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The chemokine CXCL12 promotes survival of postmitotic neurons by regulating Rb protein

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Postmitotic neurons need to keep their cell cycle under control to survive and maintain a differentiated state. This study aims to test the hypothesis that the chemokine CXCL12 regulates neuronal survival and differentiation by promoting Rb function, as suggested by previous studies showing that CXCL12 protects neurons from apoptosis induced by Rb loss. To this end, the effect of CXCL12 on Rb expression and transcriptional activity and the role of Rb in CXCL12-induced neuronal survival were studied. CXCL12 increases Rb protein and RNA levels in rat cortical neurons. The chemokine also stimulates an exogenous Rb promoter expressed in these neurons and counteracts the inhibition of the Rb promoter induced by E2F1 overexpression. Furthermore CXCL12 stimulates Rb activity as a transcription repressor. The effects of CXCL12 are mediated by its specific receptor CXCR4, and do not require the presence of glia. Finally, shRNA studies show that Rb expression is crucial to the neuroprotective activity of CXCL12 as indicated by NMDA-neurotoxicity assays. These findings suggest that proper CXCR4 stimulation in the mature CNS can prevent impairment of the Rb-E2F pathway and support neuronal survival. This is important to maintain CNS integrity in physiological conditions and prevent neuronal injury and loss typical of many neurodegenerative and neuroinflammatory conditions.

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CXCL12 (SDF-1 α) and its main receptor, CXCR4, are expressed in the brain throughout life and involved in CNS development and neuroinflammatory responses.^{1,2} Stimulation of CXCR4 by its endogenous ligand leads to activation of intracellular pathways affecting neuronal survival, migration, and neurotransmission.^{1,3} For instance, CXCR4 stimulates the PI3K/Akt pathway and regulates cell-cycle proteins in neurons.^{3–6} Under pathological conditions at least some of these essential CXCR4 functions are compromised, leading to neuronal dysfunction/death.^{1,7} Thus, a complete understanding of the effects of CXCR4 activation in the brain has important physiological and pathological implications.

Rb is a well-known transcriptional repressor, which controls cell-cycle progression, differentiation/survival, and genomic integrity. Rb is also implicated in fundamental CNS developmental processes such as, neuronal migration, differentiation, and neurite extension.^{8,9} The effects of Rb on cell survival are primarily due to regulation of members of the E2F family of transcription factors, though Rb can also directly inactivate proapoptotic proteins, such as pp32.¹⁰ E2F proteins stimulate expression of genes promoting cell-cycle progression in proliferating cells, whereas they induce apoptotic genes in differentiated cells, including neurons.¹¹ Rb is phosphorylated at several phosphoacceptor sites by specific kinases.¹⁰ Phosphorylation inactivates Rb by disrupting its interaction with E2F transcription factors and chromatin modifying

enzymes, and results in the translocation of Rb from nucleus to cytosol where it gets degraded.¹² Hence, the immediate effect of Rb phosphorylation is to remove Rb from its specific promoters, leading to inhibition of Rb-dependent gene repression. Rb phosphorylation results in enhanced E2F1 transcriptional activity culminating in death of postmitotic neurons.¹¹ Alterations of Rb/E2F pathway were reported in various neuropathologies, such as Parkinson's and Alzheimer's diseases and HIV encephalitis.^{5,13,14} Our previous studies show that CXCL12 rescues neurons exposed to proapoptotic insults that cause Rb phosphorylation and its subsequent deficit (including HIV-1 gp120).4,5 These data raise the possibility that Rb may act downstream of CXCR4 and suggest that CXCL12 promotes neuronal survival and differentiation by supporting Rb function. To test these hypotheses, the mechanisms involved in control of neuronal Rb by CXCL12 and the role of Rb in the neuroprotective action of the chemokine were investigated. To this end, we studied the effect of CXCL12 on Rb expression and transcriptional activity in cultured rat neurons; we generated GFP-Rb fusion proteins to evaluate changes in subcellular localization of Rb induced by CXCL12; and we inhibited Rb expression by RNAi and determined its consequences on CXCL12 neuroprotective action. The data show that Rb is a major player in the effects of CXCL12 on postmitotic neurons.

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Abbreviations: CDK, cyclin dependent kinase; CHX, cycloheximide; GFP, green fluorescent protein; NLS, nuclear localization signal; NMDA, N-methyl-D-aspartic acid; P13K, phosphoinositide-3-kinase; Rb, retinoblastoma; RCE, retinoblastoma control element; RNAi, RNA interference; SDF-1, stromal cell-derived factor 1; shRNA, small hairpin RNA

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Figure 1 CXCL12 increases nuclear Rb levels in cortical neurons via CXCR4 stimulation. Cortical cultures were treated with CXCL12 (20 nM) for the indicated time before collecting neuronal extracts for immunoblots. In (a) equal amounts of protein (30 μ g per lane) were loaded for each fraction – that is cytosolic extracts (CE) and nuclear extracts (NE). NeuN and actin were used as nuclear and cytosolic markers, respectively. Densitometric analysis of three independent experiments is reported in the graph as mean ± S.E.M. of band density units (*P<0.05 *versus* control). The immunoblots on the bottom (b) are from additional studies with neurons treated with the specific CXCR4 antagonist, AMD 3100 (100 ng/ml), or the protein synthesis inhibitor, cycloheximide (CHX; 1 μ g/ml). The inhibitors were added to the culture before exposure to CXCL12 (20 nM); at the end of the treatment (5 h) total cell lysates were extracted and immunoblotted for Rb and actin (b)

