

The chemokine CCL2 modulates Ca²⁺ dynamics and electrophysiological properties of cultured cerebellar Purkinje neurons

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Abstract

The chemokine CCL2 is produced at high levels in the central nervous system (CNS) during infection, injury, neuroinflammation and other pathological conditions. Cells of the CNS including neurons and glia express receptors for CCL2 and these receptors may contribute to a signaling system through which pathologic conditions in the CNS are communicated. However, our understanding of the consequences of activation of chemokine signaling in the CNS is limited, especially for neurons. In many cell types, chemokine signaling alters intracellular Ca²⁺ dynamics. Therefore, we investigated the potential involvement of this mechanism in neuronal signaling activated by CCL2. In addition, we examined the effects of CCL2 on neuronal excitability. The studies focused on the rat cerebellar Purkinje neuron, an identified CNS neuronal type reported to express both CCL2 and its receptor, CCR2. Immunohistochemical studies of Purkinje neurons *in situ* confirmed that they express CCR2 and CCL2. The effect of exogenous application on Purkinje neurons was studied in a cerebellar culture preparation. CCL2 was tested by micropressure or bath application, at high concentrations (13–100 nM) to simulate conditions during a pathologic state. Results show that Purkinje neurons express receptors for CCL2 and that activation of these receptors alters several neuronal properties. CCL2 increased resting Ca²⁺ levels, enhanced the Ca²⁺ response evoked by activation of metabotropic glutamate receptor 1 and depressed action potential generation in the cultured Purkinje neurons. Passive membrane properties were unaltered. These modulatory effects of CCL2 on neuronal properties are likely to contribute to the altered CNS function associated with CNS disease and injury.

Introduction

CCL2, previously called monocyte chemoattractive protein-1, is one of a number of chemokines that comprise a distinct class of small proteins involved in inflammatory and chemoattractive processes (Wells *et al.*, 1998). First characterized in the immune system, chemokines act through G-protein-coupled receptors and utilize several transduction pathways including inhibition of adenylate cyclase and activation of phospholipase C (PLC) (Maghazachi, 2000). Recently it was shown that chemokines and their receptors are widely expressed in the central nervous system (CNS), including in microglia, astrocytes and neurons (Horuk *et al.*, 1997; Banisadr *et al.*, 2002; Gillard *et al.*, 2002). These results suggest that chemokines may play a role in the CNS as well as in the immune system (reviewed by Bajetto *et al.*, 2001). Chemokine expression in the CNS is generally low, but elevated levels are produced in response to brain injury and disease and result in activation and chemoattraction of immune cells such as monocytes, which infiltrate the CNS (Lahrtz *et al.*, 1998; Tran & Miller, 2003). Microglia and astrocytes of the CNS are prominent sources of chemokines, although neurons can also produce chemokines (Flugel *et al.*, 2001; Little *et al.*, 2002).

A number of studies have shown that CCL2 is produced at elevated levels in the CNS during conditions associated with neuroinflammation (Conant *et al.*, 1998; Sorensen *et al.*, 1999; Galasso *et al.*, 2000; Zink *et al.*, 2001; Bajetto *et al.*, 2002; Little *et al.*, 2002; Mahad & Ransohoff, 2003; Minami & Satoh, 2003; Brouwer *et al.*, 2004). Recent studies have shown that CCR2, the receptor to which CCL2 preferentially binds, is constitutively expressed in several brain regions including the cortex, hippocampus, caudate putamen, amygdala, hypothalamus and cerebellum, and that expression is up-regulated by acute intraperitoneal lipopolysaccharide injection (Banisadr *et al.*, 2002). Studies of CCR2 knockout mice implicate several important roles for the CCL2 including chemoattraction of monocytes (Gaupp *et al.*, 2003) and neuropathic pain perception (Abbadie *et al.*, 2003). CCL2 has also been shown to have neuroprotective properties (Eugenin *et al.*, 2003). In the adult rat, injection of CCL2 into the ventricles of the brain affects motor activity, consistent with a functional role for CCL2 and its receptor in the CNS (Banisadr *et al.*, 2002).

Although CCL2 and CCR2 have been shown to be expressed in the CNS and CCL2 acting through CCR2 has been shown to alter CNS function (Plata-Salaman & Borkoski, 1994; Banisadr *et al.*, 2002; Abbadie *et al.*, 2003), few studies have investigated the effects of CCL2 on CNS neuronal physiology. To address this issue, we have tested the effects of exogenous application of CCL2 on the physiological properties of cerebellar Purkinje neurons, a CNS neuronal cell type reported to express functional CCR2s (Banisadr *et al.*, 2002; Gillard *et al.*, 2002). Additionally, we examined the expression pattern

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of CCL2 and CCR2 by immunohistochemistry in cultures or histological sections from the rat cerebellum. Results are consistent with a neuromodulatory role for CCL2 in the cerebellum during conditions associated with elevated levels of this chemokine.

Methods

Culture methods

Modified organotypic cultures were prepared from embryonic day 20 rat (Sprague–Dawley, Charles River) cerebella and maintained *in vitro* as described previously (Gruol, 1983; Nelson & Gruol, 2002). In brief, the pregnant dam was anesthetized and the uterus containing the pups removed under sterile conditions. The uterus was stored at 4 °C until the pups were removed and sacrificed, which was achieved by decapitation. The cerebellar cortices were isolated, minced and triturated in saline containing 137 mM NaCl, 5.4 mM KCl, 0.17 mM Na₂HPO₄, 0.22 mM KH₂PO₄, 27.7 mM glucose, 43.8 mM sucrose and 10 mM HEPES–NaOH (pH 7.3 with NaOH). No enzymatic treatment was used. The cell suspension was plated on glass cover slips (Fisher Scientific, Houston, TX, USA) coated with Matrigel (Collaborative Biochemical, Franklin Lakes, NJ, USA). The plating medium contained minimal essential medium (MEM) with Earle's salts and L-glutamine (Life Technologies, Grand Island, NY, USA), 10% heat-inactivated fetal calf serum (Life Technologies) and 10% heat-inactivated horse serum (Life Technologies) and was supplemented with D-glucose to a final concentration of 5.0 g/L. Medium was exchanged twice weekly, with medium as above but without calf serum. Cultures were incubated at 37 °C in a 5% CO₂ humidified atmosphere. Brief treatment with the anti-mitotic agent 5-fluorodeoxyuridine (20 µg/mL, days 4–6 *in vitro*) limited the number of non-neuronal cells in culture. Antibiotics were not used. Unless otherwise stated all chemicals were obtained from Sigma (St Louis, MO, USA). Animal care and all experimental procedures were carried out in accordance with standards set forth by the Animal Care Committee of The Scripps Research Institute.

