·基础研究·

电针督脉/夹脊穴可激活腰髓神经元提高脊髓损伤大鼠后肢运动功能

廖怡栋[1]，柯茗瀚[1]，田　淼[1]，李贝宁[1]，何冠蘅[2]，赖碧琴[1]，曾园山[1]，丁　英[1]
（1. 中山大学中山医学院，广东 广州 510080；2. 中山大学附属第一医院针灸科，广东 广州 510080）

摘　要：【目的】探究电针督脉穴或夹脊穴对全横断脊髓损伤大鼠腰髓神经元激活和瘫痪后肢运动功能恢复的影响。【方法】选取成年SD雌性大鼠20只，其中15只大鼠执行第10胸髓全横断损伤，随机分为对照（Control）组，电针督脉穴（GV）组和电针夹脊穴（JJ）组，每组5只；另外5只则为正常（Normal）组。两种电针组大鼠于术后3 d开始电针治疗，隔天治疗，每周进行Basso-Beattie-Bresnahan（BBB）行为学评分；术后8周，执行电生理皮质运动诱发电位（CMEP）检测，并灌注固定取材、冰冻切片、免疫荧光染色实验。【结果】①两种电针治疗都能激活腰髓第1节段（L1）的脊髓神经元表达兴奋性标记物c-Fos，c-Fos阳性（c-Fos$^+$）神经元主要分布在脊髓的Ⅳ、Ⅴ和Ⅶ板层，而Normal组和Control组脊髓内未见c-Fos$^+$神经元；GV组和JJ组电针激活的c-Fos$^+$神经元百分率分别为（10.23±0.15）%和（10.77±1.12）%，且两电针组无显著性差异。②vGluT1$^+$本体感觉神经纤维主要分布在L1脊髓灰质背角和中间带的Ⅶ板层。在L1脊髓背角，GV组和JJ组vGluT1$^+$神经纤维相对荧光强度大于Control组，且JJ组大于GV组；在L1脊髓中央模式发生器（CPG，支配后肢节律运动的中间神经元）所在的Ⅶ板层，GV组和JJ组的vGluT1$^+$神经纤维相对荧光强度大于Control组，且JJ组也大于GV组，并发现GV组和JJ组vGluT1$^+$神经纤维终末支配Ⅶ板层神经元并共表达兴奋性标记物c-Fos。③各组L4脊髓腹角内的乙酰胆碱酯酶（ChAT）免疫荧光染色结果显示，GV组（19.1±3.4，P=0.000 2）和JJ组（19.0±2.9，P=0.000 2）的ChAT$^+$运动神经元存活数多于Control组（9.9±1.9）。④在各组L4脊髓腹角内，与ChAT$^+$运动神经元胞体接触的vGluT2$^+$兴奋性轴突终末数、vGAT$^+$抑制性轴突终末数、vGluT2$^+$/vGAT$^+$轴突终末数比值［即反映运动神经元的兴奋/抑制（E/I）平衡］的结果分析显示，GV组和JJ组的这三个指标的数值都高于Control组，且两电针组的兴奋/抑制性轴突终末与运动神经元胞体接触的E/I比值逐渐恢复正常并与Normal组无统计学差异。⑤电生理和行为学检测结果显示，与Control组比较，GV组和JJ组的大鼠CMEP潜伏期缩短，而其波幅增高；大鼠瘫痪后肢行为学BBB评分显示，治疗8周后，GV组（4.25±1.26，P=0.023 4）和JJ组（4.75±0.96，P=0.000 2）的评分均高于Control组（1.80±1.30），但两电针组没有统计学差异。【结论】电针GV或JJ穴位均可能通过促进脊髓内本体感觉信息传入，激活L1脊髓Ⅶ板层的CPG神经元；并促进L4脊髓运动神经元存活及其兴奋/抑制平衡恢复，提高大鼠瘫痪后肢的运动功能。

关键词：脊髓损伤；电针；督脉穴；夹脊穴；运动功能
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Electroacupuncture at the Governor Vessel / Jiaji Acupoints Can Activate Lumbar Spinal Cord Neurons to Improve the Motor Function of the Hind Limbs in Rats with Spinal Cord Injury

LIAO Yidong[1], KE Minghan[1], TIAN Miao[1], LI Beining[1], HE Guanheng[2], LAI Biqin[1], ZENG Yuanshan[1], DING Ying[1]
（1. Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou 510080, China; 2. Department of Acupuncture,

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作者简介：廖怡栋，第一作者，研究方向：电针联合神经干细胞修复脊髓损伤研究，E-mail：liaoyd6@mail2.sysu.edu.cn；丁英，通信作者，教授，研究方向：电刺激/电针联合类脊髓组织修复脊髓损伤的机制研究，E-mail：dingying@mail.sysu.edu.cn

The First Affiliated Hospital of Sun Yat-sen University, Guangzhou 510080, China)
Correspondence to: DING Ying; E-mail: dingying@mail.sysu.edu.cn

Abstract:【Objective】To explore the effects of electroacupuncture at governor vessel or Jiaji acupoints on the activation of lumbar spinal cord neurons and the recovery of motor function in paralyzed hind limbs in rats with complete transection of spinal cord injury.【Methods】Twenty adult female SD rats were selected. Among them, 15 rats underwent the complete transection of the 10th thoracic spinal cord and were randomly divided into the Control group, the electroacupuncture at governor vessel acupoints (GV) group and the electroacupuncture at Jiaji acupoints (JJ) group, with 5 rats in each group. The other 5 rats served as the Normal group. Electroacupuncture treatment was initiated 3 days after surgery in the GV and JJ groups and performed every other day. Basso-Beatti-Bresnahan (BBB) behavioral score was evaluated weekly. Eight weeks after the operation, cortical motor evoked potential (CMEP) detection was performed, followed by perfusion fixation, tissue harvest, frozen sectioning, and immunofluorescence staining experiments.【Results】① Both electroacupuncture treatments activated the expression of the excitatory marker c-Fos in L1 spinal cord neurons. The c-Fos$^+$ activated neurons were mainly distributed in lamina Ⅳ, Ⅴ and Ⅶ of the L1 spinal cord, while no c-Fos$^+$ neuron was observed in the Normal group and the Control group. The percentages of c-Fos$^+$ neurons activated by electroacupuncture in the GV group and the JJ group were (10.23±0.15) % and (10.77±1.12) %, respectively, and there was no significant difference between the two electroacupuncture groups. ② vGluT1$^+$ proprioceptive afferent nerve fibers were mainly distributed in the dorsal horn and lamina Ⅶ of intermediate zone of L1 spinal cord gray matter. In the dorsal horn of L1 spinal cord, the relative fluorescence intensity of vGluT1$^+$ nerve fibers in the GV group and the JJ group were greater than that in the Control group, and that in the JJ group was greater than that in the GV group. In the lamina Ⅶ in intermediate zone of L1 spinal cord, where the central pattern generator (CPG) for hindlimb rhythmic movement is located, the relative fluorescence intensity of vGluT1$^+$ nerve fibers in the GV group and the JJ group were significantly higher than that in the Control group, and the JJ group was also higher than the GV group; The lamina Ⅶ neurons innervated by vGluT1$^+$ axon terminals were activated to express the excitatory marker c-Fos in the GV group and the JJ group. ③ The immunofluorescence staining results of acetylcholinesterase (ChAT) in the ventral horn of L4 spinal cord of each group showed that the number of surviving ChAT$^+$ motor neurons in the GV group (19.1±3.4, P=0.000 2) and JJ group (19.0±2.9, P=0.000 2) were higher than that in the Control group (9.9±1.9). ④ The quantitative analysis of the vGluT2$^+$ excitatory axonal terminals, vGAT$^+$ inhibitory axonal terminals, and the ratio of vGluT2$^+$/vGAT$^+$ axonal terminals [reflecting the excitation/inhibition (E/I) balance of motor neurons] innervating ChAT$^+$ motor neurons in the ventral horn of the L4 spinal cord revealed that all three indices were significantly higher in the GV and JJ groups than in the Control group. Moreover, the E/I ratio of motor neurons in the two electroacupuncture groups gradually reached the same level as normal and showed no statistical difference from the Normal group. ⑤ The electrophysiological and behavioral detection results showed that compared with the Control group, the latencies of CMEP in the GV group and the JJ group shortened, while their amplitudes increased. The BBB score of paralyzed hindlimbs showed that after 8 weeks of treatment, the scores of both the GV group (4.25±1.26, P=0.023 4) and the JJ group (4.75±0.96, P=0.000 2) were higher than those of the Control group (1.80±1.30), but there was no statistical difference between the two electroacupuncture groups.【Conclusion】Electroacupuncture at GV or JJ acupoints may promote the afferent of vGluT1$^+$proprioceptive nerve fibers into the spinal cord, indicating the possibility to activate CPG neurons in lamina Ⅶ of L1 spinal cord; facilitate the survival of motor neurons in the L4 spinal cord and restore their excitatory/inhibitory balance, improving the motor function of the paralyzed hindlimbs in rats.

