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Altered chemokine expression in the spinal cord and brain contributes to differential interleukin-1β-induced neutrophil recruitment

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Abstract

The pattern of neutrophil recruitment that accompanies inflammation in the CNS depends on the site of injury and the stage of development. The adult brain parenchyma is refractory to neutrophil recruitment and associated damage as compared to the spinal cord or juvenile brain. Using quantitative Taqman RT–PCR and enzyme-liked immunosorbent assay (ELISA), we compared mRNA and protein expression of the rat neutrophil chemoattractant chemokines (CINC) in spinal cord and brain of adult and juvenile rats to identify possible association with the observed differences in neutrophil recruitment. Interleukin-1β (IL-1β) injection resulted in up-regulated chemokine expression in both brain and spinal cord. CINC-3 mRNA was elevated above CINC-1 and CINC-2α, with expression levels for each higher in spinal cord than in brain. By ELISA, IL-1β induced greater CINC-1 and CINC-2α expression compared to CINC-3, with higher protein levels in spinal cord than in brain. In the juvenile brain, significantly higher levels of CINC-2α protein were observed in response to IL-1β injection than in the adult brain following an equivalent challenge. Correspondingly, neutrophil recruitment was observed in the juvenile brain and adult spinal cord, but not in the adult brain. No expression of CINC-2β mRNA was detected. Thus differential chemokine induction may contribute to variations in neutrophil recruitment in during development and between the different CNS compartments.

Keywords: chemokine, CINC, cytokine, interleukin-1β, neutrophil, spinal cord.

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In the CNS, where there is little scope for cellular repair, acute inflammation and the recruitment of neutrophils following injury has been shown in many instances to be deleterious (Matsuo *et al*. 1994; Jiang *et al*. 1995). The CNS has evolved mechanisms to regulate the potentially damaging effects of an acute inflammatory response and, in particular, to restrict the recruitment of neutrophils (Anthony *et al*. 1997). However, within the CNS, there is still marked variation in the inflammatory response to equivalent challenges at different sites (Andersson *et al*. 1991). Inflammation in the spinal cord following mechanical lesions or microinjections of tumour necrosis factor-α (TNFα) or interleukin-1β (IL-1β) is more overt when compared to that in the brain (Schnell *et al*. 1999a, 1999b). It is also clear that there are periods during development in both rats and mice, known as windows of susceptibility, when the brain is less refractory to inflammatory stimuli than it is in an adult animal (Lawson and Perry 1995; Anthony *et al*. 1997; Bolton and Perry 1998). The mechanisms that underlie the differences in neutrophil recruitment and inflammatory response between different CNS compartments and in the brain at different developmental stages are unknown. The principal inflammatory mediators, necessary for the induction of a successful inflammatory cascade, are induced in the

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Abbreviations used: BBB, blood–brain barrier; CINC, cytokine-induced neutrophil chemoattractant; GAG, glycosaminoglycan; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ELR, glutamate–leucine–arginine; GRO, growth-regulated oncogene; IL-1β, interleukin-1β; IL-8, interleukin-8; MIP-2, macrophage inflammatory protein-2; TNFα, tumour necrosis factor-α; T8, thoracic level 8.

CNS parenchyma following injury; cell adhesion molecules are either constitutively expressed on the vascular endothelium or are rapidly up-regulated upon challenge (Bell and Perry 1995; Schnell *et al*. 1999b; Bernardes-Silva *et al*. 2001), and pro-inflammatory cytokines have been observed in brain and spinal cord, either constitutively or following trauma (Pousset 1994; Glabinski *et al*. 1996; Bartholdi and Schwab 1997; Ransohoff and Tani 1998). However, it remains unclear how the neutrophil chemoattractant chemokines are regulated after acute injury to the CNS.

Chemokines are a growing family of molecules that are fundamental to the directed recruitment of leucocytes and therefore represent attractive targets for study. Despite sharing structural homology, the chemokines can be divided conveniently into two principal groups based on the relative positions of the two amino terminal cysteines. The C-x-C family comprises those members in which the first two cysteines are separated by a single amino acid, whereas the C-C family comprises those members where the cysteine residues are adjacent (for review see Moser and Loetscher 2001). The C-x-C chemokines are further divided into two subfamilies: those that contain a receptor binding glutamate–leucine–arginine (ELR) domain and those that do not (Strieter *et al*. 1995). It is only the ELR C-x-C chemokines that show specificity for recruitment of neutrophils. In humans, the principal ELR chemokines, interleukin-8 (IL-8) and growth-regulated oncogene (GRO) proteins are secreted in response to injury and infection. The rat equivalent to the human GRO ELR C-x-C chemokines are the cytokine-induced neutrophil chemoattractant (CINC) chemokines. To date, four CINC chemokines have been identified and characterized: CINC-1, CINC-2α, CINC-2β and CINC-3. There are no reports of rat CINC expression in the spinal cord following injury. A previous study examined the expression of CINC-1 and CINC-3 in the brain following injection of IL-1β into the striatum at a single time-point by a semiquantitative method, but failed to identify any differences in the expression between adult and juvenile animals (Anthony *et al*. 1998). Subsequently, new rat ELR-chemokines have been identified and more sensitive discriminating techniques for examining mRNA and protein expression have become available. The aim of the present study was to examine the temporal pattern of CINC mRNA and protein expression in the brain and spinal cord following microinjection of IL-1β to determine whether differential neutrophil recruitment between CNS compartments and between adult and juvenile animals is regulated by chemokine expression.