Immunohistochemistry

Immunohistochemical staining of cerebellar cultures and paraffin-fixed histological sections was performed according to previously published methods (Gruol & Franklin, 1987; Nelson & Gruol, 2002) using an antibody that recognizes CCR2, the primary receptor for CCL2. Additionally, paraffin-embedded fixed histological sections were tested for CCL2 immunoreactivity. The CCR2 and CCL2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were affinity-purified goat polyclonal antibodies, raised against a peptide mapping at the carboxy terminus of the C–C chemokine receptor gene type 2B (CKR-2B) of mouse origin and raised against a peptide mapping at the carboxy terminus of CCL2 of rat origin, respectively.

Cultures were rinsed with phosphate-buffered saline (PBS, 100 mM, pH 7.3) and fixed with methanol for 2 min. Endogenous biotin was blocked using the materials and methods provided in the endogenous biotin-blocking kit (Molecular Probes, Eugene, OR, USA). Cultures were incubated for 1 h at room temperature and overnight at 4 °C in PBS containing primary antibody (1 : 500 dilution) and 0.05% BSA as a blocking agent. Immunoreactivity was detected by an immunoperoxidase reaction using the materials and methods provided in the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA).

For histological sections, neonatal to adult male Sprague–Dawley rats were obtained from Harlan Winkelmann GmbH (Borchen,

Germany). At the desired age, rats were deeply anesthetized and perfused transcardially with 0.9% NaCl containing 0.2% (v/v) heparin (Leo Pharmaceutical Products, Weesp, The Netherlands) for 6 min, followed by perfusion for 8 min with 4% (w/v) freshly prepared phosphate-buffered formaldehyde (pH 7.4). The cerebella were further fixed in the same fixative by immersion overnight at 4 °C, and embedded in paraffin. Cerebella were cut on a microtome in 7-µm-thick sections. These sections were dewaxed in xylene and rehydrated in a graded series of alcohol. Endogenous peroxidase activity was reduced by incubation with 3% H₂O₂ in PBS for 30 min. In between each step, sections were rinsed in PBS. To expose antigenic sites, sections were pre-incubated for 20 min in 10 mM sodium citrate (pH 6.0), microwaved at 750 W for 7 min and incubated for 7 min in the same sodium citrate. Before incubation with antibodies, non-specific background staining was minimized by incubation with 10 mM Tris/HCl, 5 mM EDTA, 150 mM NaCl, 0.25% gelatine (v/v), 0.05% Tween 20 (w/v) (pH = 8.0) supplemented with 0.5% milk powder (Campina, Veghel, The Netherlands) for 1 h. Immunohistochemistry was then performed following the same protocol as described for the cultures (primary antibody dilutions; 1 : 500 for CCR2 and 1 : 400 for CCL2). Before mounting in Entellan (Merck, Darmstadt, Germany), the sections were dehydrated in alcohol and submerged in xylene.

Immunoblotting

Cerebellar granule cell cultures (5 days *in vitro*) from 8-day-old pups were scraped off culture plates in a buffer with 10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.5% NP-40, and a Protease Inhibitor Cocktail Tablet (Boehringer, Indianapolis, IN, USA). Protein concentrations were determined using a Protein Assay Kit (Bio-Rad). Samples of protein (10 µg) were separated by SDS-PAGE using 4–12% Novex NuPage Bis-Tris gels (Invitrogen Life Technologies), transferred overnight onto Immobilon-P membranes (Millipore, Billerica, MA, USA). Uniform transfer was confirmed by Ponceau S staining. The membranes were blocked for 1 h at room temperature in PBS, 0.05% Casein (Pierce Biotechnology, Rockford, IL, USA) and 0.1% Tween 20 and incubated for 2 h at room temperature with primary antibody. CCR2 (42 kDa) was immunolabelled using the same affinity-purified goat (diluted 1 : 3000) polyclonal antibody used for the immunohistochemistry. After washing, the membranes were incubated for 1 h with anti-goat secondary IgG coupled to horseradish peroxidase (Pierce Biotechnology), washed and visualized on photographic film (Kodak, VWR Scientific Products, San Diego, CA, USA) using the ECL chemiluminescent system (Pierce Biotechnology).

Ca²⁺ imaging

Ca²⁺ imaging experiments were performed on cultures of 14–23 days *in vitro* at room temperature in physiological bath saline that consisted of 140 mM NaCl, 3.5 mM KCl, 0.4 mM KH₂PO₄, 1.25 mM Na₂HPO₄, 2.2 mM CaCl₂, 2 mM MgSO₄, 10 mM glucose and 10 mM HEPES–NaOH (pH 7.3). Transmitter receptor antagonists were included in the saline to block synaptic responses. The antagonists used were 100 µM picrotoxin, a GABA_A receptor blocker, 1 µM CGP55845A, a GABA_B receptor antagonist (a gift from Wolfgang Froestl, Novartis, Basel, Switzerland), 25 µM D-APV, an NMDA receptor antagonist (Tocris Cookson, Ellisville, MO, USA) and 5 µM NBQX, an AMPA receptor antagonist (Tocris Cookson).

Intracellular Ca²⁺ levels were measured in the somatic region of individual Purkinje neurons using a standard microscopic fura-2

digital imaging technique (Grynkiewicz *et al.*, 1985; Gruol *et al.*, 1996). Briefly, cerebellar cultures were incubated for 30 min with the Ca²⁺-sensitive fluorescent dye fura-2/AM (3 μM; Molecular Probes) and pluronic F-127 (0.016% w/v; Molecular Probes) in physiological saline (see above). The cells were then incubated in dye-free saline solution for an additional 45 min to allow for cleavage of the acetoxymethyl (AM) ester. Live fluorescence images of one or more neurons in a microscopic field were acquired at excitation wavelengths of 340 and 380 nm using an SIT-66 video camera (DAGE-MTI; Dage, Michigan City, IN, USA) and digitized for real-time display using the MCID imaging software (Imaging Research, St-Catharines, Ontario, Canada). Data were collected at 0.8- to 1.5-s intervals. Intracellular Ca²⁺ levels were calculated by the imaging software by converting fluorescence ratios (340 nm/380 nm) to intracellular Ca²⁺ concentrations using the following formula: $[Ca^{2+}]_i = K_d[(R - R_{min}) / (R_{max} - R)]F_o/F_s$, where R is the ratio value, R_{min} is the ratio for a Ca²⁺-free solution, R_{max} is the ratio for a saturated Ca²⁺ solution, $K_d = 225$ (the dissociation constant for fura-2), F_o is the intensity of a Ca²⁺-free solution at 380 nm, and F_s is the intensity of a saturated Ca²⁺ solution at 380 nm. Calibration was performed using fura salt (100 μM) in solutions of known Ca²⁺ concentration (Molecular Probes calibration kit C-3009). Background subtraction was not used because autofluorescence was minimal. Non-cell-associated background fluorescence was also minimal and its contribution was eliminated by the calibration procedure used. In the current study, measured Ca²⁺ levels are used primarily for comparison purposes and are not intended to provide information about the absolute level of intracellular Ca²⁺.

