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Neuroadaptive Changes in Cerebellar Neurons Induced by Chronic Exposure to IL-6

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Abstract

IL-6 is an important signaling molecule in the CNS. CNS neurons express IL-6 receptors and their signal transduction molecules, consistent with a role for IL-6 in neuronal physiology. Research indicates that IL-6 levels are low in the normal brain but can be significantly elevated in CNS injury and disease. Relatively little is known about how the elevated levels of IL-6 affect neurons. In the current study we show that under conditions of chronic exposure, IL-6 induces alterations in the level of protein expression in developing CNS cells. Such changes may play a role in the altered CNS function observed in CNS conditions associated with elevated levels of IL-6 in the CNS.

1. Introduction

The production of elevated levels of the cytokine IL-6 in the developing central nervous system (CNS) during injury or disease has been widely documented and may play a role in neuroadaptive changes that result in permanent alterations in CNS function (Munoz-Fernandez and Fresno, 1998; Samuelsson et al., 2006; Smith et al., 2007). The cerebellar region of the brain is known to be highly sensitive to infections and toxic insults during the developmental period (Boltshauser, 2001), conditions that are associated with neuroinflammation and increased CNS levels of IL-6 (Gruol and Nelson, 1997). Several lines of evidence indicate that increased levels of IL-6 in the cerebellum can result in altered cerebellar function. For example, transgenic mice that chronically express elevated levels of IL-6 through genetic manipulation of astrocyte expression starting in the early postnatal period progressively develop neuroadaptive and pathological changes reflective of changes observed in CNS disease and injury (Campbell et al., 1998). As adults, the IL-6 transgenic mice exhibit cerebellar astrogliosis (Chiang et al., 1994), altered neuronal function (Nelson et al., 1999) and neurodegeneration (Campbell et al., 1993).

Results from studies of cultured cerebellar neurons also indicate the ability of IL-6 to produce neuroadaptive changes that alter neuronal function. Thus, chronic exposure of rat cerebellar granule neurons to IL-6 during their developmental period in culture results in altered neuronal properties including an increase in the magnitude of Ca^{2+} signals and voltage responses elicited by exogenous application of NMDA to the neurons and increased sensitivity to NMDA induced toxicity (Qiu et al., 1995; Qiu et al., 1998). Depending on conditions, IL-6 exposure can lead to neuronal loss in the granule neuron cultures (Conroy et al., 2004; Holliday et al., 1995). Cultured cerebellar Purkinje neurons subjected to chronic exposure to IL-6 during development also show altered neuronal function including altered

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excitability and Ca^{2+} signaling (Gruol and Nelson, 2005; Nelson et al., 2004; Nelson et al., 2002).

Although these studies indicate that chronic IL-6 exposure can produce neuroadaptive changes and neuronal loss in the cerebellum, information on the effects of IL-6 on developing neurons is still limited. To further address this issue, we have examined the effects of chronic IL-6 exposure during development on the level of proteins expressed by cerebellar cells both in cultures of cerebellar granule neurons and in cerebella obtained from 1-2 month old transgenic mice that express elevated levels of IL-6 in the CNS. The granule neuron cultures are derived from 8 day postnatal rats and have become a popular model for studies of developing neurons. The rodent cerebellum is immature at birth and developmental stages during the first three weeks postnatal reflect developmental stages occurring during the third trimester of human cerebellar development (Dobbing and Sands, 1979). IL-6, the IL-6 receptor and gp130, an essential subunit of the functional IL-6 receptor complex, are expressed in the immature cerebellum (Gadient and Otten, 1994; Ha and King, 2000). Both microglia (Hurley et al., 1999; Maslinska et al., 1998) and astrocytes (Yamada et al., 2000), important sources of IL-6 and other cytokines, are present in the immature cerebellum.

Results from our studies show that chronic IL-6 exposure during development alters the level of expression of both neuronal and glial proteins that play an important role in cerebellar function. These changes occur prior to stages when neuronal loss occurs and may reflect early neuroadaptive changes that underlie altered cerebellar function produced by adverse conditions that induce increased production of IL-6 in the CNS.

2. Materials and methods

The animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal facilities and experimental protocols were in accordance with the Association for the Assessment and Accreditation of Laboratory Animal Care.

