Chronic Interleukin-6 Exposure Alters Electrophysiological Properties and Calcium Signaling in Developing Cerebellar Purkinje Neurons in Culture

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Nelson, Thomas E., Christina L. Ur, and Donna L. Gruol. Chronic interleukin-6 exposure alters electrophysiological properties and calcium signaling in developing cerebellar Purkinje neurons in culture. J Neurophysiol 88: 475–486, 2002; 00306.2001. The cytokine interleukin-6 (IL-6) is chronically expressed at elevated levels within the CNS in many neurological disorders and may contribute to the histopathological, pathophysiological, and cognitive deficits associated with such disorders. However, the effects of chronic IL-6 exposure on neuronal function in the CNS are largely unknown. Therefore using intracellular recording and calcium imaging techniques, we investigated the effects of chronic IL-6 exposure on the physiological properties of cerebellar Purkinje neurons in primary culture. Two weeks of exposure to 1,000 units/ml (U/ml) IL-6 resulted in altered electrophysiological properties of Purkinje neurons, including a significant reduction in action potential generation, an increase in input resistance, and an enhanced electrical response to the ionotropic glutamate receptor agonist, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) compared with untreated neurons. Lower concentrations of IL-6 (100 and 500 U/ml) had no effects on these electrophysiological parameters. However, neurons exposed to 500 U/ml chronic IL-6 resulted in significantly elevated resting levels of intracellular calcium as well as an increase in the intracellular calcium signaling subunit, gp130, indicating that IL-6 may act directly on Purkinje neurons in response to AMPA, effects not observed in neurons exposed to 1,000 U/ml chronic IL-6. Morphometric analysis revealed a lack of gross structural changes following chronic IL-6 treatment, such as in the number, size, and extent of dendritic arborization of Purkinje neurons in culture. Using immunohistochemistry, we found that cultured Purkinje neurons express both the IL-6 receptor and its intracellular signaling subunit, gp130, indicating that IL-6 may act directly on Purkinje neurons to alter their physiological properties. The present data show that chronic exposure to elevated levels of IL-6, such as occurs in various neurological diseases, produces alterations in several important physiological properties of Purkinje neurons and that these changes occur in the absence of neuronal toxicity, damage, or death. The results support the hypothesis that chronic IL-6 exposure can disrupt normal CNS function and thereby contribute to the pathophysiology associated with many neurological diseases.

INTRODUCTION

The cytokine interleukin-6 (IL-6) is involved in the induction, growth, and differentiation of cells in the immune and hematopoietic systems that contribute to the initiation and coordination of inflammatory responses (Akira et al. 1990; Heinrich et al. 1990; Hirano 1992). IL-6 is also produced within the CNS by astrocytes and microglia (Benveniste et al. 1990; Frei et al. 1989) and is thought to be an important mediator of neuroimmune responses, as well as to play a more general role as a neuroprotectant and neurotrophic factor (Benveniste 1992, 1998; Gruol and Nelson 1997; Van Wagoner and Benveniste 1999). Under normal conditions the production of IL-6 in the CNS is highly regulated (Hopkins and Rothwell 1995; Van Wagoner and Benveniste 1999). However, chronic dysregulation of IL-6 expression during inflammation or infection of the CNS is thought to contribute to the neuropathological and pathophysiological features of a number of neurological disorders (Benveniste 1992; Merrill 1992). Elevated levels of IL-6 have been reported in neurological disorders associated with acquired immunodeficiency syndrome (AIDS) dementia complex (Gallo et al. 1989; Tyor et al. 1992), Alzheimer’s disease (Bauer et al. 1991), multiple sclerosis (Maimone et al. 1991), systemic lupus erythematosus (Hirohata and Miyamoto 1990), CNS trauma (Woodroofe et al. 1991), and viral (Frei et al. 1989) and bacterial meningitis (Houssiau et al. 1988).

The effects of elevated CNS expression of IL-6 have recently been studied through the use of transgenic mice in which IL-6 production is targeted to astrocytes under the control of the glial fibrillary acidic protein (GFAP) promoter (Campbell et al. 1993). The GFAP-IL6 transgenic mice exhibit anatomical, physiological, and behavioral abnormalities, particularly involving the hippocampus and cerebellum, that are reflective of CNS changes occurring in several disease states (Bellinger et al. 1995; Campbell et al. 1993; Heyser et al. 1997; Nelson et al. 1999; Steffensen et al. 1994). In our previous studies using extracellular electrophysiological recording in cerebellar slices from this mouse model, we found that cerebellar Purkinje neurons in GFAP-IL6 transgenic mice exhibited alterations in endogenous firing activity and synaptic responses (Nelson et al. 1999). These results suggest that IL-6 may be an important regulator of neuronal function under pathological conditions.

