

Cold pre-conditioning neuroprotection depends on TNF- α and is enhanced by blockade of interleukin-11

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Abstract

Cold pre-conditioning reduces subsequent brain injury in small animals but the underlying mechanisms remain undefined. As hypothermia triggers systemic macrophage tumor necrosis factor alpha (TNF- α) production and other neural pre-conditioning stimuli depend on this cytokine, we reasoned that microglia and TNF- α would be similarly involved with cold pre-conditioning neuroprotection. Also, as slice cultures closely approximate their *in vivo* counterpart and include quiescent microglia, we used rat hippocampal slice cultures to confirm this hypothesis. Furthermore, inflammatory cytokine gene screening with subsequent PCR and immunostaining confirmation of targeted mRNA and related protein changes showed that cold pre-conditioning triggered a significant rise in TNF- α that localized to microglia and a significant rise in

interleukin (IL)-11 that localized mainly to hippocampal pyramidal neurons and, more rarely, astrocytes. Importantly, co-stimulation with cold and IL-11, an anti-inflammatory cytokine that inhibits TNF- α expression, abrogated the otherwise evident protection. Instead, cold pre-conditioning coupled with blockade of IL-11 signaling further enhanced neuroprotection from that seen with cold pre-conditioning alone. Thus, physiological activation of brain pro-inflammatory cytokine signaling, and its amplification by inhibition of coincident anti-inflammatory cytokine signaling, may be opportune targets for the development of novel therapeutics that can mimic the protection seen in cold pre-conditioning.

Keywords: hippocampus, hormesis, innate immunity, interleukin-1 β , microglia, slice culture.

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Cold pre-conditioning effectively reduces brain injury in experimental animals (Nishio *et al.* 2000; Yunoki *et al.* 2002). However, no clinical pre-conditioning treatment strategies based on hypothermia have been developed to reduce neurological complications associated with general anesthesia and related surgical procedures (Moller *et al.* 1998; Bendszus and Stoll 2006; McKhann *et al.* 2009). This void likely results from the inherent difficulties in administering this form of therapy, which to date has only been applied after the onset of brain disease (for review see Schaller and Graf 2003; Tang and Yenari 2010). In addition, the underlying mechanisms of cold pre-conditioning are unknown. This precludes the development of effective cold pre-conditioning mimetics, although evidence suggests involvement of cytokines.

Fairchild *et al.* (2000) show that *in vitro* exposure of monocytes pre-activated by lipopolysaccharide to hypothermia triggers enhanced production of tumor necrosis factor alpha (TNF- α) and interleukin (IL)-1 β . Microglia, although perhaps not solely derived from monocytes (Simard and Rivest 2004; Chan *et al.* 2007), are a similar predominant

source of cytokines (Hanisch 2002) including TNF- α in uninjured brain (Hulse *et al.* 2008). Furthermore, microglia are activated by synaptic activity (Ziv *et al.* 2006), which may act as an adequate pre-activating stimulus for TNF- α production (Kraig *et al.* 2010) necessary for cold pre-conditioning to be effective. Second, a wide array of pre-conditioning stimuli evoke subsequent neuroprotection via mechanisms involving TNF- α and microglia (for review see Hallenbeck 2002; Kraig *et al.* 2010).

Accordingly, we examined brain cytokine signaling in cold pre-conditioning using hippocampal slice cultures from rats. Slice cultures are ideally suited to this purpose because, while deafferented, they are a mature and functionally intact

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Abbreviations used: IL, interleukin; TNF- α , tumor necrosis factor alpha; sTNFR1, soluble TNF receptor 1.

area of brain that remains viable and stable for weeks *in vitro*. Importantly, slice culture longevity allows microglia time to become quiescent after 10 days in culture, making the preparation ideal for study of neural immune signaling *in vitro*, where environmental conditions can be accurately controlled (Ransohoff and Perry 2008). Our results confirmed that cold pre-conditioning neuroprotection involved increased expression of TNF- α from microglia. Considerable evidence points to the involvement of TNF- α in an array of pre-conditioning paradigms. However, IL-11 inhibits TNF- α production. Accordingly, we also focused to the potential involvement of IL-11 in cold pre-conditioning. Our results provide the first evidence that removal of an anti-inflammatory cytokine, namely IL-11, enhances cold pre-conditioning protection. This work has appeared in preliminary form (Kraig *et al.* 2008; Mitchell *et al.* 2009).

Materials and methods

Culture preparation and maintenance

We prepared slice cultures and initially maintained them in media containing 23% horse serum (#26050-088; Invitrogen, Carlsbad, CA, USA; Kunkler and Kraig 1997) with transfer to serum-free media after 7 days *in vitro* and experimental use between 18 and 24 day *in vitro*. Cultures maintained in serum-free media showed ~90% vitality (Appendix S1).

Experimental manipulations

We administered cold pre-conditioning at several temperatures and over various time periods to establish dose–response patterns. Six-well trays containing serum-free media were allowed to equilibrate to hypothermic temperatures (25.5, 28, 30 and 32°C) for at least 20 min prior to cold pre-conditioning at these temperatures in an incubator (5% CO₂ balance air). Slice cultures were transferred from normal incubation conditions to cold pre-conditioning trays for 20, 40, 60, 90, 120, 150, or 180 min. Cultures were then transferred back to media equilibrated at normal incubation conditions for 24 h before excitotoxic injury (described below). With the neuroprotection response pattern established (Fig. 1), we performed all other cold pre-conditioning at 30°C for 90 min because this temperature showed the broadest effective range and 90 min was about mid-range.