Results

CXCL12 regulates Rb protein levels in neurons. Rb is involved in gene regulation and is mainly found in the cell nucleus. In line with previous reports, ^{4,15} Rb mostly localizes to the nuclei of untreated healthy cortical neurons, as also indicated by western blot studies with nuclear and cytosolic extracts (Figure 1a). Treatment of neuronal cultures with CXCL12 induces a time-dependent increase in Rb levels, which is evident in the nucleus, peaks at about 3h, and returns to basal levels after 20h (Figure 1a). Studies with total protein extracts (Figure 1b) show that the increase in

neuronal Rb triggered by CXCL12 is inhibited by the specific CXCR4 antagonist, AMD3100, and is prevented by treatment with cycloheximide (CHX; Figure 1b). These data suggest that CXCR4 activation may stimulate de novo synthesis of Rb protein in neurons. No significant changes in phospho-Rb levels were noted in these experiments (Figure 1b, upper band). This result, combined to the modest changes in cytosolic Rb protein (Figure 1a) would also indicate enhanced translocation of Rb to the nucleus of CXCL12-treated neurons. Interestingly, upregulation of Rb protein was also observed in undifferentiated and SH-SY5Y differentiated human neuronal cells (Supplementary Figure 1), which express functional CXCR4.¹⁶ Overall, these results show that stimulation of CXCR4 upregulates Rb protein levels in neuronal nuclei. These findings are in line with previous studies in cerebellar granule neurons showing that CXCL12 prevents the loss of Rb protein induced by deprivation of extracellular potassium,⁴ an in vitro model of apoptosis mimicking the absence of neuronal activity and growth factors.^{17,18}

To further study the effect of CXCL12 on Rb subcellular localization, GFP-Rb fusion proteins were generated (Figure 2a). These proteins were obtained either by cloning the full-length Rb or a truncated Rb (a.a. 1-602) lacking specific regions, in a GFP expression vector. As expression of the exogenous GFP-Rb protein is driven by a viral (CMV) promoter, changes in nuclear content of GFP-Rb induced by CXCL12 are to be ascribed to regulation of cellular import/ export mechanisms, rather than increased transcription allowing us to isolate the potential effect of CXCL12 on Rb translocation. As shown in Figure 2, GFP-Rb fusion proteins were expressed in rat cortical neurons, and localized to the nucleus (GRF-Rb 1-928) or cytosol (GFP-Rb 1-602) depending on the presence of the bipartite nuclear localization signal (NLS). Transfection of neurons with the truncated GFP-Rb construct (a.a. 1-602) lacking the pocket region and the NLS containing C-terminus, led to prominent expression of Rb outside the nucleus (Figure 2b, lower panels) - suggesting that delivery of the exogenous Rb to the nuclear compartment is regulated by the same mechanisms controlling the native protein. Expression of full-length GFP-Rb was observed in nuclei of live neurons for at least 7 days post transfection (Figure 2c), and found not to affect neuronal survival (not shown); this suggests that under these experimental conditions the exogenous protein does not interfere with the action of endogenous Rb. To test CXCL12 effects on Rb localization. HOS cells were transfected with either full-length GFP-Rb construct or truncated GFP-Rb (1-602) before treatment with CXCL12. HOS cells were used for these experiments as they homogenously express high levels of CXCR4 and uniformly respond to CXCL12 stimulation as opposed to cortical neurons, in which CXCR4 expression is more variable and restricted to subpopulations of cells.4,19,20 As reported in Figure 3, high levels of nuclear Rb are found in cells transfected with full-length GFP-Rb and treated with CXCL12 as compared to untreated cells (Figure 3, upper panels and graph). On the other hand, the truncated protein lacking the NLS (1-602) was exclusively expressed in the cytosol of both control and CXCL12-treated cells (Figure 3, lower panels). Taken together, these data suggest that the Rb protein



Figure 2 Generation of GFP-Rb fusion protein and their expression in cortical neurons. GFP-Rb fusion vectors were generated by cloning full-length Rb coding sequence as well as its truncations in a GFP expression vector at 3' of GFP coding sequence (**a**). Neurons were imaged 24 h after transfection with either the GFP-Rb 1–928 (**b**; upper panels), or the truncated Rb 1–602 lacking NLS (**b**; lower panels). A panel showing distribution of the GFP is included for comparison (large image in **b**). The neuronal marker, β -tubulin III (red) was used to identify the neuronal bodies and the nuclear dye Hoechst 33342 (blue) to visualize the nuclei (**b**). The micrograph in (**c**) shows a live neuron expressing full-length GFP-Rb in the nucleus imaged several days after transfection. Scale bar = 25 μ m

stimulated by CXCL12 accumulates into the nucleus by using the physiological mechanisms of nuclear translocation.