In the transgenic mouse model, numerous neuronal and nonneuronal targets of IL-6 action are present throughout the CNS and could contribute to the altered physiology of cerebellar Purkinje neurons through synaptic or hormonal influences or by disrupting the normal course of structural and...
histological development of the cerebellum. Therefore in the current study we have utilized a simplified model system, modified organotypic cultures of cerebellar neurons, to investigate in more detail the physiological effects of local chronic IL-6 exposure on Purkinje neurons. The culture model provides a controlled microenvironment in which the effects of IL-6 exposure on Purkinje neurons can be studied at known doses and durations of exposure. For these studies we used an exposure paradigm that simulated the IL-6 exposure period in the GFAP-IL6 mice. Glial expression of IL-6 in the transgenic model starts at about 7 days postnatal (Chiang et al. 1994), a relatively early stage in the developmental program of Purkinje neurons. The cultures are prepared from rat embryos 1 day before birth, when the cerebellum is very immature. Cerebellar neurons continue their maturation during the first 3 wk of culture, a process that parallels maturation in vivo (Gruol and Franklin 1987). IL-6 exposure by exogenous addition to the cultures was begun at 6 days in vitro (DIV), a developmental stage similar to that of the 7 day postnatal neurons in vivo. IL-6 doses that are relevant to concentrations observed in various neurological diseases (Achim et al. 1993; Frei et al. 1989; Gallo et al. 1989; Houssiau et al. 1988; Matsuzono et al. 1995) were used during the chronic exposure period.

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). In brief, cerebellar cortices were isolated, minced, and triturated in saline containing (in mM) 137 NaCl, 5.4 KCl, 0.17 Na2HPO4, 0.22 KH2PO4, 27.7 glucose, 43.8 sucrose, 10 HEPES-NaOH (pH 7.3 with NaOH). No enzymatic treatment was used. The cell suspension was plated on glass coverslips (Fisher Scientific) or tissue culture dishes coated with Matrigel (Collaborative Biochemical). The plating medium contained minimal essential medium (MEM) with Earle’s salts and l-glutamine (GIBCO-BRL), 10% heat-inactivated fetal calf serum (GIBCO-Invitrogen), and 10% heat-inactivated horse serum (GIBCO-Invitrogen) and was supplemented with p-glucose to a final concentration of 5.0 g/l. Medium was exchanged twice weekly with medium having a similar composition as above, except that the calf serum was omitted. 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, Sigma) limited the number of nonneuronal cells. Antibiotics were not used.

Chronic IL-6 treatment

Cultures from individual culture sets (i.e., sister cultures originating from a single dissection) were divided into control and chronic IL-6 treatment groups, and on the sixth day in vitro (DIV) chronic IL-6 treatment was begun. One hundred, 500, or 1,000 units/ml (U/ml; 1–10 ng/ml) of human recombinant IL-6 (hrIL-6; Roche) was added to the cultures during regular media changes, occurring twice per week (every 3–4 days) for 1–3 wk. The range of biological activity for hrIL-6 is 10–100 U/ml with 100 U/ml recommended for IL-6–dependent proliferation of B-cell hybridomas in vitro (Roche). Therefore for the current studies, 500 and 1,000 U/ml IL-6 were considered elevated levels reflective of abnormal or pathological conditions. Control cultures were not treated with IL-6 but went through normal media changes on the same schedule. To confirm that the concentrations of exogenously added IL-6 remained relatively constant during the chronic treatment period, levels of IL-6 in six of the IL-6–treated cultures were measured at multiple time points by an enzyme-linked immunosorbent assay (ELISA; Predicta Human IL-6 ELISA kit, Genzyme). ELISA measurements were given in pg/ml and converted to U/ml, as determined by the specific bioactivity (10 pg/U) of the hrIL-6. Immediately after addition of IL-6 to the cultures (during regular media changes), human IL-6 levels were 1,032 ± 75 U/ml (mean ± SE; n = 4) and 506 ± 36 U/ml (n = 2) in 1,000 and 500 U/ml IL-6–treated cultures, respectively, and, on the third day after IL-6 addition (prior to regular media changes), the human IL-6 levels were 1,148 ± 30 (n = 4) and 550 ± 41 (n = 2) U/ml, respectively. Thus the levels of exogenously added IL-6 remained relatively constant during the chronic treatment period.

Immunohistochemistry

Immunohistochemical staining of the cerebellar cultures was performed according to previously published methods (Gruol and Franklin 1987) using antibodies that recognize precursor proteins for the IL-6 receptor and gp130 (Santa Cruz Biotechnology). The IL-6 receptor antibody was an affinity-purified rabbit polyclonal antibody raised against amino acids 441–460 of the carboxy terminus of mouse IL-6R protein. The gp130 antibody was an affinity-purified rabbit polyclonal antibody raised against amino acids 895–914 of the carboxy terminus of mouse gp130 protein. In brief, cultures were rinsed with serum free MEM, fixed with paraformaldehyde (2–4%) in phosphate-buffered saline (PBS, 100 mM, pH 7.3) for 15 min, and permeabilized with 0.05% Triton X-100 in PBS for 30 min. Cultures were incubated overnight (4°C) in PBS containing primary antibody (1:100 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). As a control for nonspecific staining, prior to the immunostaining procedure, the primary antibodies were co-incubated with the respective antigenic peptides (1:10 dilution) used to produce the primary antibodies. Positive immunostaining was not observed under these conditions or when the immunostaining procedure was performed in the absence of primary antibody.

