

脊髓 Lamb1 参与 ACC-SDH 通路调控负性情绪易化神经病理性疼痛的机制

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[摘要] 目的: 神经病理性疼痛常伴焦虑、抑郁情绪, 形成疼痛-负性情绪共病状态, 其具体机制目前仍未阐明。本研究旨在探讨前扣带回皮质(anterior cingulate cortex, ACC)-脊髓背角(spinal dorsal horn, SDH)下行通路在负性情绪易化神经病理性疼痛中的调控机制, 筛选并验证其在脊髓水平发挥作用的关键分子。方法: 采用雄性 Sprague-Dawley 大鼠构建慢性不可预见性轻度应激(chronic unpredictable mild stress, CUMS)联合慢性缩窄性损伤(chronic constriction injury, CCI)模型, 用以模拟负性情绪易化神经病理性疼痛状态。其中, CUMS 持续 21 d, 每日随机给予 2 种不同应激刺激; CCI 模型通过结扎左侧坐骨神经建立。采用糖水偏好实验(sucrose preference test, SPT)和强迫游泳实验评估抑郁样行为, 采用 von-Frey 法检测机械痛阈值。通过向 SDH 注射逆行腺相关病毒(adeno-associated virus, AAV)-环化重组酶(cyclization recombination enzyme, Cre)、向 ACC 区注射 CRE 依赖型 hM4Di 病毒, 结合氯氮平 N-氧化物(clozapine-N-oxide, CNO)连续 14 d 腹腔注射给药, 特异性抑制 ACC-SDH 投射神经元活性, 并进行行为学观察。在 CCI 术后第 7 天, 取腰膨大脊髓组织进行转录组测序, 筛选差异表达基因, 结合基因本体论(gene ontology, GO)及通路富集分析明确候选分子的功能特征, 并采用蛋白质印迹法(Western blotting, WB)和免疫荧光验证候选分子层粘连蛋白 $\beta 1$ 亚基(laminin subunit beta-1, Lamb1)的表达变化、分布特征及细胞定位。进一步向 SDH 注射 AAV 敲减 Lamb1 的表达, 观察其对 ACC-SDH 通路抑制后镇痛效应及情绪相关行为的影响。结果: CUMS 处理可诱导大鼠出现明显抑郁样行为, 并进一步加重 CCI 所致机械痛敏。抑制 ACC-SDH 通路后, 自 CCI 术后第 3 天至第 10 天, 大鼠的机械痛阈值明显升高, 并且在 CCI 术后第 7 天最为显著(均 $P < 0.05$)。RNA 测序结果显示, 抑制 ACC-SDH 神经元活性后脊髓中共有 126 个差异表达基因, 其中 94 个上调、32 个下调, 主要涉及细胞外基质、突触功能、神经元结构及疼痛相关过程。Lamb1 为上调最显著的候选分子。WB 结果证实, 特异性抑制 ACC-SDH 投射神经元活性后, 脊髓中 Lamb1 蛋白的表达水平升高, 而 CUMS+CCI 组脊髓 Lamb1 表达低于 CCI+control 组(均 $P < 0.05$)。免疫荧光染色结果显示, Lamb1 在脊髓中广泛分布, 主要富集于 SDH 浅层, 并与神经元、小胶质细胞和星形胶质细胞呈现空间邻近分布。敲减脊髓 Lamb1 后, 可以下调 AAV-hM4Di 组大鼠的机械痛阈值, 并加重 CCI 大鼠的痛觉过敏。结论: Lamb1 在脊髓水平参与 ACC-SDH 通路调控负性情绪易化神经病理性疼痛的过程。本研究从脑-脊髓下行调控角度揭示了疼痛-负性情绪共病的脊髓分子基础, 为干预负性情绪相关神经病理性疼痛提供了新策略。

[关键词] 前扣带回皮质; 脊髓背角; Lamb1; 负性情绪; 神经病理性疼痛

Mechanistic role of spinal Lamb1 in negative emotion-facilitated neuropathic pain regulated by the ACC-SDH pathway

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ABSTRACT

Objective: Neuropathic pain is frequently accompanied by anxiety and depressive symptoms, resulting in a comorbid state of pain and negative emotions, the underlying mechanisms of which remain incompletely understood. This study aims to investigate the regulatory mechanism of the descending anterior cingulate cortex (ACC)-spinal dorsal horn (SDH) pathway in negative emotion-facilitated neuropathic pain and to identify and validate key molecules acting at the spinal level.

Methods: Male Sprague-Dawley rats were used to establish a chronic unpredictable mild stress (CUMS) combined with chronic constriction injury (CCI) model to mimic negative emotion-facilitated neuropathic pain. CUMS was administered for 21 days, with 2 different stressors randomly applied each day. The CCI model was established by ligation of the left sciatic nerve. Depression-like behaviors were assessed using the sucrose preference test (SPT) and forced swimming test, and the mechanical pain threshold was measured using the von Frey test. To selectively inhibit ACC-SDH projection neurons, a retrograde adeno-associated virus (AAV) expressing cyclization recombination enzyme (Cre) was injected into the SDH, while a Cre-dependent hM4Di virus was injected into the ACC. Clozapine-N-oxide (CNO) was then administered intraperitoneally for 14 consecutive days, followed by behavioral assessments. On postoperative day 7 after CCI, lumbar enlargement spinal cord tissues were collected for transcriptomic sequencing to identify differentially expressed genes. Gene ontology (GO) and pathway enrichment analyses were performed to characterize the functional features of candidate molecules. Western blotting (WB) and immunofluorescence staining were used to validate the expression changes, distribution, and cellular localization of the candidate molecule laminin subunit beta-1 (*Lamb1*). An AAV was further injected into the SDH to knock down *Lamb1* expression, and its effects on the analgesic response to ACC-SDH pathway inhibition and on emotion-related behaviors were evaluated.

Results: CUMS induced marked depression-like behaviors in rats and further aggravated CCI-induced mechanical hypersensitivity. After inhibition of the ACC-SDH pathway, the mechanical pain threshold was significantly increased from postoperative day 3 to day 10 after CCI, with the most pronounced effect observed on postoperative day 7 (all $P < 0.05$). RNA sequencing identified 126 differentially expressed genes in the spinal cord after inhibition of ACC-SDH neuronal activity, including 94 upregulated and 32 downregulated genes, mainly involving extracellular matrix organization, synaptic function, neuronal structure, and pain-related processes. *Lamb1* was the most significantly upregulated candidate molecule. WB confirmed that specific inhibition of ACC-SDH projection neuronal activity increased spinal *Lamb1* protein expression, whereas spinal *Lamb1* expression was lower in the CUMS+CCI group than in the CCI+control group (all $P < 0.05$). Immunofluorescence staining showed that *Lamb1* was widely distributed in the spinal cord, predominantly enriched in the superficial layers of the SDH, and exhibited spatial

proximity to neurons, microglia, and astrocytes. Knockdown of spinal *Lamb1* reduced the mechanical pain threshold in rats in the AAV-hM4Di group and aggravated hyperalgesia in CCI rats.

Conclusion: *Lamb1* at the spinal level participates in the regulation of negative emotion-facilitated neuropathic pain by the ACC-SDH pathway. From the perspective of descending brain-spinal cord regulation, this study reveals a spinal molecular basis underlying the comorbidity of pain and negative emotions and provides a potential basis for developing new strategies to intervene in negative emotion-associated neuropathic pain.