According to studies conducted on various cell types, Rb mainly exists in two phosphorylation states - a hypophosphorylated state where it is active and able to form specific transcription inhibitory complexes, and a hyperphosphorylated inactive state following Rb phosphorylation by cyclindependent kinases (e.g. cdk4/cdk6).¹⁰ As a result, Rb binding to inhibitory complexes is impaired and Rb-mediated transcriptional repression is lifted.¹⁰ Hyperphosphorylation of Rb is also involved in nuclear export and its cvtosolic degradation. In neurons treated with CXCL12, the levels of phosphorylated Rb (Ser780) are negligible and mostly localized to the cytosol, similarly to control neurons (Supplementary Figure 2). Moreover, protein levels of cdk4 do not change upon CXCL12 stimulation (Supplementary Figure 2). Thus, Rb should mostly be in its active form in CXCL12treated neurons. This is supported by previous studies showing that CXCL12 inhibits Rb phosphorylation on various residues induced by toxic stimuli,⁴ which is expected to facilitate its transfer to the nucleus.

CXCL12 stimulates Rb gene expression. The increase in Rb protein induced by CXCL12 (Figure 1) may be due to

enhanced Rb synthesis and/or inhibition of its degradation. To determine whether CXCL12 can stimulate Rb gene expression, neurons were transfected with a construct containing the Rb promoter cloned upstream of the firefly luciferase gene. This Rb promoter contains a specific region that binds multiple transcription factors, including E2F1. Twenty-four hours after transfection, neurons were treated with CXCL12 (Figure 4a). Luciferase activity assays show that the chemokine stimulated the Rb promoter in neurons (Figure 4a). Recent in vivo studies have shown that E2F1 represses the exogenously expressed Rb promoter in the brain,²¹ emphasizing the presence of a feedback regulatory loop in the Rb-E2F In line with these recent data, pathway. Rb promoter transcriptional activity was drastically reduced in cortical neurons transfected with an E2F1 expression vector (Figure 4b). When neurons (cotransfected with both E2F1 expression vector and Rb promoter construct) were treated with CXCL12, the inhibitory effect of E2F1 on Rb promoter was significantly diminished (Figure 4b) - resulting in upregulated Rb promoter activity as compared to untreated neurons. This shows that CXCL12 signaling counteracts E2F1 action on Rb, ultimately promoting Rb expression.





Figure 3 Effect of CXCL12 on Rb in HOS cells. HOS cells were transfected with either full-length GFP-Rb, or truncated GFP-Rb (1–602) expression vectors, treated with CXCL12 (20 nM, 3 h), fixed, and imaged. CXCL12 enhances expression of full-length GFP-Rb in the nucleus (top panel). The graph shows average pixel intensity from the nuclei of control and CXCL12-treated cells (n = 11 in each group; mean ± S.E.M.). GFP-Rb (1–602) remains in the cytosol of all cells analyzed, that is w/ or w/o CXCL12 (lower panel). Cells were stained with phalloidin (red) and Hoechst 33342 (blue). Scale bar = 25 μ m

To confirm that CXCL12 upregulates expression of the endogenous *Rb* gene, reverse transcription PCR (RT-PCR) was used to determine the effect of CXCL12 on Rb RNA. To reduce sample-to-sample variability and better evaluate differences in relative gene expression, amplification of Rb and the housekeeping gene (aldolase A) was performed in the same test tube, containing total cDNA and primers for both Rb and aldolase. As shown in the Figure 4c, CXCL12 treatment stimulates Rb RNA expression in neurons. This was confirmed by quantitative real-time PCR (qPCR) showing a twofold increase in Rb transcripts in CXCL12-treated neurons (Figure 4c, left graph). This effect of CXCL12 was maintained in neurons that overexpressed E2F1 (Figure 4c, right graph). These findings support the hypothesis that CXCL12/CXCR4 may act as regulator of endogenous Rb in neurons.

CXCL12 enhances Rb-mediated gene repression activity. Rb, along with its nuclear partners, acts as a transcriptional suppressor at specific promoter sites. Here, we determined the gene suppression activity of endogenous Rb by transfecting neurons with a luciferase-based construct

Cell Death and Differentiation

(Rb-TA-Luc) that contains a *cis*-acting element conferring repression to Rb in the promoter region. This sequence is present in the AP-1 binding site of human *fos* promoter, where Rb is known to exert its transcriptional suppression.²² Rb is able to recruit the Sp1 family of transcription factors (Sp1, Sp3) that bind to this sequence and repress promoter activity.²³ When transfected neurons were treated with CXCL12, the transcriptional activity of Rb-TA-Luc was significantly reduced (Figure 5a) indicating that CXCL12-induced Rb is transcriptionally active.

Rb silencing inhibits the effect of CXCL12 on neuronal survival. Neurons were transfected with a specific shRNA expression vector against rat Rb, psi(Rb). This shRNA has been previously shown to suppress Rb expression in neurons,²⁴ which was confirmed by our studies in both neurons and astrocytes (Figure 5b; Supplementary Figure 3). To study the role of CXCL12-induced Rb stimulation in neuronal survival, we conducted NMDA neurotoxicity assays (Figure 5c). Two sets of neurons, that is after transfection with psi(Rb) or a scrambled 'inactive' construct, were transiently (20 min) exposed to NMDA in the presence or absence of CXCL12. Neuronal survival was evaluated the next day, as we previously described. In agreement with previous studies, CXCL12 protects control neurons (i.e. transfected with scrambled sequence) from NMDA-induced death whereas the neuroprotective action of CXCL12 was abolished in neurons expressing the psi(Rb) (Figure 5c).