Electrophysiological recordings

Electrophysiological experiments were performed on cultures of 14–23 days *in vitro* under the same conditions as the Ca²⁺ imaging experiments. Intracellular current-clamp recordings were made in the somatic region of Purkinje neurons using the perforated patch method (Netzeband *et al.*, 1997). The patch recording pipette solution contained 6 mM NaCl, 140 mM K⁺-methylsulfate (Pfaltz & Bauer, Waterbury, CT, USA), 10 mM HEPES-KOH (pH 7.3) and 300 μg/mL amphotericin-B (Calbiochem, La Jolla, CA, USA). Before the patch pipettes were back-filled with amphotericin-B-containing patch solution, the tips were filled with amphotericin-B-free patch solution. Amphotericin-B stock solution (50 mg/mL) was prepared in dimethyl sulfoxide on the day of the experiment and between experiments was kept at 4 °C. Experiments were started when overshooting action potentials at stable amplitude were observed. Data acquisition was performed using the Axopatch-1C amplifier, pCLAMP (v.8.2) software and the DigiData 1320A (all from Axon Instruments, Foster City, CA, USA). Signals were additionally filtered and amplified with a CyberAmp 320 (Axon Instruments) and 60-Hz line noise was minimized using a Hum Bug (Quest Scientific, North Vancouver, BC, Canada). Recordings were monitored on a polygraph and oscilloscope. Measurements of the responses were performed using Axograph v.4.5 or Clampfit v.8.2 software (Axon Instruments). In some studies Mini analysis v.5.6.28 software (Synaptosoft, Decatur, GA, USA) was used to measure spike frequency.

Drug application

CCL2 was applied by micropressure or bath application. For micropressure application CCL2 was dissolved in bath saline (see above) and applied by brief microperfusion (10–20 s) from glass micropipettes placed near the edge of the microscopic field approxi-

mately 100 μm from the cells of interest and positioned to expose the cells of interest. The concentration of CCL2 in pipette was 250 nM. For bath application CCL2 (13–100 nM) was diluted in bath saline, added to the culture and allowed to incubate for 5–10 min before recordings were made. A higher concentration of CCL2 was used for micropressure application than was used for bath application to compensate for peptide dilution and loss that occurs with micropressure application, both of which considerably reduce the concentration of the peptide that can reach the cell surface. Peptide loss occurs because of adhesion of the peptide to the pipette glass. Peptide dilution occurs after ejection of the peptide from the pipette when the peptide is diluted with bath saline before reaching the cell surface. In some experiments tetrodotoxin (TTX; 500 nM; Calbiochem, San Diego, CA, USA) was used to block spike activity.

The mGluR1 agonist (*R,S*)-3,5-dihydroxy-phenylglycine (DHPG) (200 μM; Tocris Cookson) was also applied to the neurons by micropressure. DHPG was dissolved in bath saline (see above) and applied as for CCL2. Fast-Green was included in the DHPG solution to monitor exposure to the agonist. Fast-Green (0.03%) by itself had no effect on neuronal firing or baseline Ca²⁺ levels.

Statistical analysis

A between-cell comparison (unpaired Student's *t*-test, one group *t*-test or ANOVA and Fisher's PLSD) was used to determine the effects of chemokines on response parameters. To control for variability between culture sets, data from CCL2-treated neurons were normalized to data obtained under control conditions in neurons from the same culture set. Statistical significance was determined at a level of $P < 0.05$. Data are reported as the means ± SEM.

Results

Cerebellar Purkinje neurons express CCL2 and the CCL2 receptor, CCR2

The cerebellar cultures are comprised of several cell types found in the cortical region of the rat cerebellum. Among these are Purkinje neurons, granule neurons, inhibitory interneurons and astrocytes, which can be distinguished by morphological criteria (Gruol, 1983; Gruol & Franklin, 1987; Gruol & Crimi, 1988). Detailed information about the expression of CCR2, the predominant receptor for CCL2, in the cerebellum is limited. Therefore, the expression of CCR2 was investigated in both cerebellar cultures and paraffin sections of rat cerebellum by immunostaining with antibodies directed against the C terminal domain of the receptor. Also, to determine whether endogenous CCL2 is present in rat cerebellum, expression of CCL2 was determined in rat cerebellum paraffin sections by immunohistochemistry.

CCR2 staining was observed in the soma and dendrites of cultured Purkinje neurons (14–21 days *in vitro*) (Fig. 1A). Somatic staining was prominent in the perinuclear area, correlating with the cellular region known to be involved in peptide production and packaging (Lippincott-Schwartz, 1993). In addition, punctate surface staining was observed in both the somatic and the dendritic regions of the Purkinje neurons (Fig. 1B). Granule neurons and inhibitory interneurons in the cerebellar cultures also showed CCR2 staining (Fig. 1C). The staining was less intense than in Purkinje neurons but the pattern of staining was similar. Weak CCR2 staining was observed in astrocytes (Fig. 1C).

CCR2 expression in rat Purkinje neurons *in situ* was examined at three ages, postnatal days 11 and 24 and in the adult (2–3 months old).