Materials and methods

Animals

Twelve-week-old (> 250 g) adult or 3-week-old juvenile (40–50 g) male Wistar rats were obtained from Charles River (Kent, UK). All animals were housed under standard conditions, and fed with pelleted food and water *ad libitum*. In each experiment, at least three animals were used per group. Animal experiments were carried out with approval under UK Home Office Licence.

Reagents

Rat recombinant IL-1β was obtained from the National Institute for Biological Standards and Controls (NIBSC, Potters Bar, UK). All other reagents were obtained from Sigma (Poole, Dorset, UK) unless otherwise stated. IL-1β was dissolved in a solution of endotoxin-free saline vehicle.

Stereotaxic surgery

All surgical procedures were performed under an operating microscope Wild M650, Leitz (Leica UK Ltd, Milton Keynes, UK). The animals were anaesthetized using avertin (1.25% w/v 2,2,2-tribromoethanol; 1 mL/100 g). Stereotaxic surgery in the brain parenchyma was performed as previously described (Matyszak and Perry 1995). Briefly, anaesthetized rats were held in a stereotaxic frame and the skull exposed. A small hole was drilled in the skull and 100 Units (1 ng) of rat recombinant IL-1β or vehicle control administered in a volume of 1 μL, into the striatum through a finely drawn glass capillary according to the following co-ordinates: bregma + 1.2 mm, lateral + 3.0 mm and depth 4.5 mm for adult rats. Co-ordinates were scaled down as appropriate for juvenile rats. Stereotaxic spinal cord surgery was carried out as previously described (Schnell *et al*. 1999a). Following partial laminectomy at thoracic level (T8), a finely drawn calibrated glass capillary tube was inserted through the dura into the grey matter of the spinal cord (+ 0.4 mm laterally and to a depth of 1.4 mm). The injection of 1 μL of IL-1β (1 ng/μL) was performed through a glass capillary as described for intrastriatal injections. Animals were left to recover in a heated chamber and then killed by overdose of sodium pentobarbitone 2, 4 and 24 h post surgery as required.

Tissue collection

After saline perfusion, the brain was rapidly removed and carefully cleared of meninges, and both the injected and uninjected striata were immediately microdissected and removed. Spinal cord was dissected, cleared of meninges and the immediate area around the injection site was removed (± 0.4 cm). Tissue samples were immediately frozen in liquid nitrogen and stored at −70°C. For histology, tissue were frozen in Tissue-Tek (Miles Inc., Elkhart, IN, USA) in isopentane over liquid nitrogen in accord with standard procedures.

Identification of neutrophils

Frozen, 10-μm-thick serial sections were cut from the tissue blocks onto 3-aminopropyltrioxysilane (APS)-coated slides. Antigens were detected using a three-step indirect method (Hsu *et al*. 1981). Polymorphonuclear neutrophils were identified using the antineutrophil serum HB199, a gift from the CNS Inflammation Group (Anthony *et al*. 1998).

RNA extraction

Total RNA extraction was performed using the Qiagen RNeasy Extraction kit (Qiagen Ltd, Crawley, UK). RNA was treated with DNaseI according to manufacturer's instructions (Qiagen Ltd).

Table 1 Primers and probes

EMBL No.	Primer/probe	Sequence 5′-3′	Amplicon size (bp)
D11445	CINC-1/forward primer	ccaaaagatgctaaagggtgtcc	
	CINC-1/reverse primer	cagaagccagcgttcacca	
	CINC-1 probe	agaagatagattgcaccgatggcgtct	85 bp
D87926	CINC-2α/forward primer	caagctcccaggcttcagaa	
	CINC-2α/reverse primer	ggatcgctgctctgcttca	
	CINC-2α probe	tcttgtacggtgatggaggacctgcct	127 bp
D87928/9	CINC-2β/forward primer	cagaatggaagtgatagcagtgct	
	CINC-2β/reverse primer	agagtcctcacgcacgtagaatt	
	CINC-2β probe	agacgggaatgcaatttgtttgcatcc	77 bp
U45965	CINC-3/forward primer	ctgaacaaaggcaaggctaactg	
	CINC-3/reverse primer	ttgattctgcccgttgagg	
	CINC-3 probe	cctggaaaggaagaacatgggctcctg	71 bp

The Taqman probe consists of an oligonucleotide with a 5′-reporter dye 6-carboxyfluorescein (FAM) and a 3′-quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA). The sequences of the primers or probes for the rat CINCs, CINC-1 CINC-2α, CINC-2β and CINC-3, are shown in addition to their gene accession number and amplicon size.

Design and construction of primers/probe for real time

PCR Primers and probes for the CINCs were designed using the primer design software primer Express™ and published sequences (Table 1), and were synthesized by MWG-Biotech (UK) Ltd (Milton Keynes, UK) and Applied Biosystems (ABI) (Warrington, UK), respectively. PCR amplicons crossed an intron–exon boundary, making them cDNA-specific. Housekeeping rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control reagents were purchased from ABI. The specificity of the PCR primers was tested under normal PCR conditions in a standard thermocycler prior to quantitation. For each gene, a single DNA band of the predicted molecular size was observed following gel electrophoresis and was directly sequenced (data not shown). Primer pairs were also analysed in BLAST searches to ensure specificity for the chemokine of interest.