Though Rb and pRb were slightly increased in cytosol of NMDA-treated neurons, no significant changes of Rb/pRb were observed at nuclear level (Figures 6a and b). Overall, neuronal levels of Rb were unaffected by NMDA up to 5 h from treatment (Figure 6c; total cell extracts), indicating that the changes observed in the cytosol of NMDA-treated neurons are not associated with general Rb loss. This is in line with the ability of NMDA to induce neurotoxicity in psi(Rb) neurons. Importantly, in presence of NMDA, CXCL12 still upregulated Rb (Figures 6b and c) as expected given the survival assays results (Figure 5c).

Although Rb function was essential to CXCL12 neuroprotection against NMDA excitotoxicity, Rb depletion does not seem to affect the basal neuronal survival (Figure 5c). However, in Rb-depleted neurons treated with CXCL12 there was significant neurotoxicity as compared to the Rb-depleted controls alone, and this raises some very interesting possibilities. As CXCL12 is also neurotoxic under certain culture conditions, it can be argued that such neurotoxic effect could be attributable to the inactivation/inactivity of Rb. Taken together, the data presented here show that CXCL12mediated neuronal survival requires transcriptionally active Rb; otherwise the chemokine may act as a neurotoxic agent. These findings show that Rb is primarily involved in the neuroprotective action of CXCL12.

Discussion

This is the first report that Rb upregulation is essential to the neuroprotective action of CXCL12. Our findings show that CXCR4/CXCL12 can control Rb at transcriptional and post-translational levels, is involved in major feedback mechanisms

Role of Rb in neuronal CXCL12 function MZ Khan et al



Figure 4 CXCL12 stimulates the *Rb* promoter in neurons. Neurons were treated with CXCL12 (20 mM, 5 h), 24 h after transfection with a luciferase construct containing the human *Rb* promoter (**a**). Data from three different experiments (each run in triplicate) are reported as mean \pm S.E.M. of RLU (**P* < 0.05 *versus* control). In (**b**), neurons were transfected either with the *Rb* promoter construct alone or with both the *Rb* promoter construct and a GFP-E2F1 expression vector and then treated with CXCL12 as reported above (mean \pm S.E.M.; **P* < 0.05 *versus* control; *n* = 3). The panels in (**c**) report results from RT-PCR (top, *n* = 2) and qPCR (bottom, *n* = 4) studies performed on control neurons and neurons treated with CXCL12 (20 nM, 3 h). The qPCR graphs show relative changes in Rb expression normalized to the housekeeping gene GAPDH in the absence or presence of exogenous E2F1 (**P* < 0.01 *versus* control)

of Rb homeostasis, and can inhibit E2F1 and its downstream apoptotic effectors. The Rb increase induced by CXCL12 is due to enhanced expression of neuronal Rb gene, which results in induction of functionally active Rb. Moreover, CXCL12 neutralizes the transcriptional repression of E2F1 on the Rb promoter. Rb promoter contains a specific cluster of binding sites consisting of 26 base pairs lying 180 base pairs upstream of translation start site.²⁵ This region includes binding sites for E2F1. Sp1. Ets. and accounts for most of human/rodent Rb promoter activity in vitro.21 Recent studies have highlighted the role of E2F binding sites in vivo in the CNS.²¹ The E2F1 binding site is a repressor site.²⁶ Indeed, overexpression of E2F1 leads to transcriptional repression of the Rb promoter in neurons, which is counteracted by CXCL12. This suggests that CXCR4 activation can be part of the physiological feedback mechanisms regulating the Rb-E2F1 pathway, and is in agreement with previous evidence of CXCL12 neuroprotection against different apoptotic insults, including those involving E2F1 stimulation, such as HIV-1 proteins.3,5 However, CXCL12 might be a more general regulator of Rb, as indicated by experiments involving the retinoblastoma control element (RCE) sequence (Figure 5a).

This specific sequence includes about 30 base pairs derived from the human c-fos promoter (within AP-1 binding site).²² Rb confers transcriptional repression to it upon binding with transcription factors of the Sp1 family (Sp1, Sp3).²³ Similar RCE sequences were found in *c-myc*, *TGF-\beta1*, Fos-31, junB, and recently in CTa promoters and are linked to Rb-mediated transcriptional control of those promoters.²⁷ Though further investigation is necessary to understand the mechanisms involved, the ability of CXCL12 to reduce the activity of this promoter (along with the above E2F1 studies) shows that CXCR4 stimulation leads to expression of functionally active Rb in neurons. This is in line with CXCL12 effects on nuclear Rb level and its phosphorylation status. Underphosphorylated Rb translocates into the nucleus via specific processes involving the interaction of a bipartite NLS sequence in the Rb C-terminus with importin- α/β .²⁸ In line with this, Rb is mainly present in the nucleus of untreated, healthy neurons, whereas its phosphorylated form is preferentially found in the cytosol. Upon CXCL12 treatment, nuclear Rb is upregulated, but the levels of phosphorylated Rb (Ser780) are not increased. Indeed, CXCL12 may actually reduce Rb phosphorylation under certain experimental conditions.⁴