KEY WORDS anterior cingulate cortex; spinal dorsal horn; *Lamb1*; negative emotion; neuropathic pain

神经病理性疼痛是常见的慢性疼痛,其发病率可达5%~10%^[1]。神经病理性疼痛常伴发焦虑、抑郁等负性情绪;而长期存在的负性情绪又可进一步加重患者的疼痛感知,随着病程进展逐渐形成疼痛-负性情绪共病状态,导致神经病理性疼痛迁延难愈,并带来一系列心理、行为及社会问题^[2-3]。因此,探究负性情绪与疼痛感知之间的调控机制,对于完善对神经病理性疼痛发病机制的认识及寻找潜在治疗靶点具有重要意义。

前扣带回皮质(anterior cingulate cortex, ACC)是中枢神经系统疼痛相关皮质网络的重要组成部分,不仅参与疼痛情感成分的形成和痛觉调控,也与焦虑、抑郁等负性情绪的发生、发展密切相关^[4]。近年来研究^[5]发现,脑下行调控系统可通过改变脊髓神经元兴奋性和局部敏化水平参与疼痛的发生、发展。ACC可直接发出投射纤维至脊髓背角(spinal dorsal horn, SDH),形成ACC-SDH通路,并在疼痛易化中发挥作用^[6]。本课题组前期研究^[7]发现,在抑郁合并神经病理性疼痛的模型大鼠中,ACC-SDH投射神经元活性增强;抑制ACC-SDH通路活性可缓解负性情绪对神经病理性疼痛的易化作用。然而,该通路在脊髓水平通过何种关键分子介导上述效应,目前尚不清楚。

层粘连蛋白(laminin)是细胞外基质(extracellular matrix, ECM)的重要组成成分,属于由 α 、 β 和 γ 链组成的异源三聚体糖蛋白家族。*Laminin* $\beta 1$ 亚基(*laminin* subunit β -1, *Lamb1*)在皮层中高表达,并参与精神障碍及疼痛-抑郁共病的发生^[8-9]。然而,*Lamb1*在负性情绪易化神经病理性疼痛中的作用,尤其是其是否参与ACC-SDH通路介导的脊髓调控机制,仍缺乏直接证据。

本研究采用慢性不可预见性轻度应激(chronic unpredictable mild stress, CUMS)联合慢性缩窄性损

伤(chronic constriction injury, CCI)建立负性情绪易化神经病理性疼痛大鼠模型,并结合化学遗传学、转录组测序、蛋白质印迹法(Western blotting, WB)及免疫荧光等方法,筛选并验证ACC-SDH通路在负性情绪易化神经病理性疼痛过程中所涉及的脊髓关键分子,重点探讨*Lamb1*在其中的作用,以期疼痛-负性情绪共病机制研究及潜在干预靶点的筛选提供理论依据。

1 材料与方法

1.1 伦理声明

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1.2 实验动物及分组

体重160~180 g的雄性Sprague-Dawley大鼠购自湖南斯莱克景达实验动物有限公司,以4~5只/笼饲养,处于12 h光/暗交替周期,自由摄食、饮水。动物饲养与使用遵循美国国立卫生研究院《实验动物护理和使用指南》。所有动物行为学实验均在双盲条件下进行。CUMS实验中,假手术(Sham)+对照(control)组、Sham+CUMS组、CCI+control组、CCI+CUMS组各6只大鼠。腺相关病毒(adeno-associated virus, AAV)注射实验中,AAV-control组、AAV-化学遗传学抑制(记为hM4Di)组各13只大鼠,其中6只用于行为学检测和c-Fos表达水平验证,3只用于RNA测序、4只用于*Lamb1*的WB分析;AAV-hM4Di+乱序对照序列(记为scramble)组、AAV-hM4Di+敲减*Lamb1*(记为si*Lamb1*)组各10只大鼠,其中6只用于行为学检测,4只用于*Lamb1*的WB分析;CUMS+CCI+scramble组、CUMS+CCI+si*Lamb1*组、CCI+scramble组、CCI+si*Lamb1*组各6只大鼠。

1.3 方法

1.3.1 CUMS模型建立

按既往研究^[10]中的方法建立CUMS模型,包括食物或饮水剥夺、潮湿或干燥垫料、拥挤或单笼饲养、昼夜节律颠倒、冷水或热水强迫游泳、尾夹、笼具震荡、束缚、噪音及气味刺激等多种应激源。于21 d内每日随机给予2种不同刺激,以保证刺激的不可预见性。

1.3.2 CCI模型建立

采用异氟烷对大鼠进行全身麻醉后,建立神经病理性疼痛CCI模型^[11]。钝性分离股二头肌后暴露左侧坐骨神经,采用4-0丝线于坐骨神经周围打4个松结,结间距1 mm。假手术组仅暴露坐骨神经,不进行结扎。对于CUMS+CCI组,大鼠自手术前21 d(记为第-21 d)开始接受CUMS刺激,于手术当天(记为第0天)进行CCI手术。

1.3.3 机械痛阈值测定

大鼠机械痛阈值的测定方法参考既往文献^[12],将大鼠置于测试笼内适应30 min后,使用von-Frey针垂直刺激其左侧足底中部,针丝弯曲并保持2~3 s后,若出现抬足或舔足反应,则记为阳性。每次测量间隔5 min,记录出现阳性反应时的最小刺激强度作为机械痛阈值。

1.3.4 糖水偏好实验

糖水偏好实验(sucrose preference test, SPT)参照既往研究^[13]进行。大鼠先适应性饮用1%蔗糖溶液48 h。正式实验前禁水14 h,测试时单笼饲养,每笼放置1瓶1%蔗糖溶液和1瓶纯净水,自由饮用4 h,每2 h交换2个溶液瓶的位置。记录2种液体的摄入量,按蔗糖溶液摄入量除以总液体摄入量计算蔗糖偏好率。

1.3.5 强迫游泳实验

在强迫游泳实验中,将大鼠置于装有水的透明玻璃圆柱体中(直径20 cm,水深30 cm,室温),方法参照既往研究^[14]。前2 min为训练阶段,随后4 min为测试阶段。根据录像,当大鼠停止挣扎、呈漂浮状态时,视为不动,并记录不动时间。计算4 min测试阶段内的总不动时间。

1.3.6 转录组测序

转录组测序由数谱(上海)生物科技有限公司完成。具体实验流程:提取的总RNA样品经琼脂糖电泳和Nanodrop质检和定量后,用寡聚(dT)磁珠富集信使RNA(messenger RNA, mRNA)。使用试剂盒RNA构建测序文库,将RNA片段化后用随机引物反转录生成第一链互补DNA(complementary DNA, cDNA),加入脱氧尿苷三磷酸(deoxyuridine triphosphate, dUTP)合成第二链cDNA,双链cDNA

末端修复加A后连接Illumina匹配接头,聚合酶链反应(polymerase chain reaction, PCR)扩增得到最终文库。采用Agilent 2100对构建好的文库进行质检,并采用定量PCR(quantitative PCR, qPCR)对文库进行定量,使用Illumina HiSeq 4000测序仪进行测序。随后筛选差异表达基因并进行基因本体论(gene ontology, GO)功能显著性富集分析及通路显著性富集分析。使用Ballgown对样本水平进行定量分析,以及组间的基因表达差异分析,设定阈值为1.5倍差异,以 $P \leq 0.05$ 且组内每千个碱基的转录每百万映射读取的片段数(fragments per kilobase of transcript per million mapped reads, FPKM)均值 ≤ 0.5 来筛选差异表达基因及转录本。