2.1. Cell culture

Primary cultures of cerebellar granule neurons were prepared from cerebella of 8 day postnatal rats (Sprague-Dawley, Charles River, Wilmington, MA) by a standard protocol as described previously (Qiu et al., 1995). Cerebella were dissociated by enzymatic treatment (0.5% trypsin and 2500 units/ml DNase) and triturated in Ca^{2+} - and Mg^{2+} -free saline of the following composition (in mM): 145 NaCl, 3.5 KCl, 0.4 KH_2PO_4 , 0.33 Na_2HPO_4 , 10 glucose, 2 ethylene glycol-bis (b-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES-NaOH; pH 7.3). The granule neurons were collected by centrifugation (5 min at 70 g) and resuspended in DMEM/F12 (Invitrogen, Carlsbad, CA, USA) plus 10% horse serum (heat inactivated) supplemented with 30 mM glucose, 2 mM glutamine, 20 mM KCl, and 25 μM penicillin-streptomycin. The cells were plated at a density of 4.0×10^6 cell/ml in tissue culture plates (Falcon) and Lab-Tek slides (Nalgene Nunc International, Rochester, NY, USA) coated with Matrigel (Collaborative Biomedical Products, Bedford, MA, USA) and incubated in a standard 5% CO_2 incubator at 37°C. The antimitotic agent 5-fluorodeoxyuridine (FUDR; 20 $\mu\text{g}/\text{ml}$) was added to the medium on the first and fourth days after plating to minimize the number of non-neuronal cells in culture. To minimize toxicity that can be produced by serum containing medium in this preparation (Schramm et al., 1990), media changes were not used during the culture period. Instead, medium was refreshed every fourth day by addition of serum-free DMEM. Unless otherwise noted, chemicals were of reagent grade and were obtained from Sigma-Aldrich (Atlanta, GA, USA).

2.2 Chronic treatment of cultures

At plating, each culture set was divided into two groups, a control group and an IL-6 treatment group. The IL-6 treatment group was treated chronically with human recombinant IL-6 (Roche, Indianapolis, IN) at 5 ng/ml (500 U/ml) for 5 days. Treatment of the IL-6 group was initiated on the first day in vitro (DIV) by direct addition of IL-6 to the culture medium. A supplementary dose of IL-6 (5 ng/ml) was added at 4 DIV to ensure that biologically active IL-6 was present throughout test period. Thus, IL-6 levels increased when the media was supplemented at four DIV. The increase was approximately equal to the added dose (determined by ELISA analysis), indicating that catabolism of IL-6 was minimal during the culture period. For simplicity, the IL-6 levels are stated as the levels on the first day of treatment.

2.3 Transgenic animal

Hemizygous mice from the G167 IL-6 transgenic line were a gift from Dr. Iain Campbell. The protocol for construction of the expression vector and transgenic mice was described previously (Campbell et al., 1993). Briefly, full-length murine IL-6 cDNA was modified and inserted into the gene for glial fibrillary acidic protein (GFAP), thereby targeting expression of IL-6 to astrocytes, a cell type that normally expresses IL-6 in vivo. The fusion gene was microinjected into fertilized eggs of (C57BL/6J x SJL) F1 hybrid mice. The transgenic animals were identified by slot blot analysis of the tail DNA. Several lines were developed and have been maintained for several years. The G167 line used in this study was produced by breeding IL-6 mice (C57BL/6 strain) hemizygous for the transgene with C57BL/6 wildtype mice. Littermates that did not show expression of the transgene were used as controls. The mice were studied at 1-2 months of age, prior to the development of obvious neuropathology. The histopathological and neurological features that develop in the 167 line are similar to the line with a high level of IL-6 expression, but neuropathology occurs at an older age (12 months) and to a lesser extent in the 167 line (Campbell et al., 1993).

At 1-2 months of age, the mice were weighed, anesthetized with halothane and decapitated. Brains were rapidly removed, immersed in ice-cold artificial cerebrospinal fluid (ACSF) and allowed to cool for 2-3 min. The composition of the ACSF was (in mM): 130.0 NaCl, 3.5 KCl, 1.25 NaH₂PO₄, 24.0 NaHCO₃, 2.0 CaCl₂, 1.3 MgSO₄, and 10.0 glucose (all chemicals from Sigma). The ACSF was gassed continuously with 95% O₂/5% CO₂ (pH 7.2-7.4). The cerebellum was dissected from the rest of the brain and snap frozen on dry ice and stored at -80 °C until use. Both females and males were used.

2.4. Protein Determination

The relative level of cell proteins in granule neuron cultures and in cerebella obtained from the IL-6 transgenic (IL-6 tg) and non-transgenic littermate control (non-tg) mice was determined by standard protocols reported previously (Vereyken et al., 2007). All protein assays were done on samples from individual cultures or animals. To prepare protein samples from the cultures, the cultures were washed in ice-cold PBS, cold lysis buffer added and the cells scrapped from the dishes. The lysis buffer contained 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.5% NP-40, a Protease Inhibitor Cocktail Tablet (Boehringer Mannheim, USA), and a cocktail of phosphatase inhibitors (4.5 mM sodium pyrophosphate, 10 mM β -glycerophosphate, 1 mM sodium fluoride, 1 mM sodium orthovanadate). The lysate from each culture dish was collected and incubated on ice for 30 min. Lysates were centrifuged at 15,000 rpm (30 min) and the protein concentration of the supernatants determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA).