Production of single-species RNA for quantitation curves

PCR products for the CINC family were generated as described and analysed on a 2% agarose gel. Bands of interest were excised and gel extracted using QIAquick™ gel extraction kit (Qiagen Ltd). A T7 phage RNA polymerase promoter was ligated to the PCR product and a sense transcription template was generated by PCR using a T7 primer and the CINC 5′ gene-specific primer (see Table 1) using the Lig'n Scribe RNA polymerase promoter addition kit [AMS Biotechnology (Europe) Ltd, Cambridgeshire, UK] according to the manufacturer's instructions. Microgram quantities of single-species CINC 1, 2α, 2β or 3 RNA were then generated using the T7-MEGAshortscript *in vitro* transcription kit [AMS Biotechnology (Europe) Ltd] according to the manufacturer's instructions. Template DNA was removed by a 15-min incubation at 37°C with RNAse-free DNAse1 (2 Units). The RNA was then gel-purified on a 5% polyacrylamide gel containing 3 M urea to separate full-length transcripts from prematurely terminated transcription products as well as from unincorporated free nucleotides. RNA was eluted from the excised gel pieces, incubated at 50°C for 30 min in RNA diffusion buffer [0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA pH 8.0, 0.1% sodium dodecyl sulphate (SDS)], precipitated using 70% ethanol and resuspended in RNase-free water. The concentration of RNA in each sample was determined using the Ribogreen™ RNA quantitation kit (Molecular Probes, Cambridge Bioscience, Cambridge, UK). To determine the copy numbers of RNA-diluted transcripts, the molecular weight of each transcript was individually calculated from the following molecular weight conversion equation: M.W. = $(A_n \times 328.2) + (U_n \times 305.2) + (C_n \times 304.2) + (G_n \times 344.2) + 159$. Molecular weights were converted to copy numbers based upon Avogadro's number, i.e. 1 mol = 6.022×10^{23} molecules. Standard curves were produced by serial dilution of RNA within the range of 1 pg to 250 ag for CINC-2α (3.56–7.17 log molecules) and 200 fg to 250 ag for CINC-1 (3.72–6.62 log molecules), CINC-2β (3.47–6.37 log molecules) and CINC-3 (3.43–6.33 log molecules).

RT–PCR

Reverse transcription (RT) and PCR were carried out using the Gold RT–PCR kit (ABI) according to the manufacturer's specification. A two-step RT–PCR was performed. RT reactions used 200 ng total RNA in a total volume of 10 μL containing 1× Taqman RT buffer, 5.5 mM $MgCl_2$, 500 μM of each dNTP, 2.5 μM random hexamers, 0.4 U/μL RNAse inhibitor and Multiscribe Reverse Transcriptase. Taqman PCR was performed with 2.5 μL of RT products (50 ng total RNA), in a total volume of 25 μL of 1× Taqman mastermix (ABI) containing 100 nM forward and reverse primers and 100 nM probe. Thermocycling conditions were: 50°C for 2 min, 95°C for 10 min and run 40 cycles at 95°C for 15 s, 60°C for 1 min on the ABI PRISM 7700 Detection system (ABI). The number of mRNA copies of CINC were quantified against individual standard curves. The principles of Taqman PCR have been reported (Bustin 2000).

Normalization of Taqman PCR results

To normalize input RNA for each reaction, the housekeeping gene GAPDH was used (ABI). Results were expressed as number of copies of CINC per 1 ng input total RNA normalised to GAPDH analysed in each sample.

Protein extraction and assay

Brain or spinal cord was homogenized on ice in phosphate buffer [0.5 M NaCl; 2.5 mM NaH_2PO_4; 7.5 mM Na_2HPO_4; 0.1%

polyoxyethylene-sorbitan monolaurate (pH 7.3)] containing protease inhibitors (100 mM Amino-n-caproic acid, 10 mM Na_2 EDTA, 5 mM benzamidine, 90 mM AEBSF). Typically each dissected sample was homogenized in 600 μL of buffer. Homogenates were centrifuged (12 000 *g*) for 30 min at 4°C, and the supernatants removed and re-centrifuged for 60 min at 100 000 *g* in a Beckman TL-100 to remove any insoluble protein. Samples were assayed in duplicate using the Bio-Rad D_c protein assay against bovine serum albumin standards according to manufacturer's instructions (Bio-Rad Laboratories Ltd, Herts, UK). Samples were diluted to 0.5 μg/μL for use in ELISA.

ELISA

Reagents for the CINC-3 ELISA were obtained from R&D Systems (Abingdon, Oxon, UK) [mouse monoclonal coating antibody (2 μg/mL), goat polyclonal biotinylated antibody (2 μg/mL) and rat recombinant CINC-3 standard curve (2000–17.5 pg/mL)]. CINC-3 ELISA was performed according to manufacturer's instructions. An identical protocol was used for both CINC-2α and CINC-1 ELISA using the following reagents for CINC-1 [rabbit polyclonal anti-rat GRO/KC (Peprotech EC Ltd, London, UK) 1.2 μg/mL, goat polyclonal biotinylated anti-rat CINC-1 (R&D Systems; 0.5 μg/mL) and rat recombinant CINC-1 standard curve (R&D Systems) 2000–17.5 pg/mL] and CINC-2α [goat polyclonal anti-rat CINC-2α (Sigma; 0.5 μg/mL), goat polyclonal rat anti-CINC-2α biotinylated antibody (R&D Systems; 0.25 μg/mL) and rat recombinant CINC-2α standard curve (R&D Systems) 2000–17.5 pg/mL]. Diluent for standard curves was naïve brain homogenate (0.5 μg/μL). Results for ELISA are expressed as pg CINC/mg input total protein.

Statistical analysis

The data are presented as ± standard error of the means. Where statistical analysis has been employed data were analysed by ANOVA with Bonferroni–Dunn post-hoc tests. Results were considered statistically significant when the corresponding *p*-value was ≤ 0.0167 (0.05/No. of comparisons to eliminate type I errors).