Key words: spinal cord injury; electroacupuncture; governor vessel acupoints; Jiaji acupoints; motor function
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脊髓损伤(spinal cord injury, SCI)是一种高致残性中枢神经系统创伤性疾病,其中全横断SCI可直接中断损伤平面以下的运动、感觉及自主神经传导通路,引发神经元大量凋亡、炎症反应等一系列的继发性损伤,导致肢体瘫痪、大小便失禁等严重后遗症[1]。在中医经典古籍中将SCI归为“痿证”

“体惰”,对其治疗方法除中药方剂外,针灸是古籍记载中最为行之有效并被现代医学广为应用的治疗方式。电针督脉穴与夹脊穴是临床治疗SCI患者的首选方案[2],但其现代医学机制尚未完全阐明,是目前针灸学科迫切需要验证的重要理论之一。前期研究证实,督脉(governor vessel,GV)穴电针治疗全横断SCI可以减轻炎症,促进脊髓神经元的存活及轴突再生[3],并防止后肢肌肉萎缩,进而促进瘫痪后肢运动功能的改善[4];并揭示电针GV穴可通过刺激穴位内的神经末梢,促进背根节的传入神经末梢在脊髓内释放降钙素基因相关肽(calcitonin gene-related peptide,CGRP),作用于表达其受体的脊髓神经元,激活钙/钙调蛋白-依赖性蛋白激酶通路促进神经元合成和分泌神经营养因子-3(neurotrophin-3,NT-3)增多,促进受损伤脊髓神经元的存活及其轴突再生[5]。夹脊(Jiaji, JJ)穴作为另一种临床常用的治疗SCI的电针穴位,其穴位位置恰好紧邻督脉穴,即在脊柱背侧的督脉穴同一水平面的左右两侧。研究发现,电针夹脊穴亦能通过多通路调控促进SCI大鼠瘫痪后肢运动功能的恢复[6-8]。虽然胸段脊髓全横断SCI导致脊髓的上下行神经传导通路中断,但是腰段脊髓的中央模式发生器(central pattern generator,CPG)和后肢运动环路的结构基础仍然完整,因此,重新激活腰段脊髓CPG及运动环路的兴奋状态,可促进瘫痪后肢的运动功能恢复。Asboth等[9]研究表明,本体感觉传入增强对SCI后运动功能恢复至关重要。然而,电针GV或JJ穴是否通过增强本体感觉传入、激活腰段脊髓神经元、调控支配运动神经元的兴奋/抑制平衡,从而促进全横断SCI大鼠瘫痪后肢的运动功能改善?因此,本研究旨在探究电针GV穴或JJ穴对全横断SCI大鼠腰段脊髓神经元激活、运动神经元的兴奋/抑制平衡和后肢运动功能恢复的影响,为阐明电针GV或JJ经穴促进SCI运动功能恢复的机制提供实验和理论依据。

1 材料与方法

1.1 仪器与试剂

低频电子脉冲治疗仪(G6805-2A,上海华宜医用仪器有限公司);体式显微镜(Carl Zeiss,德国);冰冻切片机(Thermofisher NX50,美国);全自动倒置荧光显微镜(Leica,DM6B,德国);激光共聚焦显微镜(Zeiss,LSM800,德国);Alexa Fluor® 647 Anti-Synaptophysin antibody(abcam,ab196166,1∶500),Rabbit-anti-c-Fos antibody(sysy,226008,1∶2000),Mouse-anti-NeuN antibody(abcam,ab104224,1∶500),Goat-anti-ChAT-antibody(merck,ab144p,1∶250),Mouse-anti-vGAT-antibody(sysy,131011,1∶500),Rabbit-anti-vGluT2-antibody(sysy,135403,1:200),Fluo Tag®-X2 anti-vGluT1 Atto488(Nanotag,N1602-At488-L,1∶500),羊抗兔Alexa Fluor488(abcam,ab150077,1∶1 000),山羊抗兔Alexa Fluor555(abcam,ab150078,1∶1 000),羊抗兔Alexa Fluor647(abcam,ab150083,1∶500),羊抗鼠Alexa Fluor555(abcam,ab150114,1∶1 000),山羊抗鼠Alexa Fluor647(abcam,ab150119,1∶500),驴抗山羊Alexa Fluor488(abcam,ab150129,1∶1 000)。

1.2 实验动物和分组

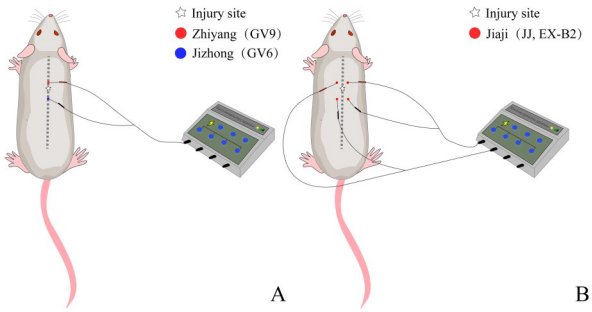
实验动物选用20只7~8周龄SPF级雌性SD大鼠,体质量200~250 g,均由中山大学实验动物中心提供,实验动物许可证号为SCXK(粤)2021-0029。大鼠饲养于恒温(22±2)℃、12 h光照/12 h黑暗循环周期的环境中,可自由进食和饮水。所有实验方案及操作均按照《实验动物福利伦理审查指南(GB/T35892-2018)》执行,并通过中山大学实验动物伦理委员会批准(伦理批号:SYSU-IACU-2023-B0741)。15只大鼠行第10胸段脊髓(T10)全横断脊髓损伤,随机分为对照(Control)组,电针督脉穴(GV)组和电针夹脊穴(JJ)组,每组5只;另外5只则为正常(Normal)组。