Soluble TNF receptor 1 (sTNFR1, 200 ng/mL, #425-R1-050; R&D Systems, Minneapolis, MN, USA) was included during and after cold pre-conditioning to abrogate TNF- α signaling. Slice cultures were initially exposed to sTNFR1 20 min prior to cold pre-conditioning and continuously exposed to sTNFR1 up until excitotoxic injury.

Slice cultures were exposed to 100 ng/mL recombinant mouse IL-11 (#418-ML; R&D Systems) in serum-free media. To examine effects of cold pre-conditioning with blockade of IL-11, slice cultures were exposed to 100 μ g/mL anti-mouse IL-11 neutralizing antibody (#MAB418; R&D Systems). Use of an isotype-specific IgG2A (100 μ g/mL) antibody (#02-6200; Invitrogen) served as a sham control. All pharmacological treatments were applied 20 min prior to cold pre-conditioning, and were maintained up until administration of excitotoxic injury.

Excitotoxic injury and quantification

Twenty-four hours after cold pre-conditioning, slice cultures were exposed to NMDA (#454575; Calbiochem, San Diego, CA, USA) and assessment of resultant CA1 pyramidal neuron area injury were performed as previously described (Hulse *et al.* 2008) with modifications.

We used NMDA-mediated excitotoxicity to model CA1 area-selective neuronal vulnerability, including that seen from ischemia. Our previous work shows that NMDA-induced injury (20–50 μ mol/L for 60 min and normal incubation conditions) produces CA1 neuronal loss analogous to that seen from oxygen glucose deprivation (Hulse *et al.* 2008).

Injury severity was registered as a level of injury minus background (i.e. the pre-screen image) via an area of interest selected around the CA1 pyramidal cell layer area for each slice culture. This practice was in contrast to previous work (Hulse *et al.* 2008) where CA1 area injury was measured as a ratio of injury compared to maximal injury (i.e. created by high doses of or lengthy exposure to NMDA with background subtracted from each image). We often could not maximally injure cultures so that those pre-conditioned by cold had maximal levels of injury comparable to control cultures. This lapse could obscure otherwise present protective effects. For example, if control injury level was 5 and maximal control injury 10, the injury ratio would be 50%. If cold preconditioned injury was 3 and associated maximal injury 6, a ratio of 50% would again be evident in spite of a 40% protection (i.e. control level of 5 vs. cold-treatment level of 3) (Mitchell *et al.* 2010).

TNF- α assay

We analyzed slice cultures for TNF- α protein content as previously described using a microsphere-based flow cytometric immunoassay (Kunkler *et al.* 2004). Total protein was determined using a BCA protein assay kit (#23235; Pierce, Rockford, IL, USA).

Gene expression studies

Techniques for RNA isolation, quantitative PCR, cloning of cDNA for IL-11, and semi-quantitative quantitative PCR array screening to probe for low-level inflammatory mediator expression changes using the RT² Profiler PCR Array (#PARN-011A) from SABiosciences (Frederick, MD, USA) followed standard procedures detailed in Appendix S1 and Mitchell *et al.* (2010).

Immunohistochemistry

Slice cultures were fixed and processed for immunostaining as previously described (Kunkler *et al.* 2005) either as whole or 20 μ m sections (Mitchell *et al.* 2010) with details described in Appendix S1.

mRNA *in situ* hybridization

Procedures for cellular localization of TNF- α and IL-11 mRNA are detailed in Appendix S1.

Statistical methods and figure preparation

Data were analyzed using SIGMASTAT (v. 3.5) software (Systat Software, Chicago, IL, USA). All data were subject to normality testing (*p*-value to reject: 0.05) and equal variance testing (*p*-value to reject: 0.05). All control values per experiment were standardized

