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1 **Influence of Cerebral Glucose Metabolism by Chronic Pain–Mediated Cognitive**

2 **Impairment in Adolescent Rats.**

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19

20 **Abstract**

21 Chronic pain during adolescence can lead to mental health disorders in adulthood, but the underlying
22 mechanism is still unclear. Furthermore, the homeostasis of cerebral glucose metabolism and
23 neurotransmitter metabolic kinetics are closely associated with cognitive development and pain
24 progression. The present study investigated changes in cognitive function and glucose metabolism in
25 adult rats, which had experienced chronic pain during their adolescence. Here, spared nerve injury (SNI)
26 surgery was conducted in 4-week-old male rats. Mechanical nociceptive reflex thresholds were analyzed,
27 and SNI chronic pain (SNI-CP) animals were screened. Based on animal behavioral tests (open field,
28 three-chambered social, novel object recognition, and the Y maze), the SNI-CP animals showed learning
29 and memory impairment and anxiety-like behaviors, compared to SNI no chronic pain (SNI-NCP)
30 animals. The cerebral glucose metabolism in the prefrontal cortex and hippocampus of adult SNI-CP
31 animals was decreased with positron emission tomography/computed tomography. GABA₂ and Glu₄
32 levels in the metabolic kinetics study were significantly decreased in the hippocampus, frontal cortex and
33 temporal cortex, and the expression of GLUT3 and GLUT4 was also significantly downregulated in the
34 prefrontal cortex and hippocampus of adult rats in the SNI-CP group. These findings suggest that the rats
35 which suffered chronic pain during adolescence have lower cerebral glucose metabolism in the cortex
36 and hippocampus, which could be related to cognitive function during the development of the central
37 nervous system.

38 **Keywords:** Neuropathic pain; Hippocampus; Cortex; Cognitive impairment; Glucose metabolism;
39 Metabolic kinetics

40

41 **Introduction**

42 Chronic pain (such as neuropathic pain, postsurgical pain, visceral pain) is recognized as a
43 multidimensional and subjective experience, which includes sensory, affective, and cognitive
44 components, and can have a serious negative impact on quality of life [1-3]. Psychiatric comorbidities
45 including anxiety [4], depression [5], poor sleep [6] and cognitive impairment [7] are always associated
46 with chronic pain. Functional cognitive disorder has also been reported as one of the most common
47 complications of chronic pain [7], which supports the notion that chronic pain and cognitive impairment
48 share some common pathogenetic mechanisms [8,9]. For example, previous studies have demonstrated
49 that chronic pain preferentially engages the regions involved in cognition and emotion modulation related
50 to pain, such as the medial prefrontal cortex (mPFC), anterior cingulate cortex (ACC), amygdala and
51 hippocampus, leading to comorbid cognitive and emotional deficits [10-13]. The development of chronic
52 pain involves long-term multiple changes, including peripheral, spinal cord and brain neural pain
53 networks [14]. The integration of pain information transmitted from peripheral to central perception leads
54 to complex neuroanatomical, neurochemical and emotional changes [15,16] Multiple brain regions that
55 are involved in processing pain are also implicated in processing affective, motivational, and emotional
56 events [17,18].

57 Chronic pain during childhood and adolescence has been shown to contribute to a heightened risk
58 of anxiety disorders, impaired emotional decision-making, and working memory loss in adulthood [19],
59 which is associated with blocked structural development in brain regions [20], abnormal cerebral
60 metabolism [21,22], hormonal changes [23], central sensitization [24], *etc.* Among these changes, the
61 variations of glucose metabolism and neurotransmitters in affective and somatosensory regions induced
62 by SNI may play a crucial role in the onset and development of cognitive and negative affective

63 components, such as anxiety and depression [25,26]. Research has shown that glucose metabolism in
64 patients with chronic pain was reduced in the dorsal prefrontal cortex and primary motor cortex, possibly
65 due to pain duration [21]. There is also increasing evidence suggesting that glutamatergic and
66 GABAergic neurons contribute to the majority of oxidative glucose metabolism in the brain [27,28].
67 Another study showed that the SNI model induced, overall, decreased activity in mPFC pyramidal
68 neurons with a subsequent reduction in glutamate levels correlated with pain-related depression-like
69 behavior and cognitive impairments [29,30]. To our knowledge, there is no study that has investigated
70 the long-term consequences of chronic pain on brain metabolism and metabolic kinetics on individuals
71 suffering from chronic pain since adolescence.

72 Here, we defined the period of adolescence in rats based on the criteria outlined in the reference
73 [31], which suggests that adolescence begins roughly around PND28. Consequently, four-week-old
74 Sprague-Dawley rats were used as experimental subjects to establish the spared nerve injury (SNI) model.
75 A comprehensive set of animal behavioral studies were implemented at the time of eight weeks after SNI.
76 Then, the cerebral glucose metabolism and glucose transporter protein expressions were initially
77 estimated, and the metabolic dynamics in glutamatergic/GABAergic neurons in the hippocampus and
78 mPFC were investigated using nuclear magnetic resonance (NMR) spectroscopy. The results showed that
79 rats which experienced chronic pain during adolescence had lower glucose metabolism and
80 neurotransmitter disorders in the hippocampus and mPFC in adulthood, which could be the mechanism
81 for chronic pain-mediated cognitive impairment during adolescence, and thus, provide basic neuronal
82 mechanisms for developing novel drugs to treat chronic pain associated with cognitive impairment.