Results

Chemokine mRNA expression is up-regulated to a higher level in the adult spinal cord than in the adult brain following IL-1β injection

To investigate whether the IL-1β-induced neutrophil recruitment that occurs in the spinal cord, but not in the brain, could be explained by differences in expression of the chemokines, we analysed the expression of the rat neutrophil chemoattractants (CINCs) by Taqman RT–PCR in response to injections of either vehicle or IL-1β in the striatum or spinal cord parenchyma (Fig. 1).

We found, in accord with previous studies (Anthony *et al*. 1998; Mennicken *et al*. 1999), that the expression of chemokines in the rat CNS is low in naïve animals. Surgery and the injection of vehicle into the CNS was associated with elevated CINC-1, CINC-2α and CINC-3 mRNA expression after 4 h in both the striatum and spinal cord parenchyma (Figs 1a–c). Of note is the general higher responsiveness of

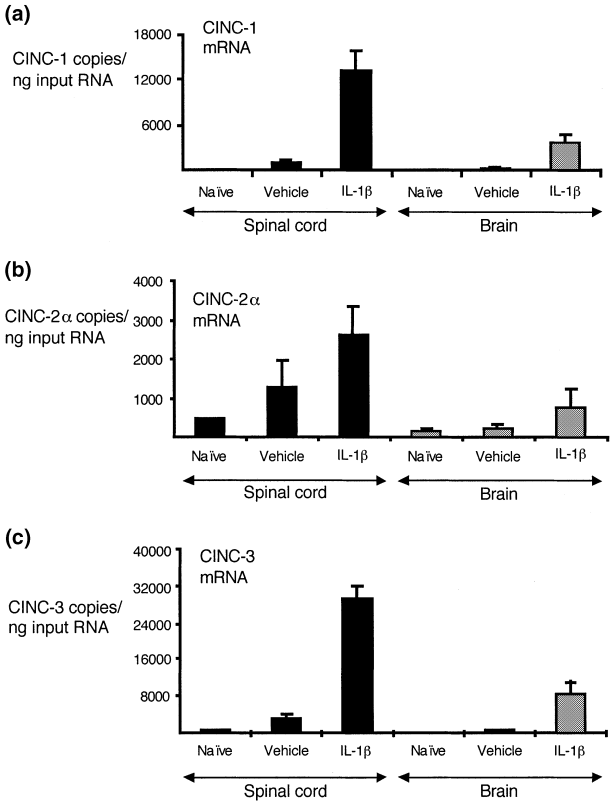

Fig. 1 Chemokine mRNA expression is up-regulated to a higher level in spinal cord than brain following IL-1β injection. Taqman PCR was used to quantitate the levels of CINC chemokines expression in the brain (grey bars) and spinal cord (black bars) 4 h following IL-1β or vehicle challenge. Note the higher mRNA expression levels of CINC-1 (a), CINC-2α (b) and CINC-3 (c) in the spinal cord compared to the brain parenchyma following either vehicle or IL-1β injection. CINC-3 is expressed at the highest level followed by CINC-1 and then CINC-2α in both the brain and spinal cord. Graphs illustrate number of CINC mRNA copies per ng input total RNA normalised to GAPDH. Values are shown as ± standard error of mean.

the spinal cord compared to the brain to equivalent insult resulting in more extensive chemokine elevations. Four hours following the injection of IL-1β into the spinal cord, a dramatic increase in CINC-1 mRNA was observed as compared to levels observed in vehicle-injected controls ($p = 0.0001$). In the striatum, CINC-1 mRNA levels were also increased in response to IL-1β injection as compared to levels observed in vehicle-injected controls, but this did not reach significance ($p = 0.06$). However, the absolute level of CINC-1 mRNA expression was higher in the spinal cord (13 267 ± 2605 copies/ng) than in the striatum (3683 ± 1076 copies/ng) in response to IL-1β ($p < 0.0001$).

Similar patterns were observed for CINC-2α (Fig. 1b) and CINC-3 (Fig. 1c) in response to injection of IL-1β into the CNS. Four hours after IL-1β injection, CINC-2α mRNA expression was further increased in the spinal cord

($p = 0.002$) and striatum ($p = 0.014$) as compared to levels observed in vehicle-injected controls. The response to IL-1β in the striatum (786 ± 253 copies/ng), however, was less than that in the spinal cord (2647 ± 416 copies/ng; $p = 0.0003$). Of all the CINC chemokines examined, CINC-3 showed the highest level of acute mRNA expression following IL-1β injection in both the spinal cord and striatum. A dramatic increase in CINC-3 mRNA (29 382 ± 2548 copies/ng) was observed in the spinal cord with respect to vehicle-injected animals ($p < 0.0001$). Smaller, but still significant elevations of CINC-3 mRNA expression were observed in the striatum (8363 ± 2614 copies/ng) following IL-1β injection as compared to vehicle injected controls ($p = 0.003$). The level of induction in the striatum was consistently lower than that observed in the spinal cord ($p < 0.0001$). The elevated CINC mRNA expression for each transcript was transient and levels had returned to control values by 24 h in both striatum and spinal cord (results not shown).

For CINC-2β the pattern was different: no CINC-2β mRNA expression was detected in either vehicle- or IL-1β-challenged striatum or spinal cord parenchyma. Furthermore, no increase in expression was observed in the uninjected hemispheres for any of the CINC mRNA species studied (results not shown).