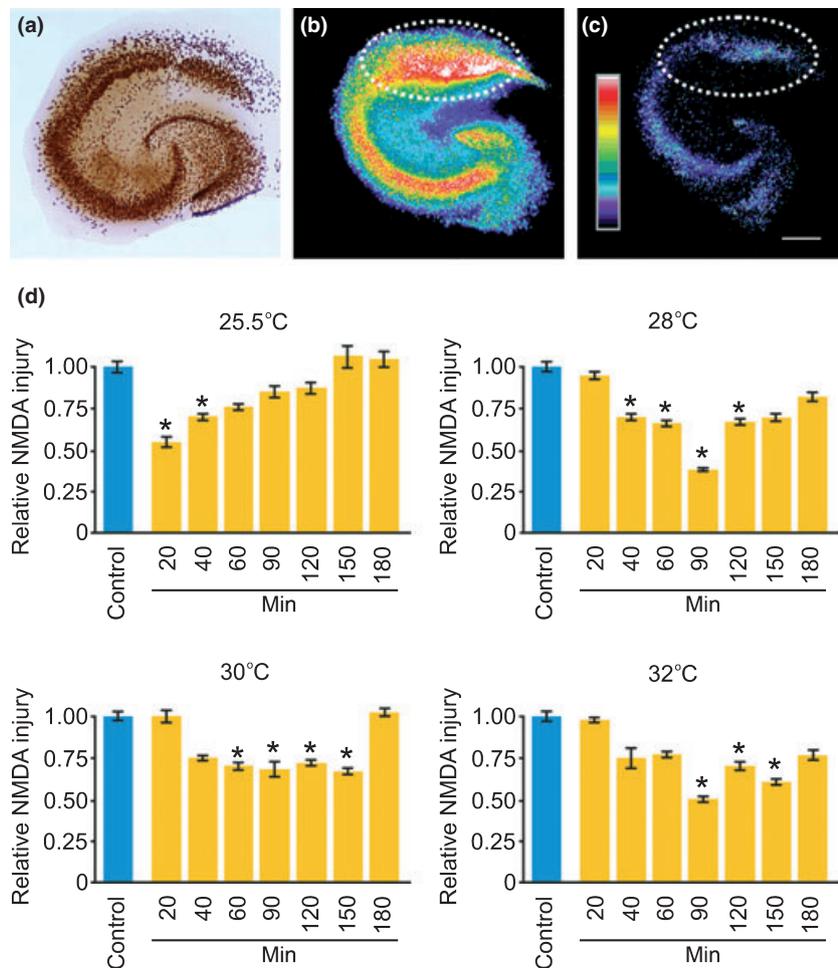


Fig. 1 Cold pre-conditioning was neuroprotective and followed a U-shaped temperature and time dose–response pattern. (a) Immunostaining for NeuN shows the typical principal neuron cytoarchitecture of a hippocampal slice culture with the CA1 pyramidal neuron area at the top, CA3 area to the left, and dentate gyrus to the lower right. (b–d) We used the fluorescent dead-cell marker Sytox to measure excitotoxic injury from NMDA exposure in the CA1 area (dotted white line area of interest) as a level of injury made relative to control injury cultures. Here, we show an exemplary image of control injury (b) and a significantly reduced level after cold pre-conditioning (25.5°C for 20 min) (c). Scale bar, 250 μ m. (d) Cold pre-conditioning produced significant neuroprotective effects that followed a U-shaped or hormetic dose–response pattern over a range of temperatures and durations. For example, cold pre-conditioning at 25.5°C evoked significant ($p = 0.002$) neuroprotection from 20 ($n = 8$) and 40 ($n = 39$)

min exposures, but not from 60 ($n = 7$), 90 ($n = 8$), 120 ($n = 4$), 150 ($n = 7$), or 180 ($n = 6$) min exposure times versus control ($n = 45$). Cold pre-conditioning at 28°C evoked significant ($p < 0.001$) protection from 40 ($n = 12$), 60 ($n = 13$), 90 ($n = 8$), and 120 ($n = 17$) min exposures, but not from 20 ($n = 7$), 150 ($n = 8$), or 180 ($n = 7$) min exposure times versus control ($n = 41$). Cold pre-conditioning at 30°C evoked significant ($p = 0.01$ at 120 min, all other times $p < 0.001$) protection from 60 ($n = 26$), 90 ($n = 13$), 120 ($n = 8$), and 150 ($n = 14$) min exposures, but not from 20 ($n = 6$), 40 ($n = 7$) or 180 ($n = 8$) min exposure times versus control ($n = 40$). Cold pre-conditioning at 32°C evoked significant ($p = 0.005$ at 120 min, all other times $p < 0.001$) protection from 90 ($n = 17$), 120 ($n = 8$), and 150 ($n = 7$) min exposures, but not from 20 ($n = 8$), 40 ($n = 8$), 60 ($n = 8$), or 180 ($n = 6$) min exposure times versus control ($n = 15$).

to 1.00 with related experimental values adjusted proportionally to allow for interexperimental analyses. ANOVA using Holm-Sidak *post hoc* testing was performed for multiple comparisons or *t*-test for comparisons. Significance was defined as $p < 0.05$.

Images were created using Coreldraw (v. X3) Photoshop (9.0.2; Adobe, San Jose, CA, USA). Confocal images were acquired using a Leica TCS SP2 AOBs laser scanning confocal microscope (University of Chicago Integrated Light Microscopy Core Facility).

Results

Characteristics of cold pre-conditioning neuroprotection

Cold pre-conditioning provided significant neuroprotection against excitotoxic injury (Fig. 1a–c). This neuroprotection followed a U-shaped response pattern for temperature and time (Fig. 1d). Other pre-conditioning stimuli involve TNF-

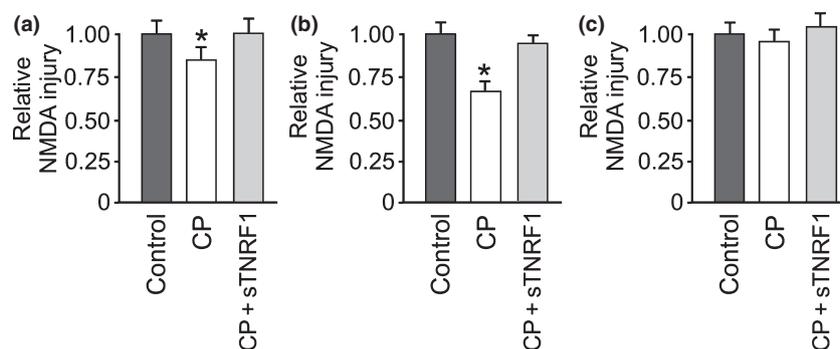


Fig. 2 Cold pre-conditioning neuroprotection depended on TNF- α and took time to develop. A, Soluble TNF- α receptor 1 (sTNFR1) blocked TNF- α signaling and abrogated the otherwise significant ($p = 0.009$) neuroprotection from cold pre-conditioning (CP). Cold pre-conditioning evoked neuroprotection at 30°C for 90 min, followed by NMDA-mediated CA1 excitotoxic injury 24 hours later ($n = 7$ –9/group). (b) Cold pre-conditioning neuroprotection ($n = 8$) remained significantly