83

84 **Materials and Methods**

85 **Animal preparation**

86 The experimental protocol was approved by the Animal Ethics Committee of Zhongnan Hospital of
87 Wuhan University (Ethics approval number: ZN2021097), and all experiments were performed in
88 accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.
89 The animal experiments were performed in the Animal Experiment Center of Zhongnan Hospital of
90 Wuhan University and complied strictly with the guidelines accepted by the International Association for
91 the Study of Pain. Male Sprague-Dawley rats were purchased from Beijing Vital River Laboratory
92 Animal Technology (4 weeks old, weighing 80-100 g). The rats were group-housed under controlled
93 temperature (23 ± 3 °C) and a 12-h light-dark cycle and had free access to food and water.

94 **Animal surgery**

95 Spared nerve injury (SNI) surgery was performed according to the experimental scheme of a
96 previous work [32]. Briefly, the rats were anesthetized with isoflurane, the fur was shaved on the lateral
97 surface of the left thigh, and the area was disinfected with iodophor. A longitudinal incision was made
98 through the skin caudal to the femur to expose the sciatic nerve and its three terminal branches: sural,
99 common peroneal and tibial nerves. The common peroneal and tibial nerves were delicately dissected
100 and separated from the surrounding tissue. Distal to the trifurcation of the sciatic nerve, sutures (4-0
101 sutures) were applied to ligate the two branches (the tibial and common peroneal nerves). Sectioned distal
102 to the ligation, 2 to 4 mm of the distal nerve stump was removed. During the entire experiment, the
103 operators were very careful not to touch the sural branch, to ensure it was left completely intact. The
104 mechanical paw withdrawal threshold (MPWT) was used to detect hyperalgesia in rats 28 days after the
105 establishment of the model. The rats were divided into two groups: the SNI chronic pain (SNI-CP) group
106 and the SNI nonchronic pain (SNI-NCP) group.

107 **Mechanical paw withdrawal threshold**

108 The mechanical paw withdrawal threshold (MPWT) was assessed one day before the surgery and
109 on Days 1, 3, 7, 14, 28, 42 and 56 postsurgery using an electronic von Frey aesthesiometer (Fig. 1). The
110 withdrawal threshold of the left hind paw was measured in response to a mechanical stimulus. The rats
111 were placed in suspended Perspex frames in a quiet environment at room temperature. The bottom of the
112 frame was covered with wire mesh that had evenly distributed round holes with a diameter of
113 approximately 0.5 cm, which gave access to the plantar surface of the hind paw. Animals were left to
114 habituate to the environment for 30 min. A plastic tip attached to the aesthesiometer was applied to the
115 lateral region of the left plantar surface, which is mostly innervated by the sural nerve through the floor
116 below. Brisk withdrawal and paw flinching were considered positive responses. The withdrawal
117 threshold was determined as the average force (g) required to withdraw the stimulated paw in three trials,
118 with 30 s intervals between the trials.

119 **Behavioral tests**

120 All animals gradually underwent different behavioral studies from postsurgery Day 56 to Day 63
121 (Fig. 1). Between tests, the boxes were wiped with 75% ethanol to eliminate olfactory cues.

122 ***Open field test***

123 The open field test was performed in a gray polyvinylene box (100 × 100 × 40 cm) as previously
124 described [33]. Rats were brought into the testing room 2 h prior to the start of testing to acclimate to the
125 environment. The box was divided into 25 equal squares virtually, with a central area (60 × 60 cm) and a
126 peripheral area (20 cm on each side). Each rat was gently placed in the center of the field and observed
127 for 5 min. The activities of the rats were recorded by a camera fixed above the box. The total distance
128 moved, and the time spent in the central zone were quantified.

129 ***Three-chambered social test***

130 The three-chambered social test was conducted in a chamber (90 × 40 × 30 cm) made from acrylic
131 resin to assess social interaction and social novelty [34]. Rats were allowed to acclimate to the
132 environment in the center chamber for 5 min and were then confined to the central compartment. For
133 social interaction testing, an empty wire cup and a wire cup with an unfamiliar sex-matched rat (S1) were
134 placed on the left or right side of the chamber, and the walls were removed to allow the testing rat to
135 explore for 10 min. For social novelty testing, another strange rat (S2) was introduced to the previous
136 empty cup, and the behaviors of the rat being tested were recorded for 10 min. The time spent in each
137 chamber was recorded. For social interaction testing, the social preference index (SPI) was calculated as
138 follows: time sniffing S1 divided by time sniffing S1 plus time sniffing empty cup (EC). For the social
139 novelty testing, the social preference index (SPI) was calculated as follows: time sniffing S2 divided by
140 time sniffing S1 plus time sniffing S2.