Morphological measurements

For morphological measurements of Purkinje neurons, cerebellar cultures were grown on Lab-Tek glass chamber slides (Nalge Nunc International) similar to the procedures outlined above. Morphological measurements of cultured Purkinje neurons were made following 9 days of treatment with 100, 500, or 1,000 U/ml IL-6. Cultures were fixed as above and immunostained using an antibody against calbindin (1:5,000; Sigma), a specific marker of cerebellar Purkinje neurons (Garcia-Segura et al. 1984). The somatic and dendritic areas of IL-6–treated and matching control Purkinje neurons were measured using the MCID imaging software (Imaging Research). In addition, the number of primary dendrites and dendritic branches were counted for each Purkinje neuron, and the density of Purkinje neurons (per unit area) was calculated for each culture. Fifteen to 20 Purkinje neurons were randomly selected for measurement in each culture. Density was calculated by counting the number of Purkinje neurons localized within a subsection of each culture defined by a central 6-mm band stretching across the entire width of the culture. This cross-sectional band represented approximately 13% of the total area of the culture.

Electrophysiological recording

Electrophysiological experiments were performed at 19–21 DIV (13–15 day IL-6 exposure) or 26–28 DIV (20–22 day IL-6 exposure). Prior to recording, the culture medium was replaced with physiological saline that consisted of (in mM) 140 NaCl, 3.5 KCl, 0.4 KH2PO4, 1.25 Na2HPO4, 2.2 CaCl2, 2 MgSO4, 10 glucose, and 10 HEPES-NaOH (pH 7.3). IL-6 was not present during recordings from chronic
IL-6-treated neurons. Intracellular current-clamp recordings were made in the somatic region of Purkinje neurons according to the nystatin perforated-patch whole cell recording method of Horn and Marty (1988). Purkinje neurons were identified by size and morphology using phase contrast or brightfield optics (×40). The patch pipette solution contained (in mM) 6 NaCl, 154 KCl–gluconate, 2 MgCl₂, 0.5 CaCl₂, 10 HEPES-KOH, and 10 glucose. Tips of patch pipettes (4–5 MΩ) were filled with nystatin-free solution, and the pipettes were back-filled with nystatin-containing solution. Nystatin was prepared as a stock solution in dimethyl sulfoxide (50 mg/ml) and diluted in the recording solution to 200 μg/ml. All recordings were made at room temperature (23–25°C).

Current-clamp recordings were performed using the Axopatch-1C amplifier, and digitized (1 kHz) using the Labmaster DMA interface and pCLAMP software (all from Axon Instruments). To compare membrane properties between control and chronic IL-6–treated neurons, current-voltage (I–V) curves were constructed by applying a series of constant current pulses (±30–150 pA; 500 ms) to each neuron at its resting membrane potential (approximately −52 mV). To compare membrane responses to the glutamate receptor agonist, (S)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA; Tocris Neuramin), recordings were acquired at a standardized membrane potential of −62 mV, achieved by injecting continuous negative current into the cell via the recording electrode. At this hyperpolarized potential, spontaneous activity was suppressed in most cells, thus allowing for clearer measurement of the AMPA-evoked responses. AMPA (1 μM) was dissolved in bath saline and applied by brief microperfusion (1 s) from glass micropipettes placed near the edge of the microscopic field. Fast Green (Sigma) was included in the AMPA solution to monitor exposure to the agonist. Fast Green (0.03%) by itself had no effect on neuronal firing or baseline Ca²⁺ levels. All recordings were monitored on a polygraph and oscilloscope. AMPA responses were recorded on FM tape (Racal Store 4DS recorder) for subsequent digitization (1 kHz) using AxoTape software (Axon Instruments). Analyses of I–V relationships and AMPA-evoked responses were done with AxoGraph software (Axon Instruments).

**Calcium imaging**

Calcium imaging experiments were performed at 14–17 DIV (8–11 day IL-6 exposure) or 20–23 DIV (14–17 day IL-6 exposure) under conditions similar to the electrophysiological experiments. Intracellular Ca²⁺ levels were measured in the somatic and dendritic regions of individual Purkinje neurons using a standard microscopic fura-2 digital imaging technique (Gruol and Curry 1995) based on the methods of Grynkiewicz et al. (1985). Briefly, cerebellar cultures were incubated 30 min with the Ca²⁺-sensitive fluorescent dye fura-2/AM (3 μM; Molecular Probes) and pluronic F-127 (0.02%; 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 a SIT-66 video camera (DAGE-MTI) and digitized for real-time display using the MCID imaging software (Imaging Research). Data were collected at 3-s intervals before and after application of AMPA (5 μM). 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: [Ca²⁺]ᵢ = \( Kₐ(R - R_{\text{min}}/(R_{\text{max}} - R))F_{\text{max}}/F_{\text{r}} \), where R is the ratio value, \( R_{\text{min}} \) is the ratio for a Ca²⁺-free solution, \( R_{\text{max}} \) is the ratio for a saturated Ca²⁺ solution, \( Kₐ = 135 \) (the dissociation constant for fura-2), \( F_{\text{max}} \) is the intensity of a Ca²⁺-free solution at 380 nm, and \( F_{\text{r}} \) 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 kit C-3009). Background subtraction was not used because autofluorescence and non-cell associated background fluorescence were found in previous studies to be minimal.