($p = 0.003$) greater than control conditions ($n = 8$) 3 days after exposure to 30°C for 90 min ($n = 8$). Inclusion of sTNFR1 (CP + sTNFR1; $n = 8$) removed this neuroprotection. (c) Finally, cold pre-conditioning at 30°C for 90 min with only 20 min of recovery before excitotoxic injury from NMDA ($n = 8$) was not protective compared to control ($n = 8$). Furthermore, inclusion of sTNFR1 (CP + sTNFR1) had no impact ($n = 8$) on injury levels.

α , a pro-inflammatory cytokine (Hallenbeck 2002; Kraig *et al.* 2010), and hypothermia triggers prolonged release of TNF- α from macrophages (Fairchild *et al.* 2000). As microglia, resident macrophages of brain, are the principal physiological source of neural TNF- α , we reasoned that TNF- α may play a similar role in cold pre-conditioning in brain. Indeed, slice cultures exposed to 30°C for 90 min ($n = 4$) and then harvested 24 h later showed significantly ($p = 0.004$) increased expression of TNF- α compared with control ($n = 7$).

We next explored whether cold pre-conditioning neuroprotection depended on TNF- α . Inclusion of sTNFR1 20 min prior to cold pre-conditioning and continuously thereafter abrogated TNF- α signaling, which removed the significant degree of protection seen in cold pre-conditioning alone (Fig. 2a).

Furthermore, we modified our experimental timeline to investigate the immediate and delayed effects of cold pre-conditioning. Delayed effects of cold pre-conditioning were examined by performing cold pre-conditioning as described above, but with results quantified using injury levels recorded 3 days after excitotoxic injury. This neuroprotection remained evident 3 days after pre-conditioning and again was completely removed by abrogation of TNF- α signaling by sTNFR1 (Fig. 2b). However, cold pre-conditioning did not produce a protective effect when excitotoxic injury occurred shortly after pre-conditioning (Fig. 2c). These results show that cold pre-conditioning neuroprotection followed a U-shaped dose–response pattern over time and temperature, involved TNF- α , and took time to develop.

PCR array evidence of inflammatory mediator changes from cold pre-conditioning

Although not all mRNAs are translated to proteins (White *et al.* 1992), cytokines are regulated at the transcriptional

level and once transcribed to RNA are faithfully translated to protein (Oppenheim and Feldmann 2001). Therefore, targeted gene analyses via PCR arrays are an efficient means to probe for inflammatory mediator changes of cold pre-conditioning (Table 1). Furthermore, PCR arrays are recognized to be a highly sensitive, reproducible method for gene screening and were therefore used to begin defining cytokine signaling in cold pre-conditioning (Supporting Information).

Cold pre-conditioning increased the expression of a number of chemokines and cytokines. Interleukin-8 receptor expression increased, a chemokine effect that could enhance leukocyte movement (Iizasa and Matsushima 2001). Moreover, the expression of several chemokines that also enhance leukocyte movement (Rollins 2001) was increased, whereas a single chemokine measured (i.e. ligand 11) was reduced. In addition, cold pre-conditioning altered the expression of cytokines. Cold pre-conditioning triggered PCR array evident changes of increased pro-inflammatory innate cytokine mRNA expression for TNF- α , IL-1 β , and its receptor IL-1R1. The expression of secreted phosphoprotein 1 (i.e. osteopontin), a cytokine with both pro- and anti-inflammatory effects (Nau 2001), was decreased. However, we focused to IL-11, a cytokine whose expression was maximally (i.e. 2.95-fold) increased by cold pre-conditioning and is directly related to TNF- α homeostasis.

Abrogation of IL-11 signaling, and not IL-11, enhanced neuroprotection

IL-11 is an anti-inflammatory mediator whose expression is stimulated by TNF- α through IL-1 β , and IL-11 inhibits expression of TNF- α (Redlich *et al.* 1996; Trepicchio *et al.* 1996). We confirmed this signaling interrelation after cold pre-conditioning using real-time RT-PCR for TNF- α , IL-1 β , and IL-11 mRNA. Consistent with the PCR array data, probing for specific amplification of these cytokines con-

