.18 ± 1.62
SNI + D-AP5	6.61 ± 1.85	5.42 ± 1.42	3.75± 1.26$^{\#▲}$	5.82 ± 0.68$^{\#▲}$	Sham SNI + D-AP5	6.73 ± 1.52	6.39 ± 1.74	5.99 ± 1.58	6.27 ± 1.49
SNI + CNQX	6.89 ± 1.36	5.16 ± 1.28	3.82 ± 0.74$^{\#▲}$	4.16 ± 0.82$^{\#▲}$	Sham SNI + CNQX	6.69 ± 1.42	6.52 ± 1.38	6.18± 1.26	6.29 ± 1.19
SNI + D-AP5 + AEA	6.53 ± 1.46	5.37 ± 1.28	3.69 ± 1. 42$^{\#▲}$	6.42 ± 1.29	Sham SNI + D-AP5 + AEA	6.71 ± 1.53	6.61 ± 1.62	6.37 ± 1.64	6.46 ± 1.29
SNI + CNQX + AEA	6.82 ± 1.29	5.37 ± 1.41	4.05 ± 1.16$^{\#▲}$	4.85 ± 1.27$^{\#▲}$	Sham SNI + CNQX + AEA	6.34 ± 1.28	6.19 ± 1.37	6.29 ± 1.52	6.31 ± 1.42
Juvenile group (P40)									
	Before operation	3 days after operation	7 days after operation	14 days after operation		Before operation	3 days after operation	7 days after operation	14 days after operation
SNI	9.46 ± 1.81	8.16 ± 1.54	5.38 ± 0.92$^{※▲}$	3.61 ± 0.82$^{※▲}$	Sham SNI	9.32 ± 1.89	9.18 ± 1.42	9.10 ± 1.32	9.03 ± 1.46
SNI + AEA	9.36 ± 1.28	8.26 ± 1.89	6.09 ± 1.56$^{※▲}$	6.61 ± 1.72$^{※▲}$	Sham SNI + AEA	9.43 ± 1.62	9.38 ± 1.46	9.09 ± 1.82	9.30 ± 1.52
SNI + D-AP5	9.51 ± 1.83	8.08 ± 1.39	5.94 ± 1.76$^{※▲}$	7.82 ± 1.21$^{※▲}$	Sham SNI + D-AP5	9.37 ± 1.29	9.03 ± 1.16	8.79 ± 1.51	9.08 ± 1.42
SNI + CNQX	9.62 ± 1.48	8.24 ± 1.72	5.94 ± 1.48$^{※▲}$	7.25 ± 1.61$^{※▲}$	Sham SNI + CNQX	9.52 ± 1.58	9.01 ± 1.56	8.79 ± 1.51	9.08 ± 1.42
SNI + D-AP5 + AEA	9.58 ± 1.81	8.46 ± 1.63	5.69 ± 1.52$^{※▲}$	8.52 ± 1.35	Sham SNI + D-AP5 + AEA	9.71 ± 1.57	9.43± 1.42	9.08 ± 1.16	9.26 ± 1.21
SNI + CNQX + AEA	9.69 ± 1.85	8.14 ± 1.54	5.19 ± 1.41$^{※▲}$	8.36 ± 1.35	Sham SNI+CNQX+AEA	9.65 ± 1.41	9.24 ± 1.63	8.92 ± 1.25	9.37 ± 1.62
Adult group (P60)									
	Before operation	3 days after operation	7 days after operation	14 days after operation		Before operation	3 days after operation	7 days after operation	14 days after operation
SNI	12.79 ± 2.52	10.71 ± 2.46	6.25 ± 1.69$^{☆▲}$	4.18 ± 0.72$^{☆▲}$	Sham SNI	12.64 ± 2.75	12.08 ± 2.64	11.98 ± 2.74	11.88 ± 2.61
SNI + AEA	12.51 ± 2.98	10.24 ± 2.46	6.61 ± 1.83$^{☆▲}$	6.97 ± 1.76$^{☆▲}$	Sham SNI + AEA	12.32 ± 2.61	12.04 ± 2.25	11.91± 2.42	12.25 ± 2.42
SNI + D-AP5	12.46 ± 2.74	10.52± 2.86	6.15 ± 2.58$^{☆▲}$	7.29 ± 1.74$^{☆▲}$	sham SNI + D-AP5	12.48 ± 2.26	11.98 ± 2.39	11.87 ± 2.61	12.12 ± 2.82
SNI + CNQX	12.36 ± 2.43	10.62 ± 2.94	6.37 ± 2.61$^{☆▲}$	8.63 ± 1.72$^{☆▲}$	Sham SNI + CNQX	12.37 ± 2.58	11.90 ± 2.26	11.89 ± 2.53	12.38 ± 2.56
SNI + D-AP5 + AEA	12.49 ± 3.14	10.86 ± 2.59	6.43 ± 1.52$^{☆▲}$	9.68 ± 1.17$^{☆▲}$	Sham SNI + D-AP5 + AEA	12.66 ± 2.74	11.89 ± 2.81	11.69 ± 2.95	12.16 ± 2.42
SNI + CNQX + AEA	12.52 ± 2.85	11.6 ± 2.71	6.57 ± 1.81$^{☆▲}$	10.96 ± 2.52	Sham SNI + CNQX + AEA	12.54 ± 2.62	11.96 ± 2.84	11.91 ± 2.72	12.42 ± 2.59

$^{\#}$, in the neonatal group, 50% PWT mechanical stimulation was compared among the SNI, SNI + AEA, SNI + D-AP5, SNI + CNQX, and SNI + CNQX + AEA groups vs. the sham SNI, sham SNI + AEA, sham SNI + CNQX, and sham SNI + CNQX + AEA groups at 7 or 14 days after the operation ($p < 0.05$); $^{※}$, in the juvenile group, 50% PWT mechanical stimulation after 14 days was compared among the SNI, SNI + AEA, SNI + D-AP5, and SNI + CNQX groups vs. the sham SNI, sham SNI + AEA, sham SNI + D-AP5, and sham SNI + CNQX groups at 7 or 14 days after the operation ($p < 0.05$); $^{☆}$, in the adult group, 50% PWT mechanical stimulation was compared after 14 days among the SNI, SNI + AEA, SNI + D-AP5, and SNI + D-AP5 + AEA groups vs. sham SNI, sham SNI + AEA, sham SNI + D-AP5, and sham SNI + D-AP5 + AEA groups at 7 or 14 days after the operation ($p < 0.05$); $^{▲}$, the 50% PWT mechanical stimulation was significantly lower at 7 or 14 days after the operation compared to pre-SNI and at 3 days after SNI in the same group ($p < 0.05$).