Figure 5 CXCL12 induces the expression of a transcriptionally active Rb that regulates its prosurvival effect in neurons. Neurons were transfected with the Rb-TA-Luc plasmid and treated with CXCL12 (20 nM; 24 h post transfection) for the indicated time (a). Data are shown as mean ± S.E.M. (*P<0.05 versus control; n = 3). Images in (b) are from neurons transfected with the Rb shRNA vector, psi(Rb), and a GFP expression vector, fixed after 24 h and immunostained for Rb (red); Hoechst 33342 (blue) was used to stain neuronal nuclei. Arrows point to an example of transfected neuron identified by GFP. Immunoblots from neurons transfected with psi(Rb) or a control vector carrying a scrambled sequence are also shown in (b). The western blot shows three separate psi(Rb) transfections in the same gel. The densitometric analysis from five independent experiments is reported in the graph (mean ± S.E.M.; *P<0.05 versus scrambled). The graph in (c) shows survival data from cortical neurons transfected with either psi(Rb) or a control vector carrying scrambled sequence (along with a GFP expression vector as transfection marker). After 24 h post transfection, the neurons were treated with NMDA (100 µM) with or without CXCL12 (20 nM); data are expressed as mean ± S.E.M. from three independent experiments containing at least three coverslips per experiment (*P < 0.01 versus control; only this is shown in graph for clarity purposes). Multiple comparison analyses showed that both scrambled and psi(Rb) NMDA are different than controls (P<0.01); the control scrambled and control psi(Rb) are not different; within the scrambled group, CXCL12 and CLCL12 + NMDA are not different than control, but different than NMDA alone (P<0.01); within the psi(Rb) group the CXCL12 and CXCL12 + NMDA are different than control (P<0.05) and not different than NMDA alone; the scrambled NMDA + CXCL12 is different than the psi(Rb) NMDA + CXCL12 and the scrambled CXCL12 is different than the psi(Rb) CXCL12 (P<0.05). Scale bars = 50 μ m

+

+ +

scrambled

+

+

+ psi(Rb) +

+

0 NMDA

CXCL12 -



Figure 6 Effect of NMDA and CXCL12 on Rb/pRb. Cytosolic and nuclear extracts (**a**, **b**) or total cell extracts (**c**) were prepared from neurons treated with NMDA (100 μ M) and/or CXCL12 (20 nM) as previously described. Neurons were exposed to NMDA for 20 min and collected at the indicated intervals (0.5–5 h) post treatment. CXCL12 was added 10 min before NMDA. Equal amount of proteins (30 μ g) were loaded in each lane; gels were blotted for total Rb, pRb (Ser 807/811), actin, and NeuN

Furthermore, in cerebellar neurons levels of phosho (Ser795)-Rb do not increase after CXCL12 stimulation, and phosphorylated Rb protein is only expressed in the cytosol.⁴ Serine 780 and 795 (along with Ser807/811) are important phosphorylation sites on the Rb C-terminus that are targeted by cyclin D-CDK4/6.¹⁰ Phosphorylation of such sites disrupts Rb interaction with transcription factors and binding of chromatin-modifying proteins with its LXCXE domain.10 Phosphorylation of Rb by cdk4 leads to the export of Rb from the nucleus to the cytosol via the exportin 1 pathway.¹² Hence, only the transcriptionally active Rb remains in the nucleus (as in CXCR4-stimulated neurons) whereas the Rb species rendered inactive by phosphorylation is shuttled to the cytosol. In line with this, previous studies showed that CXCL12 prevents Rb phosphorylation and neuronal loss under apoptotic conditions that destabilize Rb.⁴ Interestingly, studies with other chemokines activating different chemokine receptors that are dispensable for neuronal survival in vivo, suggest that these effects on Rb are restricted to CXCL12.29

The most intriguing result of this study is the discovery of Rb as a mediator of CXCL12-induced neuroprotection, which underlines the relevance of the CXCL12/CXCR4 pair in CNS physiology and pathology. However, although the role of this chemokine/receptor pair in development is relatively well established, there is still much controversy regarding their effects on mature neurons as both prosurvival and apoptotic roles of CXCL12/CXCR4 were reported. This apparent discrepancy can be explained by coupling of CXCR4 to different intracellular pathways depending on the activating ligand (i.e. CXCL12 versus viral proteins), or by the action of matrix-metalloproteases that cleave four residues from the CXCL12 N-terminal, converting it into a neurotoxic molecules.7,20,30 This proteolytic product of CXCL12 poorly binds CXCR4 and signals through a different receptor (CXCR3). Notably, in both circumstances the normal activation of CXCR4 signaling is altered, suggesting that integrity of pathways promoting Rb function is an important factor in the outcome of CXCR4 stimulation - also suggested by CXCL12 toxicity in Rb-depleted neurons (Figure 5c). Indeed, CXCR4

activation by HIVgp120 (which, unlike CXCL12, induces Rb phosphorylation) leads to stimulation of E2F apoptotic pathways in neurons.^{4,5} This would explain why Rb silencing impairs the ability of CXCL12 to protect neurons from excitotoxicity without altering basal neuronal survival.