1.3 全横断脊髓损伤模型建立

采用10 g/L戊巴比妥钠(0.4 mL/100 g)腹腔注射麻醉大鼠,剃毛备皮。以第9胸椎为中心,沿背纵切1.5 cm切口,逐层分离肌肉,暴露棘突,用咬骨钳小心去除椎板,暴露脊髓,用直尖小梁剪剪开硬脊膜后,再快速全横断T10胸段脊髓,显微镜下检测脊髓是否完全横断,并用直尖显微剪修剪保证完全横断脊髓,并截取2 mm脊髓节段出来,充分止血后,在缺损处填入2 mm的脱细胞视神经支架(decellularized optic nerves, DON)[10]。移植后,依肌层、皮下组织、皮肤顺序逐层缝合。术后每只动物连续3 d肌肉注射青霉素16万单位1 mL/d,每天人工排尿3~4次,动物术后饲养8周后进行灌注取材,期间给予保温和充分的饮食。

1.4　电针治疗

各电针组大鼠在术后第3天开始，隔天进行电针治疗（图1）。电针治疗时长为8周，共需电针28次。GV组取穴为至阳穴和脊中穴，分别位于T7~T8和T11~T12的胸椎棘突之间；JJ组取穴分别位于T7~T8胸椎棘突和T11~T12胸椎棘突中线旁开左右侧各3 mm处（大约为横突处）[11]。用针灸针通过捻针法进针，进针深度约为5 mm。然后将针灸针与低频电子脉冲治疗仪的输出线末端的电极连接。刺激频率为疏密波（50 Hz为1.05 s，2 Hz为2.85 s交替进行），刺激强度以观察到动物全身肌肉轻微颤动为度，电流≤1 mA，留针时间为20 min。



A: The schematic diagram of electroacupuncture at the GV acupoints in the SCI rat; B: The schematic diagram of electroacupuncture at the JJ acupoints in the SCI rat. GV: governor vessel; JJ: Jiaji.

图1　电针督脉穴与夹脊穴的模式图

Fig. 1　Schematic diagram of electroacupuncture at governor vessel（GV）acupoints and Jiaji（JJ）acupoints

1.5　行为学评估

术后每周分别进行行为学检测，首次检测是术后第7天（1周），末次是术后第56天（8周），一共检测了8次。Basso-Beattie-Bresnahan（BBB）评分规则参照Basso等提出的BBB评分法进行评估[12]。BBB评分时，让大鼠在开阔平地上自由爬行5 min，观察其后肢的运动情况并按照BBB评分标准进行评分。主要观察的内容包括：后肢3个关节（髋关节、膝关节和踝关节）的运动幅度和运动频率以及是否具有支撑体质量，尾部是否有运动，以及能否具有偶尔或持续的前后肢协调运动等。记录细节后，根据BBB评分表评分。

1.6　电生理检测

在术后8周，行为学BBB评分检测完后，各组实验动物进行磁刺激的大脑皮层运动诱发电位（cortical motor evoked potential，CMEP）检测。此项检测在大鼠清醒状态下进行，采用CCY-I型磁刺激治疗仪，配动物专用圆形线圈（直径为6 cm），脉冲磁场峰值强度为3 T，进行CMEP检测。将记录电极插入大鼠后肢的胫骨前肌的肌腹上，接地电极插入在大鼠的背部皮肤里，磁刺激线圈中心置于大脑头骨前囟上方，输出强度为100 %最大刺激强度，经颅磁刺激大鼠大脑运动皮质区时，在后肢胫骨前肌记录CMEP波。每只大鼠连续刺激20次（每两次刺激间隔30 s），记录CMEP的潜伏期和波幅（峰值）进行统计学分析。

1.7　动物灌注固定、取材及切片

术后8周，电生理检测完成后立即用安乐死剂量10 g/L戊巴比妥钠（0.8 mL/100 g）腹腔麻醉处死大鼠，待动物麻醉后剪开胸部肌肉及肋骨，暴露心脏。将灌注针从左心尖插入主动脉，剪开右心耳，直至流出液体澄清透明，肝脏变白。然后将灌注液换成40 g/L的多聚甲醛固定液，待观察到灌流过程中大鼠四肢抽搐抖动，尾巴翘起，肢体僵硬即灌注完全。取出腰段脊髓组织放入40 g/L多聚甲醛中后固定24 h后，分别于150 g/L和300 g/L的蔗糖中梯度脱水，随后OCT包埋脊髓组织进行冰冻切片，切片厚度为25 μm。

1.8　免疫荧光染色

取脊髓横切片，用0.01 mol/L PBS漂洗3次，每次5 min。加入100 ml/L的山羊血清，37 ℃封闭30 min。去掉封闭液，分别加入一抗（由0.03 ml/L Triton的PBS稀释配制），4 ℃过夜。隔天于37 ℃烘箱复温30 min后弃除一抗，0.01 mol/L PBS漂洗3次，每次5 min。加入相对应的荧光二抗，37 ℃孵育1 h，弃除二抗，加入0.01 mol/L PBS漂洗3次，每次5 min。加入核染料Hoechst33342，37 ℃染色10 min，常规漂洗，使用防荧光淬灭封片剂封片，于荧光显微镜或激光共聚焦显微镜下观察并拍照。L1腰段脊髓内vGluT1$^+$本体感觉神经纤维的相对荧光强度用Image J软件进行免疫荧光相对强度定量分析统计：每只大鼠选取L1腰段脊髓横切片3张，每组共15张切片，分别统计脊髓灰质背角和中间带Ⅶ板层内vGluT1$^+$累积荧光强度，再分别除以背角和中间带Ⅶ板层的面积即为vGluT1$^+$相对荧光强度值。

1.9　统计学分析

实验所得数据均采用均数±标准差（Mean±SD）表示，使用Graphpad Prism 9.0软件，两组数据用t检验进行分析，3组或以上数据用单因素方差分析

(One-way ANOVA)对计量资料进行分析,多样本均数间两两比较时符合正态分布且方差齐时采用LSD-t检验,方差不齐时采用Dunnett事后检验,以$P<0.05$为差异有统计学意义。