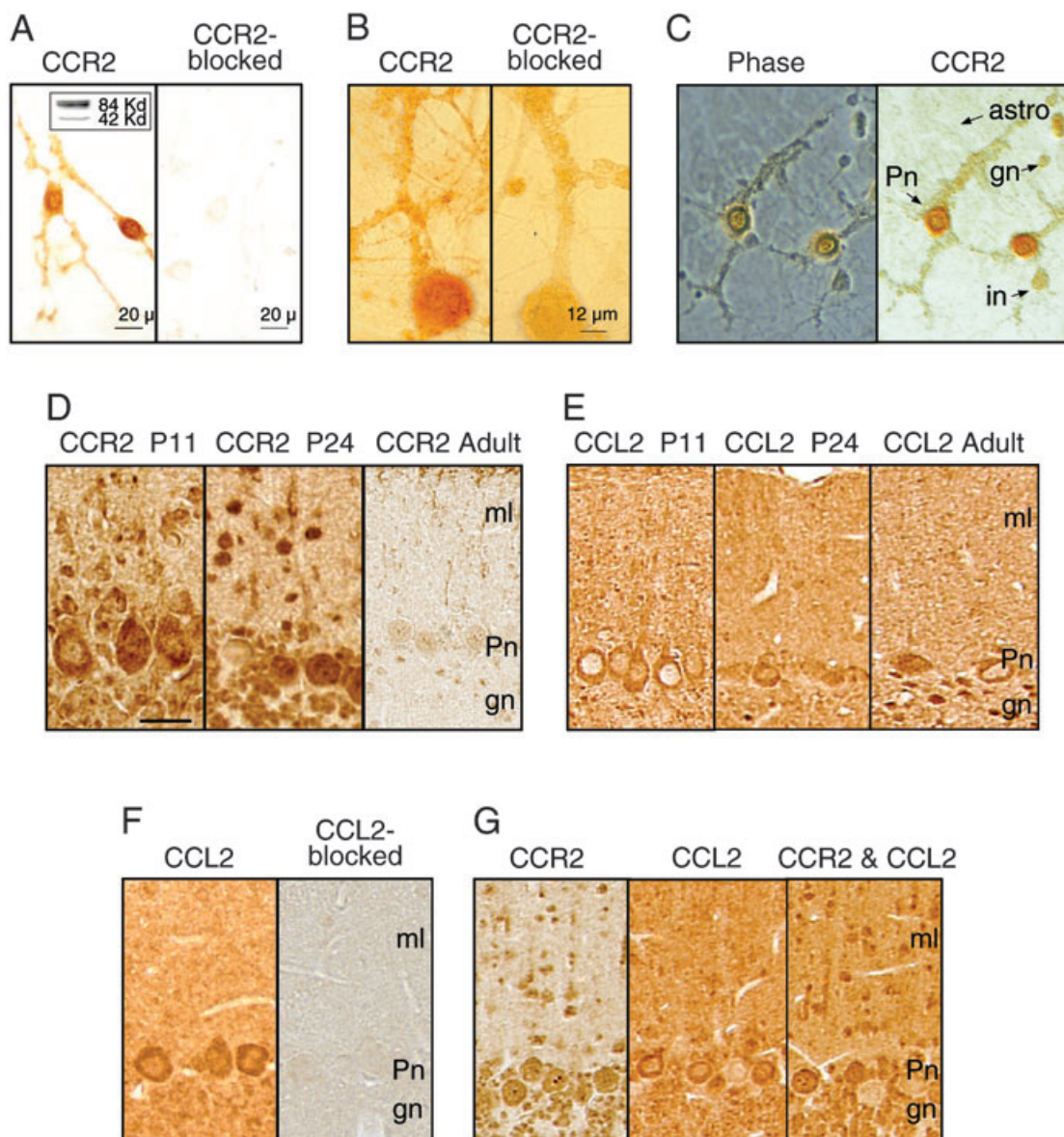


FIG. 1. Cerebellar neurons express CCR2 and CCL2. (A) Bright-field micrographs showing CCR2 immunostaining in cultured Purkinje neurons at 15 days *in vitro*. Immunostaining was blocked by pre-incubation of the antibody with the antigenic peptide. Inset: representative Western blot of CCR2 expression in cultures of cerebellar granule neurons. Two specific bands were observed in the granule neurons representing the monomer (42 kDa) and dimer (84 kDa) form of the receptor. When the denaturing step was carried out at 90 °C rather than at 70 °C, no dimer band was observed (data not shown). (B) Bright-field micrographs of punctate membrane staining in the somatic and dendritic regions of cultured Purkinje neurons. (C) Phase contrast and bright-field micrographs showing CCR2 immunostaining of the different cell types in culture. Staining was evident in Purkinje neurons (Pn), granule neurons (gn), inhibitory interneurons (in) and astrocytes (astro). (D) Micrographs showing immunostaining for CCR2 in histological sections of cerebella from postnatal day 11 (P11), 24 (P24) and adult (2–3 months) rats. gn, granule neuron layer; Pn, Purkinje neuron layer; ml, molecular layer. Scale bar, 40 µm. (E) Micrographs showing immunostaining for CCL2 in histological sections of cerebella from postnatal day 24 (P24) rats. Immunostaining was blocked by pre-incubation of the antibody with the antigenic peptide. (F) Micrographs showing immunostaining for CCL2 in histological sections of cerebella from postnatal day 24 (P24) rats. Immunostaining was blocked by pre-incubation of the antibody with the antigenic peptide. (G) Micrographs showing immunostaining for CCL2 and CCR2 alone and together in histological sections of cerebella from postnatal day 24 (P24) rats.

At postnatal day 11, similar CCR2 cytosolic staining was observed in all Purkinje neurons, granule cells and interneurons in the molecular layer of the cerebellum (Fig. 1D). Purkinje neurons showed cytosolic staining of varying intensity. Weak neuropil staining was found in the molecular layer. At postnatal day 24, moderate staining was observed in most Purkinje neurons, with again some heterogeneity. At this age, however, some Purkinje neurons were completely devoid of staining (Fig. 1D). Purkinje neuron apical dendrites in the molecular layer stained strongly. Strongest staining was observed in interneurons in the molecular layer and strong immunoreactivity was found in the

granule cells. Relatively weak neuropil staining was observed in the molecular layer. Generally, staining was more intense than in sections from postnatal day 11 cerebellum. In the adult, however, staining in granule cells and neuropil was almost completely lost. Staining of Purkinje neurons and inhibitory interneurons in the molecular layer was low (Fig. 1D). The white matter, however, showed strong staining (data not shown).

CCR2 expression in rat Purkinje neurons *in situ* was also examined at three ages, postnatal days 11 and 24 and in the adult (2–3 months old). CCL2 staining was prominent in Purkinje neurons at postnatal

day 11 (Fig. 1E). Neuropil staining was weak and no staining was observed in granule cells and inhibitory interneurons in the molecular layer. At postnatal day 24, strong CCL2 staining was observed in the somata of most Purkinje neurons, inhibitory interneurons of the molecular layer and granule cells (Fig. 1D). Some Purkinje neurons were completely devoid of staining, similar to the pattern observed for CCR2. Several scattered cells in the granule cell layer and white matter also showed strong staining. Neuropil staining in the molecular layer was moderate, whereas neuropil staining in the white matter was absent. CCL2 staining in the adult showed the same pattern as at postnatal day 24, with the exception that all Purkinje neurons showed CCL2 staining at this age (Fig. 1E).