Differential chemokine expression may explain the differences in neutrophil recruitment to the adult brain and the adult spinal cord in response to IL-1β

To determine whether altered levels of CINC mRNA expression were associated with changes to protein expression in the CNS, we measured the expression of CINC-1, CINC-2α and CINC-3 by ELISA following injection of vehicle or IL-1β into the striatum or spinal cord (Fig. 2). In agreement with the mRNA result, elevated expression of CINC-1 (Fig. 2a) was observed in the striatum and more so in the spinal cord 4 h following surgery and the injection of vehicle into the parenchyma. Four hours after the injection of IL-1β, a marked elevation of CINC-1 protein was observed in both the striatum ($p < 0.0001$) and spinal cord ($p < 0.0001$) as compared to the vehicle-injected controls. Absolute levels of CINC-1 were approximately threefold higher in the spinal cord (4772 ± 144 pg/mg) 4 h after IL-1β challenge than following the equivalent challenge in the striatum (1547 ± 122 pg/mg; $p < 0.0001$). In agreement with the profile of CINC-2α mRNA expression, the injection of vehicle into the spinal cord generated a higher responsiveness than an equivalent injection into the striatum. Four hours after the injection of IL-1β, CINC-2α protein was significantly up-regulated in the striatum ($p = 0.01$) and spinal cord ($p < 0.0001$) as compared to levels observed after the injection of vehicle (Fig. 2b). Absolute levels of CINC-2α were higher in the spinal cord (1632 ± 103 pg/mg) than in the striatum (548 ± 50 pg/mg; $p < 0.0001$). The induction of CINC-3 (Fig. 2c) following the injection of IL-1β in the CNS followed a similar profile, with elevation after 4 h in the striatum (95 ± 28 pg/mg; $p = 0.002$) and spinal cord (303 ± 9 pg/mg; $p < 0.0001$) as compared to levels observed in vehicle-injected controls. CINC-3 protein levels in the spinal cord following IL-1β challenge were threefold higher than in the striatum ($p < 0.0001$). It is of note that the absolute levels of CINC-2α and CINC-3 protein are disproportionate to the absolute level of mRNA.

To determine whether the increases in chemokine were associated with increased numbers of neutrophils into the parenchyma, we examined neutrophil numbers by immunohistochemistry 4 h following the injection of 1 ng IL-1β into the striatum or spinal cord. Florid neutrophil recruitment was observed in the spinal cord in the abluminal space of the ventro-medial vessels in response to IL-1β injection (Fig. 2d). By comparison, in the adult striatum 4 h after IL-1β challenge, no neutrophils were observed in the brain parenchyma adjacent to the injection site. However, neutrophil recruitment was observed in the adult brain, but this was restricted to the meninges (Fig. 2d).

Chemokine mRNA expression in adult and juvenile brain parenchyma are alike following IL-1β injection into the brain parenchyma

To investigate whether the differential expression of the CINC chemokines may be responsible for the differences in neutrophil recruitment observed between adult and juvenile animals, we injected IL-1β into the striatum of juvenile rats and compared the mRNA levels to those observed in adult animals (Fig. 3). We examined chemokine expression at 2 h, as well as at 4 h when the rate of neutrophil recruitment is maximal in juvenile animals (Anthony *et al*. 1997), to determine whether the temporal expression profile was altered in the juvenile animals.

In adult and juvenile rats, elevated levels of CINC-1 mRNA were observed at 2 h and more so at 4 h after the administration IL-1β compared to vehicle-treated animals (Fig. 3a). Surprisingly, there were no significant differences in CINC mRNA expression between the adult and juvenile striata. In fact, CINC-1 mRNA induction in the juvenile striatum was slightly (but not significantly) lower than in adult striatum at both 2 h ($p = 0.4$) and 4 h ($p = 0.2$). The pattern was similar for the other CINC mRNAs. Two hours after the injection of IL-1β, CINC-2α and CINC-3 mRNAs were induced in both adult and juvenile striata and were further elevated after 4 h as compared to vehicle-injected control levels. However, a trend towards greater elevation of CINC-2α (Fig. 3b) and CINC-3 (Fig. 3c) mRNA levels 4 h after IL-1β injection were observed in juvenile as compared to adult brain, but this did not reach significance. The profiles of CINC mRNA expression in response to IL-1β injection were similar in both adult and juvenile striata, with CINC-3 mRNA expressed at the highest levels, followed by CINC-1