141 ***Novel object recognition (NOR) test***

142 The NOR test was performed according to our previous experiment [33]. Before the experiment,
143 every rat was randomly placed in the apparatus (100 cm×100 cm×40 cm) for 5 min to adapt to the new
144 environment, and then the animals were allowed to freely explore two similar objects for 5 min. Twenty-
145 four hours later, one object was replaced with a novel object with a different shape and color, but the
146 other properties were the same. Every rat was allowed to explore for 5 min. A computer system recorded
147 the movement of the rats. The recognition index (RI) was calculated as follows: time spent exploring a
148 new object divided by time spent exploring a new object plus time spent exploring a familiar object.

149 ***Y maze test***

150 The Y maze test consisted of two tests to assess spatial memory [35]. In the first test, one arm was
151 randomly selected as the novel arm and was closed. The rat was placed at the starting arm and allowed
152 to freely explore the other two arms for 10 min. After 1 hour, a second test was conducted. All the arms
153 were opened, and the rats were put back in the same starting arm and allowed to move freely among the
154 three arms for 5 min. The behaviors of rats were recorded using a video camera. The time spent in each
155 arm, the total entries into the novel arm and the total distance moved were analyzed. The percentage of
156 the residence time in the new arm divided by the residence time in the new arm and in the familiar arm
157 was taken as the preference index of the new arm.

158 **¹⁸F-FDG PET/CT scan**

159 ¹⁸F-FDG PET/CT scans were performed 56 days after the surgery. The rats were fasted for 12 h
160 before scanning. Then, approximately $500 \pm 25 \mu\text{Ci}$ ¹⁸F-FDG was injected through the tail vein with 2%
161 isoflurane. After a 45-min uptake period of ¹⁸F-FDG, rats were placed on the scanning bed and subjected
162 to PET-CT scanning (Raycan Technology Co., Ltd., Suzhou, China). The PET-CT images were obtained
163 using the following parameters: PET scan mode, static; scan time, 10 min; CT scan mode, normal; scan
164 time, 3.5 min; PET reconstruction algorithm, OSEM3D/PSF; iterations, 2; subsets, 12; post filter, slightly;
165 CT reconstruction algorithm, FDK; image size, 256; TFOV scale, 1. The PET images were reconstructed
166 using the three-dimensional (3D) OSEM method with a voxel size of $0.5 \times 0.5 \times 0.5 \text{ mm}^3$. The AMIDE
167 software package (Free Software Foundation, Inc., Boston, Massachusetts, USA) was used to perform
168 the region-of-interest (ROI) analysis. The average glucose uptake of the ROI is presented as the mean
169 standardized uptake value ($\text{SUV} = \text{mean pixel value with decay-corrected ROI activity } (\mu\text{Ci/kg}) / (\text{injected}$
170 $\text{dose } [\mu\text{Ci}] / \text{weight } [\text{kg}])$).

171 **¹H-NMR**

172 **Brain sample preparation:** According to our previous study [36], before the experiment, all animals
173 were fasted overnight and only had free access to water (16-18 hours) in order to reduce endogenous
174 glucose levels. On the following experimental day, the rats were anesthetized with 2.0% isoflurane, and
175 a PE50 tube (Intech, PA, USA) was catheterized into the tail vein for the infusion of [1-¹³C] glucose
176 (Qingdao Tenglong Weibo Technology Co., LTD, Qingdao, P.R. China). After the rat had recovered for
177 approximately 15 min and began moving freely, one end of the infusion tube was connected to a swivel
178 (Instech, PA, USA), and the other side of the swivel was connected to the pump (Fusion 100, Chemyx,
179 TX, USA) using PE50 tubing. Finally, [1-¹³C] glucose was infused through the lateral tail vein in 2 min
180 at a fixed infusion rate (the dosage was based on the previous infusion protocol [37]) under awake and
181 free moving states. The rats were allowed to move freely in the cages for approximately 20 min. Then,
182 all animals were deeply anesthetized with isoflurane and euthanized using head-focused microwave
183 irradiation (Tangshan Nanosource Microwave Thermal Instrument Manufacturing Co. Ltd., China). The
184 brains were manually dissected into 12 different regions: the left frontal cortex (FC_L), right frontal cortex
185 (FC_R), left temporal cortex (TC_L), right temporal cortex (TC_R), left parietal cortex (PC_L), right parietal
186 cortex (PC_R), left occipital cortex (OC_L), right occipital cortex (OC_R), hippocampus (HIP), thalamus
187 (THA), midbrain (MID) and cerebellum (CE). The tissue was weighed and immediately frozen at -80 °C
188 for further processing.

189 **Metabolite extraction:** Briefly, the brain tissue samples were mixed with 400 μL of HCl/methanol
190 (0.1 M) and homogenized with a TissueLyser (QIAGEN, Germany) for 90 s at a frequency of 20 Hz.
191 Then, 800 μL of ethanol (60%, vol/vol) was added, and the mixture was homogenized again. The
192 homogenate was centrifuged at 14,000 g for 10 min, and the supernatant was collected. The above
193 process was repeated twice with 1200 μL of 60% ethanol for adequate extraction. Then, the supernatant

194 was collected and lyophilized (Thermo Scientific, Germany) after removal of organic solvents (methanol
195 and ethanol) in a vacuum environment at 45 °C. The dry product was dissolved with a D₂O buffer solution
196 (600 μL of D₂O with 0.2 M Na₂HPO₄/NaH₂PO₄, pH=7.2) for [¹H-¹³C]-NMR analysis, and the chemical
197 TMSP (3-(trimethylsilyl) propionic-2,2,3,3-d₄ acid sodium salt, 5 mM) was selected as the internal
198 standard chemical in the buffer.