To prepare the protein samples from cerebella, the cerebella were thawed on ice and placed in cold lysis buffer (the same buffer as used for the cultures). Each cerebellum was individually sonicated to disrupt the cells and then incubated on ice for 30 min. After incubation, the homogenates were centrifuged at 15,000 rpm (30 min), the supernatant collected and protein concentration determined using the Bio-Rad Protein Assay Kit (Bio-Rad).

IL-6 levels in the cerebellar homogenates were determined by mouse IL-6 ELISA Ready-SET-Go kit (eBioscience, San Diego, CA, USA). The levels of other cellular proteins were determined by Western blot. For Western blot, the samples (10-20 µg of protein in duplicate for cultures and 20-50 µg of protein in duplicate for cerebella) were diluted with 2× Laemmli sample buffer, separated by SDS-PAGE gel electrophoresis on 4-12% Bis-Tris NuPAGE® gels (Invitrogen, Carlsbad, CA, USA) and transferred onto Immobilon P membranes (Millipore, Billerica, MA, USA). The membranes were blocked with casein (Pierce Biotechnology, Rockford, IL, USA) and then incubated in primary antibody followed by the secondary antibody coupled to HRP (1:10000, Southern Biotech, Birmingham, AL, USA). The immunoreactive bands were detected using chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Pierce Biotechnology, Rockford, IL, USA). Quantification involved densitometry measurements using NIH image software program (<http://rsb.info.nih.gov/ni-image>). Control and IL-6 samples (from cultures or animals) were run on each gel and density measurements for the IL-6 samples were normalized to density measurements for the control samples. Summarized results are the normalized data.

2.5 Antibodies

The following antibodies were used for Western blot analysis: a monoclonal antibody to beta-actin (AC-15, 1:5000; Sigma, St. Louis, MO, USA); a purified rabbit antibody raised against a synthetic protein made to an internal region of the mouse CD11b protein (NB110-89474, 1:500; Novus Biologicals, Littleton, CO, USA); a monoclonal antibody to glial fibrillary acidic protein (GFAP; MAB360, 1:10,000; Millipore, Billerica, MA, USA); a monoclonal antibody raised against neuron specific enolase (MAB314, 1:5000; Chemicon International, Temecula, CA); a rabbit polyclonal antibody raised against a synthetic peptide to the C-terminus of rat GAD 65/67 (AB1511; 1:1000; Millipore); a purified rabbit polyclonal antibody raised against a synthetic peptide of the rat GluR1 subunit of the AMPA receptor conjugated to KLH with a cysteine added (07-660 1:500; Millipore); a purified mouse monoclonal antibody raised against the GluR2 subunit of the AMPA receptor (1-1000; #556341, BD Pharmingen, San Diego, CA); a purified rabbit polyclonal antibody raised against a 13 amino acid peptide from the carboxy terminal of human GRIK2 (kainite receptor; ab67318; 1:500; abcam); a purified rabbit polyclonal antibody raised against a carboxy terminus peptide of rat group II metabotropic glutamate receptors (mGluR2 and mGluR3; mGluR2/3) conjugated to BSA with glutaraldehyde (AB1553 1:1000; Millipore); a purified rabbit polyclonal antibody raised against a synthetic peptide corresponding to amino acids 1159-1171 of rat metabotropic glutamate receptor 1a (ab51314 1:500; abcam; San Francisco, CA, USA); a purified rabbit antibody to synapsin 1 (#51-5200, 1:5,000; Invitrogen Life Technologies); a purified goat polyclonal antibody raised against a peptide corresponding to an amino acid mapping the C-terminus of the human NMDAR1 subunit of the NMDA receptor (sc-1467, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA); a rabbit polyclonal antibody raised against a synthetic peptide from the 2nd cytoplasmic domain of human NMDAR2A conjugated to an immunogenic carrier protein (ab84181; 1:500; abcam); an affinity purified rabbit polyclonal antibody raised against a peptide corresponding to an amino acid sequence at the amino terminus of NOS1 (nNOS; 1:500 sc1025; Santa Cruz Biotechnology); a rabbit polyclonal antibody raised against a

purified peptide corresponding to residues 818-835 of the $\alpha 1C$ subunit of the rat brain voltage-gated Ca^{2+} channel (VGCC)(acc-003; 1:500; Alomone labs; Jerusalem; a rabbit polyclonal antibody raised against p42/p44 mitogen activated protein kinase (p42/44 MAPK)(1:5000, #61-7400, Zymed, Carlsbad, CA, USA); a purified rabbit polyclonal antibody raised against a synthetic phospho-peptide (KLH-coupled) corresponding to residues around Thr202/Tyr204 of human p44 MAPK (pp42/44 MAPK; #9101; 1:500; Technologies, Danvers, AM, USA); a purified rabbit polyclonal antibody raised against a synthetic peptide (KLH-coupled) corresponding to the sequence of mouse STAT3 (AB#9132; 1:1000; Cell Signaling Technologies); a purified rabbit polyclonal antibody raised against a synthetic phospho-peptide (KLH-coupled) corresponding to the residues surrounding Tyr705 of mouse STAT3 (AB#9131; 1:1000; Cell Signaling Technologies); a purified rabbit polyclonal antibody raised against a peptide mapping at the carboxy terminus of C/EBP beta C-19; 1:500; Santa Cruz; a purified monoclonal antibody raised against bovine brain calcineurin (MAB2839; 1-500; R&D Systems, Minneapolis, MN, USA).