2 结 果

2.1 电针刺激督脉穴或夹脊穴均能激活L1脊髓神经元表达c-Fos

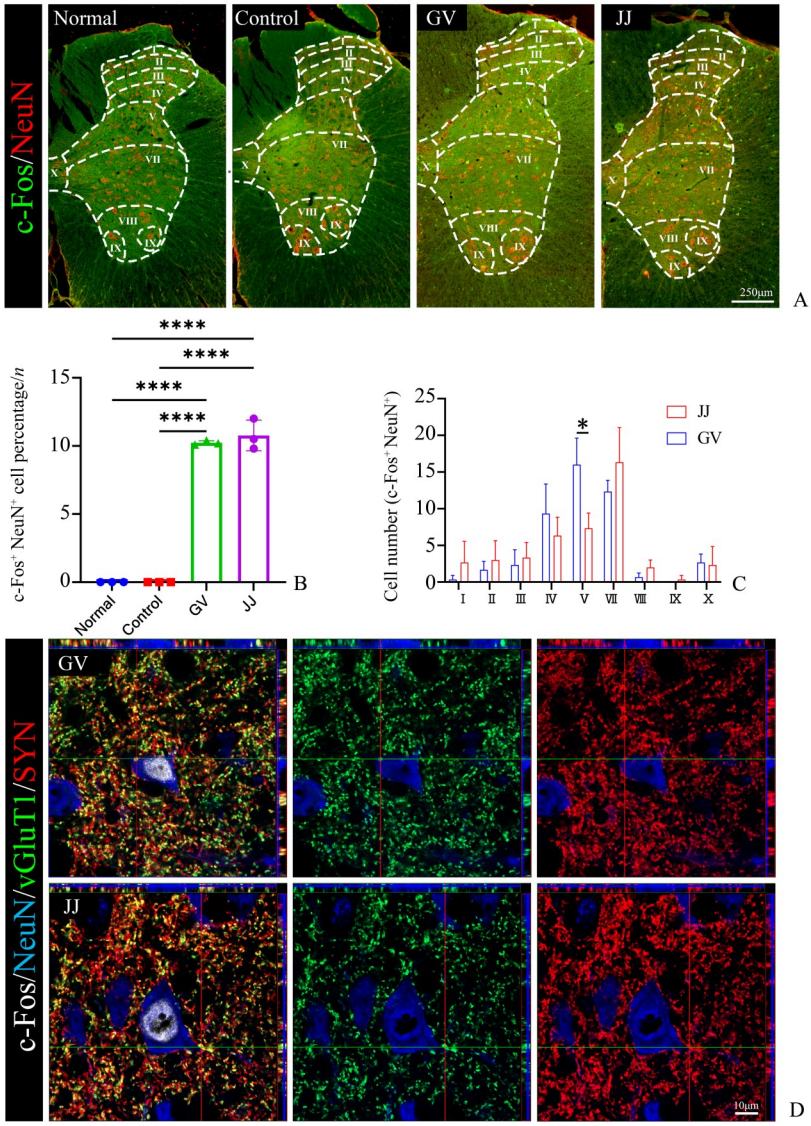
为了确定2种电针方式治疗对L1腰段脊髓神经元的激活情况,对其脊髓横切片执行神经元标记物NeuN和激活神经元兴奋性标志物c-Fos的双标免疫荧光染色,比较4组大鼠L1脊髓横切片中神经元被激活表达c-Fos(神经元兴奋性标志物)的情况(图2A)。结果显示,在Normal和Control组中未观察到c-Fos表达,而电针GV和JJ两组都可观察到c-Fos表达(图2A)。经方差分析,4组间差异有统计学意义(F=343.2,P=8.7×10^{-9})。GV组和JJ组中c-Fos$^+$神经元百分率分别为(10.23±0.15)%和(10.77±1.12)%,两电针组都显著高于其他组($P<0.000\ 1$,图2B),但GV组与JJ组无差异。通过脊髓灰质板层分析得出(图2C),两种电针治疗方式主要激活脊髓第Ⅳ、Ⅴ和Ⅶ板层的神经元,而这3个板层与感觉尤其是本体感觉的信息传递有关,这提示电针GV和JJ可刺激穴位内的感觉神经末梢,主要通过本体感觉传入神经将电针刺激信息传入脊髓内,激活大鼠L1脊髓的Ⅳ、Ⅴ和Ⅶ板层的神经元。L1脊髓的Ⅶ板层是调节后肢节律运动的CPG中间神经元所在的主要分布区,因此,推测电针GV和JJ穴位可能激活CPG内的神经元。为了证实电针可通过促进本体感觉神经纤维的信息传入来调控脊髓神经元活动,本研究采用本体感觉神经纤维标记物vGluT1、突触前成分标记物(synapsin,SYN)、神经元标记物NeuN和神经元激活标记物c-Fos的抗体执行三标免疫荧光染色。结果显示,在L1脊髓内,尤其是Ⅶ板层内,被激活的c-Fos$^+$神经元胞体周围都存在vGluT1$^+$轴突终末与SYN$^+$突触结构的共定位(图2D),这提示电针督脉穴或夹脊穴可能通过增强L1脊髓内vGluT1$^+$本体感觉神经纤维传入L1脊髓,激活其所支配的Ⅶ板层CPG神经元并使其活化。

2.2 电针治疗能增加L1脊髓内vGluT1$^+$本体感觉神经纤维密度和Ⅶ板层神经元存活

为了验证电针可促进本体感觉神经纤维传入脊髓,对各组大鼠L1脊髓背角和中间带Ⅶ板层内的vGluT1$^+$本体感觉神经纤维(图3A)进行相对荧光强度统计分析。对背角荧光强度进行方差分析,4组间差异具有统计学意义(F=31.04,P=6.6×10^{-7})。结果显示,电针治疗之后,在L1脊髓背角区域,vGluT1$^+$神经纤维的相对荧光强度在GV组(8.16±1.08,P=0.000 3)和JJ组(10.46±1.40,P=3.46×10^{-7})高于Control组(4.94±0.34),并且JJ组也高于GV组(P=0.005 8;图3C);对中间带荧光强度进行方差分析,4组间差异具有统计学意义(F=63.10,P=4.4×10^{-9})。L1脊髓中间带Ⅶ板层内的vGluT1$^+$相对荧光强度在GV组(6.30±0.55,P=1.27×10^{-5})和JJ组(8.68±0.78,P=2.1×10^{-9})高于Control组(3.62±0.23),同时JJ组大于GV组(P=5.17×10^{-5};图3D)。这些结果表明电针可以促进vGluT1$^+$本体感觉纤维传入调节脊髓神经元活性,且电针JJ效果优于GV。此外,对各组大鼠L1脊髓Ⅶ板层内NeuN$^+$神经元(图3B)进行定量分析,经方差分析,4组间差异具有统计学意义(F=22.23,P=5.97×10^{-6})。统计结果(图3E)显示,在L1脊髓Ⅶ板层内,损伤Control组的神经元数量(78.7±2.2)与Normal组(106.6±10.1)比较下降(P=5.22×10^{-6})。与Control组相比,经过GV(85.5±4.3,P=0.285 0)治疗,神经元存活数量呈增多趋势,但没有统计学差异,而经过JJ(95.1±2.8,P=0.001 9)电针治疗后,神经元数量增多,差异具有统计学意义。这表明GV电针治疗有促进L1脊髓Ⅶ板层神经元存活的趋势,JJ电针则能促进Ⅶ板层神经元存活。