Excitotoxicity is composed of rapid and delayed cell death. Immediate neurotoxicity primarily results from altered ionic homeostasis and subsequent cell swelling, and it heavily depends on ATP. Complex intracellular mechanisms are involved in delayed cell death, which would likely occur in neurons with higher ATP levels that have 'survived' the initial toxicity. These events include activation of calcium-dependent enzymes and production of reactive oxygen species that regulate downstream pathways leading to DNA damage, cleavage of structural proteins, and cell death.³¹ Though several in vivo/in vitro studies showed that CDK inhibitors prevent neuronal apoptosis induced by DNA damage or excitotoxic stress,^{31,32} the extent to which cell-cycle proteins are involved in excitotoxicity seems to depend on the insult's strength, oxidative stress, and resulting DNA damage as well as on neurons ability to overcome the acute stress.31,33 Furthermore, although certain cell-cycle components are essential to DNA repair and neuronal survival, entry into S phase and DNA replication are thought to be lethal for postmitotic neurons. 32,33 Thus, in vivo, Rb stimulation by CXCL12 could counteract the effect of toxic insults that trigger reentry of neurons into cell cycle, such as oxidative stress and DNA damage, which are the underlying cause of neuropathologies associated to excitotoxicity.34,35 However, in the present study Rb impairment (and maybe cell cycle reentry) is not essential to NMDA toxicity as the effect of NMDA is not altered by Rb silencing. The studies presented in Figure 5 indicate that NMDA still causes neuronal death in Rb-deficient neurons. Thus, Rb loss is not the cause of NMDA neurotoxicity in this case; this allowed us to investigate the effect of Rb depletion on CXCL12-induced neuroprotection independently of NMDA and it is in line with previous studies.³¹ These authors tested the effect of NMDA receptor antagonist MK801 and CDK inhibitor flavopiridol on neuronal death caused by mitochondrial toxin 3-nitroproprionic acid (3-NP), which irreversibly inhibits succinate dehydrogenase leading to loss of ATP synthesis, membrane depolarization, and activation of NMDA receptors. They showed that neurons rescued by MK-801 undergo delayed apoptosis, which can be inhibited by flavopiridol. Thus, both MK801 and flavopiridol are required to fully protect neurons from 3-NP. Though flavopiridol is not a specific CDK inhibitor, the data suggest that CDKs are involved in delayed death after block of NMDA receptors and that the component of cell death sensitive to MK801 is insensitive to flavopiridol.³¹ Additional studies are necessary to determine the precise role of cell-cycle proteins in NMDA neurotoxicity and clearly establish how other survival/death pathways intersect with CXCL12-induced Rb regulation.

An aspect deserving further consideration is the extent to which glia cells contribute to activation of different neuronal responses. Glia can alter neuronal function and signaling both directly (by releasing cytokines that interact with neurons) and/or indirectly (by regulating neurotransmitters levels and promoting neuronal differentiation). Either of these general mechanisms can modulate neuronal responses to CXCL12. Studies in our lab aim to evaluate whether glia contribute to the changes in neuronal Rb evoked by CXCL12. However, the experiments in differentiated SH-SY5Y neuronal cells indicate that CXCL12 is able to upregulate Rb levels independently of glia.

The major conclusion of this study remains that CXCL12/ CXCR4 play a crucial role in the modulation of Rb function and neuronal survival. The results suggest that CXCL12 promotes Rb function by increasing its expression, nuclear localization, and transcriptional activity in postmitotic neurons, thus protecting from cell death. Based on these data and our previous findings, we conclude that CXCL12 is an important regulator of the CDK/Rb/E2F pathway in neurons. Such a connection might lie at the basis of fundamental physiological roles of CXCR4 in the CNS, but is particularly relevant to neuropathologies involving cell-cycle proteins alterations, 5,14 such as Alzheimer's and Parkinson's diseases and neuro AIDS. Indeed, cell-cycle regulation in postmitotic neurons is essential to neuronal survival and differentiation, and failure of cell-cycle control seems to be a common cause of neurodegeneration.¹³ Thus, promoting these effects of CXCR4 may limit neuronal damage under these pathological conditions.

Materials and Methods

Cell cultures. Cortical neurons were obtained from the brains of 17- to 18-dayold rat embryos and cultured in a serum-free medium using the bilaminar cell culture system (originally described by Banker and Cowan³⁶ and modified as in Khan et al.³⁷), in which pure neuronal cultures are grown in the presence of a separate glial feeder layer supporting their growth and differentiation. This model reproduces, to a certain extent, the in vivo environment (i.e. the presence of neuronal and non neuronal cells) and offers the major advantage that neurons can be separated from the glia at any time in order to analyze neuron-specific biological responses. We have extensively used the bilaminar culture system to study expression and function of chemokine receptors in neurons, including CXCR4, and previously demonstrated that the signaling pathways induced by direct activation of neuronal CXCR4 promote neuronal survival mechanisms, even in the absence of glia. $^{\rm 3,4,19,37}$ In these studies, unless otherwise noted, CXCL12 treatments were performed in the presence of glia in order to determine the potential outcome of CXCL12 stimulation in vivo. Neurons were always separated from the glia immediately before protein or nucleic acid extraction or before performing the other assays, as indicated. Cortical neurons were plated on poly-L-lysine-coated 15 mm coverslips (35 000 cells) or 60 mm dishes (1 \times 10⁶ cells) – depending on the type of experiment. Treatments started after at least 7 days in vitro (DIV). When indicated, the CXCR4 antagonist, AMD3100 (Sigma-Aldrich, St. Louis, MO, USA), was added to the culture medium 15 min before CXCL12. Human osteosarcoma cells (HOS; CXCR4⁺) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and 1 µg/ml puromycin. Cells were serum starved before experimental treatments. HOS cells were obtained through the AIDS Research and Reference Program, Division of AIDS, NIAID, NIH (from Dr. Nathaniel Landau) as indicate previously.^{20,38} SH-SY5Y human neuroblastoma cells (obtained from ATCC) were cultured as previously described.19,16 Cells were serum-starved before experimental treatments.