2.5 Statistical analysis

All compiled data are expressed as mean $\pm$ SEM. The number of preparations analyzed is noted in figure legends. Statistical significance was determined by the one group *t* test or the paired *t* test. *p* values < 0.05 were considered to reflect a statistically significant difference.

3. Results

3.1. Effect of IL-6 on granule neuron culture characteristics

Granule neurons obtained from 8 day postnatal rats are immature at plating and differentiated during the culture period into well-developed neurons with axonal and dendritic structure by 5 days in vitro (DIV). The granule neuron cultures are comprised of ~ 95 % neurons with only a limited number of other cell types, primarily astrocytes. Therefore, these cultures are an advantageous model for assessing direct actions of IL-6 on developing neurons. Previous immunohistochemical studies showed that the cultured granule neurons express the IL-6 receptor and its intracellular signaling partner gp130 (Conroy et al., 2004). Measurements of total protein in the 5 DIV cultures showed a small (7%) but significant decrease in the level of total protein in the chronic IL-6 treated cultures (5 ng/ml) compared with control cultures (Fig. 1A), consistent with previous studies showing that chronic IL-6 exposure can cause cell loss in granule neuron cultures (Conroy et al., 2004). Western blot analysis of the relative level of enolase, a protein expressed by neurons and used as a neuronal marker, showed no significant difference between control and IL-6-treated cultures. In contrast, the level of GFAP, a structural protein localized to astrocytes and used as an astrocyte specific marker, was significantly increased in the IL-6-treated cultures (Fig. 1B), consistent with the reported ability of IL-6 exposure to induce GFAP production in astrocytes (Chiang et al., 1994).

3.2. Chronic IL-6 alters the level of p42 MAPK activation

IL-6 is known to activate both the STAT3 and MAPK signal transduction pathways (Gruol and Nelson, 1997; Spooren et al. 2011). To determine if chronic IL-6 exposure altered the level of activation of these pathways in the granule neuron cultures, the level of phosphorylated STAT3 (pSTAT3) and p44/42MAPK (pp44/pp42 MAPK) were measured by Western blot using antibodies specific for the phosphorylated form of the enzymes. p44 MAPK was not consistently detectable at a measurable level. Therefore, only p42 MAPK and pp42 MAPK were quantified. pp42 MAPK was significantly increased in the IL-6 treated cultures, whereas there was no significant effect on the level of pSTAT3 (Fig. 1C). Both p42 MAPK and STAT3 were increased in the IL-6 treated cultures but the effect was not significant (Fig. 1C). In contrast, the level of protein for the transcription factor C/EBP

beta, which is known to be upregulated by IL-6 in other cell types (Akira and Kishimoto, 1997; Cardinaux et al., 2000), was increased more than two fold in the IL-6 treated cultures (Fig. 1C).

3.3. Chronic IL-6 exposure alters the level of neuronal proteins in granule neuron cultures

Our previous studies showed that chronic exposure to IL-6 during development alters the physiological properties of the granule neurons as evidenced by a larger membrane depolarization and associated Ca^{2+} response produced by exogenous application of NMDA and by larger resting Ca^{2+} levels in the IL-6-treated neurons (Qiu et al., 1995; Qiu et al., 1998). NMDA receptors and Ca^{2+} are known to be important regulators of granule neuronal development, function and gene expression (Nakanishi and Okazawa, 2006). Therefore, through these changes or other mechanisms chronic IL-6 exposure during development could result in alteration in the levels of other important neuronal properties such as those that mediate neuronal excitability and synaptic transmission. To test this possibility, we compared the relative levels of several proteins involved in neuronal excitability and synaptic transmission in 5 DIV control and IL-6 treated cultures using Western blot analysis.

Ca^{2+} influx through L-type VGCCs plays a central role in granule neuron excitability and development (Gallo et al., 1987). For example, Ca^{2+} by activating calcineurin is an important regulator of gene expression in granule neurons (Carafoli et al., 1999; Genazzani et al., 1999; Kramer et al., 2003; Sato et al., 2005). Western blot analysis of the control and IL-6 treated cultures showed that the level of protein for L-type VGCCs was significantly reduced in the IL-6-treated cultures (Fig. 2A). Consistent with this result, several proteins whose expression is regulated by calcineurin (Genazzani et al., 1999; Kramer et al., 2003) were also reduced by IL-6 treatment (Fig. 2A). These proteins included voltage-gated K^{+} channel Kv4.2 (KCND2), the plasma membrane Ca^{2+} pump (PMAC), and the inositol 1,4,5-trisphosphate receptor (IP3R) (Fig. 2A). However, there was no difference in the level of calcineurin between control and IL-6 treated cultures (Fig. 2A). The level of the P-type VGCCs was also reduced in the IL-6 treated cultures but not significantly (Fig. 2A).