Table 1 Cold pre-conditioning regulates expression of inflammatory mediators

Ref Seq	Description	Symbol	$2^{-\Delta Ct}$		Fold up- or down-regulation CP/Control
			CP	Control	
NM_012675	Tumor necrosis factor (TNF superfamily, member 2)	Tnf	1.5E-02	5.8E-03	2.57
NM_031512	Interleukin 1 beta	Il1b	3.7E-03	2.4E-03	1.58
NM_013123	Interleukin 1 receptor, type I	Il1r1	7.5E-03	4.7E-03	1.58
NM_019310	Interleukin 8 receptor, alpha	Il8ra	2.8E-03	4.4E-03	-1.56
NM_017183	Interleukin 8 receptor, beta	Il8rb	1.5E-03	7.3E-04	2.08
NM_133519	Interleukin 11	Il11	4.6E-03	1.6E-03	2.95
NM_012881	Secreted phosphoprotein 1	Spp1	1.4E-01	2.6E-01	-1.92
XM_213425	Chemokine (C-C motif) ligand 12	Ccl12	1.0E-01	5.4E-02	1.95
NM_057151	Chemokine (C-C motif) ligand 17	Ccl17	1.2E-03	7.3E-04	1.58
NM_031530	Chemokine (C-C motif) ligand 2	Ccl2	2.6E-02	1.2E-02	2.23
NM_013025	Chemokine (C-C motif) ligand 3	Ccl3	9.1E-02	3.1E-02	2.95
NM_053858	Chemokine (C-C motif) ligand 4	Ccl4	5.2E-02	1.4E-02	3.63
NM_001004202	Chemokine (C-C motif) ligand 6	Ccl6	6.0E-02	3.5E-02	1.69
NM_001007612	Chemokine (C-C motif) ligand 7	Ccl7	3.0E-02	1.5E-02	1.95
NM_134455	Chemokine (C-X-C motif) ligand 1	Cxcl1	2.1E-02	1.3E-02	1.58
NM_182952	Chemokine (C-X-C motif) ligand 11	Cxcl11	2.6E-03	7.7E-03	-2.91
NM_053647	Chemokine (C-X-C motif) ligand 2	Cxcl2	1.7E-03	1.1E-03	1.58
NM_001007604	Ribosomal protein, large, P1	Rplp1	4.4E+00	3.9E+00	1.12
NM_012583	Hypoxanthine guanine phosphoribosyl transferase	Hprt	1.4E-01	1.4E-01	-1.03
NM_173340	Ribosomal protein L13A	Rpl13a	3.2E-01	3.0E-01	1.04
NM_017025	Lactate dehydrogenase A	Ldha	1.3E+00	1.3E+00	-1.03
NM_031144	Actin, beta	Actb	4.1E+00	4.5E+00	-1.10

Semi-quantitative PCR array analysis from control versus cold pre-conditioning CA1 area of hippocampal slices as detailed in Materials and methods. Fold-up is colored green (i.e. ≥ 1.5) and fold-down is colored red (i.e. ≤ 1.5).

firmed that cold pre-conditioning triggered a significant increase in TNF- α , IL-1 β and IL-11 mRNA compared with control conditions (Fig. 3). These PCR results further support the hypothesis that IL-11 may be involved with neuroprotection from cold pre-conditioning. However, when we applied IL-11 to slice cultures to mimic the rise from cold pre-conditioning, no significant protection was seen (Fig. 4a). Furthermore, treatment with IL-11 abrogated neuroprotection otherwise seen with cold pre-conditioning (Fig. 4b). Instead, blockade of IL-11 signaling, by use of a neutralizing antibody, more than doubled the protection from cold pre-conditioning (Fig. 4c).

Cellular origin of cold pre-conditioning cytokine signaling variables

To further detail the cold pre-conditioning cytokine signaling, we turned to identifying the cellular sources for the key cytokines involved: TNF- α and IL-11. Treatment with TNF- α , IL-1 β , or cold pre-conditioning triggered significantly greater IL-11 immunoreactivity in cells with pyramidal neuron morphology (Fig. 5). We confirmed with double-label immunohistochemistry that this increased IL-11 expression was mainly localized to pyramidal neurons (Fig. 6a-f)

and, less often, to astrocytes (Fig. 6g). Finally, we used *in situ* hybridization to further define the cellular origins of TNF- α and IL-11. Consistent with our prior work using laser dissection microscopy with slices examined under physiological conditions (Hulse *et al.* 2008), we show that TNF- α localized to microglia (Fig. 6h). Furthermore, IL-11 mRNA localized mainly to pyramidal neurons (Fig. 6i).

Discussion

This study showed that cold pre-conditioning evoked neuroprotection, which involved the pro-inflammatory cytokine TNF- α that emanated from microglia. Furthermore, the protection was dampened by coincident increased expression of IL-11, an anti-inflammatory cytokine predominantly localized to neurons, which if abrogated, significantly enhanced the protective effects of TNF- α .

Evidence points to the involvement of TNF- α in a wide range of stimuli capable of triggering brain pre-conditioning neuroprotection. When TNF- α is elevated to comparatively low levels over time by systemic stimuli applied to brain, this cytokine from the periphery serves as a pre-conditioning stimulus that initiates neuroprotection centrally (Hal-