2.3 两种电针治疗均促进L4/L5脊髓腹角运动神经元的存活

为了探究电针GV或JJ穴对全横断SCI大鼠L4/L5脊髓运动神经元的存活影响,本研究采用运动神经元特异性标记物胆碱乙酰转移酶(choline O-acetyltransferase, ChAT)进行免疫荧光染色,并对L4/L5脊髓灰质腹角外侧区ChAT$^+$运动神经元进行计数定量分析(图4A-C)。经方差分析,4组间差异具有统计学意义(F=25.84,P=2.26×10^{-6})。结果显示,与Normal组(24.0±1.9)比较,Control组L4/L5脊髓腹角外侧区的ChAT$^+$神经元数量(9.9±1.9)减少(P=1.15×10^{-6});经GV(19.1±3.4,P=0.000 2)或JJ



A: The expression of c-Fos (a marker of neuronal excitation) and NeuN in L1 spinal cord transverse sections of each group, scale=250 μm; B: The percentage of c-Fos$^+$ NeuN$^+$ neurons of each group. n=3, F=343.2, $^{****}P<0.000\ 1$; C: The number of c-Fos$^+$ NeuN$^+$ cells in each lamina of L1 spinal cord in the GV group and JJ group. n=3, t=3.606, $^{*}P<0.05$; D: There are co-localization of vGluT1$^+$ axonal terminals (puncta) and SYN$^+$ synaptic bouton surrounding the c-Fos$^+$ neurons of the Ⅶ lamina in GV group and JJ group, scale=10 μm. Values are mean ± SD. GV: governor vessel; JJ: Jiaji.

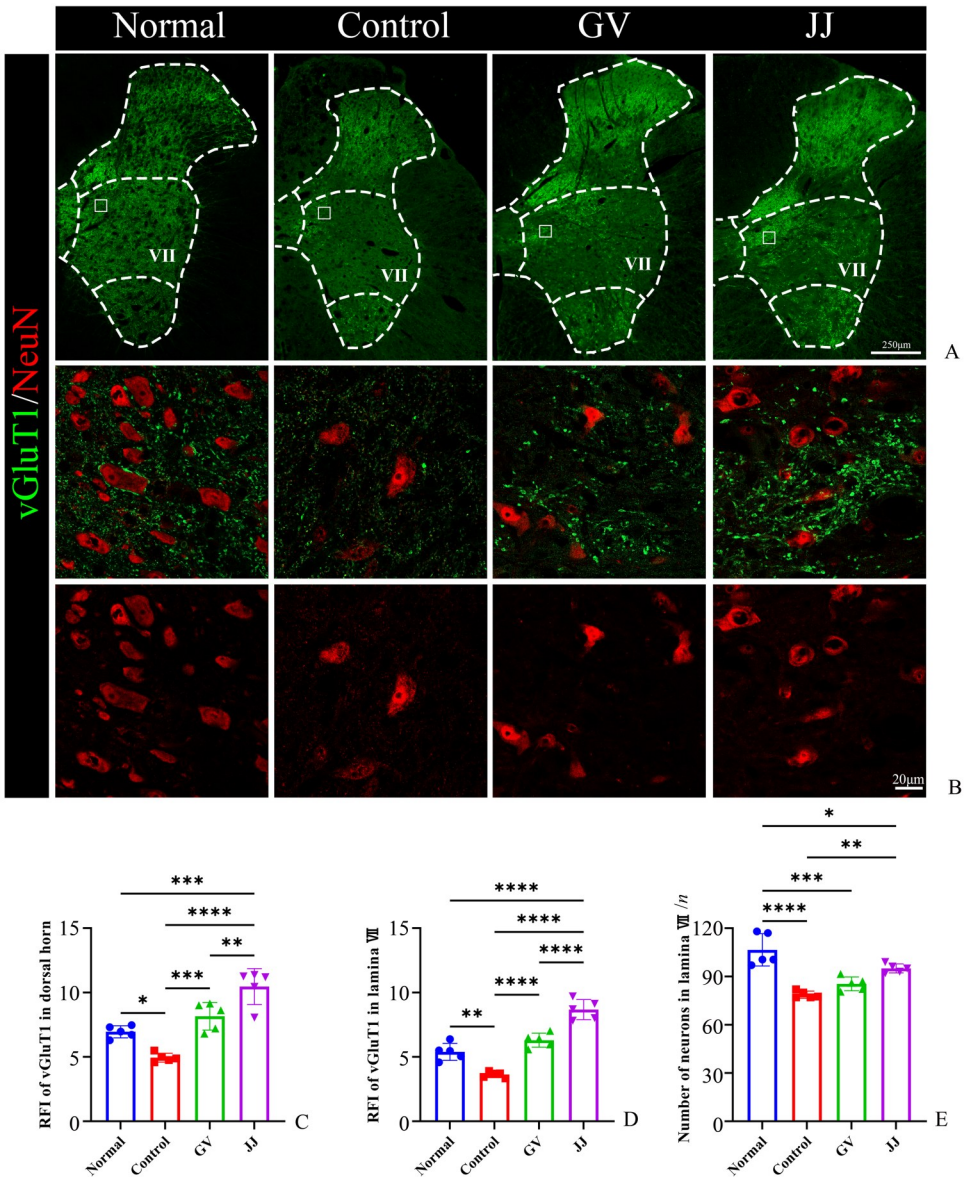
图2　电针督脉(GV)或夹脊(JJ)穴均能激活L1脊髓神经元并受vGluT1$^+$轴突终末支配

Fig. 2　Electroacupuncture at GV or JJ acupoints may activate L1 spinal cord neurons innervated by vGluT1$^+$ axonal terminals

(19.0±2.9，P=0.000 2)电针治疗后，大鼠L4/L5脊髓腹角外侧区的运动神经元存活数量多于Control组(图4B)。上述结果提示，电针GV或JJ穴均可有效抑制全横断SCI所致的腰段脊髓运动神经元凋亡，发挥显著的神经保护作用。

2.4　电针促进SCI大鼠L4/L5脊髓运动神经元的兴奋/抑制平衡恢复

为了探究两种电针治疗对全横断SCI后大鼠L4/L5脊髓运动神经元的调控作用，执行运动神经元标记物ChAT、兴奋性轴突标记物vGluT2和抑制性轴突标记物vGAT的三标免疫荧光染色(图5A)。用ZEISS ZEN软件统计脊髓腹角内与ChAT$^+$运动神经元胞体接触的vGluT2$^+$和vGAT$^+$轴突终末数量，结果分析显示，与Normal组比较，Control组内与ChAT$^+$运动神经元胞体接触的vGluT2$^+$兴奋性轴突终末数量(24.6±1.7，P=1.61×10^{-6})和vGAT$^+$抑制性轴突终末数量(45.2±3.0，P=2.81×10^{-5})都下降；GV组(36.7±3.2，P=0.000 2)和JJ组(41.0±3.2，P=4×