199 *NMR spectrum acquisition:* All NMR spectra were obtained at 298 K with a BrukerAvance III 500
200 MHz NMR spectrometer (BrukerBioSpin, Germany). With POCE (proton-observed carbon editing, ¹H-
201 [¹³C]-NMR) pulse sequence, the ¹³C enrichment of metabolites was presented as the subtraction between
202 one spin-echo measurement without inversion pulse applied at the ¹³C frequency (the total metabolite
203 concentrations, ¹²C+¹³C) and the other with the inversion pulse (the difference of metabolites, ¹²C-¹³C).
204 The collection parameters used were described in our previous study: number of scans, 64; repetition
205 time, 20 s; sweep width, 20 ppm; acquisition data, 64 K; and echo time, 8 ms.

206 *NMR data processing:* The phase and baseline correction of the NMR spectra were manually
207 performed in Topspin (Version 2.1, Bruker BioSpin, Germany). Then, all the spectra were automatically
208 processed and analyzed with the homemade software NMRSpec based on MATALAB (freely available
209 by request: jie.wang@wipm.ac.cn). After the peak alignment in NMRSpec, the ¹³C enrichment in
210 different kinds of metabolites was automatically integrated and calculated.

211 **Western blotting**

212 The rats in different groups were anesthetized with isoflurane, and the frontal cortex and
213 hippocampus were collected at 4 °C and stored at -80 °C. Tissue proteins were extracted using RIPA
214 total protein lysate with proteinase inhibitor. The protein concentrations were measured by the BCA
215 Protein Assay Kit. Then, the mixed loading buffer samples were denatured in 95 °C water for 10 minutes.

216 Equal amounts of protein samples were separated by electrophoresis on 8-15% SDS-PAGE gels and
217 transferred onto PVDF membranes (Aspen). After incubation with western blotting-specific blocking
218 solution (5% skimmed milk powder (AS1107, Aspen, China) diluted in TBST), the PVDF membranes
219 were incubated with anti-GLUT4 (1:1000, AGT-024, Alomone), anti-(extracellular) GLUT3 (1:1000,
220 AGT-023, Alomone) and anti- β -actin (1:10,000, Aspen) antibodies at 4 °C overnight. Then, the blots
221 were washed, incubated with HRP-conjugated secondary antibody for 1.5 h and detected by a
222 chemiluminescent imaging system (Tanon, China).

223 **Immunofluorescence**

224 The rats were anesthetized with isoflurane, transcardially perfused with PBS and subsequently
225 incubated with 4% paraformaldehyde. The fixed brain was then rapidly removed, postfixed in
226 paraformaldehyde at 4 °C for 48 h, embedded, and sectioned for immunofluorescence. The tissue sections
227 were washed and incubated with IF-specific blocking solution (10% goat serum (AR0009, Booster,
228 China) diluted in PBS) for 30 min. The sections were incubated with anti-GLUT3 (1: 100, AGT-023,
229 Alomone) and anti-GLUT4 (1: 200, AGT-024, Alomone) overnight at 4 °C. After three washes with PBS,
230 the sections were incubated with Cy3 goat anti-rabbit secondary antibody (1:50, AS-1109, Aspen) at
231 37 °C for 2 h in the dark. The sections were imaged using an Aperio VERSA 8 microscope (Leica,
232 Germany), and the fluorescence intensity of the images was analyzed using imaging software.

233 **Statistical analysis**

234 Statistical analysis was performed using SPSS 21.0 statistical software (IBM, New York, USA).
235 Student's t-test was used to compare the differences between the 2 groups. Two-way analysis of variance
236 was applied to calculate the values of the mechanical paw withdrawal threshold (MPWT). $P < 0.05$ was
237 considered statistically significant. All results are expressed as the mean \pm SEM.

238

239 **Results**

240 **Chronic pain and nonchronic pain following SNI**

241 Mechanical hyperalgesia was assessed one day before the surgery and on Days 1, 3, 7, 14, 28, 42
242 and 56 postsurgery using the electronic von Frey aesthesiometer. The mechanical paw withdrawal
243 threshold (MPWT) was used to separate the rats with chronic pain from those with nonchronic pain at
244 28 days after establishment of the model. All animals were divided into two groups: the SNI chronic pain
245 (SNI-CP) group and the SNI nonchronic pain (SNI-NCP) group. The results suggest that mechanical
246 hypersensitivity did not develop continuously in approximately one-quarter of the rats, and MPWT
247 gradually recovered to baseline within one week. The rats in the SNI-CP group developed marked
248 hypersensitivity to stimulation with von Frey filaments from the first day after surgery that lasted until
249 two months after SNI surgery (Fig. 2a).