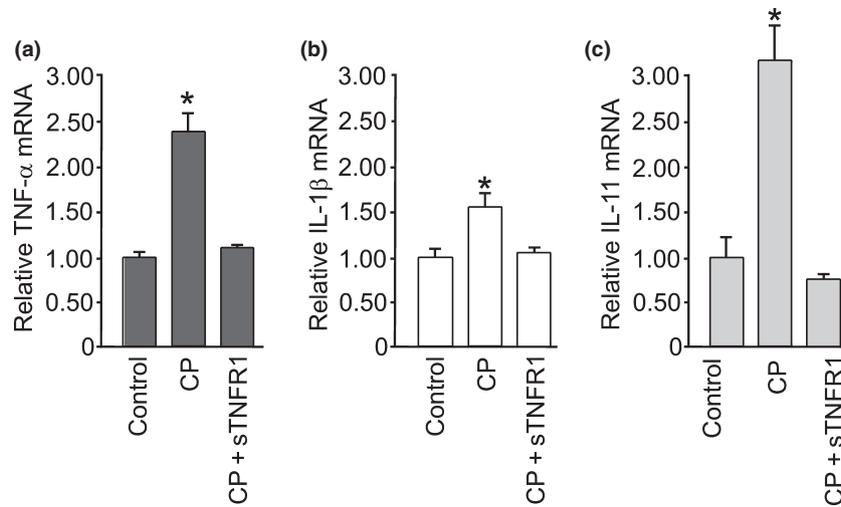


Fig. 3 Cytokine mRNA changes further suggest TNF- α involvement in cold pre-conditioning neuroprotective signaling. (a) Cold pre-conditioning (CP) at 30°C for 90 min with 2 h recovery triggered significantly ($p < 0.001$) increased relative TNF- α mRNA expression compared to control. Cold pre-conditioning plus blockade of TNF- α signaling by inclusion of sTNFR1 (CP + sTNFR1) returned relative TNF- α mRNA expression to a non-significant difference ($n = 3$ /group). (b) Cold pre-conditioning at 30°C for 90 min with 2 h recovery also triggered a significant ($p = 0.01$) increase in relative IL-1 β mRNA expression

compared to control and cold pre-conditioning. In addition, blockade of TNF- α signaling (CP + sTNFR1) returned IL-1 β mRNA expression to control levels ($n = 3$ /group). (c) Finally, cold pre-conditioning at 30°C for 90 min with 2 h recovery triggered significantly ($p = 0.002$) increased relative expression of IL-11 mRNA compared to control whereas cold pre-conditioning with blockade of TNF- α signaling (CP + sTNFR1) triggered a non-significant change in relative IL-11 mRNA expression compared to control ($n = 3$ per group).

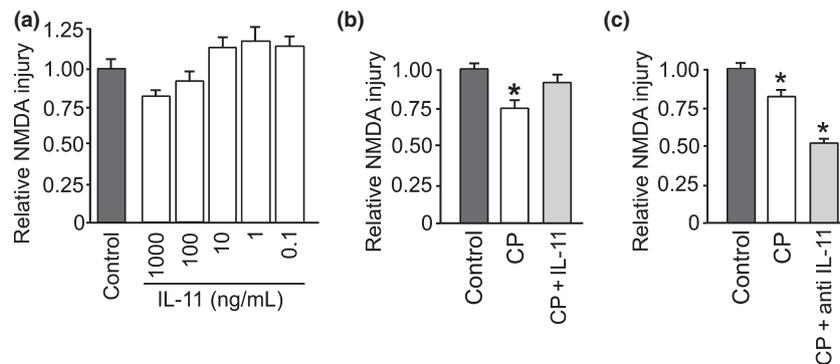


Fig. 4 Interleukin-11 counteracted TNF- α involvement in cold pre-conditioning neuroprotection. (a) Pre-conditioning with IL-11, an anti-inflammatory cytokine that inhibits TNF- α , at 1000 ($n = 4$), 100 ($n = 14$), 10 ($n = 16$), 1 ($n = 10$), and 0.1 ($n = 8$) ng/mL for 24 h did not significantly protect against subsequent NMDA injury versus control ($n = 16$). (b) Furthermore, cold pre-conditioning (CP; 30°C for 90 min) with IL-11 (CP + IL-11; 100 ng/mL; $n = 21$) abrogated the otherwise significant ($p = 0.002$) neuroprotection from CP ($n = 22$) versus control ($n = 24$). (c) In contrast, cold pre-conditioning plus blockade of IL-

11 signaling (CP + anti-IL-11; $n = 17$) by pre-treatment for 24 h with anti-IL-11 neutralizing antibody (100 μ g/mL) significantly ($p < 0.001$) enhanced neuroprotection from cold pre-conditioning alone ($n = 13$) with both conditions providing significant ($p < 0.001$) protection over control ($n = 25$). Finally, the specificity of this IL-11 blocking effect was confirmed by comparison to the effect of cold pre-conditioning plus an isotype, but otherwise non-specific, antibody (CP + IgG2A; 100 μ g/mL; $n = 9$), which did not significantly ($p = 0.401$) enhance neuroprotection from cold pre-conditioning alone.

lenbeck 2002; Dirnagl *et al.* 2009). Similar protective effects of TNF- α occur after stimuli from within brain that trigger TNF- α production. Increased synaptic activity from environmental enrichment as well as treadmill activity alone (Ding *et al.* 2005) triggers pre-conditioning neuroprotection and increased brain production of TNF- α (Kraig *et al.*

2010). For our purposes here, neuronal activity was a priming signal for cold-induced microglial TNF- α production analogous to the lipopolysaccharide used by Fairchild *et al.* (2000), which, when coupled to reduced temperature exposure resulted in enhanced TNF- α expression from monocytes.