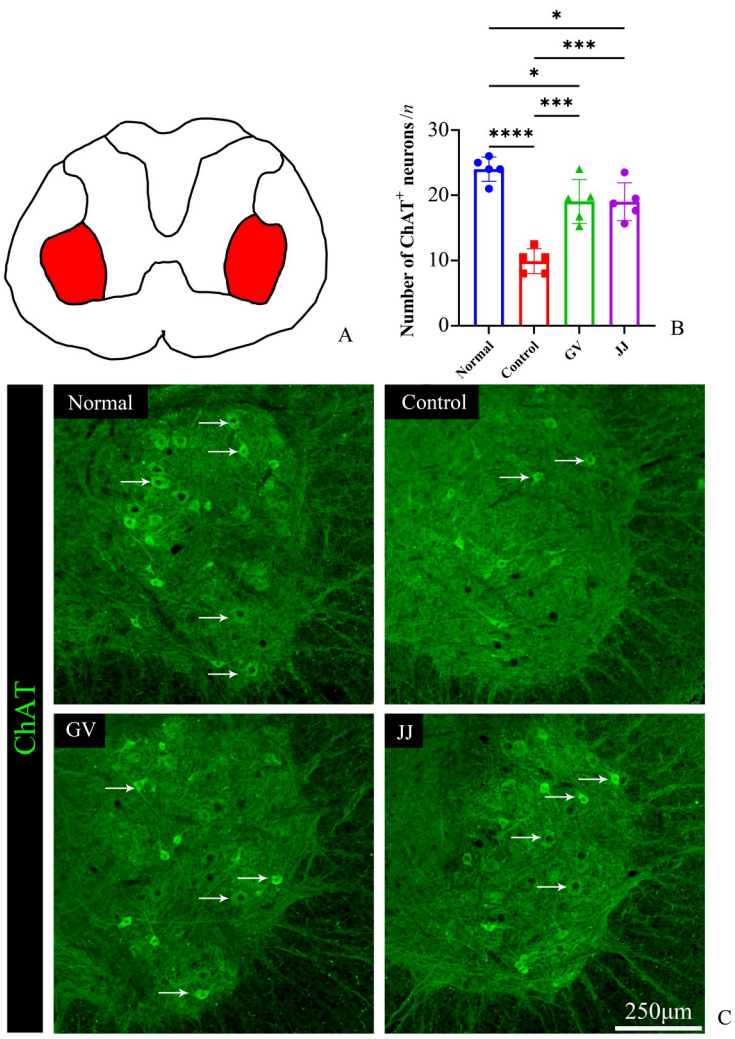


A: The distribution of vGluT1$^+$ proprioceptive nerve fibers in the L1 spinal cord, scale=250 μm; B: High-magnification images from the solid box of the distribution of vGluT1$^+$ nerve fibers and number of NeuN$^+$ neurons in lamina Ⅶ of L1 spinal cord, scale=20 μm; C: The statistical result of relative fluorescence intensity(RFI) of vGluT1$^+$ in dorsal horn of L1 spinal cord, F=31.04; D: The statistical result of relative fluorescence intensity(RFI) of vGluT1$^+$ in lamina Ⅶ of L1 spinal cord, F=63.10; E: The statistical analysis of the NeuN$^+$ neurons count in lamina Ⅶ, F=22.23. n=5, $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.000\ 1$. Values are mean ± SD. GV: governor vessel; JJ: Jiaji.

图3 电针增加L1脊髓vGluT1$^+$神经纤维密度和Ⅶ板层神经元存活

Fig. 3 Electroacupuncture increases the density of vGluT1$^+$ nerve fibers in L1 spinal cord and the survival of neurons in lamina Ⅶ

10^{-6})内与运动神经元胞体接触的兴奋性轴突终末数量高于Control组(图5B);同时,与Control组比较,GV组(57.2±1.9,P=0.009 8)和JJ组(67.0±8.5,P=2.81×10^{-5})电针治疗也增加了与运动神经元胞体接触的抑制性轴突终末数量,并且JJ组的抑制性轴突终末数量多于GV组(P=0.037 7,图5C)。进一步分析支配运动神经元的vGluT2$^+$兴奋性/vGAT$^+$抑制性轴突终末比值来反映运动神经元的兴奋/抑制(excitatory/inhibitory,E/I)平衡情况,经方差分析,4组间差异具有统计学意义(F=13.00,P=0.000 1)。结果(图5D)显示,Control组的运动神经元的E/I比值(0.480±0.037)比Normal组的(0.589±0.018)显著降低(P=0.000 8)。与Control组相比,GV组(0.546±0.015,P=0.041 1)或JJ组(0.608±0.055,P=0.000 2)



A: The schematic diagram of the ChAT$^+$ motor neuron counting region in the ventral horn of the L4/L5 spinal cord; B: The statistical result of the number of ChAT$^+$ motor neurons. n=5, F=25.84, *P < 0.05, ***P < 0.001, ****P < 0.000 1; C: The representative images of ChAT immunofluorescence staining of motor neurons in L4/L5 spinal cord ventral horn in 4 groups, scale=250 μm. Values are mean ± SD. GV: governor vessel; JJ: Jiaji.