250 **Effect of chronic pain in adolescence on animal behaviors**

251 The open field test was used to evaluate emotion-related behaviors. The results indicate that SNI-
252 CP rats spent less time in the central zone than SNI-NCP rats did (Fig. 2b). The three-chamber social test
253 was used to assess the sociability and social novelty of the animals. For the sociability test, compared
254 with that of SNI-NCP rats, the SNI-CP group exhibited a significantly lower social preference index
255 (SPI). For the social novelty test, rats in the SNI-CP group did not show a preference for the Stranger 2
256 chamber (S2), and the social preference index (SPI) in the SNI-CP group was lower than that in the SNI-
257 NCP group (Fig. 2c). The novel object recognition test (NOR) and Y maze test were used to estimate the
258 recognition memory and spatial memory of the rats. In the novel object recognition test, the recognition
259 index (RI), which is calculated as the time spent exploring a new object divided by the time spent

260 exploring a new object plus the time spent exploring a familiar object, was used as a measure of NOR.
261 Compared to that of the SNI-NCP group, the SNI-CP group exhibited a significantly lower RI (Fig. 2d).
262 In the Y maze test, the SNI-CP group spent less time in the novel arm than the SNI-NCP rats did (Fig.
263 2e). These data suggest that the chronic pain in adolescence induced anxiety-like behaviors, social
264 deficits and recognition and spatial memory deficits in adulthood.

265 **Effect of chronic pain in adolescence on cerebral glucose metabolism**

266 To evaluate the changes in regional glucose metabolic activities between the two groups, we
267 performed an ^{18}F -FDG PET/CT scan to detect glucose uptake at 56 days postsurgery (n=4), and the data
268 were semi-quantitatively analyzed from the images after attenuation correction. Representative brain
269 images of ^{18}F -FDG PET/CT of the two groups are shown in Fig. 3a (coronal section, sagittal section and
270 horizontal section, from left to right). The results show that SUVs in the prefrontal cortex (PFC), motor
271 cortex (MC), somatosensory cortex (SC), and hippocampus (HIP) of both the left and right sides were
272 lower in SNI-CP rats than in SNI-NCP rats (Fig. 3b). We also compared the SUVs between the left and
273 right brain regions from the SNI-NCP and SNI-CP groups, and the results demonstrate that there was no
274 difference between the left and right brain regions in the SNI-NCP group and the SNI-CP group (Fig. 3c,
275 $p>0.05$).

276 **Effect of chronic pain in adolescence on cerebral metabolic kinetics**

277 Here, a POCE NMR pulse sequence was employed to investigate the metabolic composition of the
278 brain extracts. Examples of NMR spectra from the prefrontal cortex in two different groups are illustrated
279 in Fig. 4a. The relative concentrations of the metabolites were obtained from the PQN normalized
280 nonedited spectrum (upper two spectra), and the total concentrations of ^{13}C -labeled metabolites were
281 calculated by subtracting the two series of spectra in POCE data, which are shown in the lower two

282 spectra. The NMR spectrum demonstrate that the enrichment rates of metabolites in SNI-CP were lower
283 than those in SNI-NCP. The differences in metabolites between the two groups are shown by the heat
284 map (Fig. 4b), and the statistical t values of metabolites for different brain regions between the two groups
285 are illustrated by the color of every grid in the heat map, in which the grid with a white point is considered
286 statistically significant ($p < 0.05$). The heat map directly indicates the types of metabolites that are
287 statistically significant between the two groups in the studied brain regions. These results show that it is
288 necessary to further analyze the data in order to obtain more detailed implications.

289 With the infusion of $[1-^{13}\text{C}]$ -glucose, glucose-related metabolites were gradually labeled through
290 tricarboxylic acid (TCA) cycle flux in neurons and astrocytes. In the first cycle, Glu₄ was labeled in
291 glutamatergic neurons, GABA₂ in GABAergic neurons and Gln₄ in astroglia. Compared with that of the
292 SNI-NCP group, the results confirm that Glu₄ enrichment in the SNI-CP group was significantly
293 decreased in the FC_L, FC_R, TC_L, TC_R, HIP, THA, MID and CE (Fig. 4c), while GABA₂ enrichment in
294 the SNI-CP group was decreased in the FC_L, FC_R, TC_L, TC_R, HIP, THA and CE (Fig. 4d). Glx₃ (Glu +
295 Gln) enrichment in the SNI-CP group was significantly lower in the FC_L, FC_R, TC_L, TC_R, HIP and THA
296 than the enrichment in the SNI-NCP group (Fig. 4e). Gln₄ enrichment was significantly decreased in the
297 FC_L, FC_R, TC_L, TC_R, HIP, THA and CE in the SNI-CP group relative to the SNI-NCP group (Fig. 4f).
298 We also compared the differences in metabolites between the left and right frontal cortex (FC), temporal
299 cortex (TC), parietal cortex (PC) and occipital cortex (OC) in the SNI-NCP and SNI-CP groups, and the
300 results indicate that there was no significant difference in metabolites between the left and right cortex
301 in SNI-NCP and SNI-CP rats (Fig. 5a, b, $p > 0.05$), which is also similar to the former PET study.