The IP3R is an important downstream target of group I metabotropic glutamate receptors (mGluR1), a G-protein coupled receptor. When activated by glutamate, mGluR1 initiates Gq activation of phospholipase C resulting in the production of the second messengers, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 initiates release of Ca^{2+} from intracellular Ca^{2+} stores controlled by the IP3R. DAG and Ca^{2+} activate phospholipase C beta (PLC β). In addition to IP3R, the level of protein for both the alpha subunit of mGluR1 and PLC β were reduced in the IL-6-treated cultures compared with control cultures (Fig. 2B). Thus, several components of the mGluR1 signal transduction pathway were reduced by the chronic IL-6 exposure (mGluR1, IP3R, PLC β). Western blot analysis also showed that the level of protein for other glutamate receptor subunits is reduced by IL-6 treatment of the cultures. These receptors include the GluR2 subunit of the AMPA receptor and the alpha subunit of mGluR2/3 (Fig. 2B).

3.4. mGluR1 activation of pCREB is reduced in IL-6-treated cultures

Activation of mGluR1 can result in downstream activation of transcription factors such as CREB (cAMP-responsive element binding protein) and regulation of gene expression (Mao et al., 2008). The lower level of mGluR1 in the IL-6-treated cultures could impact the normal function of this pathway. To test this possibility, we examined the effect of exogenous application of the selective mGluR1 agonist 3,5-dihydroxyphenylglycine (DHPG) on the level of activated CREB in the granule neuron cultures. For these studies, CREB activation was determined by Western blot and immunohistochemistry using an antibody that recognizes the phosphorylated (i.e., activated) form of the enzyme. Western

blot analysis showed that under baseline conditions, pCREB levels were significantly higher in the 5 DIV IL-6-treated cultures compared with control cultures (Fig. 3A), perhaps due to the higher resting Ca^{2+} levels observed in IL-6-treated granule neurons (Qiu et al., 1995). Exposure to DHPG significantly increased the levels of pCREB in control cultures but produced only a non-significant increase in the IL-6-treated cultures (Fig. 3A). Results from studies using semi-quantitative measurement of pCREB immunostaining in the granule neuron cultures were consistent with these results. Granule neurons in IL-6-treated cultures showed stronger immunostaining compared with neurons in control cultures under baseline conditions (Fig. 3B). A significant increase in pCREB immunostaining was produced by exposure to DHPG in the control cultures but not the IL-6-treated cultures.

pCREB can also be activated by Ca^{2+} influx through VGCCs. Granule neurons express L-, N- and P-type Ca^{2+} channels, which can be activated by K^{+} depolarization resulting in Ca^{2+} influx and activation of pCREB. K^{+} depolarization produced an increase in pCREB immunostaining in the cultured granule neurons that was larger than observed with DHPG exposure, but the increase was similar for control and IL-6 treated cultures (Fig. 3B). This result is consistent with our previous studies showing that the magnitude of Ca^{2+} signal produced by K^{+} depolarization was similar for control and IL-6 treated granule neurons (Qiu et al., 1998). Unlike K^{+} depolarization, we were unable to detect a Ca^{2+} signal when DHPG was applied to the granule neurons, perhaps due to a small size of the Ca^{2+} signal produced by activation of the mGluR1 pathway.

3.1. Chronic IL-6 exposure in vivo alters the level of neuronal proteins in IL-6 transgenic mice

The above studies provide evidence the IL-6 exposure during development can alter the level of neuronal proteins involved in granule neuron function. To determine if such changes occur in an in vivo model, we examined protein expression by Western blot analysis of the cerebellum of young (1-2 months of age) hemizygous transgenic (IL-6 tg) mice that express elevated levels of IL-6 in the CNS. Non-transgenic (non-tg) littermates were used as controls. Expression of the transgene starts about 1 week postnatal in the transgenic mice, with levels of IL-6 mRNA (total brain mRNA) increasing progressively with age until about 3 months of age (Chiang et al., 1994). Therefore, the cerebellar neurons in vivo were exposed to IL-6 during the main period of neuronal development, as was the case for the cultured granule neurons (Conroy et al., 2004). Measurement of IL-6 levels in IL-6 tg and non-tg cerebellar homogenates by ELISA revealed that IL-6 could be detected in both non-tg and IL-6 tg mice, but IL-6 levels were significantly higher in the IL-6 tg cerebellum. Mean values ($\pm$ SEM) were 62 ± 8 (n=4) pg/ml for the IL-6 tg cerebella compared to 24 ± 4 (n=4) pg/ml for the non-tg cerebella (unpaired *t*-test; $p > 0.05$).