**Statistical analyses**

A between-cell comparison (unpaired t-test, Mann-Whitney U test, or ANOVA + Fisher’s LSD or Tukey/Kramer post hoc tests) was used to determine the effects of chronic IL-6 exposure on the response parameters. To control for variability between cultures sets, some of the data were normalized to the mean of the controls from each culture set. Statistical significance was determined at a level of \( P < 0.05 \). Data are reported as the means ± SE.

**Results**

Expression of interleukin-6 receptor and gp130 in cerebellar culture

The cerebellar cultures contain cell types present in the cortical region of the cerebellum (Purkinje neurons, granule neurons, inhibitory interneurons, and astrocytes), which can be distinguished by morphological criteria (Gruol 1983; Gruol and Crimi 1988; Gruol and Franklin 1987). Immunohistochemical assay of the cerebellar cultures was used to determine the cell types capable of responding to IL-6, as evidenced by expression of IL-6 receptors and gp130, the intracellular signal transduction subunit of the IL-6 receptor (Fig. 1, A and B). The expression of the IL-6 receptor and gp130 was determined over a range of developmental time points in culture (9–36 DIV). IL-6 receptor– and gp130-immunoreactive Purkinje neurons were observed in both control and IL-6–treated cultures, and the immunoreactivity was evident in the somata and primary processes of Purkinje neurons.

**FIG. 1.** Interleukin-6 (IL-6) receptor and gp130 immunocytochemistry in primary cultures of rat cerebellum. A: IL-6 receptor expression in primary cerebellar cultures at 21 days in vitro (DIV). B: gp130 expression at 21 DIV. Cell types expressing the IL-6 receptor and gp130 were identified using morphological criteria. Expression of the IL-6 receptor and gp130 was observed in the somata (P) and dendrites (arrowheads) of Purkinje neurons as well as in granule neurons (g). Astrocitc expression (*) of the IL-6 receptor (A) and gp130 (B) was low in the cerebellar cultures. C and D: specific IL-6 receptor (C) and gp130 (D) immunoreactivity was blocked when the primary antibodies were preincubated with their respective antigenic peptides. Scale bars = 50 μm.
dendrites of both immature and mature Purkinje neurons. Levels of immunoreactivity of both the IL-6 receptor and gp130 were similar across the developmental time points, and the immunostaining was comparable in control and IL-6–treated cultures. In addition, cerebellar granule neurons and interneurons were immunoreactive for the IL-6 receptor and gp130. Relative to neuronal staining, IL-6 receptor–positive and gp130-positive immunostaining of astrocytes was low in the cerebellar cultures.

Chronic interleukin-6 does not alter the development of Purkinje neurons in cerebellar culture

To determine whether chronic IL-6 exposure altered the development of cerebellar Purkinje neurons in culture, we measured the somatic and dendritic area of calbindin-immunostained neurons after 9 days of chronic IL-6 exposure (100, 500, and 1,000 U/ml) and compared with matching controls (i.e., untreated cultures in the same culture sets). During the chronic treatment period, Purkinje neurons completed the main period of dendritic growth and maturation (Gruol and Franklin 1987). Following chronic IL-6 treatment, the morphological appearance of Purkinje neurons was similar to matching controls (Fig. 2, A–D). Although a small reduction (~10%, not significant) in somatic and dendritic area was evident following chronic exposure to either 500 or 1,000 U/ml IL-6 (Fig. 2E), no significant differences in dendritic arborization (e.g., the number of primary dendrites or dendritic branches) were observed between control and IL-6–treated Purkinje neurons (Fig. 2F). The general appearance of the control and IL-6–treated cultures was similar, and there was no difference in the density of Purkinje neurons between treatment groups (Fig. 2G).

Chronic interleukin-6 exposure alters intrinsic electrophysiological properties of cultured cerebellar Purkinje neurons

Using intracellular recording techniques, we determined whether chronic exposure to IL-6 altered the intrinsic electrophysiological properties of cerebellar Purkinje neurons for three doses (100, 500, and 1,000 U/ml) and two different durations (14 and 21 days) of IL-6 exposure.

To test for chronic IL-6–induced changes in action potential generation and passive membrane properties of Purkinje neurons, the evoked spike frequency, I-V relationship, and input resistance were determined using a standardized series of depolarizing and hyperpolarizing current pulses (±30–150 pA). The general appearances of the responses were similar in control and IL-6–treated neurons. Injection of depolarizing current pulses evoked repetitive firing in the cultured neurons (Figs. 3-4, A and B), whereas hyperpolarizing current pulses produced an electrotonic potential with a time-dependent sag (Fig. 4, A and B).