1.3.7 WB

WB按既往研究^[15]中的方法进行。取大鼠腰膨大段脊髓组织,匀浆后提取总蛋白质。每孔加入30 μ g蛋白质,经凝胶电泳分离后转至聚偏二氟乙烯(polyvinylidene fluoride, PVDF)膜。加入5%脱脂奶粉,于室温下封闭1 h后,在4 $^{\circ}$ C孵育一抗过夜,所用一抗包括兔抗Lamb1抗体(稀释比1:2 000)和兔抗Tubulin抗体(稀释比1:5 000),均购自美国Proteintech公司;洗膜后加入相应二抗,在室温下孵育1 h,采用化学发光法显影并进行灰度分析。

1.3.8 免疫荧光实验

免疫荧光实验按既往研究^[16]中的方法进行。大鼠在异氟烷麻醉下经心脏灌注磷酸盐缓冲液(phosphate buffered saline, PBS),随后灌注4%多聚甲醛进行固定。取大鼠ACC或腰膨大段脊髓组织,于4%多聚甲醛中固定过夜,再置于30%蔗糖溶液脱水,制备冰冻切片。切片经PBS漂洗后,以5%正常驴血清封闭1 h,加入兔抗Lamb1抗体(稀释比1:100,美国Proteintech公司)于4 $^{\circ}$ C孵育过夜;部分切片进一步与小鼠抗神经元核抗原(neuronal nuclei, NeuN;稀释比1:200,英国Abcam公司)、小鼠抗离子钙结合衔接分子1(ionized calcium binding adapter molecule 1, Iba1;稀释比1:200,英国Abcam公司)、小鼠抗神经胶质细胞原纤维酸性蛋白(glial fibrillary acidic protein, GFAP;稀释比1:200,英国Abcam公司)及小鼠抗c-Fos(稀释比1:200,英国Abcam公司)进行双标染色。次日加入相应荧光标记二抗,于室温下孵育2 h。最后封片并在Leica显微镜下观察。所有图像在相同显微镜参数下采集,采用统一背景扣除和阈值设定原则进行分析。

1.3.9 病毒立体定位注射及化学遗传学操作

在深度异氟烷麻醉下,将大鼠固定于立体定位仪中,于右侧ACC注射400 nL病毒,速度为50 nL/min,坐标为前囟前2.0 mm、中线右侧0.5 mm、

硬膜下 2.0 mm。脊髓注射时暴露 $T_{13}\sim L_1$ 椎体, 于左侧 SDH 注射 400 nL AAV, 坐标为距中线 0.5 mm、距背根入区表面深度 0.2 mm。为特异性干预 ACC 中投射至 SDH 的兴奋性神经元, 向 ACC 注射 rAAV-CaMK II α -DIO-hM4Di-mCherry 或 rAAV-CaMK II α -DIO-mCherry, 向 SDH 注射逆行病毒 rAAV-hSyn-EGFP-Cre。3 周后, 自 CCI 手术开始起连续 14 d 腹腔注射氯氮平 N-氧化物(clozapine-N-oxide, CNO) 10 mg/kg 或载体, 给药后 30 min 进行行为学测试。为敲减脊髓中 *Lamb1* 的表达, 向 SDH 注射 *Lamb1* 敲减病毒 rAAV-U6-siLamb1-CMV(5'-GCTACAGGTGTCTGGA-GAACA-3')或对照病毒, 于注射病毒 3 周后通过 WB 验证 *Lamb1* 敲减效果。采用逆行病毒示踪联合化学遗传学方法, 向脊髓注射 AAV-Cre-EGFP 逆行病毒, 向 ACC 注射 Cre 依赖型 AAV-hM4Di-Cherry 病毒。实验所用病毒均购自武汉枢密脑科学技术有限公司。

1.4 统计学处理

采用 GraphPad Prism 9 软件进行数据统计和分析。计量资料以均数 $\pm$ 标准差表示。组间比较在满足正态分布且方差齐性时采用独立样本 *t* 检验; 不满足条件时采用 Mann-Whitney *U* 检验。重复测量数据采用双因素重复测量方差分析, 事后比较采用 Tukey 或 Sidak 法。 $P<0.05$ 为差异有统计学意义。

2 结果

2.1 化学遗传学抑制 ACC-SDH 投射神经元可缓解负性情绪易化的机械性痛敏

行为学结果显示, CUMS 处理后大鼠出现明显抑郁样行为, 表现为强迫游泳实验中总不动时间延长及蔗糖偏好率下降(均 $P<0.05$, 图 1A、1B)。与 CCI+control 组相比, CCI+CUMS 组大鼠的机械痛阈值在术后 3 至 7 d 进一步降低(均 $P<0.05$, 图 1C)。逆行病毒示踪联合化学遗传学结果(图 1D~1F)显示, ACC 中出现逆行示踪信号并与 hM4Di 病毒荧光存在共定位, 提示 ACC-SDH 投射神经元标记成功。腹腔注射 CNO 后, 与 AAV-control 组相比, AAV-hM4Di 组 mCherry 阳性 ACC-SDH 投射神经元中 c-Fos 阳性比例明显降低($P<0.05$, 图 1G~1H), 提示 CNO 可有效抑制 hM4Di 标记的 ACC-SDH 投射神经元活性。CCI 术后第 3 天至第 7 天, AAV-hM4Di 组大鼠的机械痛阈值均高于 AAV-control 组, 其中 CCI 术后第 7 天 $[(5.889\pm 1.544) \text{ g vs } (2.544\pm 0.584) \text{ g}]$ 差异最显著($P<0.05$, 图 1I), 提示抑制 ACC-SDH 通路可减轻负性情绪对神经病理性疼痛的易化作用。采用化学遗传学手段抑制 ACC-SDH 通路后, 大鼠在强迫游泳实验中的总不动时间及蔗糖偏好率均无明显改变(均 $P>0.05$, 图 1J、1K), 提示该干预主要影响痛觉敏化, 而未显著改善抑郁样行为。