Western blot analysis of the level of β -actin, a house keeping protein, showed no difference between IL-6 tg and non-tg cerebella, suggesting that cell loss was absent of minimal at these young ages (1-2 month; Fig. 4A). This result is consistent with previous studies showing that cerebellar pathology is absent at young ages and is not evident at a significant level until after 6 months of age (Heyser et al., 1997; Samland et al., 2003). The level of p44 MAPK, a prominent cellular protein, was also comparable for IL-6 tg and non-tg cerebella, although the level of p42 MAPK was somewhat lower in the IL-6 tg cerebellum (Fig. 4A). We were unable to measure the phosphorylated form of 42/44 MAPK in the cerebellar samples. Unlike the cerebellar cultures, the level of calcineurin A was significantly increased in the IL-6-tg cerebellum. This increase may reflect astrocyte levels of calcineurin A, as the increase was similar to that observed for the astrocyte proteins GFAP and glutamine synthetase (Fig. 4A,B). The levels of the transcription factors C/EBP beta and STAT3 were also significantly higher in the IL-6 tg cerebellum compared with the non-tg cerebellum (Fig. 4A). The level of pSTAT3 was undetectable in the non-tg cerebellum but

was prominent in the IL-6 tg cerebellum (Fig. 4A). Studies by others have shown increased levels of pSTAT in the IL-6-tg cerebellum reflects STAT activation in astrocytes (Sanz et al., 2008).

Analysis of the levels of cell specific proteins showed that the level of neuron specific enolase, a marker for neurons, GAD65/67, the synthetic enzyme for the inhibitory transmitter GABA and a marker for inhibitory neurons, and nNOS, a protein expressed in neurons, were all reduced in the IL-6 tg cerebellum compared with the non-tg cerebellum (Fig. 4B). The level of protein for the astrocyte marker GFAP was significantly increased in the IL-6 tg cerebellum compared with the non-tg cerebellum, as reported previously for these mice (Chiang et al., 1994), as was the astrocyte protein glutamine synthetase (Fig. 4B). The level CD11b, a microglial marker, showed no difference between non-tg and IL-6-tg cerebellum (Fig. 4B).

As was observed in the granule neuron cultures, the levels of protein for mGluR1, IP3R, PLC β and L-type VGCC were reduced in the IL-6 tg cerebellum compared with the non-tg cerebellum (Fig. 4C). The level of protein for synapsin I, a synaptic protein and marker for presynaptic terminals, was also reduced in the IL-6 cerebellum (Fig. 4C), as reported previously for cultured granule neurons (Conroy et al., 2004). In addition to mGluR1, the levels of protein for other glutamate receptors were also reduced in the IL-6 tg cerebellum including the NR1 and NR2A subunits of the NMDA receptor, the GluR1 and GluR2 subunits of the AMPA receptor, the alpha subunit of mGluR2/3 and the kainite receptor (Fig. 4C). The lower level of NR1 in the IL-6 transgenic cerebellum is in contrast to the larger responses to NMDA in the granule neurons cultures, but this difference may reflect NR1 levels in the inhibitory neurons in the cerebellum in vivo, which are not present in the granule neuron cultures.

4. Discussion

The current studies show that chronic exposure of cerebellar neurons to IL-6 during development in vivo and in vitro alters the levels of cellular proteins in the cerebellum and that the effects of IL-6 vary for the different cell types and different proteins. In most cases, the levels of neuronal proteins were reduced by IL-6 exposure. In contrast, the levels of two proteins expressed by astrocytes, GFAP and glutamine synthetase, were increased by chronic exposure to IL-6. The level of CD11b, a microglial protein, was not altered by chronic IL-6 exposure. These results are consistent with cell type specific effects of enhanced levels of IL-6 in the developing cerebellum.

Studies in the granule neuron cultures showed that proteins involved in mGluR1 signal transduction pathway were reduced by chronic IL-6 treatment including mGluR1, PLC β and IP3R. To determine if there was a functional correlate to these changes, mGluR1 was activated in granule neuron cultures with the selective agonist DHPG and a downstream consequence of this activation, phosphorylation of CREB, was measured. Exposure to DHPG significantly increased the levels of pCREB in control cultures but produced only a non-significant increase in the IL-6-treated cultures, consistent with reduced functionality of the mGluR1 pathway in the IL-6-treated granule neurons. The down regulation of the mGluR1 pathway could have important consequences for granule neuron function. In addition to a role in synaptic responses, the mGluR1 pathway is coupled to G-protein activation of PLC and the production of two second messengers, IP3 and DAG. IP3 activates the IP3R initiating release of Ca²⁺, another second messenger, from intracellular stores. DAG and Ca²⁺ activate PKC. Thus, cellular functions regulated by DAG, Ca²⁺ and PKC could all be influenced by the effect of IL-6 on the mGluR1 signaling pathway. This down regulation of the mGluR pathway produced by IL-6 may be a neuroadaptive change

that has a neuroprotective function under some conditions. Blockade of the mGluR1 pathway by antagonists has been shown to reduce neuronal degeneration and behavioral deficits after traumatic brain injury in rats and to improve outcome after experimental brain trauma (Faden et al., 2001; Lyeth et al., 2001), two conditions in which IL-6 levels are known to be elevated (Spooren et al., 2011).