302 **Effect of chronic pain in adolescence on GLUT3 and GLUT4 protein expression in the**
303 **hippocampus and frontal cortex.**

304 Glucose metabolism in neurons is closely related to glucose transporters, especially for GLUT3 and
305 GLUT4; thus, the influence of these two transporters on chronic pain in adolescence was further
306 investigated to disclose the reason for the suppression of glucose utilization and metabolic kinetics in the
307 hippocampus and frontal cortex in adult rats. As shown in Fig. 6a-c, immunofluorescence staining
308 revealed a significant decrease in GLUT3 and GLUT4 levels in the hippocampus and prefrontal cortex
309 of the SNI-CP group compared with the SNI-NCP group. Western blot analysis showed that the SNI-CP
310 group rats had lower GLUT3 protein expression (Fig. 6d, e) and GLUT4 protein expression (Fig. 6d, f)
311 in both the hippocampus and prefrontal cortex than that of SNI-NCP rats.

312

313 **Discussion**

314 Clinical studies have demonstrated that the majority of children and adolescents who suffer from
315 chronic pain also experience cognitive impairment and mood disorders [38-42], but the underlying
316 mechanisms remain largely unclear. Some studies have proposed neuroplasticity, competing limited
317 resources, or dysregulated neurochemistry to explain the potential mechanisms involved in pain-related
318 cognitive impairment [43,9]. In this study, we found that chronic pathologic pain during adolescence
319 resulted in the impairment of emotion-related cognition (anxiety-like behaviors and social interaction
320 deficits), working memory and spatial memory loss (novel object recognition test and Y maze test) in
321 adult rats, which was further supported by the finding that the SNI-CP group had reduced glucose uptake
322 and transport in the PFC and hippocampus and inhibited ¹³C enrichment of GABA₂ and Glu₄ in the PFC
323 and HIP. Our results offer greater evidence of chronic pain, not peripheral nerve injury or transient pain,
324 mediating cognitive impairment and mood disorders, which could be attributed to energy deficiency and
325 disorder of neurotransmitters.

326 In addition to pain sensory symptoms, neuropsychological functioning is affected by SNI [25], and
327 the complex forebrain network is considered the substrate for negative affective states and cognitive
328 impairments [44]. However, in young animals that do not have a mature immune system, tactile allodynia
329 and microglial activation are not evident and do not develop until the rats are at least 4 weeks old at the
330 time of SNI induction [45,46]. Here, we screened pain and no pain animals in 4-week-old SNI rats and
331 tested whether behavioral function in adulthood was influenced by chronic pain. We found that the
332 adolescent rats with chronic pain exhibited anxiety and depression-like behaviors and cognitive
333 impairment on Day 56 after SNI surgery. Interestingly, no behavioral abnormalities were observed in
334 adult SNI-NCP animals who showed initial mechanical hypersensitivity immediately after surgery that
335 returned to baseline thresholds within one week.

336 The brain is an organ with the most abundant energy metabolism in the human body. The high
337 energy consumption in the brain dominantly derived from glucose metabolism makes it vulnerable to
338 impaired energy metabolism [22]. Deficits in glucose metabolism heavily affect human brain health,
339 especially cognitive function [47]. Substantial evidence has also shown that in aging subjects,
340 performance deficits on a series of cognitive tasks during training are due to insufficient cerebral glucose
341 supply [48,49]. Previous studies proposed that decreased regional glucose metabolism was closely
342 correlated with cognitive impairments [50]. ¹⁸F-FDG PET imaging revealed that patients with cognitive
343 impairments exhibited significantly lower metabolism in the right cerebellar posterior lobe, left cerebellar
344 anterior lobe, bilateral thalamus and left limbic lobe and hypometabolic changes in the temporoparietal
345 and posterior cingulate cortices [51]. Individuals with regional brain glucose hypometabolism have an
346 increased risk of incident cognitive dysfunctions compared to those with normal glucose metabolism
347 [48]. Impaired brain glucose metabolism compromises transmembrane ion transport, vesicle recycling,

348 neurotransmitter release and synaptic signaling, leading to hyperexcitability, excitatory-inhibitory
349 imbalance and functional impairment of cortical networks, which further compromises the brain's energy
350 efficiency [52]. Insufficient neuronal glucose and mitochondrial energy generation compromise the
351 clearance of neurotoxic proteins from the brain. Energy deficits and neurotoxic protein accumulation
352 mutually aggravate one another in a vicious cycle, accelerating the development of cognitive dysfunction
353 [53]. It was demonstrated that glucose metabolism was contralaterally reduced in the dorsal prefrontal
354 cortex and primary motor cortex in patients with chronic pain who were diagnosed with complex regional
355 pain syndrome (CRPS) [21]. In SNL rats, regional cerebral glucose metabolism was decreased in the
356 thalamus, left primary somatosensory cortex and right cerebellum [54]. In the present study, chronic pain
357 impaired glucose metabolism in the PFC and hippocampus, with no difference between left and right
358 glucose metabolism, leading to more profound task-associated depletion of local brain glucose correlated
359 with impaired cognitive performance. Increased local glycolytic metabolism is key to supporting the
360 energy demands of the Na^+/K^+ ATPase and promoting enhanced cellular excitability and synaptic
361 plasticity.