GFP-Rb fusion proteins. The Rb coding sequence was excised from plasmid pRb-BS (gift of Dr. Giordano, Temple University) by digestion with restriction enzymes *BamHI/Sall*. All restriction enzymes described in this section and the Klenow fragments of DNA polymerase were from New England Biolabs (lpswich, MA, USA). The digestion product corresponding to the Rb gene was isolated from agarose gel, purified with Qiaex II kit (Qiagen, Valencia, CA, USA), following the manufacturer protocol, and ligated to the pEGFP-C1 vector (Clontech, Mountain View, CA, USA), previously digested with restriction enzymes *BglI/Sall*, using the TaKaRa DNA ligation solution I (version 2). The resulting construct contains the Rb gene fused to the 3' end of the GFP coding sequence; this product is referred to as GFP-Rb, and numbers following GFP-Rb in the recombinant plasmids described below indicate the Rb amino-acid fragments expressed by each

1671

construct. Truncations of the GFP-Rb chimera were generated by enzymatically removing DNA sequences between restriction sites located in the Rb sequence. GFP-Rb 1–602 was obtained by digesting the GFP-Rb plasmid with enzymes *Pst*l and *Sma*l, the 3' overhangs left by *Pst*l activity were removed by Klenow treatment to form blunt ends, and the resulting fragment was self-ligated after purification from agarose gel. Cells were transfected with up to 2 μ g per coverslip of GFP-Rb plasmids using the calcium phosphate method (efficiency of transfection in HOS cells was approximately 45%) or LipofectamineTM 2000 (Invitrogen, Carlsbad, CA, USA). Transfection efficiency in the latter case was 60–70% in HOS cells and about 35% in primary neurons.

Immunocytochemistry. Neurons were fixed in 4% paraformaldehyde. For neuronal marker staining, anti- β tubulin III (clone TUJ1, 1:500, from Covance, Berkeley, CA, USA) was used, followed by a cy3-conjugated secondary antibody (Figure 2b). For Rb staining, anti-Rb (clone G3-245, 1:50, BD Biosciences PharMingen, San Jose, CA, USA) was followed by a cy3-conjugated secondary antibody (Figure 5b). Nuclear staining was obtained using Hoechst 33342 (0.5 μ l/ml). Actin was stained using red cy3-phalloidin (Figure 3). Cells were observed under an epifluorescent microscope (Olympus IX70, Melville, NY, USA) connected to a CCD camera (Micromax, Trenton, NJ, USA), and images were taken using Metamorph software (Molecular Devices, Sunnyvale, CA, USA).

Western blots. For cytosolic and nuclear protein fractions, cells were washed with ice-cold balanced salt solution and were scraped in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 2 mM MgCl₂, 0.5 mM dithiothreitol, 1 mM AEBSF, 5 µg/ml aprotinin, 5 µg/ml pepstatin A, and 5 µg/ml leupeptin) containing 0.1% IGEPAL-CA-630 detergent for 15 min on ice, vortexed vigorously for 15 s, and centrifuged at 14000 r.p.m. for 1 min. The supernatant was collected and kept as cytosolic fraction. The pelleted nuclei were resuspended in buffer B (20 mM HEPES, pH 7.9, 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM AEBSF, 5 μ g/ml aprotinin, and 5 μ g/ml leupeptin). After 45 min on ice, lysates were centrifuged at 14 000 r.p.m. for 10 min. For total cell lysates, cells were scraped in lysis buffer (25 mM Tris, 150 mM NaCl, 5 mM NaF, 1 mM EDTA, 1 mM DTT, 1% IGEPAL-CA-630, 5 μg each of aprotinin, leupeptin, and pepstatin, 1 mM AEBSF, and 1 mM vanadate) as reported previously.^{19,37,39} The protein concentration of cell lysates was determined using the bicinchonic acid assay from Pierce (Rockford, IL, USA). Equal amounts of proteins (30 µg) were loaded in each lane, separated by SDS-PAGE and transferred to PVDF membranes for immunoblotting. The following primary antibodies were used: anti-Rb (1:4000, G3-245; BD Biosciences PharMingen), anti-Phospho Rb, Ser 780 (1:2000; Cell Signaling Technology, Danvers, MA, USA), and Ser 807/811 (1:1000; Cell Signaling Technology), anti-cdk4 (1: 1000, C-22; Santa Cruz Biotech., Santa Cruz, CA, USA), anti-NeuN (1:1000, MAB377; Chemicon, Temecula, CA, USA), and anti-Actin (1:5000, A2066; Sigma-Aldrich). The last two antibodies were used as specific markers for nuclear and cytoplasmic protein fractions, respectively. Bands were detected by chemiluminescence using the Pierce SuperSignal West Femto maximum sensitivity kit, according to the manufacture's instructions, and analyzed using the FluorChem 8900 apparatus from Alpha Innotech (San Leandro, CA, USA).