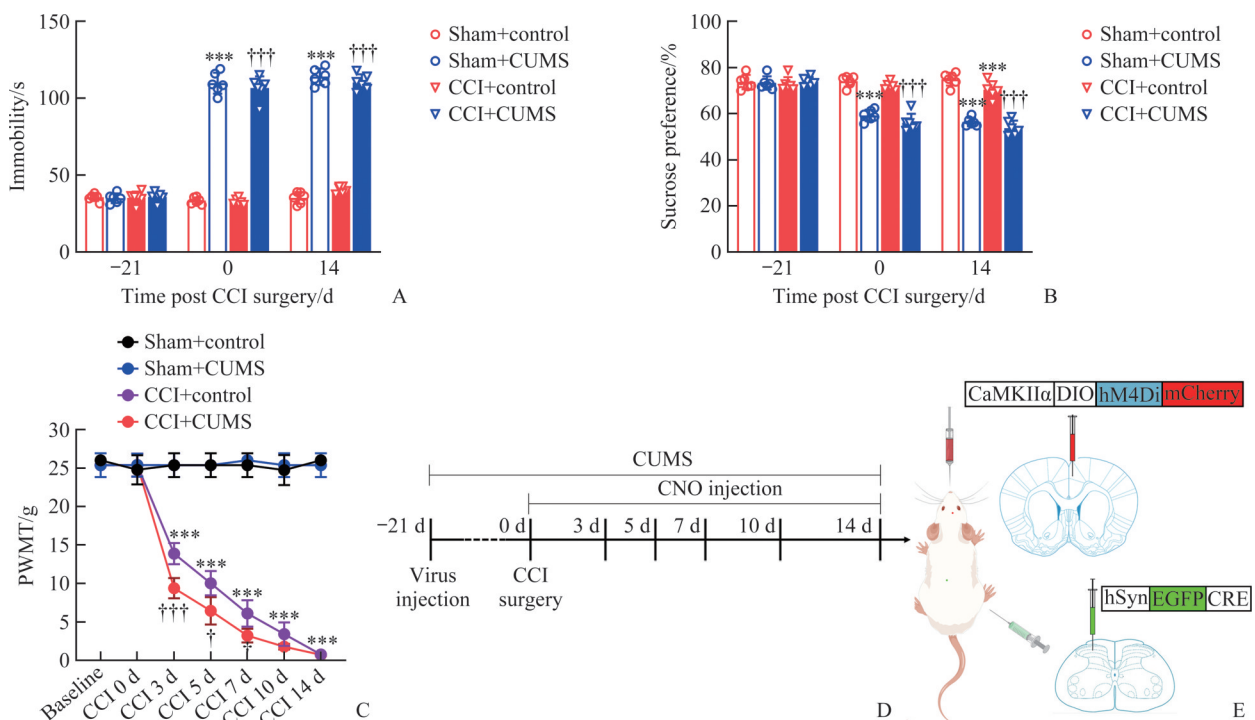


图1 抑制ACC-SDH神经活性可缓解CUMS对CCI大鼠神经病理性疼痛的易化作用

Figure 1 Inhibition of ACC-SDH neuronal activity alleviates CUMS-facilitated neuropathic pain in CCI rats

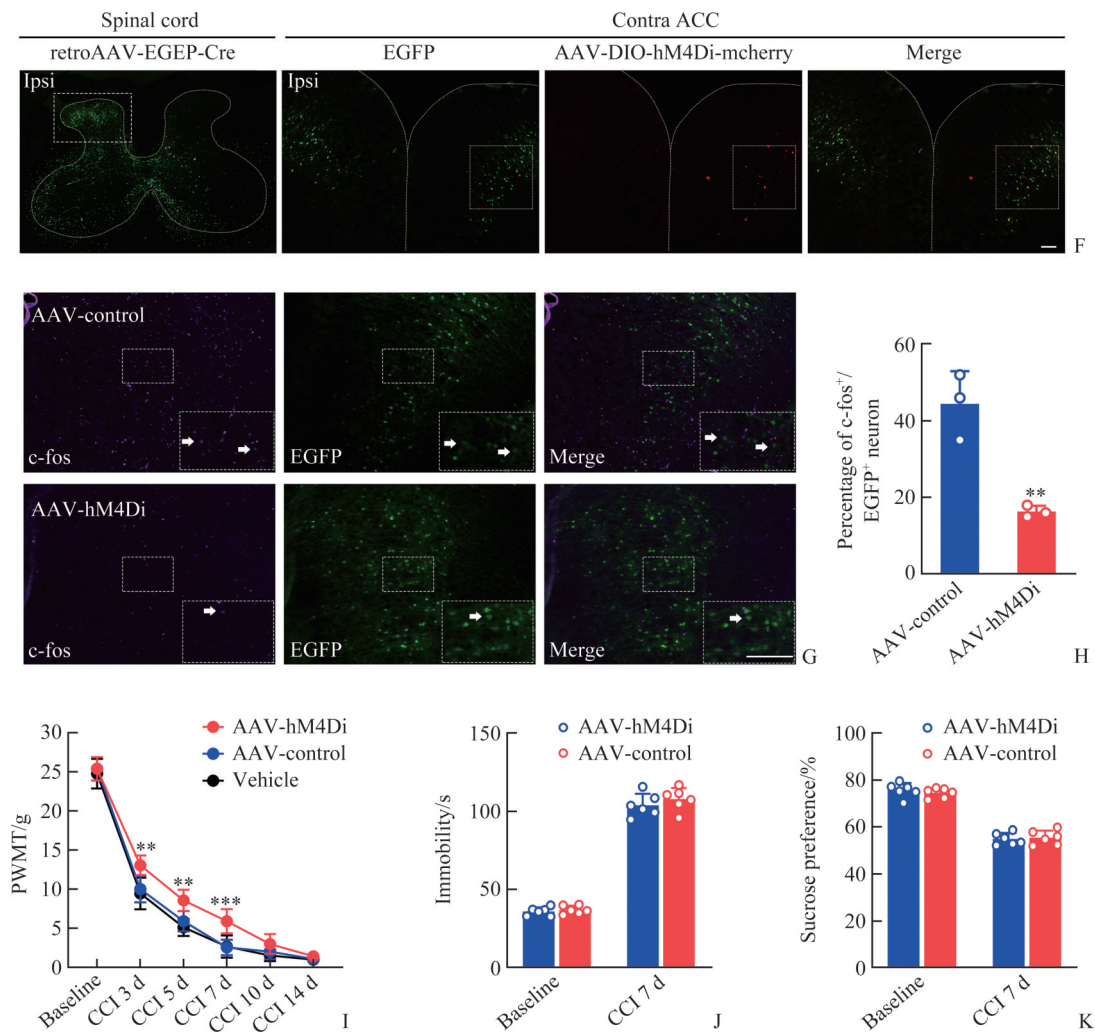


图1 抑制ACC-SDH神经元活性可缓解CUMS对CCI大鼠神经病理性疼痛的易化作用(续)

Figure 1 Inhibition of ACC-SDH neuronal activity alleviates CUMS-facilitated neuropathic pain in CCI rats (continued)

A and B: After 21 d of CUMS exposure, rats exhibited depression-like behaviors, characterized by increased immobility time in the forced swimming test (A) and decreased sucrose preference (B). $n=6$. Compared with the Sham+control group, $***P<0.001$; Compared with the CCI+control group, $\dagger\dagger\dagger P<0.001$. C: Changes in mechanical pain thresholds across groups. CCI reduced the mechanical threshold in non-stressed rats, while CUMS further facilitated CCI-induced mechanical hypersensitivity. $n=6$. Compared with the Sham+control group, $***P<0.001$; Compared with the CCI+control group, $\dagger P<0.05$, $\dagger\dagger\dagger P<0.001$. D: Timeline of viral tracing and chemogenetic experiments. E: Schematic diagram of viral injection strategy: retrograde tracer virus (retrograde rAAV-hSyn-EGFP-Cre) injected into the SDH, and chemogenetic virus (rAAV-CaMKII α -DIO-hM4Di-mCherry) injected into the contralateral ACC. F: Immunofluorescence validation of viral expression in the ACC and spinal cord 21 d after injection. Scale bar=200 μm . Dotted-boxed regions represent the spinal cord dorsal horn and the ACC region, respectively. G and H: Representative double immunostaining images (G) and the statistical bar charts (H) show that injection of CNO induced a decrease in c-Fos expression in the ACC-SDH projecting neurons. Scale bar=100 μm . $n=3$. Compared with the AAV-control group, $**P<0.01$. I: Chemogenetic inhibition of ACC-SDH activity increased the mechanical pain threshold in CUMS+CCI rats. $n=6$. Compared with the AAV-control group, $**P<0.01$, $***P<0.001$. J–K: Chemogenetic inhibition of ACC-SDH activity did not affect immobility time in the forced swimming test (J) or sucrose preference (K). $n=6$. AAV-control: CUMS+CCI+AAV-control+CNO; AAV-hM4Di: CUMS+CCI+AAV-hM4Di+CNO; Vehicle: CUMS+CCI+AAV-control+vehicle. CUMS: Chronic unpredictable mild stress; CCI: Chronic constriction injury; PWMT: Paw withdrawal mechanical threshold; CNO: Clozapine-N-oxide; AAV: Adeno-associated virus; retroAAV: Retrograde AAV; rAAV: Recombinant AAV; hSyn: Human synapsin I promoter; CaMKII α : Calcium/calmodulin-dependent protein kinase II α ; DIO: Double-floxed inverted open reading frame; Cre: Cyclization recombination enzyme; EGFP: Enhanced green fluorescent protein; mCherry: Monomeric cherry; Ipsi: Ipsilateral; ACC: Anterior cingulate cortex; SDH: Spinal dorsal horn; hM4Di: Gi-coupled human M4 muscarinic DREADD receptor.