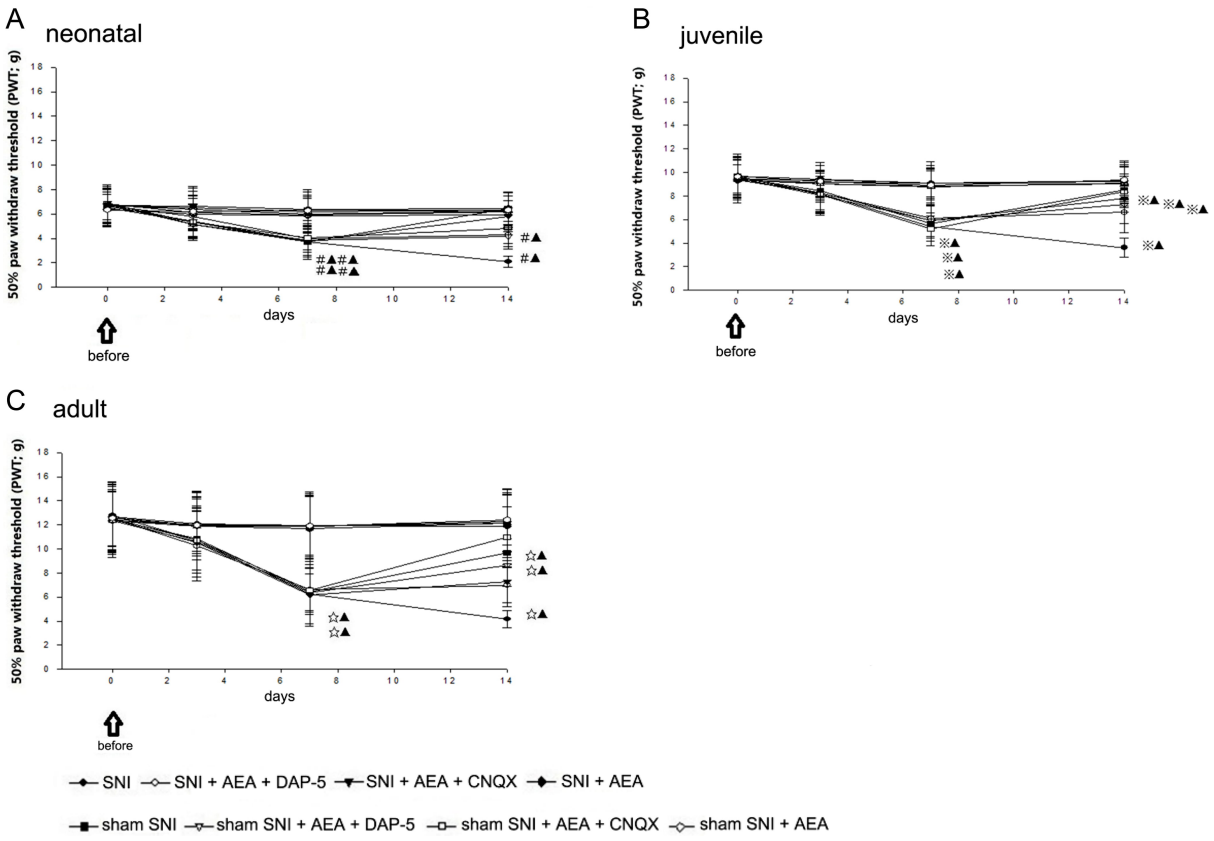

Fig. 2. 50% PWT before and after SNI in rats of different ages.

2.3 Estimated of 50% PWT after Mechanical Stimulation

50% PWT was measured before the injury and at 3, 7, and 14 days after the SNI operation. Morphology was observed and 50% PWT was determined in rats as follows. The animals were placed in a von Frey^{TM} resin glass cage (10 cm × 10 cm × 15 cm) (UGO Basile, Comerio, Italy) at 25 °C ± 1 °C, and allowed to adapt to the environment for 30 min before the experiment. According to Chaplan *et al.* [14], a series of stimulations were performed on the dorsal skin of the right foot of rats of different ages using calibrated von Frey silk (Stoelting Company, IL, USA). The initial stimulation intensity started from 2.041 g. In order to avoid agitation or pain to animals caused by frequent stimulation, the interval between two stimulations was set to 5 min, and each pulse lasted for 6–8 s. A sharp twitch of the foot during von Frey silk stimulation was considered a positive response. The formula was 50% PWT = (10lgX + kδ)/10000, where X is the value of the final stimulus using von Frey filaments; k is the tabular value of the positive/negative response pattern, and δ is the average difference between stimulations.

2.4 Electrophysiological Experiment

According to the *in vitro* electrophysiological technology [15], the sciatic nerve of P20, P40, and P60 rats received ankylosing electrical stimulation at 14 days after SNI to induce LTP in the superficial layer of the spinal dorsal horn, respectively. The field potential amplitude was processed on the RM6240 multi-channel signal acquisition system (RM6240 model: RM6240BD, VBD5246, CZ 01000158) and subjected to single stimulation; P20, P40, and P60 animals were stimulated with 3 V, 5 V, and 8 V, respectively. In the sham operation groups, P20, P40, and P60 animals were stimulated with 5 V, 10 V, and 15 V, respectively. Then, the evoked waves of spinal dorsal horn A or/and C fibers (basic values) were induced. After high-frequency stimulation (HFS) at 6 V, 10 V, and 12 V, respectively, the sham operation group received four levels of electrical stimulation at 10 V, 20 V, and 30 V, frequency 100 Hz, wave width 0.5 ms, string length 1 s, and string interval 10 s. The evoked waves of spinal dorsal horn A and C fibers were induced through the sciatic nerve to produce field potential changes that were recorded for at least 60 min; 14 days after sham SNI, CNQX, D-AP5, and AEA was injected intravenously 6 hours later, and behavioral tests were carried out after 2 h. Subsequently, the electrophysiological experiments were carried out to observe the evoked field potentials of A and C fibers as well as the potential amplitude changes in AMPAR- and/or NMDAR-mediated FPs (expressed in %) (Fig. 2 and Table 2).

Table 2. Potential amplitudes (μv) evoked by A and C nerve fibers during LTP formation in the spinal dorsal horn of SNI rats of different ages.

	A			C		
	Neonatal	Juvenile	Adult	Neonatal	Juvenile	Adult
Before ankylosis in the SNI group	31.62 ± 5.71	32.65 ± 5.82	34.19 ± 5.27	5.68 ± 1.49	9.27 ± 2.51	15.49 ± 3.24
After ankylosis	80.15 ± 6.48$^{\diamond\bullet}$	76.29 ± 7.31$^{\diamond\bullet}$	71.63 ± 7.94$^{\diamond\bullet}$	6.35 ± 1.67	22.75 ± 4.83$^{\diamond\bullet}$	27.29 ± 6.71$^{\diamond\bullet}$
Before ankylosis in the sham SNI group	30.48 ± 4.59	31.76 ± 4.28	34.49 ± 5.61	5.52 ± 1.49	9.63 ± 2.85	16.08 ± 4.52
After ankylosis	42.81 ±5.36$^{\diamond\triangledown}$	38.82 ± 5.64$^{\diamond\triangledown}$	36.14 ± 5.25	5.56 ± 1.83	12.48 ± 4.27$^{\diamond\triangledown}$	23.94 ± 6.28$^{\diamond\triangledown}$
Before ankylosis in the SNI + AEA group	30.58 ± 5.71	30.85 ± 5.26	34.26 ± 5.74	5.98 ± 1.95	9.75 ± 2.68	15.76 ± 5.72
After ankylosis	50.31 ± 5.46$^{\square\bullet}$	42.59 ± 6.72$^{\star\bullet}$	46.09 ± 6.53$^{\star\bullet}$	6.49 ± 1.37	12.27 ± 4.95$^{\star\bullet}$	20.74± 6.08$^{\square\bullet}$
Before ankylosis in the sham SNI + AEA group	30.94 ± 6.52	32.13 ± 5.36	34.47 ±5.92	5.36 ± 1.69	9.52 ± 2.74	15.46 ± 4.59
After ankylosis	34.38 ± 5.13*	32.82 ± 5.57	35.08 ± 5.75	5.75 ± 1.68	11.97 ± 5.86*	17.31 ± 6.05*
Before ankylosis in the SNI + D-AP5 group	30.63 ± 4.67	30.82 ± 4.92	34.16 ± 5.26	5.82 ± 1.95	10.57 ± 1.74	15.81 ± 5.67
After ankylosis	49.37 ± 8.79$^{\blacktriangle\bullet}$	56.08 ± 6.94$^{\divideontimes\bullet}$	50.59 ± 8.51$^{\divideontimes\bullet}$	6.25 ± 1.73	13.82 ± 3.68$^{\divideontimes\bullet}$	18.75 ± 6.54$^{\divideontimes\bullet}$
Before ankylosis in the sham SNI + D-AP5 group	30.75 ± 5.29	31.53 ± 4.62	33.94 ± 5.36	5.41 ± 2.53	9.86 ± 3.12	15.64 ± 4.28
After ankylosis	33.28 ± 5.68$^{\blacktriangle}$	34.83 ± 6.27	35.52 ± 6.74	5.85 ± 2.47	11.73 ± 5.02$^{\blacktriangle}$	17.82 ± 5.46$^{\blacktriangle}$
Before ankylosis in the SNI + CNQX group	31.82 ± 4.36	32.48 ± 5.27	33.27 ±5.19	5.69 ± 1.65	9.85 ± 2.94	15.78 ± 5.41
After ankylosis	52.49 ± 6.91$^{\#\bullet}$	50.16 ± 6.85$^{\blacksquare\bullet}$	48.23 ± 6.28$^{\blacksquare\bullet}$	5.87 ± 1.64	12.69 ± 4.13$^{\blacksquare\bullet}$	17.64 ± 5.37$^{\blacksquare\bullet}$
Before ankylosis in the sham SNI + CNQX group	31.46 ± 4.58	30.52 ± 5.82	34.07 ± 4.61	5.29 ± 1.75	9.46 ± 3.72	14.69 ± 6.28
After ankylosis	36.08 ± 5.27$^{\blacksquare\blacktriangledown}$	32.64 ± 5.49$^{\blacksquare}$	35.75 ± 4.98	5.09 ± 1.82	11.86 ± 3.59$^{\blacksquare}$	15.51 ± 5.74$^{\blacksquare}$
Before ankylosis in the SN I + D-AP5 + AEA group	30.64 ± 4.2	30. 86 ± 3.58	33.57 ± 4.61	5.35 ± 1.47	10.09 ± 2.52	14.35 ± 4.93
After ankylosis	31.95 ± 4.29	33.15 ± 6.48	37.49 ± 5.71$^{\blacklozenge\bullet}$	5.57 ± 1.89	11.28 ± 3.64	15.59 ± 4.83
Before ankylosis in the sham SNI + D-AP 5 + AEA group	30.73 ± 4.52	32.25 ± 3.82	33.18 ± 3.35	5.16 ± 1.29	10.64 ± 2.73	14.45 ± 3.61
After ankylosis	31.65 ± 4.37	33.41 ± 4.26	33.61 ± 4.27	5.42 ± 1.63	11.57 ± 2.62	14.95 ± 4.38
Before ankylosis in the SNI CNQX + AEA group	30.25 ± 3.17	31.96 ± 4.53	34.1 ± 4.68	5.21 ± 1.29	10.51 ± 2.65	14.83 ± 5.17
After ankylosis	35.91 ± 5.28$^{\blacklozenge\bullet}$	33.79 ± 4.85	34.86 ± 6.17	5.64 ± 1.73	10.69 ± 3.45	14.74 ± 5.16
Before ankylosis in the sham SNI + CNQX + AEA group	32.18 ± 4.29	31.52 ± 4.26	33.72 ± 4.09	5.19 ± 1.62	10.73 ± 2.49	14.65 ± 3.38
After ankylosis	33.41 ± 4.87	33.65 ± 4.95	34.59 ± 5.46	5.47± 1.58	10.92 ± 3.15	14.82 ± 4.69