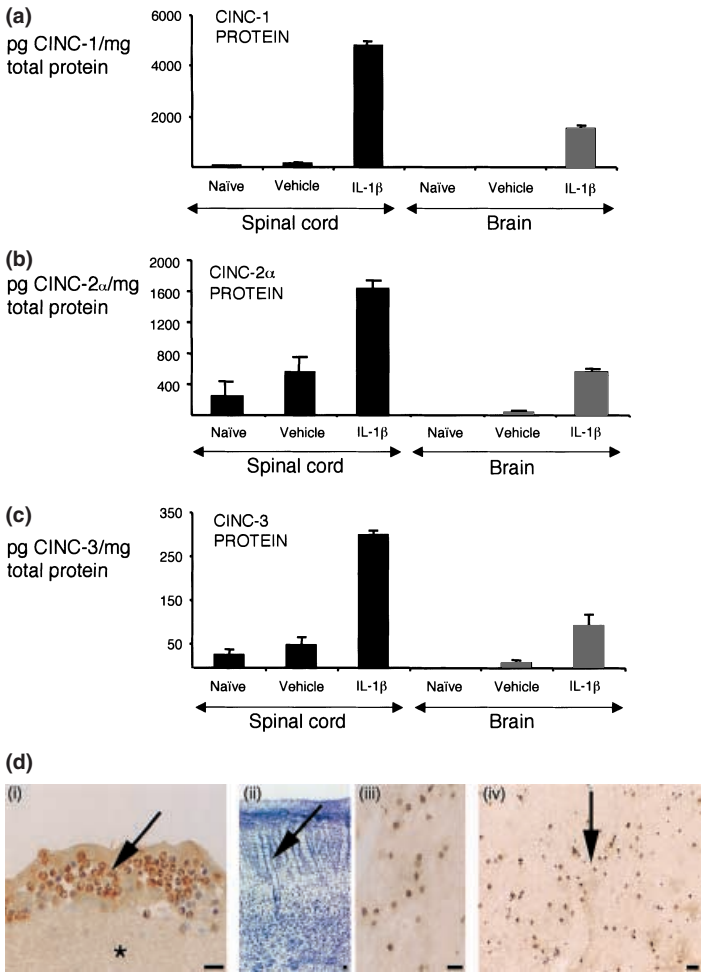

Fig. 2 Differential chemokine expression may explain the differences in neutrophil recruitment to the brain but not the spinal cord in response to IL-1β. Quantitative ELISA specific for CINC-1 (a), CINC-2α (b) or CINC-3 (c) was used to assay protein levels in rat brain or spinal cord 4 h following either surgery and the injection of vehicle or the injection of IL-1β (1 ng). Note that CINC-1, CINC-2α and CINC-3 proteins are elevated in both the spinal cord (black bars) and brain (grey bars) following 1 ng IL-1β injection, but that the chemokine response in the spinal cord is higher. ELISA results are expressed as pg of chemokine per mg total protein. Values are shown as ± standard error of mean. Neutrophils [d(i)], identified by immunohistochemistry with HB199, in the meninges (black arrow) 4 h after the injection of IL-1β (1 ng) into the adult brain. Note the absence of neutrophils in the brain parenchyma (*). Cresyl-violet-stained section [d(ii)] close to the injection site 4 h after the injection of IL-1β (1 ng) into the adult rat spinal cord. Black arrow indicates marked neutrophil recruitment to the abluminal space around the ventro-medial vessels. Neutrophil recruitment to the spinal cord parenchyma [d(iii)] as revealed by HB199 immunohistochemistry. HB199-stained neutrophils in juvenile brain parenchyma 4 h after the injection of IL-1β [1 ng; d(iv)]. A black arrow indicates the injection site. Scale bars represent 20 μM.

and then CINC-2α mRNA. No expression of CINC-2β mRNA was observed in either adult or juvenile rats at any time following IL-1β injection (results not shown).

Differential CINC protein expression may explain the differences in neutrophil recruitment in the developing rat in response to IL-1β

CINC-1, CINC-2α and CINC-3 protein levels were analysed in juvenile striatum 4 h after surgery and injection of either vehicle or IL-1β and compared to levels observed after the equivalent challenge in adult rats (Fig. 4). In agreement with the mRNA data, very low or undetectable amounts of CINC-1 protein (Fig. 4a) were observed in naïve juvenile rats or following surgery and injection of vehicle. There was significant elevation of CINC-1 protein following IL-1β challenge as compared to levels observed in vehicle-injected juvenile striatum ($p < 0.0001$). However, after this IL-1β challenge the CINC-1 observed (1533 ± 89 pg/mg) was not different to that observed in the adult animals (1546 ± 122 pg/mg; $p = 0.884$).

In agreement with CINC-2α mRNA data, injection of vehicle into the juvenile rat striatum was associated with

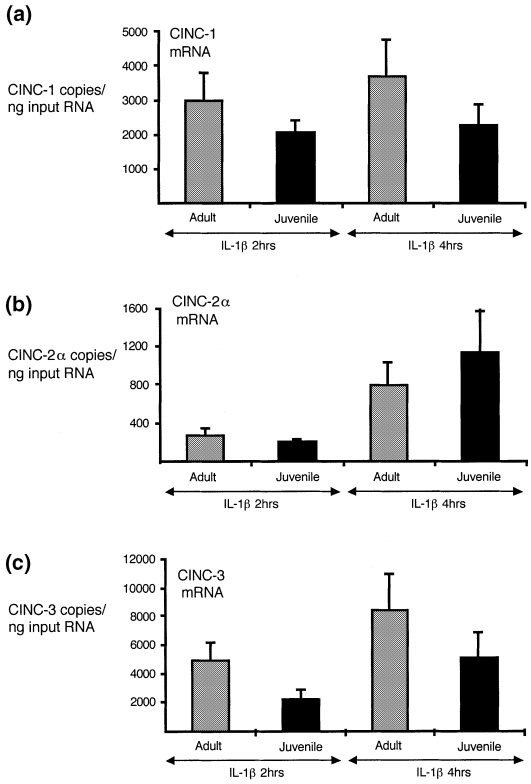

Fig. 3 Chemokine mRNA expression in adult and juvenile brain parenchyma are alike following IL-1β intrastriatal challenge. Taqman PCR was used to quantitate the levels of CINC chemokine expression in brain parenchyma 2 or 4 h following IL-1β intrastriatal injection in either adult (grey bars) or juvenile (black bars) rats. Note that levels of CINC-1 (a), CINC-2α (b) and CINC-3 (c) are not significantly different at either 2 or 4 h post-challenge. CINC-3 is expressed at the highest level followed by CINC-1 and then CINC-2α. Graphs illustrate number of CINC mRNA copies per ng input total RNA normalised to GAPDH. Values are shown as ± standard error of mean.