362 The developing rat brain undergoes a series of functional and anatomic changes that affect its rate
363 of cerebral glucose utilization. The cellular expression of glucose transporter proteins is rate limiting for
364 cerebral glucose utilization during early postnatal development in rats [55]. Glucose transport across the
365 plasma membrane is the most upstream and one of the most important processes in cellular glucose
366 metabolism, and evidence indicates that GLUT3 and GLUT4 are altered in cognitive development
367 [56,57], which could also explain the cellular machinery fueling the transmembrane transport of
368 glutamate through glycolytic enzymes and glucose transporters in a model of chronic pain. The increase
369 in GLUT3 expression is further seen in the brain regional variation in the GLUT3 expression pattern

370 with maturation, which coincides with the increased need for the supply of fuel to meet these demands
371 [58]. Our results show that chronic pain in adolescent rats decreased both GLUT3 and GLUT4 expression
372 in the hippocampus and mPFC. Demonstrations of the decreased glucose transport and glycolysis provide
373 further confirmation of cognitive impairment associated with chronic pain.

374 Glucose is required to provide precursors for neurotransmitter synthesis. Glucose metabolism
375 provides fuel for physiological brain function through the generation of ATP, the foundation for neuronal
376 and nonneuronal cellular maintenance, as well as the generation of neurotransmitters [28]. A proposed
377 mechanism of chronic pain is dysregulation between the main inhibitory (GABA) and excitatory
378 (glutamate) neurometabolites of the central nervous system [59]. GC J's studies showed that pain-related
379 plasticity in the BLA and synaptic inhibition of mPFC pyramidal cells were glutamate driven, which
380 impairs mPFC function and produces cognitive decision-making deficits [60]. Pain-related inhibition of
381 mPFC neurons depends on mGluR1-mediated endogenous activation of GABA(A) receptors [61]. At the
382 same time, hippocampal glutamatergic synapses were significantly impaired after peripheral nerve injury
383 [62]. Human brain imaging studies have revealed consistent cortical and subcortical networks that are
384 activated by pain, including the primary somatosensory cortex (S1), secondary somatosensory cortex
385 (S2), anterior cingulate cortex (ACC), insula, prefrontal cortex (PFC), thalamus and cerebellum [11].
386 Findings from studies using proton magnetic resonance spectrometry show increased glutamate levels
387 and decreased neuronal marker N-acetyl aspartate in the frontal cortices of patients with chronic back
388 pain and fibromyalgia. In our study, we found that the dynamics information of Glu4 (FC_L, FC_R, TC_L,
389 TC_R, HIP, THA, MID, and CE), GABA₂ (FC_L, FC_R, TC_L, TC_R, HIP, THA, and CE), Glx₃ (FC_L, FC_R, TC_L,
390 TC_R, HIP, and THA) and Gln₄ (FC_L, FC_R, TC_L, TC_R, HIP, THA, and CE) were significantly reduced in
391 SNI-CP rats. This can quantitatively be observed by considering the relationship between

392 neurotransmitter glutamate-glutamine cycling and glucose metabolism determined over a large range of
393 neural activities.

394 Furthermore, lactate acts as a signaling molecule in the brain linking metabolism, substrate
395 availability, blood flow and neuronal activity [63]. It can modulate various neuronal functions, including
396 excitability, plasticity and memory consolidation [64], and homeostatic functions [65]. Unfortunately,
397 the present study is an *in vitro* study, the lactate concentration and its metabolism are very unstable
398 [66,67], and its concentration is significantly increased due to post-mortem changes. To decrease these
399 consequences, the head focused microwave method [67] was utilized to fix the brain and immediately
400 cease the metabolism. Thus, the changes of lactate kinetics in the brain regions were not studied in this
401 study.

402 In conclusion, this study confirmed that animals experience chronic pain in adolescence with
403 induced anxiety and social deficits, as well as recognition and spatial memory defects. GABA and
404 glutamate neurometabolites in the mPFC and hippocampus were impacted by glucose transporters and
405 metabolism, which could be the key parameters of cognitive impairment associated with chronic pain in
406 adolescence. Our findings suggest that elevated glucose metabolism in neurons should be considered a
407 new effective strategy for the treatment of cognitive impairment in patients with chronic pain.

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411

412 **Author Contributions**

413 Yuanyuan Fang, Chang Chen, Jie Wang, and Zongze Zhang designed the study; Yuanyuan Fang,

414 Qi Zhong, Lirong Wang, Zhu Gui, and Jinpiao Zhu performed the experiments; Yuanyuan Fang, Chang
415 Chen, Jie Wang, and Fuqiang Xu contributed to the data. Yuanyuan Fang, Chang Chen, Jie Wang,
416 Zongze Zhang, and Anne Manyande wrote the manuscript. The content of this manuscript has been
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422

423 **Availability of data and materials**

424 All raw data and materials during the current study are available from the corresponding author
425 upon reasonable request.

426 **Compliance with Ethical Standards**

427 All animal procedures were carried out in accordance with the National Institutes of Health
428 Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Ethics
429 Committee of Zhongnan Hospital of Wuhan University (Ethics approval number: ZN2021097).