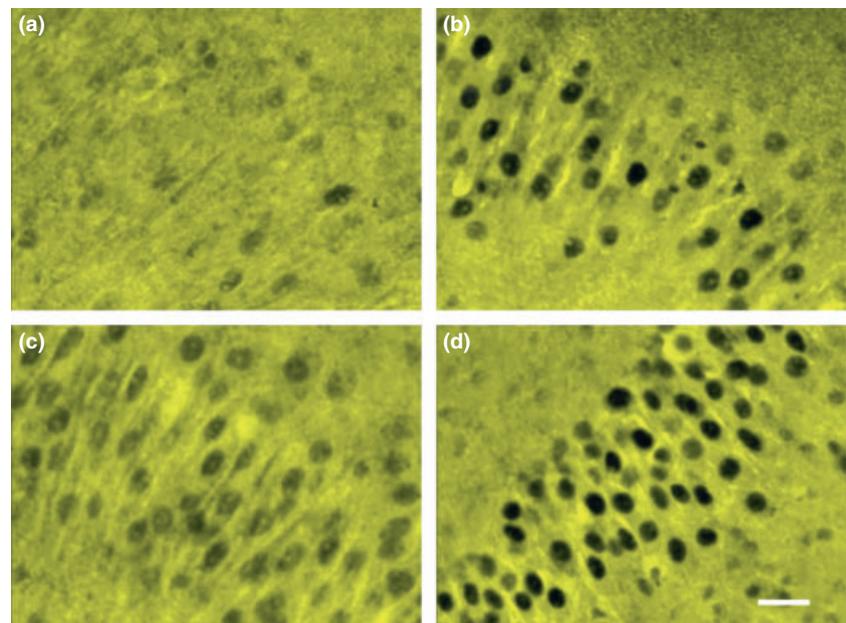


Fig. 5 Cold pre-conditioning and related effector cytokines increased hippocampal pyramidal layer interleukin-11 immunoreactivity. (a–d) We observed the most IL-11 immunoreactivity at the pyramidal cell layer in all groups. Here, we show faint and sparsely distributed IL-11 immunostaining under control conditions (a) that markedly increased 24 h after cold pre-conditioning CP (30°C for 90 min) (b), TNF- α exposure (TNF- α ; 100 ng/mL) (c), and IL-1 β exposure (IL-1 β ; 100 ng/mL) (d). (e) CP, TNF- α , and IL-1 β ($n = 9$ per group) significantly ($p < 0.001$) increased the number of IL-11-immunopositive cells at the pyramidal cell layer. Scale bar, 50 μ m.

Cold pre-conditioning results here provide further support for the ability of brain itself to employ pro-inflammatory functional and structural changes to trigger adaptive nutritive changes. First, we showed that cold pre-conditioning depended on TNF- α . Second, the cold induced TNF- α change came from activated microglia. Third, the protective effects of cold pre-conditioning were not immediately evident and the resultant neuroprotection showed a U-shaped dose–response pattern. These latter characteristics are consistent with physiological conditioning hormesis (Calabrese *et al.* 2007), a dose–response pattern seen with all neuroprotective agents reported to date (Calabrese 2008). Importantly, hormesis involves two basic tenets – namely, an initiating irritative (i.e. pro-inflammatory) stimulus must be sufficient to evoke a response and that sufficient time must elapse for a nutritive effect to take place (for review see Kraig *et al.* 2010). Here, the initiating, irritative stimulus that ultimately provides protection involves the pro-inflammatory cytokine TNF- α . Furthermore, our data show for the first time that reduced anti-inflammatory effects, that is, from IL-11, add to the initiating irritative impact of pro-inflammatory

changes needed for adaptive neuroprotection from cold pre-conditioning.

Anti-inflammatory effects play an important role in the mechanisms by which hypothermia, after the onset of disease, is protective (Schaller and Graf 2003; Tang and Yenari 2010). This likely helps explain why IL-11, an anti-inflammatory cytokine, is protective after the onset of disease. For example, treatment with IL-11 enhances survival and reduces TNF- α production after radiation-induced thoracic injury involving macrophages (Redlich *et al.* 1996). IL-11 also reduces oxidant-mediated injury of endothelial cells (Waxman *et al.* 2003) and developing lung cells (Chetty *et al.* 2008). IL-11 reduces ischemia/reperfusion injury in heart, protecting cardiac myocytes against oxidative damage (Kimura *et al.* 2007). Although less studied in brain, Zhang *et al.* (2006) show that astrocytes in white matter from patients with multiple sclerosis express IL-11 and this expression supports oligodendrocyte vitality.

In contrast, the current results show that IL-11 has no protective effect before the onset of disease. IL-11 did not improve cold pre-conditioning neuroprotection against