SNI and sham SNI groups: $^{\diamond}$, $p < 0.01$: SNI (sham) groups vs. SNI (sham) + AEA, SNI (sham) + DAP5, SNI (sham) + CNQX, SNI (sham) + DAP5 + AEA, and SNI (sham) + CNQX + AEA groups. *, $p < 0.05$; $^{\square}$, $p < 0.01$: SNI (sham) + AEA groups vs. SNI (sham) + D-AP5, SNI (sham) + CNQX, SNI (sham) + DAP5 + AEA, and SNI (sham) + CNQX + AEA groups. $^{\blacktriangle}$, $p < 0.05$; $^{\divideontimes}$, $p < 0.01$: SNI (sham) + D-AP5 groups vs. SNI (sham) + CNQX, SNI (sham) + DAP5 + AEA, and SNI (sham) + CNQX + AEA groups. $^{\blacksquare}$, $p < 0.05$; $^{\#}$, $p < 0.01$: SNI (sham) + CNQX vs. SNI (sham) + DAP5 + AEA and SNI (sham) + CNQX + AEA groups. $^{\blacklozenge}$, $p < 0.05$: SNI + DAP5 + AEA vs. SNI + CNQX + AEA groups. Sham SNI groups: SNI group vs. sham SNI group: $^{\bullet}$, $p < 0.01$ vs. sham groups.

2.5 Assessment of CB1 Receptor Expression in the Spinal Dorsal Horn of Rats with LTP Formation in Immature Rat SNI Models

Animal Perfusion and Tissue Section Preparation

After the electrophysiological experiment, the chests of the animals were opened, the right atrial appendage was cut off, and 100–150 mL of normal saline and 250 m of 4% paraformaldehyde fixation solution were injected successively and rapidly through the ascending aorta (4 °C). Then, the perfusion rate was slowed down for 30 min. Subsequently, the L4 spinal cord segments of the rats were collected and fixed in 4% paraformaldehyde for 4 h and then immersed in 30% sucrose (4 °C, 48 h). POTC was used for embedding the tissue and sectioning.

2.6 Immunohistochemistry Analysis of CB1 Receptor Expression in spinal Cord Tissue Samples after SNI Detection

After the electrophysiological experiment, the tissue sections were analyzed according to the above operation, followed by phosphate-buffered saline (PBS) hydration, and sealing with skimmed milk. Hematoxylin and eosin (H&E) staining was performed for histological analysis. The cytoplasm stained red with strong reflectivity, and the color changed with SNI, electrical stimulation, and medication. At room temperature, anti-CB1 receptor antibodies (Abclonal, A1447) were added and incubated for 2 h, followed by incubation with secondary antibodies (A0208,1:1000, Beyotime, Shanghai, China) for 1 h. After three PBS washes, the film was sealed and observed under a light microscope.

The Mshot High Definition Color Pathological Graphic Report Management System (Mshot MD50) was used to acquire the images of lumbar 2 and 3 segments of the spinal cord of neonatal, juvenile, and adult rats, respectively. Under a 200× light microscope, the superficial region of the spinal cord's posterior horn from rat sections was selected for H&E staining. Densitometric analysis was performed using Image ProPlus 6.0 image analysis system (Media Cybernetics). The optical density (OD) reflected the positive staining intensity of the tissue; the higher the OD, the stronger the staining. All OD measurements were conducted under the same optical conditions.

2.7 Detection of CB1 Receptor Expression by Western Blot

After the above treatments in various groups, spinal cord tissue samples were obtained, snap frozen with liquid nitrogen, homogenized, and tissue protein extract was obtained by centrifugation. An equivalent of 35 μg/well total protein was analyzed by SDS-PAGE and transferred to PVDF membranes. After probing with anti-CB1 receptor primary antibodies, the membranes were incubated with secondary antibodies. Enhanced chemiluminescence (ECL) solution was used for color development, and a direct gel imaging CCD system was used for image analysis of CB1 receptor signal expression at 14 days after nociceptive stimulation.

The ImageJ software (version 1.8.0, LOCI, University of Wisconsin, Madison, WI, USA) was used to analyze the gray value of strips. GAPDH (66009-1-Ig, Proteintech, Company) was used as an internal reference for normalization. The secondary antibodies were goat anti-mouse (SA00001-1, Proteintech Inc., Chicago, IL, USA) and goat anti-rabbit (SA00001-2, Proteintech Inc, Chicago, IL, USA).

2.8 Statistical Analysis

Measurement data were analyzed using the SPSS 13.0 software (IBM Corp., Chicago, IL, USA) and expressed as mean $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) was performed for multiple group comparisons, and the treatment and the corresponding sham groups were compared using *t*-test (PWT) and ANOVA (LTP, immunohistochemistry assessment and Western blot). $p < 0.05$ indicated statistical significance.