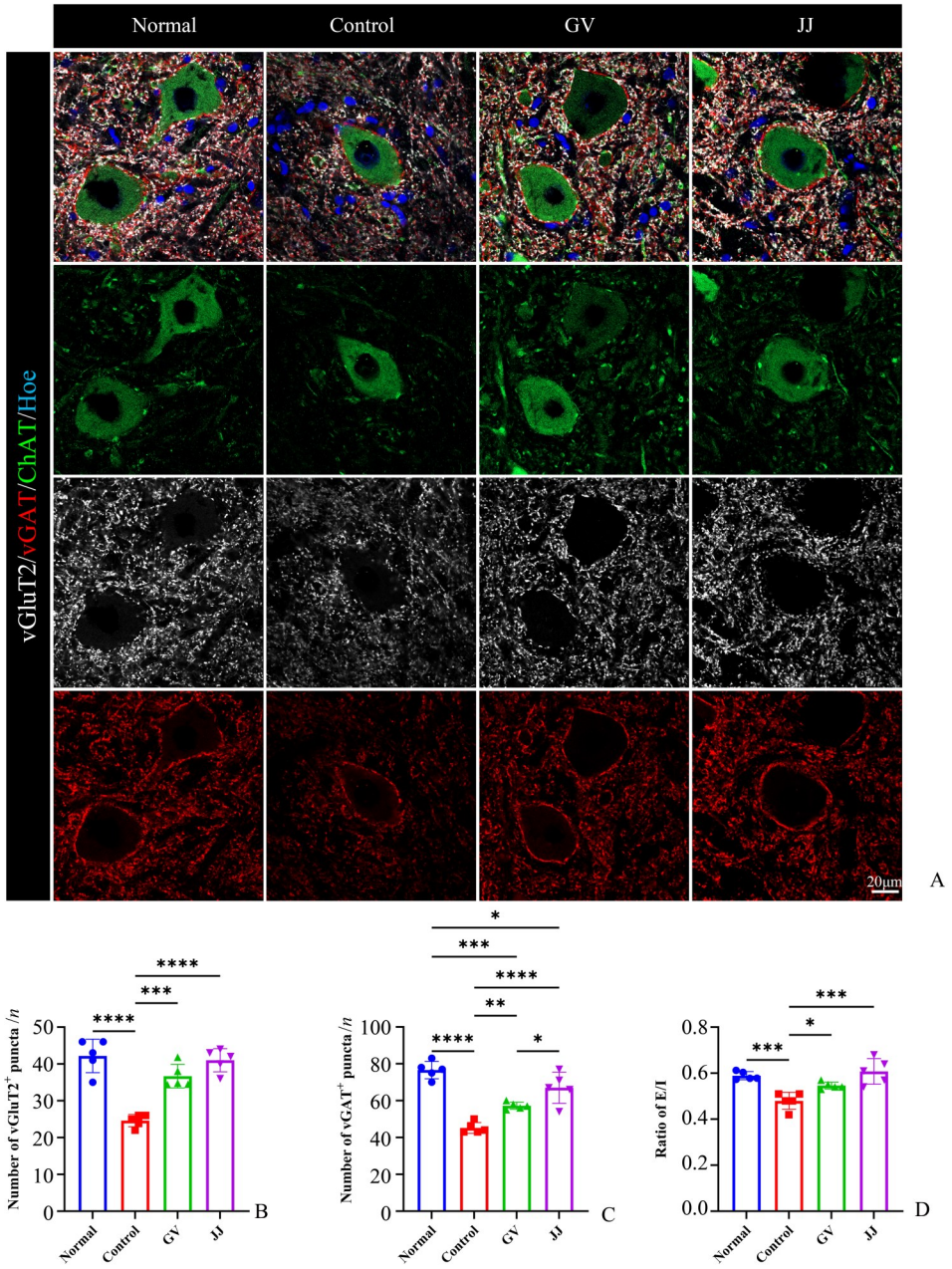
图4　电针促进SCI大鼠L4/L5腰段脊髓运动神经元存活

Fig. 4　Electroacupuncture promotes the survival of motor neurons in the L4/L5 spinal cord of SCI rats

的E/I比值提高（图5D）。这些结果表明，全横断SCI导致支配L4/L5脊髓运动神经元的兴奋性和抑制性轴突终末数减少、E/I比值显著下降和E/I失衡，提示这些运动神经元处于沉默状态；而经电针GV或JJ治疗后，可重塑脊髓运动神经元的兴奋/抑制平衡，进而激活SCI导致的沉默的运动神经元。

2.5　电针促进瘫痪后肢运动功能的改善

为了探究电针GV或JJ穴对全横断SCI大鼠后肢运动功能恢复的影响，SCI后大鼠每周进行BBB评分，并在第8周终止实验前大鼠执行CMEP检测。CMEP检测结果显示，与Control组比较，GV组和JJ组大鼠CMEP的潜伏期缩短并且波幅升高（图6A）。对潜伏期数据进行方差分析，3组间差异具有统计学意义（F=15.62，P=0.004 2）。各组SCI大鼠CMEP潜伏期统计分析显示，与Control组[（8.62±0.35）ms]比较，GV组[（7.67±0.39）ms，P=0.040 8）]和JJ组[（7.00±0.33）ms，P=0.003 4）]的CMEP潜伏期缩短（图6B）。对波幅数据进行方差分析，3组间差异具有统计学意义（F=46.85，P=0.000 2）。各组SCI大鼠CMEP波幅统计分析显示，GV组[（197.5±11.6）μV，P=0.002]和JJ组[（257.8±36.2）μV，P=0.000 2]的CMEP波幅高于Control组[（85.6±4.3）μV；图6C）。行为学BBB评分结果显示（图6D），在全横断SCI之前大鼠后肢的运动功能正常，BBB评分均为21分，在执行全横断SCI手术后一周，BBB评分为0分，随着时间的延长，各组大



A: The representative images of ChAT, vGluT2 and vGAT triple-labeling immunofluorescence staining in the L4 spinal cord ventral horn in 4 groups, scale=20 μm; B: The statistical result of the number of vGluT2$^+$ axonal terminals (puncta) surrounding the ChAT$^+$ neuronal soma, F=29.43; C: The statistical result of the vGAT$^+$ puncta surrounding the ChAT$^+$ neuronal soma, F=34.05; D: The statistical result of the E/I ratio (vGluT2$^+$ puncta / vGAT$^+$ puncta), F=13.00. n=5, $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.000\,1$. Values are mean ± SD. GV: governor vessel; JJ: Jiaji.

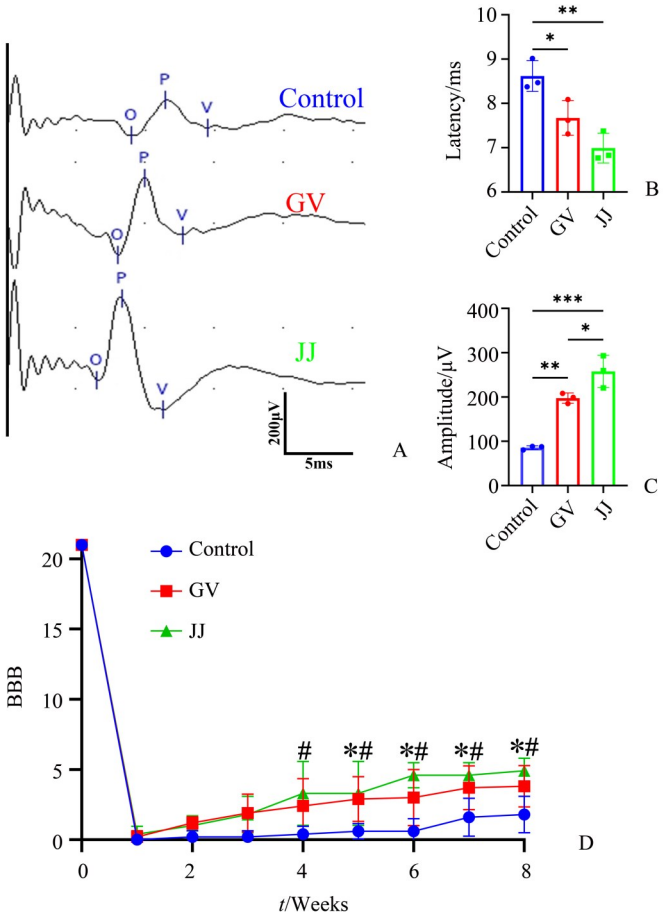
图5 电针促进SCI大鼠L4/L5脊髓运动神经元的兴奋/抑制平衡恢复

Fig. 5 Electroacupuncture promotes the recovery of excitation/inhibition balance of motor neurons in the L4/L5 spinal cord of SCI rats

鼠BBB评分逐渐增加,到术后8周两电针组BBB评分约为5分,表现为髋关节大幅度运动,膝关节、踝关节轻微运动;并且从术后5周到8周,GV组和JJ组大鼠BBB评分均有统计学意义的高于Control组($P<0.05$)。上述结果表明,电针GV或JJ穴能改善SCI大鼠瘫痪后肢的运动功能。

3 讨 论

完全性全横断SCI不仅会引起脑投射到腰段脊髓的下行运动神经通路中断,造成后肢运动瘫痪,也导致支配后肢运动的腰段运动神经环路的沉



A: The representative images of CMEP in the Control, GV and JJ groups at 8 weeks post SCI; B: The statistical result of latency of CMEP among groups, n=3, F=15.62; C: The statistical result of amplitude of CMEP among groups, n=3, F=46.85. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; D: The BBB scores of hindlimbs locomotor during 8 weeks after SCI. $^{\#}P<0.05$, GV group compared with control group. $^{*}P<0.05$, JJ group compared with control group. n=5. Values are mean ± SD. GV: governor vessel; JJ: Jiaji; BBB: Basso-Beattie-Bresnahan.