Following 14 days of exposure to 100 U/ml IL-6, Purkinje neurons showed only a small decrease in evoked action potential generation (Fig. 3E) as compared with a significant in-
crease in action potential firing following 21 days of exposure to IL-6 (Fig. 3, A, C, and E). Evoked action potential activity was not altered significantly following either 14 or 21 days of exposure to 500 U/ml IL-6 (Fig. 3E). After 14 days of exposure to 1,000 U/ml IL-6, Purkinje neurons exhibited a significant reduction in action potential firing relative to controls (Fig. 3, B, D, and E), whereas, after 21 days of IL-6 exposure, Purkinje neuron action potential generation was similar in 1,000 U/ml IL-6–treated and matching control cultures (Fig. 3E).

Resting membrane potentials were not significantly different from matching controls at any of the chronic IL-6 doses or treatment durations tested (Fig. 3F). The mean resting membrane potential for all Purkinje neurons was $-52.4 \pm 0.5$ mV ($n = 60$).

Effects of chronic IL-6 on passive membrane properties were similar for the two durations of IL-6 treatment (14 and 21 days IL-6), and the results have been combined. At doses of 100 (Fig. 4, A and C) or 500 U/ml IL-6 (not shown), the average $I-V$ relationship and the corresponding mean input resistance were similar between chronic IL-6–treated and control Purkinje neurons (Fig. 4C). However, at 1,000 U/ml chronic IL-6 a significant enhancement in the membrane depolarization in response to positive current injection was observed (Fig. 4, B and D) corresponding to a small, although nonsignificant increase ($^*\text{trend, } P = 0.06$) in the mean input resistance of chronic IL-6–treated neurons compared with matching controls (Fig. 4D).

**Chronic interleukin-6 exposure alters AMPA-induced membrane responses of cultured cerebellar Purkinje neurons**

Excitatory transmission to Purkinje neurons, as well as most CNS neurons, is mediated predominantly by the AMPA subtype of glutamate receptor. To assess possible chronic IL-6–induced changes in glutamate receptor–mediated activity, we compared the responses of Purkinje neurons to the ionotropic glutamate receptor agonist AMPA (1 $\mu$M) in control and
treated cultures following 2–3 wk of exposure to 100, 500, or 1,000 U/ml IL-6.

Brief (1 s) application of AMPA resulted in a rapid depolarization of the Purkinje neuron and usually initiated high-frequency firing of action potentials followed by a slow, prolonged afterhyperpolarization (AHP; Fig. 5, A and B). Responses to AMPA were quantified by measurement of the peak amplitude and duration of the depolarization as well as the AHP amplitude. Results for each chronic IL-6 dose were similar at both durations of exposure (14 and 21 days), and data have been combined. Figure 5, C and D, shows the mean peak amplitude (left) and response duration (right) for different doses of chronic IL-6 exposure. Following chronic exposure to either 100 (Fig. 5, A and C) or 500 U/ml IL-6 (not shown), peak amplitude and response duration were similar to responses in matching control Purkinje neurons. Following exposure to 1,000 U/ml chronic IL-6 (Fig. 5, B and D), a significant enhancement of both the peak amplitude and duration of the AMPA response was observed relative to matching controls, an effect likely to result from the higher input resistance in this membrane potential range (see Fig. 4D). In addition, the mean AHP amplitude was significantly larger following chronic IL-6 treatment at this dose (3.9 ± 0.5 mV) compared with matching controls (2.8 ± 0.4 mV; Fig. 5, E and F).

FIG. 4. Effects of chronic IL-6 on intrinsic membrane properties of cerebellar Purkinje neurons in primary culture. A and B: representative voltage (V) recordings of depolarizing and hyperpolarizing responses to injection of ±150 pA current (I) in Purkinje neurons following 100 U/ml chronic IL-6 treatment and in matching control cultures. C: chronic exposure to 100 U/ml IL-6 had no effect on the current-voltage (I-V) relationship or input resistance (inset) of cerebellar Purkinje neurons. D: chronic exposure to 1,000 U/ml IL-6 resulted in an enhanced depolarization in response to positive current injection that corresponded to a small enhancement of the input resistance in the depolarizing direction (inset). I-V curves were plotted from the sustained hyperpolarizations and depolarizations of the membrane potential (dashed lines in A) resulting from a series of negative and positive current pulses (±30–150 pA), respectively. The input resistance of each neuron was calculated from the slope of the I-V relationship in both the hyperpolarizing and depolarizing directions. Number of cells in parentheses. * P < 0.05; † trend (P = 0.06).
Chronic interleukin-6 exposure alters intracellular $\text{Ca}^{2+}$ homeostasis and AMPA-induced calcium signaling in cultured cerebellar Purkinje neurons

Cytosolic $\text{Ca}^{2+}$ levels are elevated in neurons as a result of sufficient AMPA receptor–mediated depolarization that initiates $\text{Ca}^{2+}$ entry through voltage-sensitive $\text{Ca}^{2+}$ channels (VSCCs). $\text{Ca}^{2+}$-induced $\text{Ca}^{2+}$ release (CICR) from intracellular stores can contribute to elevated cytosolic $\text{Ca}^{2+}$ levels if the $\text{Ca}^{2+}$ influx activates the ryanodine receptor (Gruol et al. 1996). To assess chronic IL-6–induced changes in AMPA-mediated $\text{Ca}^{2+}$ signaling, we measured intracellular $\text{Ca}^{2+}$ levels in response to brief applications (1 s) of 5 μM AMPA. A higher concentration of AMPA was used in the calcium imaging experiments than in the electrophysiological experiments to ensure that sufficient depolarization was achieved to initiate rises in intracellular $\text{Ca}^{2+}$. Two doses of chronic IL-6 (500 and 1,000 U/ml) and two durations of IL-6 exposure (8–11 and 14–17 days) were examined.