3. Results

3.1 PWT Changes in SNI Rats

The present study evaluated the right paw. No significant difference was detected in 50% PWT between the sham SNI and SNI groups before and 3 days after SNI. In neonatal rats, 50% PWT in sham SNI rats (including those injected with D-AP5, CNQX, and AEA) and the SNI + D-AP5 + AEA group was significantly different from that in the SNI + AEA and SNI + CNQX + AEA groups at 7 and 14 days postoperatively ($p < 0.05$, Table 1). In juvenile rats, 50% PWT in the sham SNI (including injection groups), the SNI + CNQX + AEA, and SNI + D-AP5 + AEA groups differed significantly compared to the SNI, SNI + AEA, SNI + D-AP5, and SNI + CNQX groups ($p < 0.05$, Table 1). In adult rats, 50% PWT of the sham SNI (including injection groups) and SNI + CNQX + AEA groups showed significant differences compared to the SNI, SNI + AEA, SNI + D-AP5, SNI + D-AP5 + AEA, and SNI + CNQX groups ($p < 0.05$, Table 1). These results indicated that CNQX or D-AP5 inhibits PWT in neonatal, juvenile, and adult rats and enhanced the inhibitory effect of AEA on LTP. Specifically, D-AP5 and CNQX exerted a significant inhibitory effect on PWT in neonatal rats and adult rats, respectively. In addition, D-AP5 and CNQX inhibited PWT in juvenile rats and enhanced the inhibitory effect of AEA (Fig. 2).

3.2 Changes in C and A Fiber-evoked Potentials Induced by SNI

An amplitude 20% greater than the baseline value and lasting for >30 minutes is considered the plasticity threshold for inducing LTP (C or A nerve fibers) evoked by spinal dorsal horn nerve fibers, resulting in long-term enhancement of evoked potential in the spinal dorsal horn. In sham rats, low-intensity (5 V, 10 V, and 15 V for neonatal, juve-

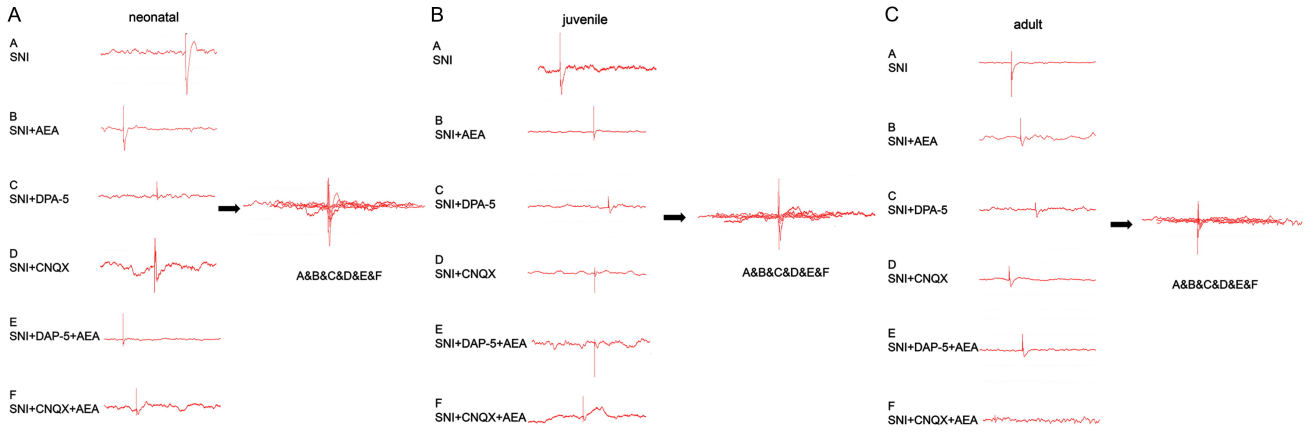

Fig. 3. Changes in C and A fiber-evoked potentials induced by SNI in rats of different ages under various treatments. Neonatal (A), juvenile (B), and adult (C) animals were assessed under various treatments.

nile, and adult groups, respectively) electrical stimulation could not induce LTP in the spinal dorsal horn's C-fiber evoked (or A-fiber evoked) field potential (Fig. 3), while electrical stimulation with high amplitude (30 V) induced LTP. In the SNI group, low-intensity electrical stimulation induced LTP of C-fiber evoked (or A-fiber evoked) field potential in the spinal dorsal horn (Fig. 3). These findings suggested that SNI decreases the plasticity threshold of the C-fiber evoked (or A-fiber evoked) field potential evoked by the dorsal horn of the spinal cord and increased the amplitude of the C-fiber or/and A-fiber evoked potentials. However, after D-AP5 and CNQX administration, the amplitude of this potential was suppressed compared to that in the SNI group. After treatment with AEA (incomplete inhibitor dose), the inhibitory effects of LTP (FPs) on A and C fibers were distinct on field potential at 14 days. Among them, D-AP5 in the neonatal group inhibited the LTP of A fibers. Since C fibers do not play a major role in the superficial layer of the dorsal horn function, LTP formed by A fibers was inhibited ($p < 0.01$, Table 2), and the wave and change in C fibers was not obvious ($p > 0.05$, Table 2). D-AP5 and CNQX inhibited the LTP of A and C nerve fibers in the juvenile group ($p < 0.05$, Table 2), which was enhanced by AEA ($p < 0.01$, Table 2). In the adult group, the A and C nerve fibers were mainly inhibited after CNQX administration, and completely inhibited by AEA. The comparison between the SNI and sham SNI groups revealed that the amplitudes of A fibers after ankylosis differed significantly in the neonatal and juvenile groups ($p < 0.05$ or $p < 0.01$, Table 2), and those of C fibers were significantly different in the juvenile and adult groups ($p < 0.05$ or $p < 0.01$, Table 2). The comparison of the SNI + AEA and sham SNI + AEA groups after ankylosis showed that the amplitudes of A fibers were significantly different ($p < 0.05$, Table 2), and those of C fibers differed significantly in the juvenile and adult groups ($p < 0.05$ or $p < 0.01$, Table 2). The comparison of the SNI + D-AP5 and sham SNI + D-AP5 groups did not reveal any significant difference in the amplitude of A fibers after ankylosis in the neonatal period ($p > 0.05$, Table 2), while the amplitude of C fibers had significant differences between the juvenile and adult groups ($p < 0.05$ or $p < 0.01$, Table 2). The comparison between the SNI + D-AP5 + AEA and sham SNI + D-AP5 + AEA groups after ankylosis did not show any significant differences in the amplitudes of A and C nerve waveforms ($p > 0.05$, Table 2). The comparison between the SNI + CNQX and sham SNI + CNQX groups after ankylosis showed that the amplitudes of A fibers in the neonatal period differed significantly ($p < 0.05$, Table 2), and those of C fibers differed in the juvenile and adult groups ($p > 0.05$, Table 2). The comparison of the SNI + CNQX + AEA and sham SNI + CNQX + AEA groups did not show any significant differences in the amplitudes of A and C nerve waveforms ($p > 0.05$, Table 2). In order to present the alteration of LTP, we calculated the area of evoked field potentials (μv s) evoked by A and C nerve fibers during formation and quantification of LTP (Table 3), which was consistent with the results shown in Table 2.

3.3 Histology and Changes in CB1 Protein Expression after 14 Days of SNI

H&E and immunohistochemistry staining showed eosinophils in the dorsal horn of the spinal cord after electrical stimulation at 14 days after SNI, with bright red, strong, and dark-brown signals. Immunohistochemistry showed that spinal cord cells had brownish-red and dark brown signals at 14 days after SNI. On day 14, the signals were significantly lower in the SNI + D-AP5 + AEA group of neonatal rats compared to the SNI group ($p < 0.01$) and lower in the SNI + CNQX + AEA and SNI + AEA groups compared to the SNI group ($p < 0.05$). The signals were lower in the SNI + CNQX + AEA, SNI + D-AP5 + AEA, and SNI + AEA groups in juvenile rats ($p < 0.05$ or $p < 0.01$), SNI + CNQX + AEA group in adult rats ($p < 0.01$), and SNI + D-AP5 + AEA and SNI + AEA groups compared to the SNI group ($p < 0.05$) (Fig. 4).