The IL-6-induced down regulation of mGluR1 in the cultured granule neuron cultures is consistent with studies of Purkinje neurons in cerebellar cultures subjected to similar IL-6 exposure (Nelson et al., 2004). The relative level of mGluR1 α , the predominant metabotropic glutamate receptor splice variant expressed by Purkinje neurons (Casabona et al., 1997), determined by a semiquantitative immunocytochemical technique was reduced in chronic IL-6-treated cultured Purkinje neurons compared with Purkinje neurons in control cultures. However, the level of the IP3R determined by a semiquantitative immunocytochemical technique was not reduced by the chronic IL-6 treatment of the cultured Purkinje neurons (Nelson et al., 2004). This difference from results in the studies of cultured granule neurons may relate to the developmental stage at which IL-6 treatment was started, which was at a later developmental stage for the cultured Purkinje neurons (when the soma was well-developed). Although the level of mGluR1 was reduced in the Purkinje neurons, the Ca²⁺ signal produced by activation of mGluR1 was increased due to increased Ca²⁺ release from intracellular stores (Nelson et al., 2004). We were unable to measure a Ca²⁺ signal due to mGluR1 induced Ca²⁺ release from intracellular stores in the granule neurons. However, our previous studies of Ca²⁺ signals produced by activation of NMDARs in the cultured granule neurons showed that Ca²⁺ release from intracellular stores is increased in the IL-6-treated granule neurons (Qiu et al., 1995). In these studies, IL-6-treated granule neurons were more sensitive to pharmacological agents that alter intracellular stores than control granule neurons. These pharmacological agents included caffeine, which depletes intracellular stores, and dantrolene, which is an antagonist at ryanodine receptors that control Ca²⁺ induced Ca²⁺ release. These results indicate that mechanisms linked to Ca²⁺ homeostasis are important targets of chronic IL-6 exposure.

The level of protein for other molecules that contribute to Ca²⁺ homeostasis in the granule neurons was also altered by the chronic IL-6 exposure including PMAC, L-type VGCCs and GluR2. The reduced levels of PMAC, which pumps Ca²⁺ out of the cell and thereby helps to establish the resting Ca²⁺ levels, could be a contributing factor in the higher resting Ca²⁺ levels of the IL-6 treated granule neurons. A reduction in L-type VGCCs would be expected to result in reduced Ca²⁺ influx during membrane depolarization, whereas reduced GluR2 would increase Ca²⁺ influx through AMPA receptors. The GluR2 subunit controls the Ca²⁺ permeability of AMPA receptors and a decreased level results in increased Ca²⁺ permeability of AMPA receptors. Reduced Ca²⁺ influx through L-type VGCCs could be a contributing factor to the lower levels of Kv4.2 (KCND2), PMAC, and IP3R, which are regulated by calcineurin, an enzyme activated by Ca²⁺ influx through L-type VGCCs in granule neurons (Carafoli et al., 1999).

The mechanisms mediating the altered protein levels in the granule neuron cultures remain to be determined and are likely to be complex, however p42 MAPK or C/EBP beta may be involved. Western blot analysis showed that the level of active p42 MAPK was increased in the cultures, as was the level of the C/EBP beta. The level of active STAT3 (pSTAT3) was not increased in the cultures, consistent with previous immunohistochemical studies of IL-6 tg cerebellum showing that pSTAT3 was localized to astrocytes and not to the neuronal population (Sanz et al., 2008). Studies using In situ hybridization showed prominent C/EBP beta mRNA expression in Purkinje neurons and granule neurons of the cerebellum (Sterneck and Johnson, 1998), consistent with a localization of the increased C/EBP beta to the granule neurons. C/EBPs can either transactivate or repress gene expression. Interestingly,

recent studies have identified numerous neuronal proteins that are regulated by C/EBP beta in the hippocampus and have also shown that C/EBP beta is a negative regulator of gene expression for many hippocampal proteins (Kfoury and Kapatos, 2009). Thus, the elevated level of C/EBP both in vitro and in vivo could be an important contributing factor to the reduced levels of neuronal proteins observed in the current studies.