Prior to AMPA application, measurements of the baseline resting levels of intracellular $\text{Ca}^{2+}$ were made. Mean resting $\text{Ca}^{2+}$ levels were significantly increased in both the somata and dendrites of Purkinje neurons treated with 500 U/ml chronic IL-6 for either 8–11 (Fig. 6A) or 14–17 days (Fig. 6B) compared with age-matched controls. However, resting $\text{Ca}^{2+}$ levels were not different from control at either timepoint following chronic exposure with 1,000 U/ml IL-6 (Fig. 6, C and D).

Application of 5 μM AMPA to cultured Purkinje neurons resulted in a rapid increase in both somatic and dendritic intracellular $\text{Ca}^{2+}$ levels that lasted between 1 and 2 min (Figs. 7 and 8, A, B, D, and E). Measurements of the amplitude of the $\text{Ca}^{2+}$ signal in response to AMPA were made at the peak of the response and 60 s after AMPA application. Resting $\text{Ca}^{2+}$ levels were subtracted from the peak and 60-s timepoints to compare only the AMPA-induced $\text{Ca}^{2+}$ signal in control and chronic IL-6–treated Purkinje neurons. The results were then normalized to the mean of the control neurons for each experiment, as above.

Eight to 11 days of exposure to 500 U/ml chronic IL-6 resulted in a significant enhancement of the mean amplitude of the $\text{Ca}^{2+}$ signal at the peak of the AMPA response (Fig. 7, A–C). A similar enhancement of the peak was observed in the somata (Fig. 7A) and dendrites (Fig. 7B) of Purkinje neurons (Fig. 7C). Longer periods of exposure to 500 U/ml IL-6 (14–17 days) also resulted in altered $\text{Ca}^{2+}$ signaling in response to AMPA (Fig. 7, D–F), although the effect of chronic IL-6 appeared to be different after longer durations of exposure to IL-6 than after shorter exposure periods. Rather than an enhancement of the peak of the signal, the effect of 14–17 days of chronic IL-6 exposure on the AMPA-induced $\text{Ca}^{2+}$ signal
FIG. 6. Effects of chronic IL-6 exposure on resting Ca\(^{2+}\) levels in cultured cerebellar Purkinje neurons. A and B: chronic exposure to 500 U/ml IL-6 for either 8–11 days (A) or 14–17 days (B) resulted in significant elevations in resting somatic and dendritic Ca\(^{2+}\) levels in Purkinje neurons relative to control. C and D: in contrast, 1,000 U/ml IL-6 had no effect on resting Ca\(^{2+}\) levels after either 8–11 (C) or 14–17 days (D) of chronic exposure. Number of cells in parentheses. *P < 0.05, †trend (P = 0.08).

FIG. 7. Effects of chronic exposure to 500 U/ml IL-6 on AMPA-induced intracellular Ca\(^{2+}\) signaling in cultured cerebellar Purkinje neurons. A and B: mean Ca\(^{2+}\) responses to AMPA (5 µM; 1-s application at arrows) measured in the somata (A) and dendrites (B) of Purkinje neurons in cultures treated for 8–11 days with 500 U/ml IL-6 and in control sister cultures. C: 8–11 days of chronic exposure to 500 U/ml IL-6 resulted in a significantly enhanced peak amplitude of the Ca\(^{2+}\) signal in response to AMPA. Data are normalized to the mean peak amplitude of the Ca\(^{2+}\) response of the control neurons in each culture set. D and E: mean AMPA-induced Ca\(^{2+}\) responses in the somata (D) and dendrites (E) of Purkinje neurons in cultures treated for 14–17 days with 500 U/ml IL-6 and in control sister cultures. F: in contrast to the shorter duration of exposure, 14–17 days of 500 U/ml IL-6 treatment resulted in a significant increase in the Ca\(^{2+}\) signal at 60 s following AMPA application. Number of cells/dendrites in A–C and D–F in parentheses. *P < 0.05.
was characterized by an elevation in both the somatic (Fig. 7D) and dendritic (Fig. 7E) amplitude at 60 s after AMPA application (Fig. 7F), suggesting that chronic IL-6 altered the recovery phase of the Ca\(^{2+}\)/H\(_{11001}\) signal at this stage. In addition, control Purkinje neuron responses to AMPA were generally larger at this later developmental stage (20–23 DIV; Figs. 7 and 8, D and E). In contrast to 500 U/ml IL-6, neither 8–11 days nor 14–17 days of chronic exposure to 1,000 U/ml IL-6 had any effect on the somatic (Fig. 8, A and D) or dendritic (Fig. 8, B and E) AMPA-induced Ca\(^{2+}\)/H\(_{11001}\) signal of cultured Purkinje neurons (Fig. 8, C and F).