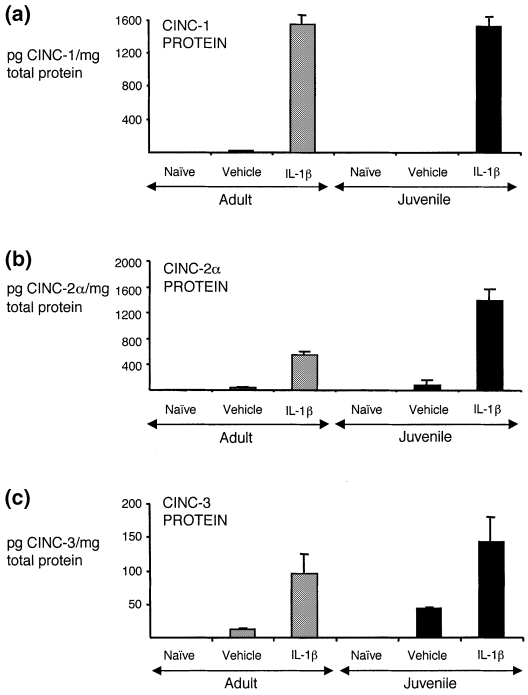

Fig. 4 Differential CINC expression cannot explain the differences in neutrophil recruitment in the developing rat in response to IL-1β. Quantitative ELISA specific for CINC-1 (a), CINC-2α (b), or CINC-3 (c) was used to assay protein levels in adult (grey bars) or juvenile rat (black bars) brain 4 h following either surgery and the injection of vehicle or the injection of IL-1β (1 ng). Note that CINC-2α proteins is elevated significantly more in the juvenile striatum compared to the adult striatum following 1 ng IL-1β. Results are expressed as pg of chemokine per mg total protein. Values are shown as ± standard error of mean.

small elevations of CINC-2α protein that were significantly elevated 4 h after the injection of IL-1β ($p < 0.0001$). However, in contrast to CINC-2α mRNA induction, which was only slightly elevated in response to IL-1β, analysis of CINC-2α protein revealed significantly higher levels in the juvenile striatum (1379 ± 188 pg/mg) as compared to levels observed in the adult striatum (548 ± 50 pg/mg) 4 h following an equivalent IL-1β injection ($p < 0.0001$).

A similar pattern was observed with CINC-3 (Fig. 4c) where the intrastriatal injection of vehicle in juvenile rats resulted in increased CINC-3 levels (44 ± 1 pg/mg) that were further elevated by challenge with IL-1β (142 ± 38 pg/mg). Elevation of CINC-3 in response to injection of IL-1β was higher in the juvenile striatum (142 ± 38 pg/mg) as compared to levels observed in the adult striatum (96 ± 28 pg/mg) following an equivalent challenge, but this did not reach statistical significance. As with adult brain, despite high levels of CINC-3 mRNA in the juveniles there were remarkably low levels of detectable CINC-3 protein.

Neutrophil recruitment to the adult and juvenile brain was measured by immunocytochemistry 4 h following the injection of IL-1β into the brain parenchyma. Following IL-1β injection, florid neutrophil recruitment was observed in juvenile brain that was not restricted to the meninges as was observed in the adult brain, but neutrophils were identified in the parenchyma, not only adjacent to the injection site but throughout the striatum [Figs 2d(i) and d(iv)]. The different levels of CINC-2α and CINC-3 chemokine expression in the adult and juvenile rat striata 4 h after IL-1β injection may be associated with the observed differences in neutrophil recruitment.

Discussion

Previous results from this laboratory have described a profound inflammatory response following delivery of the cytokine IL-1β to the spinal cord with florid neutrophil recruitment and blood–brain barrier (BBB) breakdown, which is not observed in the striatum following an equivalent challenge (Schnell *et al*. 1999a). However, the intracerebral

injection of IL-1β is sufficient to induce intense meningitis around the injected hemisphere with breakdown of the blood–cerebrospinal fluid barrier. In the striatum of a juvenile rodent, microinjection of 1 ng IL-1β results in neutrophil recruitment to both the brain parenchyma and the meninges with associated breakdown of the BBB (Anthony *et al*. 1997). These observations highlight the fact that the dose of IL-1β used (1 ng) is physiologically sufficient to initiate an acute inflammatory response and, as such, other factors must function to limit neutrophil recruitment in the adult brain parenchyma. The difference in the inflammatory response to IL-1β or TNFα in different CNS compartments and at different ages is not limited to cytokine-induced inflammation (Schnell *et al*. 1999a). Mechanical trauma to the spinal cord also exhibits a more florid inflammatory response than mechanical trauma to the brain (Schnell *et al*. 1999b). However, the inflammatory response to an intraparenchymal injection of excitotoxin is more pronounced in immature rodents when compared to adults (Lawson and Perry 1995; Bolton and Perry 1998). The results from these earlier studies suggest that different CNS compartments and rodents at different stages of development mount different inflammatory responses.

Previous studies have demonstrated that the most marked neutrophil recruitment and associated BBB breakdown observed in spinal cord was at 4 h after an IL-1β-injection (Schnell *et al*. 1999a). In this study, we confirm this result and compared the local mRNA expression of the neutrophil chemoattractants, the CINCs, at the IL-1β injection site in the striatum and spinal cord parenchyma. We found that CINC-1, CINC-2α and CINC-3 mRNA levels were up-regulated in both CNS compartments in a similar pattern although not at similar levels, with CINC-3 showing the highest expression levels, followed by CINC-1 and CINC-2α. CINC-2β mRNA was invariably undetectable in both the striatum and spinal cord. The absence of CINC-2β was not unexpected as other groups studying this isoform in a rat model of bronchopulmonary infection describe high levels of CINC-2α mRNA in the absence of CINC-2β expression, suggesting differential transcriptional regulation of the CINC-2 isoforms (Amano *et al*. 2000).