图6　电针促进SCI大鼠后肢运动功能的恢复

Fig. 6　Electroacupuncture promotes the functional recovery of hindlimbs locomotor in rats with spinal cord injury

默[13]。电针作为一种中医临床上常用的治疗神经系统损伤的方法，已被证明电针GV和JJ穴位可以改善损伤患者的运动功能，但其作用机制尚未完全明确。本研究结果发现，电针GV穴或JJ穴均能激活全横断SCI大鼠L1脊髓神经元兴奋，并伴随vGluT1^{+}本体感觉神经纤维密度和Ⅶ板层神经元存活数的增加；同时两种电针治疗也促进L4/L5脊髓运动神经元（直接支配后肢肌肉运动）存活及其兴奋/抑制平衡恢复，从而提高了瘫痪后肢的运动功能。

本研究发现，GV与JJ这2种临床常用穴位电针治疗全横断SCI大鼠均可激活L1脊髓神经元兴奋表达c-Fos，并且这些被激活的神经元主要定位于L1脊髓灰质的第Ⅳ、Ⅴ、Ⅶ板层，同时vGluT1^{+}本体感觉神经纤维密度和Ⅶ板层神经元存活数的增加。第Ⅳ板层主要接收并处理皮肤低阈值机械感受器传入的触觉、压觉、振动觉信息，同时整合部分关节及肌肉本体感觉传入信号；第Ⅴ板层整合后肢皮肤、肌肉、关节及盆腔脏器的感觉输入，在介导后肢深度疼痛与强烈机械刺激中发挥关键作用；第Ⅶ板层的神经元主要为支配L4/L5脊髓运动神经元池的中间神经元。第Ⅶ板层中央大部分区域的神经元参与姿势与运动的调节，长下行运动通路并不直接支配运动神经元，而是通过与第Ⅶ板层的中间神经元建立连接来控制运动神经元的输出信息。另外，CPG是脊髓内可自主产生周期性节律运动模式的神经网络，主要存在于大鼠颈膨大和腰膨大脊髓灰质Ⅶ和Ⅷ板层，其中L1~L3脊髓的CPG神经元可通过调控L4/L5脊髓前角运动神经元的放电输出，进而介导后肢节律性运动的产生与控制。脊髓

损伤后,外部电刺激腰段脊髓CPG可诱导后肢产生节律性屈伸运动,有效促进SCI患者运动功能恢复[14]。本研究中电针激活的神经元主要分布于L1脊髓第Ⅳ、Ⅴ和Ⅶ板层,与CPG中间神经元的主要分布范围部分重叠,提示这两种电针治疗方式可能通过增强L1脊髓的本体感觉信息传入,激活L1脊髓Ⅶ板层CPG神经元,进而调控L4/L5脊髓运动神经元(直接支配后肢肌肉运动)存活及其兴奋/抑制平衡,从而提高全横断SCI大鼠瘫痪后肢的运动功能。

本体感觉信息传入对于SCI患者运动功能恢复是至关重要的,本体感觉的丧失会导致严重的步态缺陷[15]。本研究中本体感觉神经纤维标记物vGluT1染色结果发现,2种电针治疗均可增加L1脊髓内vGluT1$^+$本体感觉信息,并且JJ组vGluT1$^+$神经纤维密度大于GV组,提示JJ组电针在募集本体感觉信息方面的效果优于GV组。该差异可能与JJ组所用电针刺激穴位数量为GV组的2倍,其电针刺激产生的电流强度更高有关;此外,也可能与电针JJ穴位可直接电刺激竖直肌,引发更多本体感受器肌梭兴奋,将更多的本体感觉信息传入脊髓有关,其具体机制仍需进一步实验验证。

脊髓损伤导致大鼠腰段脊髓运动神经环路沉默[9],我们的研究也发现,在SCI后,大鼠L4/L5脊髓内支配运动神经元的兴奋性和抑制性轴突终末数量都减少,并且其兴奋/抑制(E/I)平衡也受到破坏,E/I比值降低,这也证明了支配大鼠后肢运动的神经环路沉默。但经过GV或JJ穴位电针治疗8周后,均发现2种电针治疗均可促进L4/L5脊髓运动神经元存活、增加了支配运动神经元的兴奋性和抑制性轴突终末数量,并使E/I比值恢复至接近正常鼠水平,这提示电针治疗可调节运动神经元的兴奋/抑制性突触可塑性,促进E/I平衡,使得沉默的运动神经环路被重新激活,这也被CMEP和BBB的结果进一步证实。电生理CMEP检测结果和BBB行为学评分表明,2种电针治疗均可提高SCI大鼠瘫痪后肢的运动功能。尽管从行为学BBB评分结果来看,JJ组与GV组没有显著差异,但电生理CMEP数据提示JJ电针比GV电针治疗效果稍优,这可能与JJ组电针刺激穴位数量为GV组的2倍,其电针刺激产生的电流强度更高,促进更多的下行神经纤维再生长入损伤区并中继脑源性信息下行重新支配后肢的运动。

综上所述,本研究证实电针GV或JJ穴均可能通过促进本体感觉信息传入、激活L1脊髓Ⅶ板层的CPG神经元,并恢复L4/L5脊髓运动神经元的兴奋/抑制平衡等机制,改善全横断SCI大鼠瘫痪后肢的运动功能。这些发现为临床选用GV和JJ经穴电针治疗SCI的作用机制提供实验支撑,也为后续联合治疗研究奠定了理论基础。目前临床针灸医生常采用多穴位配伍(如督脉穴配伍夹脊穴、足三里穴等)电针治疗SCI患者[2]。单一治疗效果十分有限,联合治疗已成为SCI研究的热点[16-19]。已有研究表明,自重跑步机训练、康复训练、经颅磁刺激等与电刺激、导电材料或干细胞源性神经网络组织移植联合应用,均能促进SCI的运动功能恢复[1,16-21]。近年来,随着人工智能技术发展,脑机接口作为SCI治疗的突破性技术,可使脑部意识信号跨越损伤区,直接支配靶肌或外骨骼设备运动;此外,脑机接口与脊髓功能性电刺激结合可构建闭环系统,绕过受损神经通路,强化腰段脊髓运动神经环路可塑性[22]。有研究发现,经皮脊髓电刺激可提高脑机接口的神经信号稳定性,加速神经调节技能学习,实现对脑机接口的精准控制[23]。基于此,未来将特定穴位电刺激与脑机接口联合应用,有望在促进神经通路重建、激活并重建SCI沉默的运动神经环路的基础上,显著提升SCI的修复效果。

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