**DISCUSSION**

In the present study we have shown that chronic exposure to the cytokine IL-6 can alter the electrical properties and intracellular Ca\(^{2+}\) dynamics of CNS neurons. Chronic IL-6 treatment induced dose- and duration-dependent changes in evoked action potential generation, membrane biophysical properties, resting Ca\(^{2+}\) levels, and the membrane and Ca\(^{2+}\) signaling responses to glutamate receptor agonists of cerebellar Purkinje neurons maintained in cell culture. These alterations in neurophysiological properties occurred in the absence of gross morphological changes of these neurons. Moreover, we found that Purkinje neurons in culture express both the IL-6 receptor and its intracellular signaling subunit, gp130.

The cerebellar cultures were treated with a range of IL-6 doses (100–1,000 U/ml, equivalent to 1–10 ng/ml) that were based on concentrations reported in the cerebrospinal fluid (CSF) during a variety of neurological diseases (Achim et al. 1993; Frei et al. 1989; Gallo et al. 1989; Houssiau et al. 1988; Matsuzono et al. 1995). Although it is difficult to ascertain the actual levels of IL-6 within the brain parenchyma by measuring CSF concentrations, one can assume that IL-6 levels are likely similar, if not considerably higher, in the parenchyma than in the CSF. Depending on the study, CSF IL-6 concentrations in neurological patients have been reported to range from several pg/ml to several ng/ml, which corresponds to the lower end of the concentration range we have used in the current study. Thus we have used doses of IL-6 that are relevant to the expression of IL-6 within the CNS during various neuroinflammatory conditions.

The characteristics of chronic IL-6--induced changes in the physiological properties of Purkinje neurons were dependent on the dose of IL-6 and the duration of exposure. However, the relationship between the dose/duration of IL-6 and the effect was not always straightforward. For example, 14 days of 100–1,000 U/ml IL-6 resulted in dose-dependent reductions in
evoked spike firing with significant suppression of spike firing occurring after exposure to 1,000 U/ml chronic IL-6. In contrast, after 21 days of IL-6 treatment, Purkinje neurons in cultures treated with 100 U/ml IL-6 exhibited enhanced spike firing relative to those in matching control cultures, whereas spike firing returned to control levels in Purkinje neurons treated with 1,000 U/ml IL-6. Such differences in the effects of various doses and durations of IL-6 exposure may represent neuroadaptive changes in the spike firing properties of Purkinje neurons to compensate for prolonged exposure to IL-6 (e.g., development of tolerance to IL-6). Thus the differences may reflect a balance between the early, dose-dependent effects of chronic IL-6 (at 14 days of exposure) and the induction of negative regulatory pathways that lead to neuroadaptive changes in response to prolonged IL-6 exposure (i.e., 21 days). In addition, there may be changes in the sensitivity of Purkinje neurons to IL-6 during development in culture.

The effects of chronic IL-6 on the membrane properties of cerebellar Purkinje neurons were also dependent on the dose of IL-6. Low doses (100 U/ml) of IL-6 had no effect on the membrane responses to current injection or input resistance, whereas a higher dose (1,000 U/ml) resulted in an increase in membrane input resistance and a consequent enhancement of the current-induced membrane depolarization. Unlike the effects of chronic IL-6 exposure on spike firing activity, membrane depolarizations were not affected by the duration of IL-6 exposure; i.e., the results were similar for both long and short durations of IL-6 exposure. A larger depolarization in chronic IL-6–treated neurons would be predicted to result in an increased excitability of Purkinje neurons. However, a suppressive effect on action potential generation was instead observed in the same neurons. Thus it is likely that the effects of 1,000 U/ml chronic IL-6 exposure on spike generation and input resistance are independent effects.

The input resistance of a neuron is a determining factor in the magnitude of a neuronal response to synaptic input. Thus chronic IL-6–induced alterations in the input resistance of cerebellar Purkinje neurons would be expected to result in changes in their responses to activation of glutamate receptors. Chronic IL-6–treated Purkinje neurons exhibited an augmentation of AMPA-induced depolarization compared with matching controls. The enhanced AMPA response was similar in magnitude to the increased membrane depolarization in response to current injection, suggesting that the effects of IL-6 on the response of Purkinje neurons to glutamate receptor activation are related primarily to changes in the biophysical properties of the cell membrane rather than IL-6–induced modulation of AMPA receptor properties.