To investigate whether Purkinje neurons *in situ* that were deficient for CCR2 were also deficient for CCL2, primary antibodies were combined to the final concentrations described earlier. Staining revealed approximately the same amount of unstained Purkinje neurons, showing that Purkinje neurons that did not express CCR2 also did not express CCL2 (Fig. 1G). As a control for non-specific staining, primary antibodies were co-incubated with the respective antigenic peptides (1 : 10 dilution) used to produce the primary antibodies. No specific immunostaining was observed in the cultures or histological sections under these conditions (Fig. 1A and F) or when the immunostaining procedure was performed in the absence of primary antibody. The expression of CCR2 in rat cerebellum was also confirmed in cerebellar granule neuron cultures by Western blot determination (Fig. 1A).

CCL2 increases intracellular Ca^{2+} levels in culture Purkinje neurons

To determine if CCL2 activates neuronal signaling coupled to Ca^{2+} dynamics, we tested the effect of micropressure and bath application of CCL2 on intracellular Ca^{2+} levels in the cultured Purkinje neurons (14–23 days *in vitro*) using fura-2-based Ca^{2+} imaging. We used micropressure application to determine if CCL2 induced rapid changes in intracellular Ca^{2+} . Rapid changes could be missed with bath application for technical reasons. For micropressure application, CCL2 was tested at a pipette concentration of 250 nM. Although the pipette concentration is known with this method of application, the actual amount ejected and the concentration at the cell surface after dilution in the bath saline is unknown. In contrast, with bath application the concentrations at the neuronal surface are known. We used bath application of CCL2 at concentrations ranging from 13 to 100 nM. The K_d for CCR2 has been reported to be ~ 1 nM (Sorensen *et al.*, 1999; Andjelkovic & Pachter, 2000; Andjelkovic *et al.*, 2002) and the range of biological activities reported for the recombinant rat CCL2 used in our studies is 0.7–7 nM (measured for chemoattraction of monocytes, ProTech, Inc., Rocky Hill, NJ, USA). Therefore, the higher concentrations of CCL2 used in our studies are likely to simulate CNS levels during pathological conditions such as occur during injury or disease.

Brief micropressure application of CCL2 (250 nM, 10–20 s) produced a small, prolonged Ca^{2+} response in the somatic and dendritic regions of the cultured Purkinje neurons (Fig. 2A). In cells tested by bath application, CCL2 (13–100 nM) also produced an increase in resting Ca^{2+} levels (Fig. 2B), consistent with the effects produced by micropressure application. Measurements of resting Ca^{2+} levels were made 5–60 min after addition of CCL2 to the bath and the elevated levels were maintained during this period. A significant effect of bath-applied CCL2 on resting Ca^{2+} levels occurred at all concentrations of CCL2 tested, with the largest effect occurring at 25 nM CCL2. The mean resting Ca^{2+} level (\pm SEM) after addition of

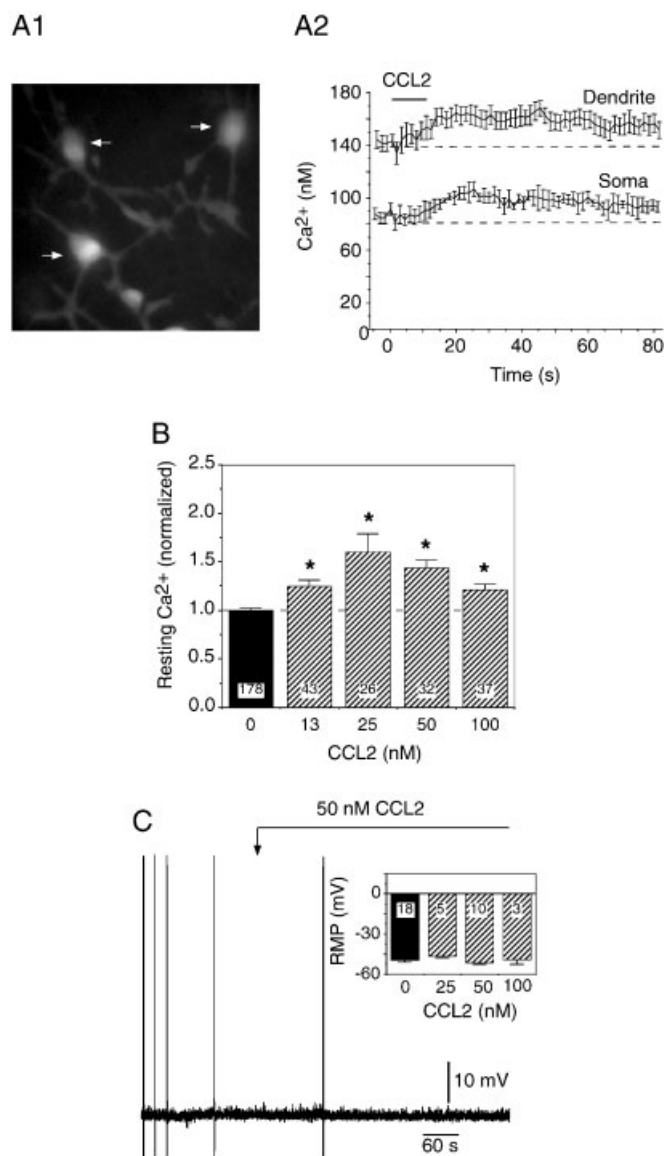


FIG. 2. CCL2 increases intracellular Ca^{2+} levels. (A) Representative response to CCL2 applied for 10 s by micropressure. A digitized greyscale image of the fura-2 neurons (arrows) used for the recording is shown in A1. Mean values (\pm SEM) for the intracellular Ca^{2+} levels in the soma and dendrites of the three neurons during the recording period are shown in A2. (B) Mean values (\pm SEM) for resting Ca^{2+} levels in Purkinje neurons exposed to different doses of CCL2 (13–100 nM) applied by bath application. Data are normalized to control values (no CCL2) for the population of neurons studied before addition of CCL2 to the bath. Resting Ca^{2+} levels under control conditions generally ranged from 50 to 150 nM. All CCL2 concentrations tested produced a significant increase (one group *t*-test; $P < 0.05$) in resting Ca^{2+} with the largest effect produced by 25 nM CCL2. Numbers in the bars indicate the number of neurons studied. (C) Bath or micropressure (not shown) application of CCL2 did not alter resting membrane potential when examined during current clamp recordings. A representative current clamp recording with bath application of CCL2 is shown. Inset: mean values (\pm SEM) for resting membrane potential (RMP).