Chemokine mRNA and proteins are typically co-induced with no measurable delay before translation. There is, however, evidence for post-transcriptional control of chemokine expression (O'Hehir *et al*. 1996; Tran *et al*. 1996; von Luettichau *et al*. 1996). We investigated whether differential post-transcriptional regulation could account for the developmental and compartmental differences in neutrophil recruitment in the CNS using specific ELISAs. Although the pattern of CINC-1, CINC-2α and CINC-3 protein expression was similar to the pattern of mRNA induction, the absolute protein levels were markedly different. CINC-3 mRNA, which was induced to the highest level, gave rise to protein levels that were 20-fold lower than those produced by CINC-1 mRNA. It seems likely that CINC-1 and CINC-2α, rather than CINC-3 (as indicated by our mRNA data), are the principal C-x-C chemokines responsible for the acute recruitment of neutrophils to the spinal cord and brain.

In the spinal cord, we observed significantly higher levels of CINC mRNAs following an IL-1β challenge than we observed in adult or juvenile striatum. Indeed, the injection of vehicle alone induces some chemokine expression and, as Schnell *et al*. (1999a) report, the recruitment of neutrophils, which together illustrate the permissiveness of the spinal cord compared to the striatum. Therefore, the differential expression of the CINCs in the striatum and spinal cord may account for the differences observed in neutrophil recruitment between these CNS compartments. We found that CINC mRNA expression had declined to basal levels by 24 h, indicating that the response was transient. This finding is consistent with previous work in the spinal cord, which indicated that 24 h post-IL-1β injection there was reduced neutrophil infiltration, whilst monocytes continue to be recruited (Schnell *et al*. 1999a).

A key role of the CINC proteins as neutrophil chemoattractants in the CNS has been clearly demonstrated in studies where the intrinsic resistance of the brain to neutrophil recruitment has been overcome by administration of high doses of CINC proteins. Indeed, intrastriatal injection of 0.5 μg of recombinant CINC-1 (Anthony *et al*. 1998) or adenoviral vector expression of murine macrophage inflammatory protein-2 (MIP-2; Bell *et al*. 1996) is associated with neutrophil recruitment. Further, transgenic animals overexpressing CINC targeted to oligodendrocytes exhibited neutrophil selective recruitment to perivascular, meningeal and parenchymal sites of the brain and spinal cord (Tani *et al*. 1996). We have shown that, in response to administration of recombinant CINC-1 into the spinal cord, there is neutrophil recruitment, BBB breakdown and axonal damage (unpublished observations). Administration of neutralizing CINC-1 antisera following an ischaemic lesion or following the microinjection of IL-1β can eliminate the recruitment of neutrophils to the brain parenchyma (Yamasaki *et al*. 1997; Anthony *et al*. 1998), which highlights the importance of CINC in neutrophil recruitment. Despite these studies describing CINC-1 and CINC-3 proteins as key modulators of neutrophil recruitment in the brain we were unable to find any differences in the protein expression of these chemokines that would account for the differential neutrophil recruitment patterns observed between adult and juvenile animals. However, we did find that CINC-2α is produced in the striatum in response to IL-1β in juvenile animals at two and a half times the level than is found in the striatum of adult animals.

Previous investigations have been undertaken to look for differences in the pathway to neutrophil recruitment in the adult and juvenile CNS. Adhesion molecule expression (selectins and integrins) is observed at equivalent levels in adult and juvenile rodents after equivalent pro-inflammatory challenges (Bernardes-Silva *et al*. 2001). The differences in

permeability to neutrophils cannot be attributed simply to an immature BBB as a tight BBB is formed prior to parturition (Butt *et al*. 1990; Moldofsky 1994). CINC mRNA has been shown in this study to be effectively produced following intrastriatal injection of IL-1β. In accordance with others, we have shown CINC-1 (Anthony *et al*. 1998) and now CINC-3 to be translated into protein at similar levels in the adult and juvenile CNS. Thus the differential expression of CINC-2α is the only dissimilarity between adult and juvenile animals likely to contribute to the window of susceptibility phenomenon. However, this finding does not rule out a role for other contributory factors. The charge on vascular endothelial cells of the brain is important in regulating permeability and cellular traffic, and has been shown *in vitro* to be related to the age of cells (dos Santos *et al*. 1995); whether this age-related difference is also conserved *in vivo* is not known. Furthermore, the electrochemical charge on brain endothelium is higher than that observed on endothelium derived from the aorta (dos Santos *et al*. 1996), where florid neutrophil recruitment is observed following pro-inflammatory challenge. The appropriate presentation of chemokines on the luminal endothelial surface is also essential to attract passing leucocytes from the circulation (Andjelkovic *et al*. 1999a, 1999b). Differential chemokine presentation may be a factor in the differential neutrophil recruitment. Interactions between cell surface glycosaminoglycans (GAGs) and the chemokines drive the formation of haptotactic or immobilized gradients. The affinity of chemokine binding to GAGs has been observed to be dependent on the profile of GAG expression (Kuschert *et al*. 1999). The profile of GAGs in adult and juvenile rats and their effectiveness in producing haptotactic gradients is unknown. Interestingly, the ratio of the local to systemic chemokine levels has recently been demonstrated to be critical in determining the flow of neutrophils (Call *et al*. 2001), which represents another possible route of investigation. Whether the differences in neutrophil recruitment patterns can also be attributed, in part, to the mechanisms described above or whether a novel, developmentally regulated inhibitor may be present in adult animals requires further investigation.

Differences in expression of the neutrophil chemoattractants such have been described in this study, may, at least in part, explain the site-specific neutrophil recruitment patterns in defined CNS compartments and at different stages of development. These findings also have important implications for the treatment of spinal cord injury where the modulation of chemokine expression and thus neutrophil recruitment may minimize secondary neuronal death caused by inflammation.

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