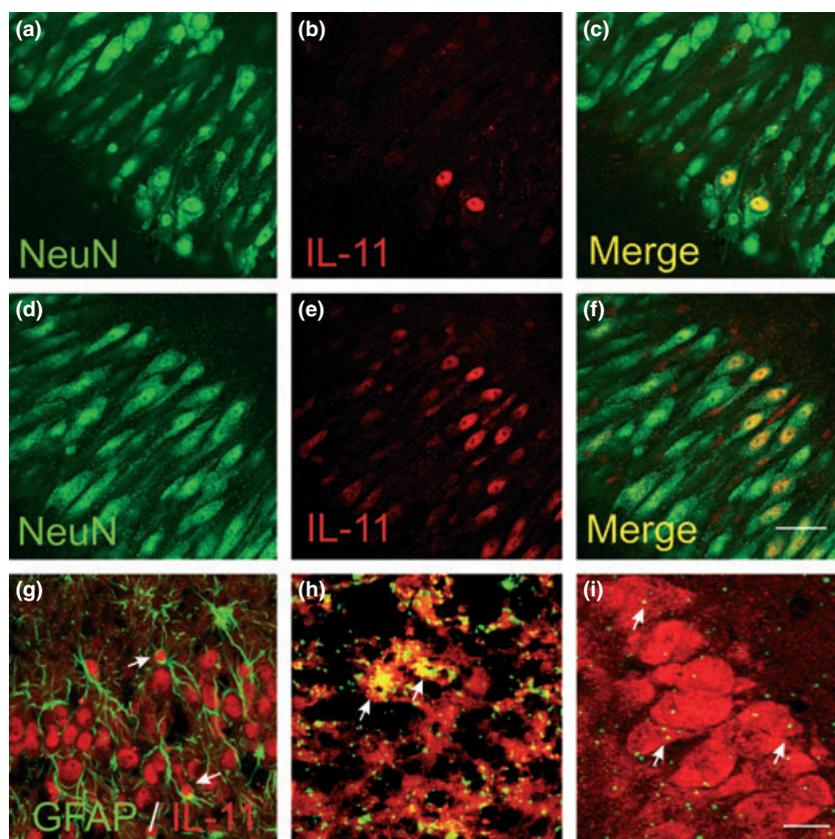


Fig. 6 Cellular localization of cold pre-conditioning $\text{TNF-}\alpha$ and IL-11 changes. (a–c) Control hippocampal slice culture immunostaining with neuronal marker NeuN (a) followed by IL-11 immunostaining (b) showed that the predominant cell type positive for IL-11 immunoreactivity were pyramidal neurons (c). (d–f) Exposure to $\text{TNF-}\alpha$ (100 ng/mL) for 24 h dramatically increased neuron-specific IL-11 immunostaining. Here, we show exemplary confocal photomicrographs ($n = 5$ per group). Scale bar, 50 μm . (g) Image shows double-labeling with glial fibrillary acidic protein (GFAP) and IL-11 immunostaining at the pyramidal cell layer from an exemplary hippocampal slice culture 24 h after cold pre-conditioning (30°C for 90 min). We observed IL-11-immunopositive astrocytes (arrows) in only a minority of photomicro-

graphs ($n = 5$). (h) Image shows that mRNA for $\text{TNF-}\alpha$ (green) 3 h after cold pre-conditioning (90 min at 30°C) localized to microglia, immunostained with CD11b (red). The resultant exemplary (from $n = 5$) image shows green spots overlying red cells or the optical conversion of these spots to yellow when both *in situ* and immunostaining probes are blended at the same plane of focus (arrows). (i) Twelve hours after analogous cold pre-conditioning ($n = 5$), mRNA for IL-11 (green) localized mainly to pyramidal neurons immunostained with NeuN (red). Arrows point to exemplary *in-focus in situ* and immunostaining probe markings. Although sparse, these probe markings for IL-11 mRNA concentrate to the pyramidal neuron layer. (g–i) Scale bar, 25 μm (g and h) and 50 μm (i).

subsequent NMDA-mediated excitotoxic injury. Furthermore, IL-11 abrogated the protective effects of cold pre-conditioning neuroprotection, further supporting the notion that a sufficient, initiating pro-inflammatory stimulus (i.e. $\text{TNF-}\alpha$) is needed for the protection. This suggestion was confirmed by blockade of IL-11 signaling, which significantly enhanced the protective effects of cold pre-conditioning. $\text{TNF-}\alpha$ stimulates production of IL-11 via IL-1 β and IL-11, in return, inhibits $\text{TNF-}\alpha$ expression (Redlich *et al.* 1996; Trepicchio *et al.* 1996), effects which are coincident and consistent with cytokine network behavior. We suggest IL-11 serves to mitigate the impact of $\text{TNF-}\alpha$ rise. Thus, blocking IL-11 signaling enhances the physiological impact of $\text{TNF-}\alpha$ to trigger neuroprotection before the onset of disease.

Although previous work shows IL-11 is expressed in astrocytes within white matter, our work shows that neurons predominantly express IL-11 in gray matter. As noted, IL-11 is expressed in astrocytes from myelinated borders of active and silent multiple sclerosis lesions (Zhang *et al.* 2006) and in primary astrocytic cultures (Bsibsi *et al.* 2006). Our work confirms and extends these findings: we found that astrocytes could express IL-11, but neurons were the principal source of this cytokine in gray matter. This neuronal localization is consistent with *in situ* hybridization work that shows IL-11 mRNA is distributed in the granular layer of the dentate gyrus and the pyramidal cell layers of the hippocampus (Du *et al.* 1996).

Taken together, our results suggest that physiological activation of brain pro-inflammatory cytokine signaling, and

its amplification by inhibition of coincident anti-inflammatory cytokine signaling, may be opportune targets for the development of novel therapeutics that can mimic the protection seen in cold pre-conditioning.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Appendix S1. Supplementary Materials and methods.

Table S1. Complete inflammatory gene expression array for response to cold pre-conditioning.

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