Table 3. Area of evoked field potentials (μv s) evoked by A and C nerve fibers during LTP formation in the spinal dorsal horn of SNI rats of different ages.

	A			C		
	Neonatal	Juvenile	Adult	Neonatal	Juvenile	Adult
Before ankylosis in the SNI group	42.72 ± 5.28	41.98 ± 5.64	45.27 ± 4.92	7.22 ± 1.85	10.86 ± 3.94	20.58 ± 5.71
After ankylosis	92.75 ±7.19$^{\diamond\bullet}$	88.57 ± 6.85$^{\diamond\bullet}$	86.45 ± 7.49$^{\diamond\bullet}$	7.85 ± 1.97	28.17 ± 5.28$^{\diamond\bullet}$	29.84 ± 7.62$^{\diamond\bullet}$
Before ankylosis in the sham SNI group	40.64 ±6.78	41.94 ± 6.25	44.76 ± 7.59	7.36 ± 1.56	10.43 ±2.97	19.83 ±5.86
After ankylosis	53.81 ± 6.24$^{\diamond\nabla}$	45.68 ± 6.17$^{\diamond\nabla}$	46.51 ± 6.18	7.43 ± 1.51	18.54 ± 4.71$^{\diamond\nabla}$	25.96 ± 5.41$^{\diamond\nabla}$
Before ankylosis in the SNI + AEA group	40.59 ± 6.35	42.73 ± 5.84	45.62 ± 6.37	7.62 ± 1.74	10.58 ± 2.36	19.84 ± 5.02
After ankylosis	58.16 ± 5.72$^{\square\bullet}$	51.58 ± 6.92$^{\star\bullet}$	54.72 ± 6.64$^{\star\bullet}$	7.91 ± 1.53	18.86 ±5.16$^{\star\bullet}$	23.27 ± 6.63$^{\square\bullet}$
Before ankylosis in the sham SNI + AEA group	41.29 ± 5.46	42.67 ±5.47	43.32 ± 5.06	7.59 ± 1.81	10.32 ± 3.42	20.65 ± 5.58
After ankylosis	44.68 ± 5.29*	43.92± 6.38	45.83 ± 5.37	7.98 ± 1.69	15.28 ± 3.51*	22.82± 5.74*
Before ankylosis in the SNI + D-AP5 group	40.73 ± 4.48	40.42 ± 5.16	43.57 ± 5.29	7.64 ± 1.84	10.81 ± 4.16	20.29 ± 6.24
After ankylosis	58.85 ± 8.37$^{\blacktriangle\bullet}$	62.49 ± 8.52$^{\divideontimes\bullet}$	60.24 ± 9.15$^{\divideontimes\bullet}$	8.05 ± 1.49	16.75 ± 3.24$^{\divideontimes\bullet}$	23.52 ± 6.09$^{\divideontimes\bullet}$
Before ankylosis in the sham SNI + D-AP5 group	41.25 ±6.13	41.06 ± 5.72	43.82 ± 6.63	7.29 ± 1.53	10.72 ± 3.27	20.58 ±6.85
After ankylosis	49.59 ± 6.31$^{\blacktriangle}$	42.18 ± 7.42	44.59 ± 7.35	7.81 ± 1.84	13.94 ± 4.58$^{\blacktriangle}$	22.17 ± 5.57$^{\blacktriangle}$
Before ankylosis in the SNI + CNQX group	41.83 ± 6.51	42.06 ± 6.27	44.28 ± 6.51	7.52 ± 1.41	10.89 ± 2.59	20.15 ± 5.27
After ankylosis	60.72 ± 7.84$^{\#\bullet}$	57.41 ± 7.85$^{\blacksquare\bullet}$	55.28 ± 8.31$^{\blacksquare\bullet}$	7.89 ± 1.82	15.29 ± 4.82$^{\blacksquare\bullet}$	24.52 ± 6.75$^{\blacksquare\bullet}$
Before ankylosis in the sham SNI + CNQX group	40.75 ± 6.04	41.94 ± 6.31	43.36 ± 6.84	7.94 ± 1.58	10.68 ± 4.39	20.27 ± 6.31
After ankylosis	55.49 ± 6.64$^{\blacksquare\blacktriangledown}$	42.86 ± 4.83$^{\blacksquare}$	44.75 ± 3.26	8.15 ± 1.72	13.49 ± 3.82$^{\blacksquare}$	23.28 ± 5.85$^{\blacksquare}$
Before ankylosis in the SN I + D-AP5 + AEA group	40.58 ± 6.71	41. 28 ± 6.18	45.09 ± 6.28	7.17 ± 1.86	11.42 ± 3.52	20.82 ± 4.6
After ankylosis	44.29 ± 6.26	43.46 ± 6.58	57.82 ± 6.71$^{\blacklozenge\bullet}$	7.98 ± 1.74	12.42 ± 3.62	22.49 ± 3.62
Before ankylosis in the sham SNI + D-AP 5 + AEA group	40.72 ± 6.48	42.26 ± 6.81	43.25 ± 6.39	7.14 ± 1.26	10.1 ± 2.26	13.26 ± 3.8
After ankylosis	41.25 ± 6.28	43.41 ± 6.59	44.08 ± 7.46	7.36 ± 1.67	12.62 ± 2.28	21.73 ± 4.58
Before ankylosis in the SNI CNQX + AEA group	40.47 ± 6.39	41.58 ± 5.82	42.06 ± 4.73	7.21 ± 1.28	10.27 ± 2.51	20.61 ± 5.09
After ankylosis	45.27 ± 6.46$^{\blacklozenge\bullet}$	43.18 ± 6.05	43.52 ± 6.38	7.39 ± 1.69	10.86 ± 3.68	20.89 ± 5.57
Before ankylosis in the sham SNI + CNQX + AEA group	41.85 ± 6.38	40.86 ± 6.72	43.32 ± 6.82	7.35 ± 1.59	10.12 ± 2.62	20.36 ± 3.75
After ankylosis	43.63 ± 6.76	43.27 ± 6.85	44.93 ± 7.62	7.42 ± 1.82	10.65 ± 3.86	20.82 ± 3.91

SNI and sham SNI groups: $^{\diamond}$, $p < 0.01$: SNI (sham) groups vs. SNI (sham) + AEA, SNI (sham) + DAP5, SNI (sham) + CNQX, SNI (sham) + DAP5 + AEA, and SNI (sham) + CNQX + AEA groups. *, $p < 0.05$; $^{\square}$, $p < 0.01$: SNI (sham) + AEA groups vs. SNI (sham) + D-AP5, SNI (sham) + CNQX, SNI (sham) + DAP5 + AEA, and SNI (sham) + CNQX + AEA groups. $^{\blacktriangle}$, $p < 0.05$; $^{\divideontimes}$, $p < 0.01$: SNI (sham) + D-AP5 groups vs. SNI (sham) + CNQX, SNI (sham) + DAP5 + AEA, and SNI (sham) + CNQX + AEA groups. $^{\blacksquare}$, $p < 0.05$, $^{\#}$, $p < 0.01$: SNI (sham) + CNQX vs. SNI (sham) + DAP5 + AEA and SNI (sham) + CNQX + AEA groups. $^{\blacklozenge}$, $p < 0.05$: SNI + DAP5 + AEA vs. SNI + CNQX + AEA groups. Sham SNI groups: SNI group vs. sham SNI group: $^{\bullet}$, $p < 0.01$ vs. sham groups.