2.2 RNA 测序筛选获得 ACC-SDH 通路调控的脊髓差异表达基因

图 2A 为差异表达基因火山图, 共检测到 14 328 个基因, 其中 94 个基因表达上调, 32 个基因表达下调。图 2B 展示了上调基因的通路显著性富集分析结果, 差异基因主要涉及胶质瘤相关通路、生物节律相关通路、ErbB 受体酪氨酸激酶家族(ErbB family of receptor tyrosine kinases, ErbB)信号通路及钙信号通路等。对上调的差异表达基因进行 GO 富集分析(图 2C)。其中, 生物学过程分析显示, 这些上调的差异表达基因主要参与调控学习记忆、囊泡融合及疼痛反应等功能。细胞组成分析结果显示, 上调的差异表达基因主要位于轴突、突触前膜、突触囊泡、神经元胞体及 ECM 中。分子功能分析结果显示, 差异表达基因主要参与钙离子依赖性磷脂结合、网格蛋白结合、突触融合蛋白结合等过程。进一步分析发现, 在抑制 ACC-SDH 投射神经元活性后, *Lamb1* 基因表达上调最为显著(图 2D)。进一步的 WB 验证结果显示, AAV-hM4Di 组脊髓 *Lamb1* 蛋白表达水平明显高于 AAV-control 组($P<0.05$, 图 2E), 与 RNA 测序结果一致。

2.3 CUMS 处理后 CCI 大鼠脊髓 *Lamb1* 表达下调

WB 结果显示, CUMS+CCI 组大鼠脊髓中 *Lamb1* 的表达水平显著低于 CCI+control 组($P<0.05$, 图 3A)。

免疫荧光结果显示, *Lamb1* 在脊髓中广泛分布, 主要表达于 SDH 浅层, 且其变化趋势与 WB 结果变化相一致(图 3B)。免疫荧光双标空间定位结果显示, *Lamb1* 与 NeuN、Iba1 和 GFAP 标记区域呈空间邻近或部分重叠分布(图 3C)。

2.4 敲减脊髓 *Lamb1* 可部分逆转抑制 ACC-SDH 通路的镇痛作用

WB 结果显示, AAV-hM4Di+si*Lamb1* 组大鼠中脊髓 *Lamb1* 的表达水平较 AAV-hM4Di+scramble 组显著下调($P<0.05$, 图 4A、4B), 提示病毒敲减成功。行为学结果显示, 与 AAV-hM4Di+scramble 组相比, AAV-hM4Di+si*Lamb1* 组大鼠的机械痛阈值在 CCI 术后第 5 及第 7 天均下降(均 $P<0.05$, 图 4C), 提示该镇痛效应在 CCI 术后第 5 及第 7 天减弱。敲减 *Lamb1* 后, 大鼠强迫游泳实验中的总不动时间和蔗糖偏好率均无明显变化(均 $P>0.05$, 图 4D、4E), 提示脊髓 *Lamb1* 主要参与 ACC-SDH 通路对痛觉敏化的调控, 而对抑郁样行为的影响有限。除此之外, 与 CCI+scramble 组相比, CCI+si*Lamb1* 组在术后第 3 和第 5 天的机械痛阈值均明显降低(均 $P<0.05$, 图 4G), 而 CUMS+CCI+si*Lamb1* 组的机械痛阈值与 CUMS+CCI+scramble 组相比差异无统计学意义($P>0.05$, 图 4F)。

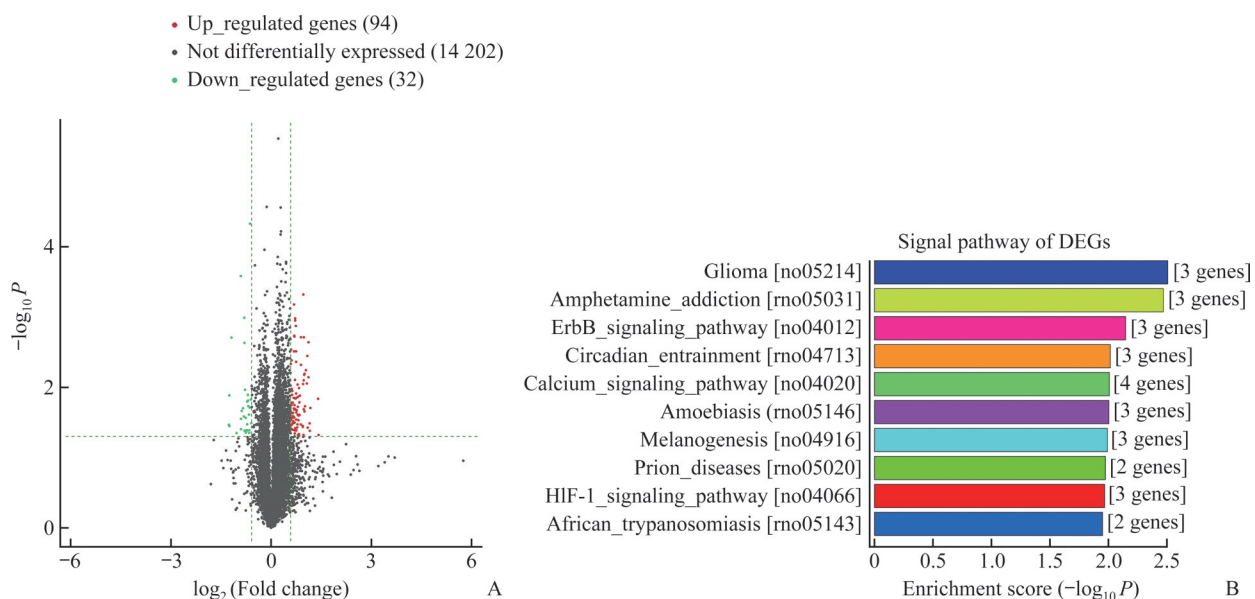


图2 抑制 ACC-SDH 投射神经元活性后脊髓差异表达基因筛选及 *Lamb1* 表达验证

Figure 2 Identification of differentially expressed genes in the spinal cord after inhibition of ACC-SDH projection neurons and validation of *Lamb1* expression