25 nM CCL2 to the bath was 145 ± 17 nM ($n = 26$) in the population of neurons tested compared with a mean resting Ca^{2+} level of 90 ± 5 nM ($n = 28$) prior to adding the CCL2. Current clamp recordings were carried out during bath application of CCL2 to determine if the change in intracellular Ca^{2+} was associated with a change in resting membrane potential. No effect on resting membrane

potential was observed at any of the concentrations of CCL2 tested (Fig. 2C).

CCL2 alters the firing pattern of cultured Purkinje neurons

About 50% of Purkinje neurons recorded during electrophysiological studies exhibited sustained spontaneous action potential firing, a normal characteristic of this neuronal type. In these neurons, we measured the effect of CCL2 on the frequency of spontaneous spiking. Bath application of CCL2 (50 nM) produced a small (~15%) but significant decrease in the frequency of spontaneous spiking (Fig. 3A and B). CCL2 regulation of spike firing was also examined under more controlled conditions by determining its effect on current-evoked spike firing. In these experiments, resting membrane potential was held at a standardized level of -62 mV by continuously injecting negative holding current. By hyperpolarizing the membrane potential to -62 mV the spontaneous spike activity, which could complicate analysis of the results, was eliminated. In addition, this protocol ensured that the neurons were at a similar membrane potential relative to the threshold for spike activation. Depolarization of the membrane by a standardized current pulse ($+120$ pA, 500 ms) evoked spike activity under baseline conditions and after the addition of CCL2 to the bath. CCL2 at 50 and 100 nM caused small but significant decreases in current evoked spike firing, consistent with the effect of CCL2 on spontaneous spike firing (Fig. 3B). Input resistance, a relative measure of the activity of membrane ion channels that are the primary determinants of the resting membrane potential, was unaltered by CCL2 (Fig. 3C).

CCL2 alters DHPG-evoked Ca^{2+} signals and spiking firing

In many cell types, chemokines use a transduction pathway that alters intracellular Ca^{2+} levels by activation of PLC, production of inositol 1,4,5-triphosphate (IP3) and Ca^{2+} release from IP3-sensitive intracellular Ca^{2+} stores (Rollins, 1997; Wu *et al.*, 2000). In Purkinje neurons, the transduction pathway utilized by the metabotropic glutamate receptor 1 (mGluR1) also couples to activation of PLC with the production of inositol IP3 and Ca^{2+} release from IP3-sensitive intracellular Ca^{2+} stores (Netzeband *et al.*, 1997). mGluR1 is expressed in abundance in Purkinje neurons and plays an important role in synaptic transmission and motor learning. Therefore, it was of interest

to determine if interactions could occur between these two pathways, as such interactions could have important consequences for cerebellar function if both receptors were simultaneously activated. To test this possibility, we investigated the effect of bath-applied CCL2 on the Ca^{2+} response produced by pressure application of the selective mGluR1 agonist DHPG. Activation of mGluR1 by brief (1 s) micropressure application of DHPG produces both a Ca^{2+} and a membrane response in Purkinje neurons (Fig. 4A) (Netzeband *et al.*, 1997). The membrane response consists of a depolarization that typically evokes spike firing and is followed by an after-hyperpolarization (Fig. 4A). The Ca^{2+} response consists of an increase in intracellular Ca^{2+} that peaks during the depolarizing phase of the membrane response and returns to baseline during the hyperpolarizing phase (Fig. 4A).

The Ca^{2+} response produced by DHPG in the presence of CCL2 was similar in general form to that observed under control conditions (Fig. 4, B2). The Ca^{2+} responses were quantified by measurement of peak amplitude (resting levels subtracted), time to peak and duration (at half maximal peak amplitude). Addition of 13–25 nM CCL2 to the bath significantly increased the peak amplitude of the Ca^{2+} response produced by DHPG. In contrast, 50 nM CCL2 did not alter the peak amplitude and 100 nM CCL2 produced a significant decrease in the peak amplitude (Fig. 4, B1). Other measures of the Ca^{2+} responses including time to peak (~4 s) and response duration (~13 s) were not altered by CCL2 (data not shown).

The CCL2-induced change in the peak amplitude of the Ca^{2+} response produced by DHPG could involve an alteration in the associated membrane depolarization or spike firing produced by DHPG. To assess the potential contribution of these mechanisms, current clamp experiments were carried out. To facilitate measurement of the membrane depolarization, spontaneous and DHPG-evoked spiking were blocked by TTX (500 nM). Membrane responses to DHPG were similar in general form under control conditions and after addition of CCL2 (25–50 nM) to the bath (Fig. 5). The peak amplitude of the depolarization was not significantly altered by CCL2 (25–50 nM) (Fig. 5), indicating that a change in the membrane depolarization produced by DHPG did not account for CCL2-induced changes in the amplitude of the Ca^{2+} response.

The effect of CCL2 on DHPG-evoked spiking was assessed in the absence of TTX by measuring spike frequency during the first few seconds of DHPG-evoked spiking, a time that was associated with the peak amplitude of the Ca^{2+} response. Bath application of CCL2 (50 nM) produced a small but significant reduction of the DHPG-

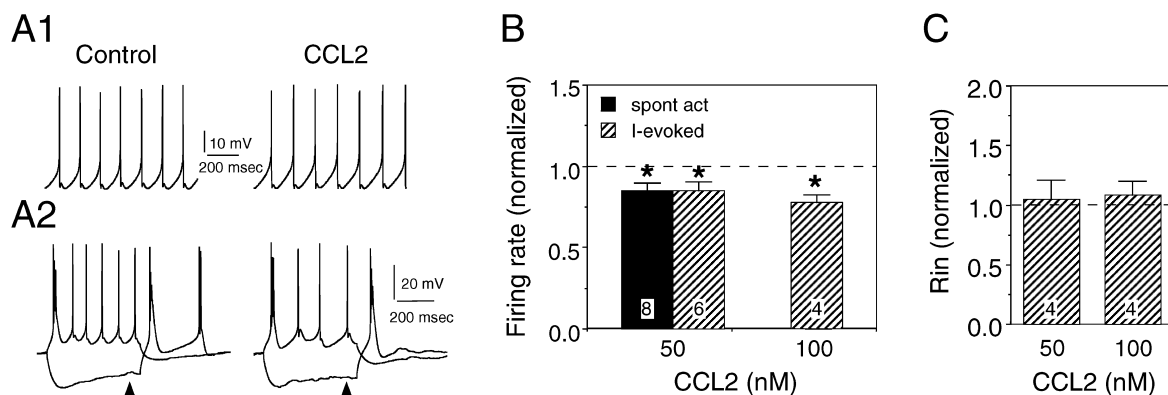


FIG. 3. CCL2 reduces spontaneous and current-evoked spike firing. (A) Representative recordings of spontaneous (A1) and current-evoked (A2) spike firing under control conditions and after bath application of CCL2 (50 nM). The membrane response to a hyperpolarizing current pulse is also shown in A2. Mean (\pm SEM) normalized values for firing rate are shown in B. The dotted line represents control values. (C) Mean (\pm SEM) normalized values for input resistance. Under control conditions input resistance ranged from 82 to 187 M Ω (128 ± 7 M Ω , $n = 16$) as determined from the slope of the current–voltage relationship for the sustained region (arrow) of the voltage response to hyperpolarizing current pulses. Numbers in the bars indicate the number of neurons studied.