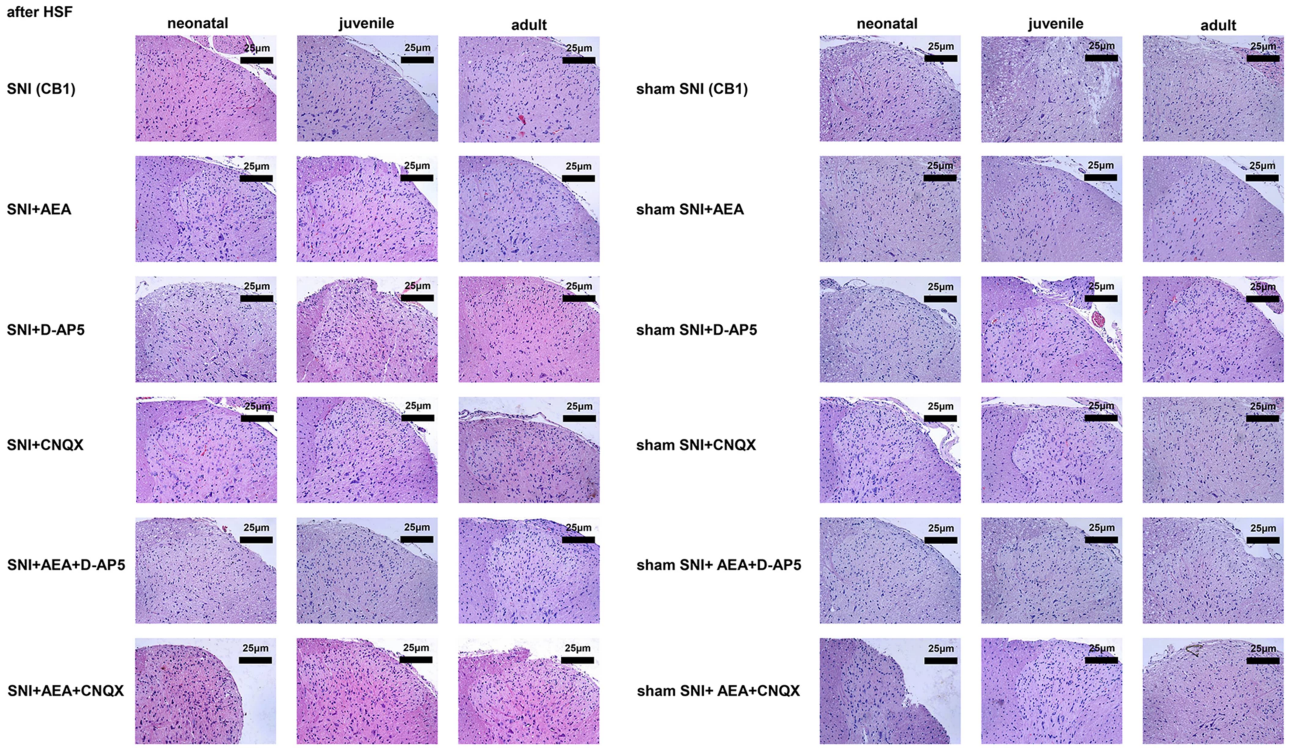

Fig. 4. Histological analysis of samples collected before or after SNI in rats of different ages under various treatments.

The CB1 signals in the SNI groups of neonatal, juvenile, and adult rats were bright red and dark brown and significantly higher than those of the corresponding sham SNI groups on day 14 ($p < 0.05$). After treatment with CNQX or D-AP5, the red and dark brown signals became lighter, indicating decreased expression levels of CB1, which was further decreased by AEA. Strikingly, after treatment with D-AP5 and AEA, the CB1 levels were significantly lower in neonatal rats than the SNI + AEA, SNI + D-AP5, SNI + CNQX, and SNI + CNQX + AEA groups ($p < 0.05$ or 0.01). After treatment with CNQX or D-AP5 and AEA, the expression levels of CB1 in juvenile rats were significantly lower than those of the SNI + AEA, SNI + D-AP5, SNI + CNQX, SNI + D-AP5 + AEA, and SNI + CNQX + AEA groups ($p < 0.05$ or 0.01). After treatment with CNQX and AEA, the expression levels of CB1 in adult animals were significantly lower than those of the SNI + AEA, SNI + D-AP5, SNI + CNQX, and SNI + CNQX + AEA groups ($p < 0.05$ or 0.01) (Table 4, Fig. 5). After treatment with CNQX or D-AP5 and AEA, the CB1 levels in the sham SNI + DAP5 + AEA and sham SNI + CNQX + AEA groups were significantly lower than the corresponding SNI groups ($p < 0.05$).

3.4 Expression Levels of CB1 in LTP Formation after SNI as Determined by Western Blot

Western blot was performed to detect the level of CB1 protein in rats after treatment with AEA, CNQX, and D-AP5 at 14 days after SNI. The changes in CB1 levels were consistent with those detecte3d by immunohistochemistry analysis (Fig. 6). The CB1 protein levels were normalized against the GAPDH density, an internal control (Table 5).

4. Discussion

The present study showed that activated CB1 receptors in neonatal rats produce analgesic effects, mainly through NMDA receptors giving rise to the inhibitory activity of FPs. In juvenile and adult rats, this effect was produced through NMDA and AMPA receptors, respectively.

The endogenous cannabinoid system controls the activity of NMDA receptor [16], and CB1 activation produces lasting functional changes in glutamic acid metabolism. The discovery of the endogenous cannabinoid AEA [17] also confirmed the existence of an endogenous cannabinoid system in the body. Subsequently, two endogenous cannabinoid receptors, CB1 and CB2, were cloned; both are G-protein coupled receptors (GPRs). Cannabinoid-derived CB1 receptors are one of the leading targets in the treatment of chronic pain, and the antinociceptive effects of endogenous and exogenous cannabinoids effectuate via CB1 receptors and are widely present in the central and peripheral nervous systems. The immunostaining of CB1 in the dorsal horn of the spinal cord revealed that the receptors are expressed on the internal axons of spinal neurons and that the main sites of antinociceptive action are the A and C fibers of the dorsal horn of the spinal cord. The endogenous ligand concentrations of cannabinoid receptors at 1 mM inhibited mEPSCs, and cannabinoid-activated receptors reduced the synaptic activity.

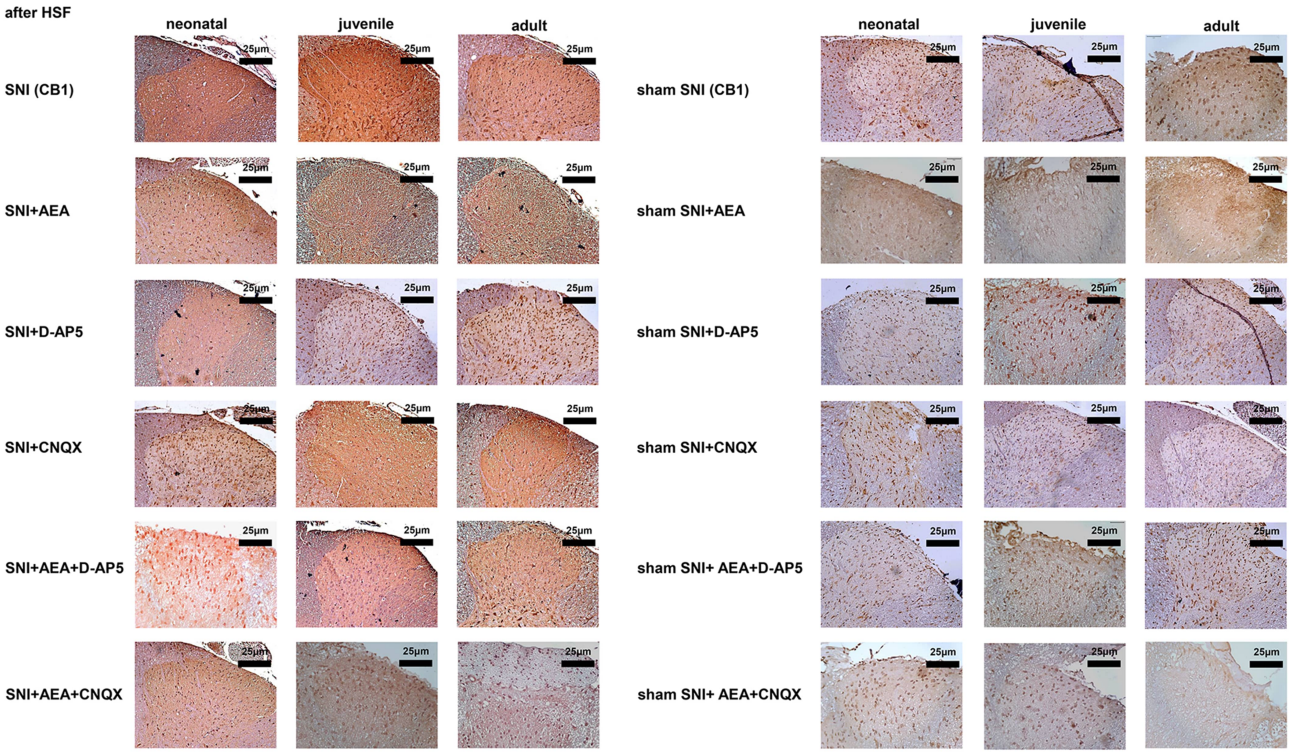

Fig. 5. CB1 protein levels detected by immunohistochemistry in samples collected before or after SNI in rats of different ages under various treatments.