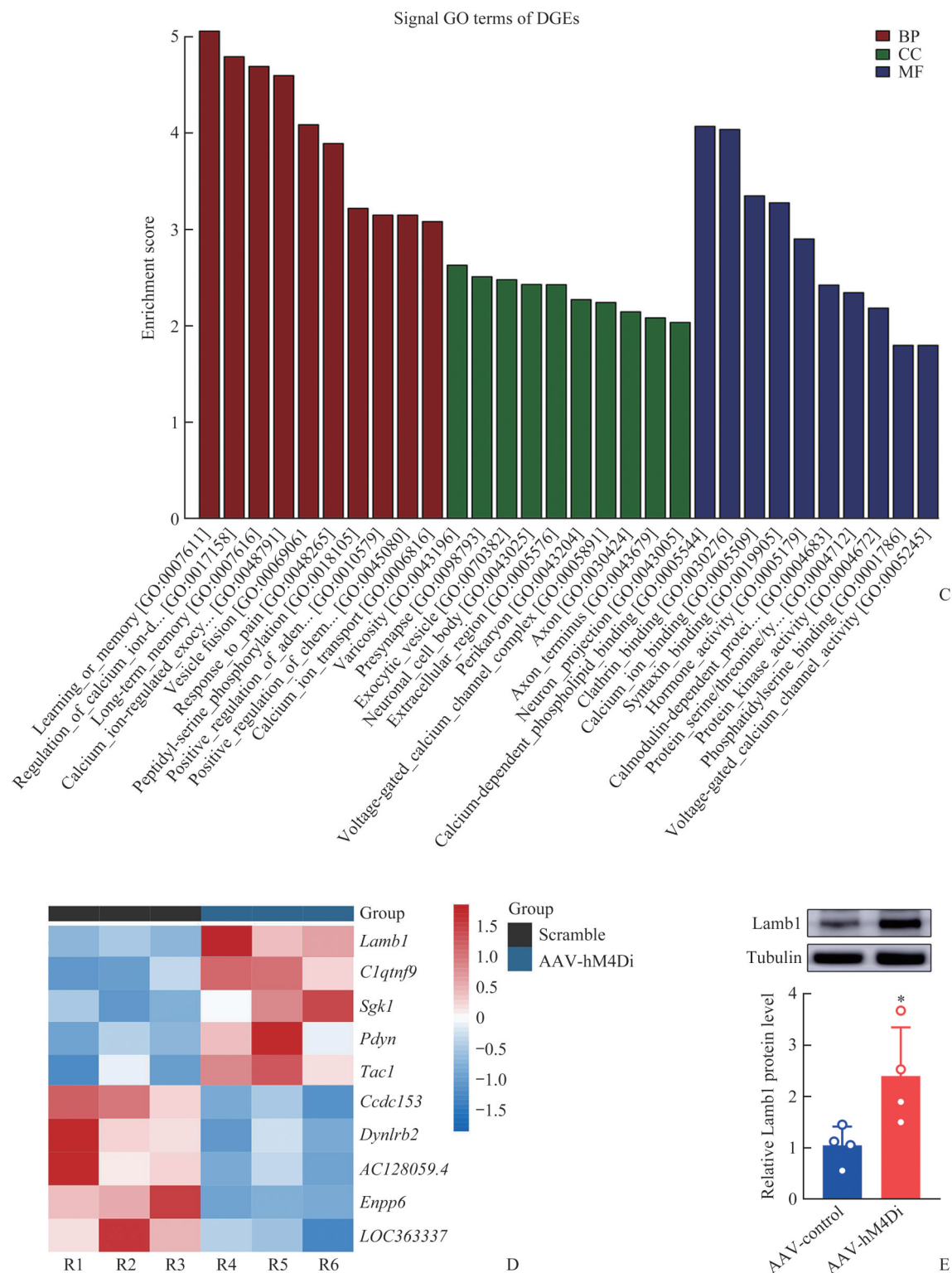


图2 抑制ACC-SDH投射神经元活性后脊髓差异表达基因筛选及Lamb1表达验证(续)

Figure 2 Identification of differentially expressed genes in the spinal cord after inhibition of ACC-SDH projection neurons and validation of Lamb1 expression (continued)

A: Volcano plot of DEGs. B: KEGG pathway enrichment analysis of upregulated genes. C: GO enrichment analysis of upregulated genes. D: Heatmap showing the top 5 up-regulated and top 5 down-regulated DEGs. E: WB analysis validating changes in spinal Lamb1 expression following inhibition of ACC-SDH activity. $n=4$. Compared with the AAV-control group, $*P<0.05$. GO: Gene ontology; DEGs: Differentially expressed genes; KEGG: Kyoto Encyclopedia of Genes and Genomes; BP: Biological process; CC: Cellular component; MF: Molecular function; WB: Western blotting.

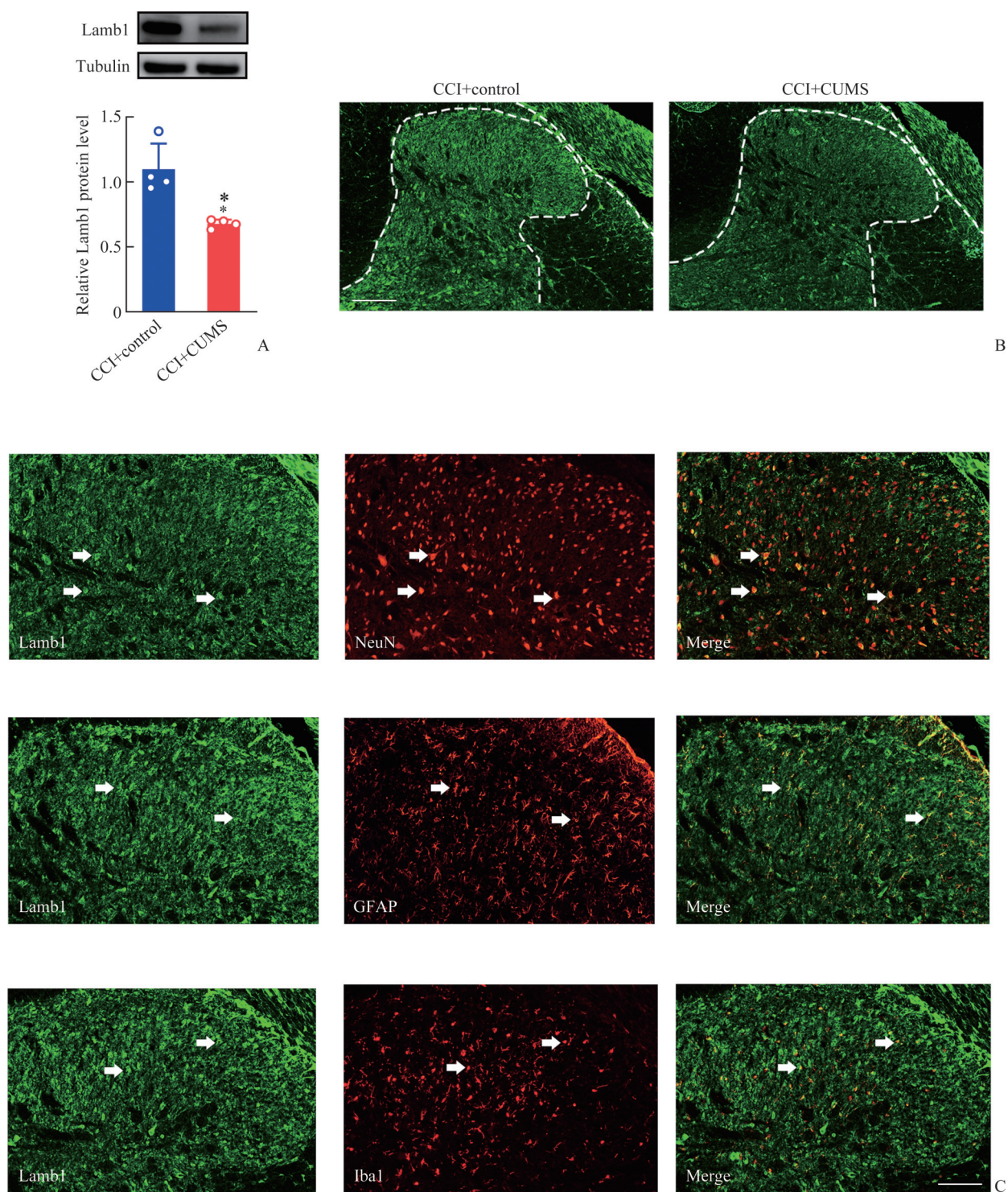


图3 CUMS处理后CCI大鼠脊髓Lamb1表达变化及其细胞定位

Figure 3 Changes in spinal Lamb1 expression in CCI rats after CUMS exposure and its cellular localization in the spinal dorsal horn