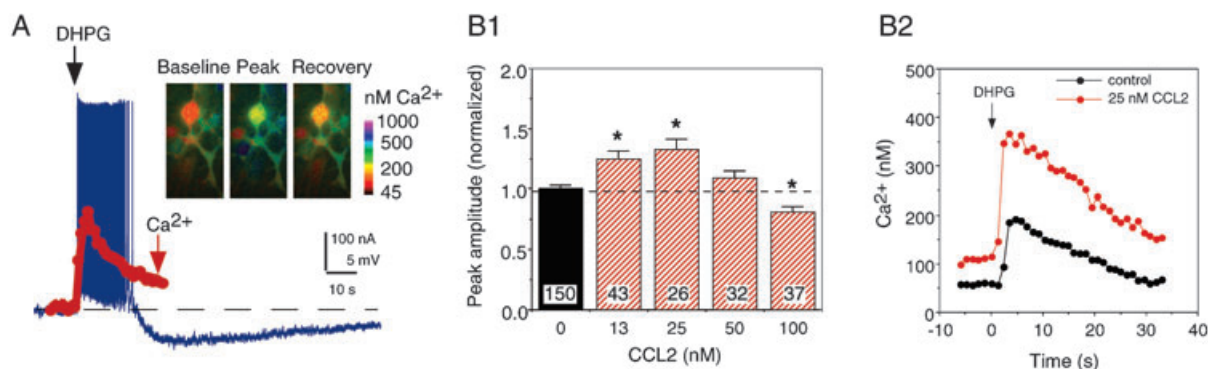


FIG. 4. CCL2 enhances the Ca^{2+} signal produced by DHPG. (A) Representative Ca^{2+} signal and electrophysiological response produced by brief (1 s) DHPG application under control conditions. Pseudocolor images of the recorded neuron are shown in the inset. (B1) Mean values (\pm SEM) for the peak amplitude of the Ca^{2+} signal produced by brief DHPG application (1 s at the arrow) to cultured Purkinje neurons in the absence and presence of CCL2 in the bath. Data are normalized to the peak amplitude of the Ca^{2+} signal to DHPG under control conditions. Numbers in the bars indicate the number of neurons studied. CCL2 at 25 nM caused a significant ($P < 0.05$) increase in peak Ca^{2+} , whereas CCL2 at 100 nM caused a significant decrease in peak Ca^{2+} . (B2) Representative Ca^{2+} signal produced by brief (1 s) DHPG application under control conditions and in the presence of CCL2. $*P < 0.05$.

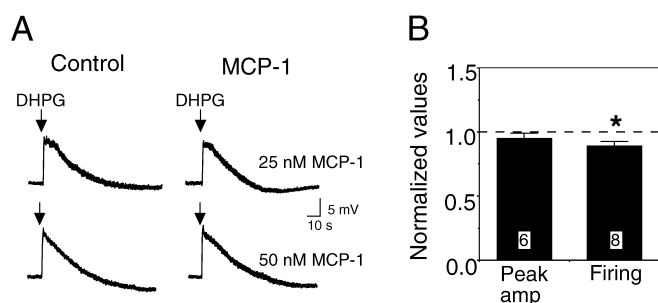


FIG. 5. CCL2 does not alter the membrane depolarization produced by DHPG. (A) Representative recordings of depolarizations produced by micro-pressure application (1 s) of DHPG (arrow) under control conditions and in the presence of CCL2 (25 and 50 nM). There was no significant effect of CCL2 on the peak amplitude of the depolarization produced by DHPG. Mean values (\pm SEM) for the peak amplitude are shown in B. TTX was present in the recording saline to block spike activity. In the absence of TTX (B, right panel), spike firing (Firing) during the depolarization produced by DHPG was reduced by CCL2 (50 nM tested). Numbers in the bars indicate the number of neurons studied. Data are normalized to the respective measures under control conditions. $*P < 0.05$.

evoked spiking ($\sim 11\%$; Fig. 5), an effect of CCL2 similar to that observed for spontaneous and current-evoked spiking.

Discussion

Data on the function of chemokines and their receptors has come primarily from studies on immune cells. However, chemokines and their receptors are also expressed in other cell types, including microglia, astrocytes and neurons of the CNS (Horuk *et al.*, 1997; Banisadr *et al.*, 2002). Chemokines are up-regulated in the brain during several pathological states (Bajetto *et al.*, 2002; Mahad & Ransohoff, 2003), raising the possibility that they may play a role in the pathological or repair process. Our results show that at concentrations that reflect pathologic conditions the chemokine CCL2 is neuroactive. Brief micropressure application of CCL2 elicited a small, prolonged Ca^{2+} response in cultured cerebellar Purkinje neurons and bath application of CCL2 increased resting Ca^{2+} levels in an inverse dose-dependent manner. These results complement the recent Ca^{2+} imaging studies by Gillard *et al.* (2002), who also

showed that exogenous application of CCL2 increases intracellular Ca^{2+} in cultured Purkinje neurons.

In addition to the effects on resting Ca^{2+} levels, our studies showed that CCL2 alters Ca^{2+} responses associated with activation of mGluR1, a glutamate receptor that plays an important role in the physiology of Purkinje neurons (Ito, 1993). We also showed that CCL2 depresses neuronal excitability, as evidenced by the depression of spike firing produced by CCL2. These effects of CCL2 were relatively modest, consistent with CCL2 acting as a neuromodulator at pathophysiological concentrations. A physiological role for CCR2 in the cerebellum is not clear, CCR2 $^{-/-}$ mice do not show any motor impairment (Abbadie *et al.*, 2003). However, other chemokine receptors, such as CXCR4, can play an important role in normal cerebellar development. CXCR4-deficient mice, for example, express numerous deficiencies and develop irregularly organized cerebellar layers (Ma *et al.*, 1998).

