SUPPLEMENTARY MATERIAL

METHODS

TUNEL and Fluoro-Jade B staining. Naïve rats and sham- and CCI-treated rats at 14 d after nerve injury were deeply anesthetized with pentobarbital and perfused transcardially with fixative at 6 h after single injection of fluorocitrate or vehicle or 4 h after injection of recombinant cytokines in the RVM. RVM sections (n=3 rats per group) were collected in cold 0.1 M PB and distributed into three alternate series. One series was Nissl-stained with 0.1% cresyl violet. The second series was reacted for the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) staining with *In situ* cell death detection kit (Fluorescein, Roche, Mannheim, Germany) as described previously (Wei et al, 2003). The third series was used for Fluoro-Jade B staining as described previously (Schmued and Hopkins, 2000). Negative control sections were processed similarly, but the TdT enzyme or Fluoro-Jade B was omitted. In addition, immunofluorescence double labeling with TUNEL staining and a neuronal marker, as detected with an anti-NeuN antiserum, or glial marker GFAP and CD11b, was used to assess neuronal apoptosis or glial loss after microinjection of fluorocitrate, respectively. TUNEL/NeuN labeled cells were counted from epicenter to 2 mm rostral and caudal to the needle track on RVM sections of fluorocitrate-treated, sham and naïve rats (n=4-6 per group). For Fluoro-Jade staining, slide-mounted sections were immersed in a solution containing 1% sodium hydroxide in 80% alcohol for 5 min, and then in 70% alcohol for 2 min and in distill water for 2 min. The sections were transferred to a solution of 0.06% potassium permanganate for 2-10 min, rinsed and stained for 10 min in 0.0001% solution of Fluoro-Jade B (Histo-Chem Inc., Jefferson, AR) dissolved in 0.1 % acetic acid. After rinsing and drying, the sections were coverslipped without dehydration. To confirm whether neuronal necrosis in

the RVM after fluorocitrate injection, a double label combining Fluoro-Jade staining with NeuN immunostaining was also performed.

FIGURE LEGEND

Figure S1. Dose dependent effects of glial toxin fluorocitrate on neuronal damage and glial functions. **A. a**: An example of Nissl-stained section showing the track (black star) of intra-RVM microinjection. **b-d**: Few TUNEL labeled cells (white arrowheads) are observed in the RVM of normal rats at 6 h after injection vehicle (**b**, 0.5 µl volume) or low dose of fluorocitrate (FC, 100 fmol) (**c**). However, microinjection of high dose of fluorocitrate (10 pmol) produces massive apoptosis in the RVM (**d**) around the track of injection needle (white stars) at 6 h. **B**: Double staining shows that TUNEL labeled cells are also immunoreactive to NeuN (white arrows), a biomarker for neurons in the RVM treated by fluorocitrate (10 pmol), indicating neuronal apoptosis occurred after local administration of high doses of fluorocitrate in the RVM. **C**: Summary of the neuronal apoptosis after microinjection of vehicle and fluorocitrate in normal rats. There is a significant increase in number of TUNEL labeled neurons at 6 h only after a high dose (10 pmol) but not a low dose (100 fmol, $p < 0.001$) of fluorocitrate (n=4 per group), compared with the naïve animals. ***, $p < 0.001$. **D**. Fluoro-Jade B (FJB) staining shows many cells underlie necrosis in the microinjection sites of RVM sections treated by fluorocitrate (10 pmol) but not (100 fmol). Double staining with NeuN further indicates FJB positive cells are glia-like cells and not neurons, suggesting glial necrosis occurred after local administration of high doses of fluorocitrate in the RVM. **E**: Double immunostaining demonstrates that there is little neuronal apoptosis (**d**) at 6 h after injection of fluorocitrate (100 pmol) compared to vehicle (**a**). Meanwhile, this dose of fluorocitrate attenuates CCI-induced increases in GFAP expression at 14 d

(**e**) but not CD11b expressions at 3 d after CCI (**f**) in the RVM, compared to vehicle treatments (**b** and **c**, respectively). Scale bars: 0.5 mm (A a), 0.2 mm (A b-d), 0.05 mm (B) and 0.1 mm (D and E). 7n, facial nerve; NGC, nucleus reticularis gigantocellularis; NRM, nucleus raphe magnus; Py, pyramidal tract.