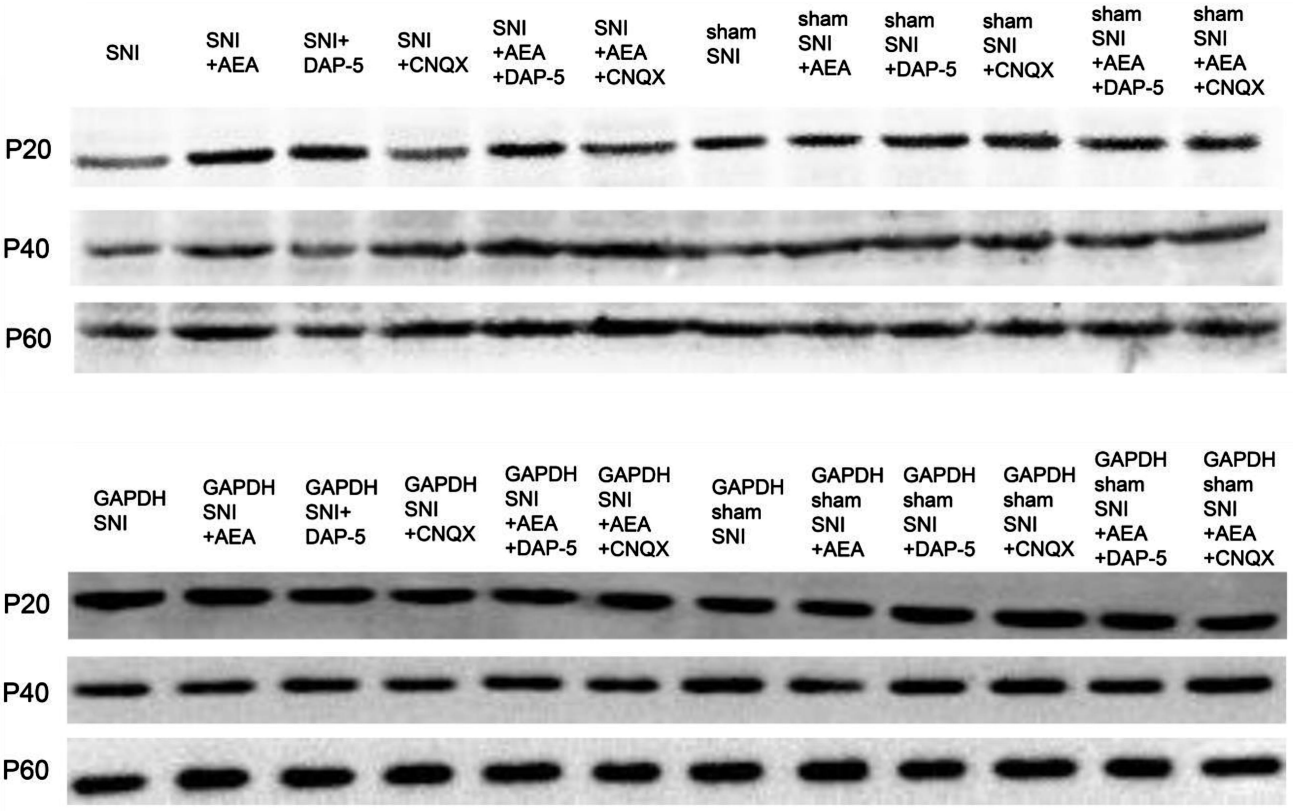

Fig. 6. CB1 protein levels detected by Western blot in samples collected before or after SNI in rats of different ages under various treatments.

Table 4. Immunohistochemistry of CB1 receptor expression in the SNI spinal dorsal horn of rats of different ages during LTP formation (OD value of positive signals).

	Neonatal	Juvenile	Adult		Neonatal	Juvenile	Adult
SNI	$120.48 \pm 44.61^{\blacklozenge\bullet}$	124.69 ± 34.28	130.84 ± 35.18	sham SNI	102.25 ± 31.75	$105.24 \pm 36.41^{\triangledown}$	$108.51 \pm 39.24^{\triangledown}$
SNI + AEA	$149.75 \pm 32.74^{\#\bullet}$	$152.54 \pm 23.82^{\blacksquare\bullet}$	$159.16 \pm 36.47^{\blacksquare\bullet}$	sham SNI + AEA	104.57 ± 33.52	$106.52 \pm 31.64^{\triangledown}$	$102.72 \pm 36.58^{\triangledown}$
SNI + D-AP5	$144.86 \pm 38.65^{\blacklozenge\bullet}$	135.28 ± 53.49	138.63 ± 32.91	sham SNI + D-AP5	106.52 ± 32.24	102.79 ± 39.43	101.08 ± 33.46
SNI + CNQX	$149.86 \pm 40.76^{\blacktriangle\bullet}$	$153.94 \pm 55.27^{\bullet}$	$156.72 \pm 43.74^{\divideontimes\bullet}$	sham SNI + CNQX	104.65 ± 37.05	108.48 ± 34.29	109.14 ± 31.75
SNI + D-AP5 + AEA	$159.85 \pm 37.9^{\square\bullet}$	$151.19 \pm 42.15^{\star\bullet}$	$147.24 \pm 45.61^{\star\bullet}$	sham SNI + AEA + D-AP5	103.53 ± 38.41	101.36 ± 36.18	100.29 ± 44.26
SNI + CNQX + AEA	$160.28 \pm 60.04^{\diamond\bullet}$	$171.49 \pm 51.67^{\diamond\bullet}$	$175.83 \pm 42.18^{\diamond\bullet}$	Sham SNI + AEA + CNQX	100.73 ± 32.54	102.06 ± 31.72	104.86 ± 36.92

SNI group, SNI group and sham group: $^{\diamond}$, $p < 0.01$: SNI group vs. SNI + AEA, SNI + DAP5, SNI + CNQX, SNI + DAP5 + AEA, and SNI + CNQX + AEA groups. *, $p < 0.05$; $^{\square}$, $p < 0.01$: SNI + AEA group vs. SNI + D-AP5, SNI + CNQX, SNI + DAP5 + AEA, and SNI + CNQX + AEA groups. $^{\blacktriangle}$, $p < 0.05$; $^{\divideontimes}$, $p < 0.01$: SNI + D-AP5 group vs. SNI + CNQX, SNI + DAP5 + AEA, and SNI + CNQX + AEA groups. $^{\blacksquare}$, $p < 0.05$, $^{\#}$, $p < 0.01$: SNI + CNQX group vs. SNI + DAP5 + AEA and SNI + CNQX + AEA groups. $^{\blacklozenge}$, $p < 0.05$: SNI + DAP5 + AEA group vs. SNI + CNQX + AEA group. Sham SNI test groups: $^{\triangledown}$, $p < 0.05$, sham SNI group vs. sham SNI + DAP5 + AEA and sham SNI + CNQX + AEA groups; $^{\bullet}$, $p < 0.01$, SNI group vs. sham SNI group vs. sham groups.