CCL2 effects on Purkinje neurons did not involve synaptic transmission, which was blocked by the addition of receptor antagonists to the recording saline, suggesting that CCL2 acts directly on Purkinje neurons. Consistent with this possibility, we showed using immunohistochemistry that the receptor for CCL2, CCR2, is expressed by the cultured cerebellar cells and throughout the rat cerebellum *in situ*, including Purkinje neurons, granule neurons and interneurons. At postnatal day 11, all Purkinje neurons *in situ* express immunostaining for CCR2, although quite heterogeneously. At postnatal day 24, most but not all Purkinje neurons *in situ* express immunostaining for CCR2, while strongest staining was observed in inhibitory interneurons. In adult rats, most CCR2 staining in the cerebellum is lost, except for strong staining in the white matter. These results are consistent with a loss of sensitivity of Purkinje neurons to CCL2, a change that could affect susceptibility to pathological conditions. However, we cannot eliminate the possibility that the interaction of the antibody with CCR2 expressed by the neurons is affected by age and therefore immunostaining is reduced in the adult cerebellum.

Cellular staining for CCR2 in Purkinje neurons *in situ* was distributed throughout the cytosol but most prominently in the perinuclear region and presumably reflected synthesis and storage of non-functional receptors. A similar pattern of staining was observed in the cultured Purkinje neurons. In the cultured Purkinje neurons, a large portion of the neuronal surface can be clearly visualized, enabling an assessment of surface expression of CCR2. Punctate surface staining

for CCR2 was observed on the somatic and dendritic regions of the cultured Purkinje neurons and presumably reflected sites of functional receptors. CCR2 was also observed in immunoblots of cultured granule neurons as two specific bands, one for the monomer and one at 84 kDa for the dimer. It has been reported previously by heterodimerization studies (Rodríguez-Frade *et al.*, 1999; Mellado *et al.*, 2001) that chemokine receptors such as CCR2 usually function as dimers, either homodimers or heterodimers (especially CCR2 with CCR5).

In addition to the expression of CCR2, we examined the expression of CCL2 in the rat cerebellum *in situ* by immunohistochemistry (expression in cultures not examined). These studies showed endogenously expressed CCL2 in most cell cerebellar types at all ages studied (postnatal days 11 and 24, adult). CCL2 immunostaining in Purkinje neurons of fetal human brain has been reported previously (Meng *et al.*, 1999). CCL2 has also been shown to be expressed by glial cells derived from the CNS (microglia and astrocytes) (Rezaie *et al.*, 2002). Interestingly, Purkinje neurons that did not express CCR2 were also deficient for CCL2, a correlation that may reflect a paracrine/autocrine role for CCL2.

Micropressure or bath application of CCL2 increased resting intracellular Ca^{2+} levels in the cultured Purkinje neurons, an effect that occurred without a change in resting membrane potential. This result suggests that the increase in resting Ca^{2+} produced by CCL2 did not involve activation of membrane ion channels because such an effect would alter resting membrane potential. Our results also showed that the increase in resting Ca^{2+} produced by CCL2 did not involve an increase in spike firing. An increase in spike firing could result in a greater influx of Ca^{2+} due to the activation of voltage-sensitive Ca^{2+} channels involved in spike firing. Instead CCL2 decreased cell firing, an effect expected to reduce Ca^{2+} influx. Therefore, it is unlikely that Ca^{2+} influx is the primary mechanism mediating the increase in resting Ca^{2+} produced by CCL2. In non-neuronal cells, chemokine receptors are linked to the PLC/IP3 transduction pathway, which regulates Ca^{2+} release from intracellular stores controlled by the IP3 receptor. Such a mechanism could underlie the increase in resting Ca^{2+} produced by CCL2 in the Purkinje neurons.

Co-application of CCL2 and the mGluR agonist DHPG resulted in an interaction that altered the amplitude of the DHPG-evoked Ca^{2+} response. At the lower ranges of CCL2 concentrations tested (e.g. 25 nM), CCL2 enhanced the DHPG-evoked Ca^{2+} response, while at the high concentration tested (100 nM) CCL2 caused a decrease in the DHPG-evoked Ca^{2+} response. The mechanisms responsible for these dose-dependent changes have yet to be identified. CCL2 at 25 and 50 nM did not alter the amplitude of the membrane depolarization produced by DHPG and depressed DHPG-evoked spike firing. Therefore, changes in Ca^{2+} influx are unlikely to underlie the enhancing effect of CCL2. A CCL2 depression of spike firing could contribute to the smaller Ca^{2+} response produced by DHPG when the neurons were simultaneously exposed to 100 nM CCL2. The time course of the DHPG-evoked Ca^{2+} response was unaltered by CCL2, making it unlikely that uptake pumps contribute to this modulatory action of CCL2. Therefore, interactions at the level of the transduction pathway appear to be the most likely mechanism. At 25 nM, CCL2 may prime the PLC/IP3 transduction pathway, whereas high concentrations (100 nM) and the prolonged exposure (min) period used in our studies could result in desensitization of the pathway (Ferguson, 2001). For example, the IP3 receptors could be desensitized (Honda *et al.*, 1995; Scherer *et al.*, 2001; Willars *et al.*, 2001) by the prolonged activation of the PLC/IP3 pathway by CCL2, resulting in an attenuated DHPG response. Interactions between G-protein-coupled receptors that result in increased Ca^{2+} responses have been reported and a number of mechanisms are being pursued to explain these

interactions, including convergence at PLC, sensitization of the IP3 receptor by phosphorylation, receptor dimerization and protein-protein interactions (Werry *et al.*, 2003). Further studies will be necessary to determine a potential role for these mechanisms in the interaction observed in the current study.

In summary, our results show that CCR2 is widely expressed throughout the cerebellum and when activated by CCL2 modulates intracellular Ca^{2+} levels and reduces neuronal excitability. Passive membrane properties including resting membrane potential and input resistance were not modulated by CCL2. CCL2 has recently been shown to be neuroprotective during NMDA- or HIV-induced apoptosis (Eugenin *et al.*, 2003) and lowering excitability might play an important role in this neuroprotective effect. However, in the case of long-term chronic exposure, effects of CCL2 on Ca^{2+} dynamics are likely to be detrimental and to contribute to the neuropathology associated with chronic disease states.

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Abbreviations

CNS, central nervous system; DHPG, (R,S)-3,5-dihydroxy-phenylglycine; IP3, inositol 1,4,5-triphosphate; mGluR1, metabotropic glutamate receptor 1; PLC, phospholipase C; TTX, tetrodotoxin.

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