A: WB results show that spinal Lamb1 expression was downregulated in CCI rats following CUMS treatment. $n=4$. Compared with the CCI+control group, $*P<0.05$. B: Immunofluorescence analysis of spinal Lamb1 expression. Scale bar=100 μ m. C: Co-localization of Lamb1 with neuronal marker (NeuN), microglial marker (Iba1), and astrocytic marker (GFAP) in the spinal cord of Sham rats. Scale bar=50 μ m. NeuN: Neuronal nuclei; Iba1: Ionized calcium binding adapter molecule 1; GFAP: Glial fibrillary acidic protein.

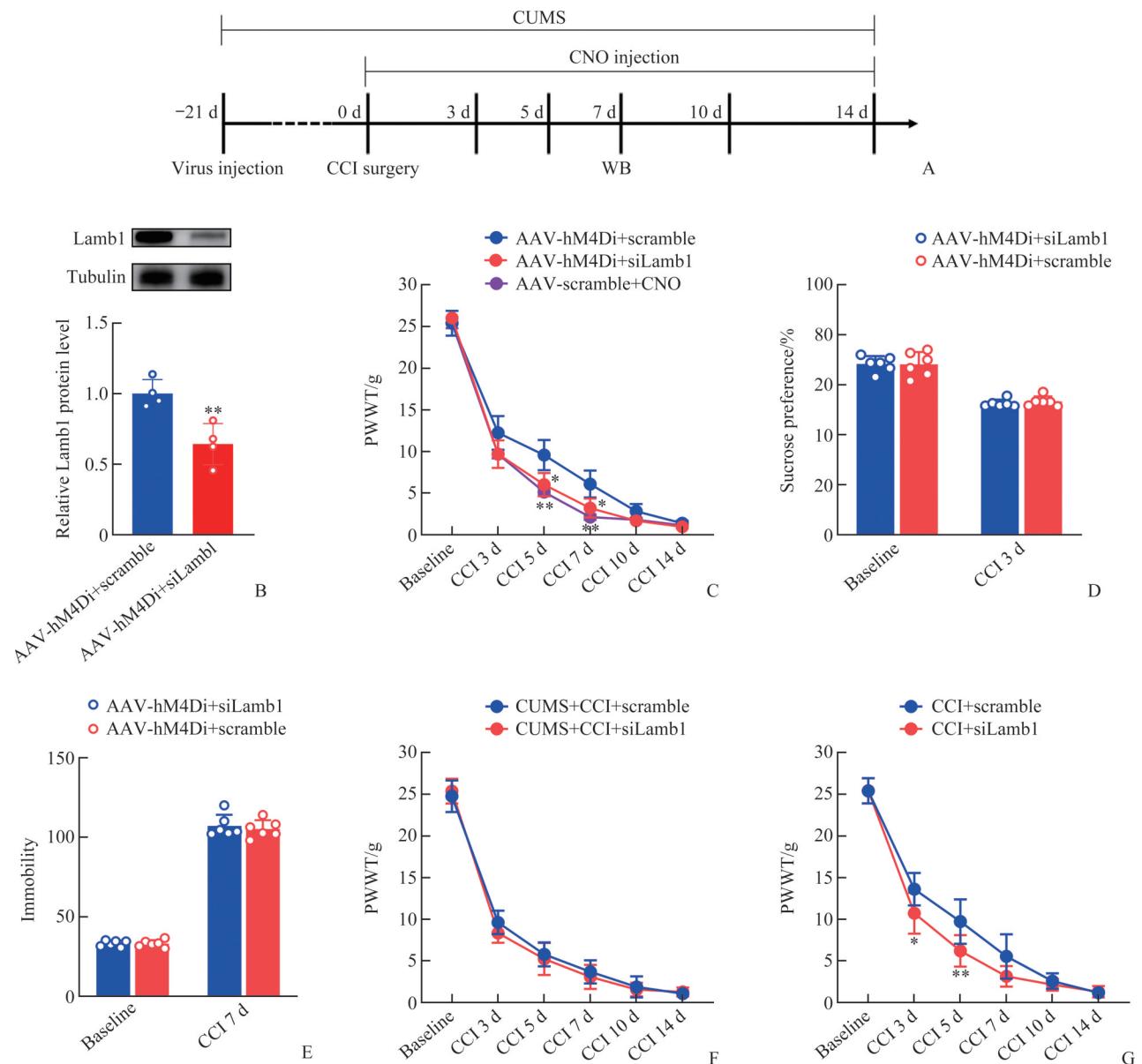


图4 敲减脊髓 *Lamb1* 可以部分逆转抑制 ACC-SDH 通路所致的镇痛作用

Figure 4 Knockdown of spinal *Lamb1* partially reverses the analgesic effect induced by inhibition of the ACC-SDH pathway
A: Experimental schematic. B: WB validation of spinal *Lamb1* knockdown efficiency. $n=4$. Compared with the AAV-hM4Di+scramble group, $**P<0.01$. C: Changes in mechanical pain thresholds after *Lamb1* knockdown in different groups. $n=6$. Compared with the AAV-hM4Di+scramble group, $*P<0.05$, $**P<0.01$. D–E: *Lamb1* knockdown did not affect immobility time in the forced swimming test (D) or sucrose preference (E). $n=6$. F and G: After knockdown of spinal *Lamb1*, changes in the mechanical pain threshold were observed in CCI-treated rats (G) and CCI+CUMS-treated rats (F). $n=6$. Compared with the CCI+scramble group, $*P<0.05$, $**P<0.01$. siLamb1: *Lamb1* knockdown small interfering RNA.

3 讨论

本研究采用 CUMS 联合 CCI 大鼠模型, 结合化学遗传学、转录组测序、WB 及免疫荧光等方法, 探讨了 ACC-SDH 下行通路在负性情绪化神经病理性疼痛中的脊髓分子机制。结果显示, CUMS 可诱导出明显的抑郁样行为, 并进一步加重 CCI 所致的机械性

痛敏; 抑制 ACC-SDH 投射神经元活性后, 大鼠机械痛阈值升高, 提示该通路参与负性情绪对神经病理性疼痛的易化作用。进一步转录组筛选及分子验证结果显示, 脊髓 *Lamb1* 可能是该通路的重要下游分子之一。