430

431 **Conflict of interest**

432 All authors claim that there are no conflicts of interest.

433

434 **Consent to Participate** Not applicable.

435

436 **Consent for Publication**

437 All authors have read the manuscript and agreed for its publication.

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645 **Figure legends**

646 **Fig. 1 Illustration of the experimental design.** SNI surgery was performed on 4-week-old male SD rats
647 at D1. The mechanical paw withdrawal threshold (MPWT) was assessed at D0, D1, D3, D7, D14, D28,
648 D42 and D56. PET/CT and NMR were performed at D56 (n=8). We harvested brain tissue (hippocampus
649 and prefrontal cortex) at D56 for western blot (WB) and immunofluorescence (IF) experiments. Two
650 groups of rats (n=8) were used for behavioral tests from D56 to D63.

651

652 **Fig. 2 Chronic pain in adolescence induces anxiety-like behaviors and cognitive impairment in**
653 **adulthood. (a)** The mechanical paw withdrawal threshold was measured by an electronic Von Frey. **(b)**
654 Representative traces in two groups in the open field test (left panel). Quantification of the time spent in
655 the central zone (right histogram). **(c)** Quantification of the social preference index (SPI) in the social
656 interaction test (left histogram); quantification of the social preference index (SPI) in the social novelty
657 test (right histogram). **(d)** Representative traces in the two groups during the testing phase of the novel
658 object recognition test (left panel). Quantification of the recognition index (RI) (right histogram) during
659 the testing phase. **(e)** Representative tracing heatmap during the testing phase of the Y maze test (left
660 panel). Quantification of novel arm preference (right histogram) during the testing phase. Data are
661 presented as the mean \pm SEM. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, compared to the SNI-NCP group, n=
662 8 per group.
663

664 **Fig. 3 The effect of chronic pain in adolescence on glucose metabolism in adulthood. (a)**
665 Representative images of ¹⁸F-FDG PET/CT of a rat brain in each group (coronal section, sagittal section
666 and horizontal section, from left to right). **(b)** Quantification of glucose metabolism in different brain
667 regions from the two groups. **(c)** Comparison of SUVs in the left and right brain regions from the SNI-
668 NCP group (left histogram); comparison of SUVs in the left and right brain regions from the SNI-CP
669 group (right histogram). Data are presented as the mean \pm SEM. * $p < 0.05$, compared to the SNI-NCP
670 group, n= 4 per group.
671

672 **Fig. 4 The effect of chronic pain in adolescence on cerebral metabolic kinetics in adulthood. (a)**
673 Examples of NMR spectra for total metabolites ($^{12}\text{C}+^{13}\text{C}$, upper panel) and ^{13}C -labeled metabolites
674 ($2*^{13}\text{C}$, lower panel) from the hippocampus in the two groups. Note: Asp: aspartate; Cre: creatine; GABA:
675 γ -aminobutyric acid; Gln: glutamine; Glu: glutamate; Glx: glutamine + glutamate; NAA: N-
676 acetylaspartate; Subscript number: ^{13}C labeled positions in different metabolites. **(b)** The difference in
677 metabolites between the two groups in the heat map; the statistical t value of the metabolites in different
678 brain regions between the two groups is illustrated by the color of every grid in the heat map in which
679 the grid with a white origin was considered statistically significant between the two groups ($p < 0.05$). **(c-**
680 **f)** The enrichment of Glu₄ **(c)**, GABA₂ **(d)**, Glx₃ **(e)** and Gln₄ **(f)** in different brain regions in the two
681 groups. Values represent the mean \pm SEM. * $p < 0.05$, ** $p < 0.001$, compared to the SNI-NCP group. $n = 8$
682 per group.
683

684 **Fig. 5 Comparison of metabolites between the left and right cortex (FC, TC, PC, and OC) in the**
685 **SNI-NCP group and the SNI-CP group. (a)** Comparison of metabolites between the left and right
686 cortex (FC, TC, PC, and OC) in the SNI-NCP group. **(b)** Comparison of metabolites between the left and
687 right cortex (FC, TC, PC, and OC) in the SNI-CP group. Data are presented as the mean \pm SEM. n= 8
688 per group. Note: FC: frontal cortex; TC: temporal cortex; PC: parietal cortex; OC: occipital cortex.
689

690 **Fig. 6 The protein expression of GLUT3 and GLUT4 in the hippocampus and prefrontal cortex.**
691 **(a)** Fluorescent images showing GLUT3 expression in neurons of the hippocampal CA1 area and
692 prefrontal cortex (scale bars, 50µm). **(b, c)** Quantification of GLUT3 and GLUT4 intensity in the CA1
693 region of the hippocampus and prefrontal cortex in the two groups. **(d)** WB analysis of GLUT3 and
694 GLUT4 protein expression in the hippocampus and prefrontal cortex in the two groups. **(e, f)** Histograms
695 showing the quantification of GLUT3 and GLUT4 blots in the hippocampus and prefrontal cortex. n= 4
696 per group. * $P < 0.05$ versus the SNI-NCP group. Data are presented as the mean \pm SEM.
697