Table 5. CB1 levels assessed by Western blot in the formation of LTP in the spinal dorsal horn of rats of different ages.

	Neonatal	Juvenile	Adult		Neonatal	Juvenile	Adult
SNI	$0.54 \pm 0.07^{\diamond\bullet}$	$0.59 \pm 0.12^{\diamond\bullet}$	$0.67 \pm 0.16^{\diamond\bullet}$	sham SNI	0.62 ± 0.12	0.60 ± 0.08	0.61 ± 0.14
SNI + AEA	$0.98 \pm 0.18^{\star\bullet}$	$1.32 \pm 0.19^{\star\bullet}$	$1.50 \pm 0.24^{\star\bullet}$	sham SNI + AEA	0.61 ± 0.12	0.65 ± 0.10	0.68 ± 0.15
SNI + D-AP5	$0.84 \pm 0.13^{\blacktriangle\bullet}$	$0.42 \pm 0.08^{\divideontimes\bullet}$	$0.62 \pm 0.05^{\divideontimes\bullet}$	sham SNI + D-AP5	0.62 ± 0.14	0.61 ± 0.18	0.63 ± 0.15
SNI + CNQX	$0.78 \pm 0.08^{\#\bullet}$	$1.03 \pm 0.21^{\blacksquare\bullet}$	$1.35 \pm 0.38^{\blacksquare\bullet}$	sham SNI + CNQX	0.64 ± 0.16	0.62 ± 0.18	0.64 ± 0.15
SNI + D-AP5 + AEA	1.25 ± 0.18	0.99 ± 0.14	$0.94 \pm 0.23^{\blacklozenge\bullet}$	sham SNI + D-AP5 + AEA	0.69 ± 0.18	0.64 ± 0.16	0.62 ± 0.13
SNI + CNQX + AEA	$0.89 \pm 0.18^{\blacklozenge\bullet}$	2.06 ± 0.21	2.92 ± 0.25	sham SNI + CNQX + AEA +	0.63 ± 0.17	0.66 ± 0.13	0.68 ± 0.14

SNI group, SNI group and sham group: $^{\diamond}$, $p < 0.01$: SNI group vs. SNI + AEA, SNI + DAP5, SNI + CNQX, SNI + DAP5 + AEA, and SNI + CNQX + AEA groups. *, $p < 0.05$; $^{\square}$, $p < 0.01$: SNI + AEA group vs. SNI + D-AP5, SNI + CNQX, SNI + DAP5 + AEA, and SNI + CNQX + AEA groups. $^{\blacktriangle}$, $p < 0.05$; $^{\divideontimes}$, $p < 0.01$: SNI + D-AP5 group vs. SNI + CNQX, SNI + DAP5 + AEA, and SNI + CNQX + AEA groups. $^{\blacksquare}$, $p < 0.05$; $^{\#}$, $p < 0.01$: SNI + CNQX group vs. SNI + DAP5 + AEA and SNI + CNQX + AEA groups. $^{\blacklozenge}$, $p < 0.05$: SNI + DAP5 + AEA group vs. SNI + CNQX + AEA group. Sham groups: SNI group vs. sham SNI group: $^{\bullet}$, $p < 0.01$ vs. sham groups.

Endogenous cannabinoid signals are key regulators of synaptic transmission in the brain. AEA is a partial agonist of CB1 and CB2 receptors [18] and a seven-time transmembrane Gi/o type GPR. The inhibitory effect of AEA on mEPSCs could be blocked completely by the selective CB1 receptor antagonist SR141716A; therefore, endogenous cannabinoids maintain the inhibitory effects in the developing spinal cord [19]. The present study provided evidence that cannabinoids mediate presynaptic inhibition of excitatory synapses and nociceptive input in the superficial dorsal layer of the spinal cord, which might be one of the mechanisms of analgesia. CB1 receptor in the central nervous system is activated and other cannabinoid-like effects are produced or the degradation of the endogenous cannabinoid system is inhibited. Therefore, activated CB1 receptors inhibit the development of spinal cord plasticity (FPs) by regulating the content of NMDA in neonatal and juvenile rats.

D-AP5 is a specific inhibitor of NMDA receptor. The primary characteristics of D-AP5 in adult rats are rapid desensitization of agonists and a long-term inactivation phase. However, the NMDA receptors have less activated and weakened AMPA receptor activation stages in neonatal and juvenile rats than in adult rats, which might be related to the developmental process of rats [20]. The excitatory amino acids participate in complex physiological processes and synaptic plasticity changes of the central nervous system through NMDA and AMPA receptors, including the induction and maintenance of LTP. The NMDA receptor activation induces LTP [21], which in turn enhances the excitatory postsynaptic potential. Thus, it could be speculated that ion channels, including AMPA and NMDA receptor channels, mediating this potential should be modified chemically. Only then the receptor conductivity could be changed, enhancing the postsynaptic potential. During LTP induction in adult rats, the sensitivity of NMDA and AMPA receptors to glutamic acid increases rapidly [22], which could be related to the phosphorylation at tryptophan, threonine, and tyrosine residues in the receptors. Several protein kinases can phosphorylate the receptors; for example, PKC phosphorylates both NMDA and AMPA receptors. The increased sensitivity of NMDA and AMPA receptors is also related to synaptic structure changes [23]; Ca^{2+} influx and its activated cascade reaction, especially the interaction between PKC and calmodulin, alters the composition of cytoskeleton, changes the synaptic structure, increases the synaptic surface area and postsynaptic membrane density, and enhances prominent plasticity in various chronic animal models. AMPA receptor activity is higher than that of NMDA in SNI adult rats. However, AMPA receptors in young and newborn rats are imperfectly developed, and MNDA receptors are active due to continuous stimulation and excitation. CNQX represents the most frequently applied AMPA receptor antagonist in the CNS [24]. In the present study, the LTP levels of the A and C nerves were relieved after D-AP5 or CNQX treatment in young and adult animals, with a pronounced blocking effect observed with CNQX, indicating a higher involvement of AMPA compared to NMDA in this process.

In this study, neuropathic pain models at different ages were studied. PWT, LTP, and CB1 immunohistochemistry and Western blot signals were stronger in juvenile rats than in neonatal and adult animals; also, the stress and pain were controlled by NMDA receptor blockers in neonatal and juvenile rats. Nonetheless, the current study only analyzed the above parameters from a performance perspective but no positive or negative controls in terms of chemical compounds. Such deficiencies would be avoided and improved in the future studies, and additional experiments are required to determine all the differences.

5. Conclusions

CB1 receptor activation in neonatal rats produced analgesic effects through NMDA receptor to realize FP inhibitory effects. Conversely, in juvenile and adult animals, FPs were mainly inhibited through the activation of NMDA and AMPA receptors. Nonetheless, the receptor ratio was altered during development.

Abbreviations

FP, field potential; GPR, G protein coupled receptor; LTD, long-term depression; LTP, long-term potentiation; OD, optical density; PWT, paw withdrawal threshold; SNI, spinal nerve preservation injury; AEA, Anandamide; TRPV1, transient receptor potential cation channel, subfamily V, member 1; NMDA, N-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid.

Author Contributions

JC—Experiment operation, Manuscript writing and Editing; XC—Experiment operation and Data collation; HX, FK, LW—Data collation; LZ—Supervise; JW—Experimental operation and Review. All authors have read and agreed to the published version of the manuscript.

Ethics Approval and Consent to Participate

The animals were purchased from Unilever in Beijing, and the experiments were conducted at the Laboratory A III of Center for Animal Experiment of Wuhan University (Approval Number: 2019073).

Acknowledgment

Not applicable.

Funding

This work was supported by the Hubei Provincial Health and Family Planning Commission (grant number WJ2019F008).

Conflict of Interest

The authors declare no conflict of interest.

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