Intracellular Ca$^{2+}$ is an important signaling molecule involved in numerous cellular functions, including various aspects of neuronal physiology and development. In the current study, we found that both resting Ca$^{2+}$ levels and the Ca$^{2+}$ signal in response to AMPA receptor activation were altered following chronic treatment with 500 U/ml IL-6. However, 1,000 U/ml chronic IL-6 had no effect on either the resting Ca$^{2+}$ levels or the Ca$^{2+}$ signal in response to AMPA receptor activation, suggesting that higher doses of IL-6 may activate negative feedback mechanisms that result in a reduction of IL-6–mediated effects. One such potential mechanism is the suppressor of cytokine signaling (SOCS) family of proteins that are induced by cytokines and that inhibit cytokine signaling mediated by the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signal transduction pathway (Endo et al. 1997; Naka et al. 1997; Nicholson et al. 1999; Starr et al. 1997). A recent study has reported the expression of SOCS-1 and SOCS-3 mRNA in Purkinje neurons of the mouse cerebellum (Polizzotto et al. 2000). Such regulatory mechanisms would likely play important roles in the ability to lessen the effects of IL-6 on various physiological processes, including neuronal properties, during periods of elevated cytokine production such as inflammatory responses, whereas failure of these regulatory mechanisms could also contribute to the detrimental effects of prolonged chronic IL-6 exposure. Additionally, IL-6 receptor/gp130 activation has been linked to the mitogen-activated protein (MAP) kinase pathway that is activated independently of the STAT pathway (Hirano et al. 1997). Differences in SOCS-induced regulation of the STAT and MAP kinase pathways may thus provide a basis for the varied dose- and duration-dependent effects of chronic IL-6 on various physiological parameters of neurons.

In a previous study we found that Purkinje neurons in cerebellar slices taken from GFAP-IL6 transgenic mice exhibited altered firing properties and synaptic responses compared with littermate control mice (Nelson et al. 1999). For example, Purkinje neurons in slices from GFAP-IL6 mice had a significantly lower spontaneous firing rate. Results from the present study, which utilized a simplified model of chronic IL-6 exposure of CNS neurons, are consistent with this change. Although measurements of spontaneous activity were not done in the neuronal cultures, after 2 wk of exposure to IL-6, Purkinje neurons exhibited a reduction in current-evoked action potential generation relative to controls, consistent with the reduced spontaneous action potential generation in the GFAP-IL6 mice. Interestingly, after longer IL-6 exposure, IL-6–treated Purkinje neurons did not exhibit this reduction in action potential generation, suggesting that over time the neurons adapted to the continued presence of IL-6 in the cultures. A similar trend toward a less dramatic reduction of spontaneous activity was observed in cerebellar slices from older GFAP-IL6 mice (Nelson et al. 1999).

In addition to changes in spontaneous activity, Purkinje neurons from the GFAP-IL6 mice exhibited changes in synaptic responses, specifically a longer duration of the “climbing fiber pause,” a period of quiescence in the spontaneous firing activity of Purkinje neurons following synaptic activation via the climbing fiber afferent input (Nelson et al. 1999). The climbing fiber pause is caused principally by activation of Ca$^{2+}$–dependent K$^{+}$ conductances that hyperpolarize the neuron as a result of Ca$^{2+}$ influx that occurs during the initial depolarizing phase of the climbing fiber synaptic response (Hounsgaard and Midggaard 1989; Llinas and Sugimori 1980a,b). Chronic IL-6 exposure induced an enhancement of the AHP component of the membrane response to AMPA, a component that is mediated by Ca$^{2+}$–dependent K$^{+}$ conductances. The larger AHP in the cultured neurons is likely to result from the chronic IL-6–induced enhancement of the Ca$^{2+}$ signal evoked by AMPA. Enhancement of the AHP may also promote oscillatory firing in the neurons and could thus contribute to the higher incidence of oscillatory firing patterns observed in GFAP-IL6 mice. Moreover, the results from the current study suggest that Ca$^{2+}$ influx is increased during the climbing fiber response in the GFAP-IL6 mice.
Previously, we showed that cerebellar Purkinje neurons express the IL-6 receptor and its intracellular signaling protein, gp130, in both wild-type mice and GFAP-IL6 transgenic mice (Nelson et al. 1999). We have extended this observation to rat Purkinje neurons in cerebellar culture in the current study. In addition, nonneuronal cells in the cerebellar cultures appeared to express very low levels of the IL-6 receptor and gp130, indicating that these cells are unlikely to be responsive to IL-6. Moreover, two studies have shown that cerebellar Purkinje neurons express STAT3, a transcription factor activated by receptors linked to gp130 (Planas et al. 1997; Stromberg et al. 2000). Taken together, these results strongly suggest that the effects of chronic IL-6 on Purkinje neurons are exerted directly on these cells as a result of their expression of the receptors and intracellular machinery necessary for IL-6–mediated actions.

The results of the present study demonstrate that chronic exposure to elevated levels of IL-6, such as occurs in various CNS disease states, can alter important aspects of CNS neuronal physiology. Such IL-6–induced neurophysiological changes would be expected to contribute to the deficits in the overall function of the CNS, as evidenced by the decline in cognitive and motor skills of GFAP-IL6 mice (Campbell et al. 1993). Alterations in Purkinje neuron physiology would likely result in considerable alterations in information processing by the cerebellum in vivo, thus disrupting the output of the cerebellum to descending motor systems. Such disruption of cerebellar function can produce symptoms of discoordinated movement and ataxia, which are common to both the GFAP-IL6 transgenic mice and a number of neurological disorders with cerebellar function can produce symptoms of discoordinated movement and ataxia, which are common to both the GFAP-IL6 transgenic mice and a number of neurological disorders with...