To determine if chronic in vivo exposure to IL-6 produced changes in protein levels similar to those observed in culture, Western blot studies of IL-6 tg cerebella were carried out. The IL-6 tg cerebellum contains a variety of neuronal types (e.g., Purkinje neurons, stellate cells, golgi cells) besides granule neurons and the exposure duration and IL-6 concentrations in the IL-tg cerebellum differed from the exposure paradigm used for the cultures. In spite of these differences, many of the changes observed in the granule neuron cultures were also observed in the IL-6 tg cerebellum. As in the granule neuron cultures, the level of protein for L-type Ca^{2+} channels, mGluR1, IP3R, PLC β and GluR2 were all reduced in the IL-6 tg cerebella compared with the non-tg controls. The level of GFAP was significantly increased in the IL-6 tg cerebellum, as was observed in the granule neuron cultures. In contrast, the level of NR1 protein was reduced in the IL-6 tg hippocampus, whereas our previous studies of cultured granule neurons indicate an increase in functional NMDA receptors (Qiu et al., 1998). Also reduced in the IL-6 cerebella were enolase, a neuronal marker, nNOS and GAD65/67, a marker for inhibitory neurons.

The reduced levels of neuronal proteins in the IL-6-tg cerebellum raise the possibility of neuronal loss. However, this explanation seems unlikely. Studies of histological sections of cerebella from the low expressor 167 line used in the current studies showed normal histogenesis and architecture of the cerebellar at 1-2 months of age (Brett et al., 1996). Except for abnormal blood barrier formation and vascular proliferation, the cerebellum appeared normal until 6-12 months of age (Brett et al., 1996) Therefore, the changes in protein levels observed in the current study are likely to reflect changes in protein expression within cells rather than cell loss. Studies of hippocampus from IL-6 tg mice also showed no cell loss at 2-3 months of age (Heyser et al., 1997; Samland et al., 2003). Our studies of protein levels in the hippocampus of the transgenic mice at 1 month of age showed no effect of the in vivo IL-6 exposure on the level of beta actin and enolase but reduced levels of the L-type VGCC and mGluR2/3 (Vereyken et al., 2007). The levels of other neuronal proteins (synapsin, NMDAR1, GluR2) were similar in non-tg and IL-6 tg hippocampus at 1 month of age (Vereyken et al., 2007). IL-6 expression in the transgenic mice is known to be highest in the cerebellum (Campbell et al., 1993; Chiang et al., 1994), which could explain the greater effect of IL-6 on neuronal proteins the cerebellum. The reduction of proteins in cerebellar neurons identified in the current study may reflect early changes in the neuroadaptive processes that result in altered neuronal function in the cerebellum of the IL-6 tg mice. Similar changes could also occur in cerebellar injury or disease when increased levels of cerebellar IL-6 are present in the cerebellum.

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Figure 1.

Effects of chronic IL-6 exposure on protein levels in granule neuron cultures. A. Graph showing mean±SEM values for total protein levels in control and IL-6 treated cultures. B. Graphs showing mean±SEM values (normalized to control) for the level of the neuron specific protein enolase and the glial specific protein GFAP in control and IL-6 treated cultures determined by Western blot. C. Graphs showing mean±SEM values (normalized to control) for the level of signal transduction molecules in control and IL-6 treated cultures. In all graphs, the number of cultures measured is shown in the boxes. Representative Western blots are shown above the graphs. Dotted line reflects control values (control = 1). * = significantly different from control (one group t-test; $p < 0.05$).

Figure 2.

Effects of chronic IL-6 exposure on the level of proteins that play a role in granule neuron physiology. A,B. Graph showing mean $\pm$ SEM values (normalized to control) for the levels of cellular proteins in granule neuron cultures determined by Western blot. Dotted line reflects control values (control = 1). The number of cultures measured is shown in the boxes. Representative Western blots are shown above the graphs. *= significantly different from control (one group t-test or paired t-test; $p < 0.05$).

Figure 3.

Effect of chronic IL-6 exposure on the level of pCREB in granule neuron cultures. A, B. Level of DHPG-induced CREB phosphorylation measured by Western blot (A) and Immunohistochemistry (B). Dotted line reflects control values (control = 1). In B, a representative cluster of immunostained granule neurons is shown in the image above the graphs. In A, the number of cultures measured is shown in the boxes. In B, 100-200 neurons were measured for each condition. * = significantly different from baseline for control group (one group t-test; $p < 0.5$). @ = significantly different from baseline for IL-6 group (unpaired t-test; $p < 0.5$).

Figure 4. Effects of chronic in vivo IL-6 exposure on the level of cellular proteins in the cerebellum. A-D. Graph showing mean±SEM values (normalized to non-tg) for the level of cellular proteins determined by Western blot in cerebella from IL-6-tg and non-tg mice. Dotted line reflects non-tg values (control = 1). A. Structural and signal transduction proteins. B. Cell specific proteins. C.,D. Proteins involved in excitability, Ca²⁺ homeostasis and synaptic transmission. Representative Western blots are shown above the graphs. The number of animals measured is shown in the boxes. *= significantly different from non-tg (one group t-test; p<0.5).