ACC 是疼痛与负性情绪相互作用的重要中枢节点, 既参与疼痛情感成分的加工, 也参与焦虑、抑

郁等负性情绪的调控^[17]。本研究发现,抑制ACC-SDH通路可减轻CUMS对CCI所致机械性痛敏的促进作用,但对强迫游泳总不动时间和蔗糖偏好率无明显影响,这与本课题组前期研究^[7]结果一致。这些结果提示,脊髓Lamb1更可能参与伤害性感受信息在脊髓水平的整合及敏化,而不是直接调控抑郁样行为。与ACC等上位脑区相比,SDH主要承担初级感觉信息的传入、突触整合和痛觉敏化调控功能;而抑郁样行为更依赖ACC、前额叶皮质、海马、伏隔核等情绪调节环路^[18-19]。此外,脊髓Lamb1作为ECM相关分子,其作用更可能集中在SDH突触结构稳定、神经元-胶质细胞微环境及痛觉传入整合等方面,因而更容易影响机械痛阈值,而不一定直接调控快感缺失、行为绝望等复杂情绪行为。该现象也提示疼痛感觉维度与情绪维度虽然相互影响,但可能由部分可分离的神经环路和分子机制调控。

RNA测序结果显示,抑制ACC-SDH通路后,脊髓差异表达基因主要涉及ECM、突触功能及神经可塑性相关过程。这提示ACC-SDH通路可能通过调控脊髓局部细胞外环境和神经可塑性,参与负性情绪易化神经病理性疼痛的形成与维持。在众多差异表达基因中,Lamb1上调最为显著,且该结果经WB进一步证实。此外,在CUMS+CCI模型中,脊髓Lamb1表达下调,提示Lamb1表达水平变化与负性情绪促进疼痛敏化的过程具有一致性。上述结果提示,Lamb1表达水平变化不仅是ACC-SDH通路调控后的伴随性改变,Lamb1本身也可能是该通路在脊髓水平发挥作用的重要下游效应分子。值得注意的是,本研究观察到CUMS+CCI状态下脊髓Lamb1表达下降,而抑制ACC-SDH投射神经元活性后Lamb1表达上调。两者并不矛盾,而可能反映Lamb1在脊髓ECM稳态中的保护性作用。负性情绪与神经损伤共同作用时,脊髓Lamb1下降可能削弱ECM对突触结构和神经元兴奋性的稳定作用,从而促进痛觉敏化;相反,抑制ACC-SDH下行易化通路后,Lamb1表达上调可能有助于恢复SDH ECM稳态,限制异常突触可塑性,并最终表现为机械痛阈值升高。因此,Lamb1很可能更适合被理解为脊髓ECM稳态的“刹车分子”或保护性调节因子。

免疫荧光结果显示,Lamb1在脊髓中广泛分布,主要富集于SDH浅层。SDH浅层是初级伤害性感受信息传入和整合的重要区域,因此Lamb1在该区域富集表达提示其可能参与局部突触结构重塑及神经元兴奋性调节。免疫荧光双标结果显示,Lamb1与NeuN、Iba1和GFAP标记区域存在空间邻近或部分重叠,考虑到Lamb1是laminin $\beta 1$ 亚基,一般认为其

主要分布于基膜及ECM,本研究不将该结果简单解释为Lamb1在上述细胞内表达,而倾向于认为Lamb1可能位于神经元及胶质细胞周围的ECM微环境中。结合敲减实验结果,脊髓Lamb1下调后,抑制ACC-SDH通路所产生的镇痛效应被部分逆转,进一步说明Lamb1并非单纯反映病理状态变化的标志物,而是ACC-SDH下行调控在脊髓水平的重要功能分子。值得注意的是,本研究发现敲减脊髓Lamb1仅影响机械痛阈值,而未显著改变强迫游泳总不动时间和蔗糖偏好率,这与既往在ACC内Lamb1敲减可同时影响疼痛和情绪行为的结果并不完全一致^[20]。这一差异提示,同一ECM分子在不同解剖水平可能具有不同的功能分工。SDH中的Lamb1更多承担脑下行调控信号在脊髓局部的执行作用,主要影响痛觉敏化这一感觉维度。因此,脊髓Lamb1更可能是负性情绪易化神经病理性疼痛过程中下行调控的末端效应分子,而非抑郁样行为本身的决定因素。此外,本研究发现敲减脊髓Lamb1可加重CCI大鼠的机械痛敏,但并未改变CUMS+CCI大鼠的机械痛阈值。这一结果提示,脊髓Lamb1的作用具有明显的状态依赖性:在单纯神经损伤背景下,Lamb1更可能限制痛觉敏化;而在负性情绪叠加背景下,CUMS诱导的广泛下行易化和应激网络可能部分掩盖其局部效应。

Lamb1作为laminin的重要亚基,可能通过调节ECM-整合素(integrin)受体信号影响脊髓突触可塑性。已有研究提示,Lamb1可调控胞外信号调节激酶(extracellular signal-regulated kinase, ERK)/丝裂原激活的蛋白激酶(mitogen-activated protein kinase, MAPK)、血清和糖皮质激素诱导的蛋白激酶1(serum and glucocorticoid-induced protein kinase 1, SGK1)及细胞骨架相关信号通路,从而调控突触传递和长时程增强^[21]。在ACC中,Lamb1下调可增强谷氨酸能突触传递,并加重疼痛敏化及焦虑/抑郁样行为^[20-22]。结合本研究的转录组富集结果,抑制ACC-SDH通路后的差异表达基因涉及ECM、突触功能、神经元结构及疼痛相关过程,推测脊髓Lamb1上调可能通过integrin受体相关信号,如integrin/原癌基因酪氨酸蛋白激酶Src(proto-oncogene tyrosine-protein kinase Src, Src)/Ras同源家族成员A(ras homolog family member A, RhoA)/rho相关卷曲螺旋形成蛋白激酶1(rho associated-coiledcoil forming protein kinase, ROCK)或ERK/MAPK通路,影响SDH兴奋性突触结构、突触后致密区蛋白分布及胶质细胞-神经元相互作用,从而限制痛觉敏化。未来可进一步检测integrin $\beta 1$ 、磷酸化Src(phosphorylated Src, p-Src)、RhoA/ROCK、磷酸化ERK(phosphorylated ERK, p-ERK)、SGK1及

囊泡谷氨酸转运体2(vesicular glutamate transporter 2, VGLUT2)与突触后致密区95(postsynaptic density-95, PSD95)的共定位等指标,以明确Lamb1调控脊髓突触可塑性的下游机制。

本研究仍存在一定局限性。首先,因Lamb1编码序列较大,难以直接包装进入常规AAV载体,限制了Lamb1的特异性过表达或功能恢复实验。本研究未完成脊髓Lamb1过表达或功能恢复实验,后续可考虑采用慢病毒、双AAV分段表达、重组蛋白局部递送,或靶向Lamb1功能结构域的干预策略以进行正向验证。其次,本研究主要采用雄性大鼠,尚未评价雌性动物的性激素周期对负性情绪-疼痛的交互作用及Lamb1表达水平的影响。鉴于慢性疼痛和情绪障碍均具有明显性别差异,未来需纳入雌性动物并分层分析。最后,本研究对Lamb1下游信号通路的验证仍不充分,未来应结合免疫荧光、电生理、突触超微结构观察及通路阻断实验,进一步明确Lamb1是否通过integrin/Src/RhoA、ERK/MAPK、SGK1等信号调控脊髓突触可塑性。

综上,本研究证实ACC-SDH下行通路参与负性情绪易化神经病理性疼痛的易化过程,脊髓Lamb1是该通路发挥作用的重要下游分子之一。负性情绪状态下,脊髓中的Lamb1表达下调,抑制ACC-SDH通路后伴随脊髓Lamb1表达上调,敲减Lamb1可部分削弱ACC-SDH通路的镇痛效应。本研究从脑-脊髓下行调控角度为阐明疼痛与负性情绪共病机制提供了新的实验依据,也为相关干预靶点的筛选提供了参考。

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