Reverse transcription-PCR. Total RNA was extracted using the RNEASY[®] mini kit (Qiagen). Random hexamers (Roche Applied Science, Indianapolis, IN, USA) were used to synthesize cDNAs. The following primers were used to amplify rat Rb: 5'-CTGGAATTCCCATTAATGGTTCACCT-3' and 5'-GCGGAATTCGCAAACTTCTCTTTAAAGAT-3'. For rat aldolase A, the following primers were used: 5'AACCAATGGCGAGACCACTAC-3' and 5'-AATTTCAGGC TCCACAATGG-3'. PCR was performed as follows: 4 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 61°C, and 60 s at 72°C.

Quantitative real-time PCR. Total RNA was isolated from control or CXCL12-treated primary rat neurons and cDNA was synthesized as mentioned above. The rat-specific primers for qPCR were as follows: Rb forward, 5'-GAAG CAGACGGGAGTAAACATCTC-3'; Rb reverse, 5'-CCTTGTTTGAGATTTCCATG CTATC-3'; GAPDH forward, 5'-CCTGCCAAGTATGATGACATCAA-3'; GAPDH reverse, 5'-AGCCCAGGATGCCCTTTAGT-3'. The amplification was performed with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on ABI PRISM 7300 Sequence Detection System (Applied Biosystems). The thermal cycle profile for all transcripts was as follows: the reaction was initiated at 95°C for 15 min. A total number of 40 PCR cycles were performed with 30 s of denaturing at

95°C, then 30 s at 55°C for annealing and 30 s at 72°C for extension. Following amplification, a melting curve analysis of the amplified DNA was done. A standard curve for each gene was generated to determine the linear range and amplification efficiency of each sample. The $\Delta\Delta$ CT method was used to analyze the data. Level of expression of Rb was expressed relative to that of the housekeeping gene GAPDH and expressed as a relative fold change. Real-time PCR quantifications were performed in triplicate with cDNAs from four independent experiments. In selected experiments, a GFP-tagged E2F1 expression plasmid (pmaxE2F1) was transfected by using lipofectamine, as reported below.

Luciferase assays. Rat cortical neurons (7 DIV) were cultured in six-well plates and transfected with the pGV-B-mRb promoter (-1353/-85) plasmid (a gift from Dr. Hiroshi Handa, Tokyo Institute of Technology, Yokohama, Japan) containing the luciferase cDNA sequence downstream of the human Rb promoter;40 neurons were transfected with 5.6 μg of the Rb promoter plasmid per well along with the EGFP plasmid (0.5–1 µg per well), using Lipofectamine[™] 2000 (Invitrogen). A GFP-tagged E2F1 expression plasmid (pmaxE2F1) was transfected with the other plasmids where indicated (7.6 µg per well). In order to measure Rb transcriptional activity, pRb-TA-Luc vector (Clontech) was used at 7.6 µg per well. The Bright-Glo™ (Promega, Madison, WI, USA) reagents were used to detect luciferase activity as indicated by the manufacturer, using a spectrophotofluorometer (Wallac 1420 multilabel counter; PerkinElmer, Wellesley, MA, USA). Expression of GFP, by either pEGFP or pmaxGFP vectors, was measured to determine transfection efficiency and all luciferase measurements were normalized accordingly. Transfection efficiency in these experiments was 35%. Neurons were treated with CXCL12 (20 nM) 24 h after the transfection.

siRNA and survival assays. Double-stranded shRNA against rat, psi(Rb), was created following the GeneSuppressor[™] System protocol (IMG-800; from Imgenex, San Diego, CA, USA) using the following oligos; 5'-TCGAGGAGCAC GAGTGTAATGTATTCAAGAGATACATTACACTCGTGCTCCTTTTT-3' and 5'-CT AGAAAAAGGAGCACGAGTGTAATGTATCTCTTGAATACATTACACTCGTGCTC C-3'. This shRNA vector has already been shown to knockdown Rb expression in rat neurons²⁴ and we verified silencing of Rb in rat glia cultures and in neurons (Supplementary Figure 3; Figure 5b). Both glia cells and neurons were transfected using electroporation by Amaxa as indicated by the manufacturer, using 2 and 6 μ g of vectors per four million cells, respectively. Transfection efficiency was 55% (neurons) and 65% (glia). For survival assays, rat cortical neurons (50 000 cells per coverslip) were divided in two groups. One group was transfected with psi(Rb), whereas the other group was transfected with the negative control plasmid carrying a scrambled sequence (IMG-800-6; from Imgenex), using Lipofectamine[™] 2000 reagent (Invitrogen), 2 μ g of plasmid per coverslip. A GFP expression vector was used as transfection control in both groups. NMDA neurotoxicity assay was conducted as described before, ¹⁹ and only the cells expressing GFP were analyzed. Transfection efficiency in these experiments was about 35%.

Materials. Unless otherwise specified, tissue culture media are from Invitrogen. CXCL12 (SDF-1 α) was purchased from PeproTech Inc. (Rocky Hill, NJ, USA); chemokine was reconstituted in PBS/0.1% BSA, stored as stock solution at -20° C, and used at a final concentration of 20 nM. AMD3100 and CHX were obtained from Sigma-Aldrich, reconstituted in water, and used at final concentrations of 100 ng/ml and 1 μ g/ml, respectively. All oligonucleotides were purchased from Operon Biotech (Huntsville, Alabama, USA).

Statistical analysis. Student's *t*-test or ANOVA were used for statistical analysis (a $P \leq 0.05$. was considered statistically significant). All data are reported as mean \pm S.E.M. Experiments were performed at least three times.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)