

Role of Cell Cycle Proteins in CNS Injury

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Abstract Following trauma or ischemia to the central nervous system (CNS), there is a marked increase in the expression of cell cycle-related proteins. This up-regulation is associated with apoptosis of post-mitotic cells, including neurons and oligodendrocytes, both in vitro and in vivo. Cell cycle activation also induces proliferation of astrocytes and microglia, contributing to the glial scar and microglial activation with release of inflammatory factors. Treatment with cell cycle inhibitors in CNS injury models inhibits glial scar formation and neuronal cell death, resulting in substantially decreased lesion volumes and improved behavioral recovery. Here we critically review the role of cell cycle pathways in the pathophysiology of experimental stroke, traumatic brain injury and spinal cord injury, and discuss the potential of cell cycle inhibitors as neuroprotective agents.

Keywords Brain injury · Flavopiridol · Proliferation · Review · Spinal cord injury · Stroke

Introduction

Up-regulation of cell cycle proteins occurs after central nervous system (CNS) injury [1–6], and contributes to apoptosis of post-mitotic cells such as neurons and oligodendroglia [2, 6–8]. Cell cycle activation also plays an important role in post-traumatic gliosis and microglial activation [1, 5, 9, 10]. Inhibition of cell cycle pathways

reduces injury-induced neuronal death in vitro [11] and after CNS injury [12], as well as decreases glial scar formation and release of microglial associated inflammatory factors [1, 10].

Cell cycle activation induces caspase-dependent neuronal apoptosis [13] in a variety of in vitro [8, 14, 15] and in vivo models [16, 17]. Mechanisms of neuronal apoptosis and cell cycle activation share common regulatory elements such as retinoblastoma protein (Rb), E2F, and p53. Generally, post-mitotic cells such as neurons do not engage in cell cycle progression once they differentiate and cell cycle proteins are usually down-regulated in these cells [18]. However, mature neurons have the capacity to re-enter the cell cycle, resulting in apoptosis rather than neuronal proliferation [19, 20]. In contrast, mitotic cells such as astrocytes or microglia within the CNS, respond to cell cycle activation by proliferating, leading to increased inflammation and glial scar formation [1, 5, 10].

The cell cycle

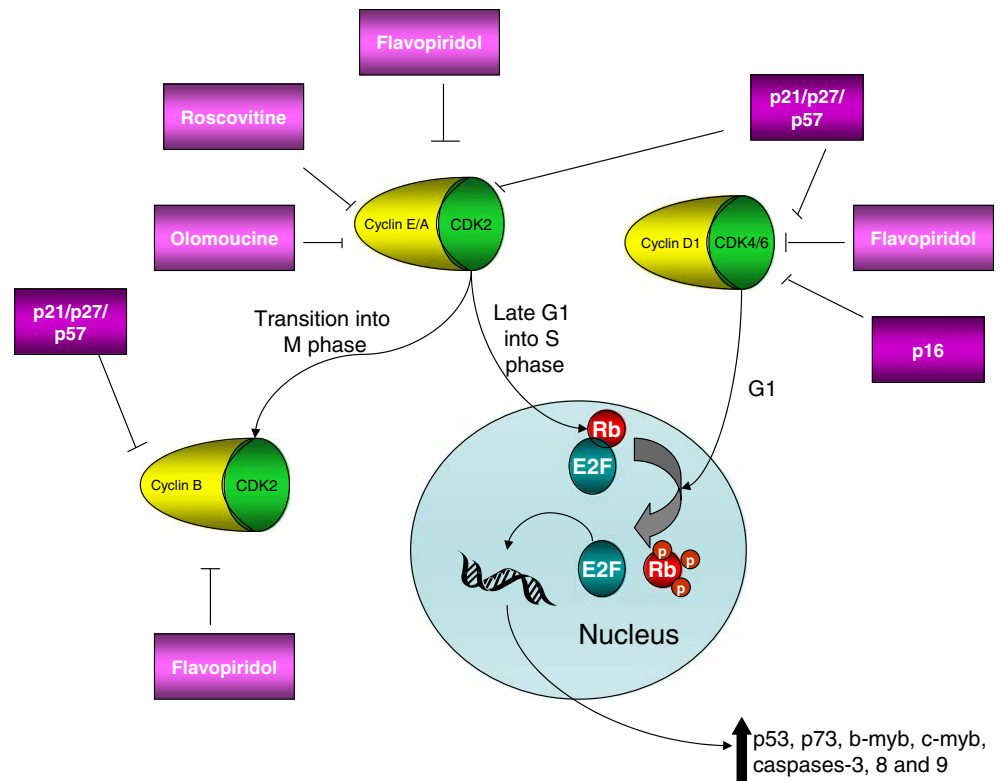
Progression through the cell cycle is controlled by the interaction of numerous factors [21] including cyclins [22], cyclin-activated kinases (CDKs) [23, 24] and cyclin-dependent kinase inhibitors (CDKIs) [25, 26] (Fig. 1). In response to stimulation, D-type cyclins are synthesized and bind CDK4 and CDK6, which prompts cells to enter the G1 phase [27]. In the nucleus, CDK4/6 phosphorylates Rb on specific residues including Ser780 and Ser795 [28]. Phosphorylation of Rb causes release of E2F transcription factors, activating their transcriptional activity and the transcription of genes required for the next phase of the cycle [28–31]. Cyclin E is induced later in the G1 phase, and its association with CDK2 is required for transition

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Fig. 1 Cell cycle diagram. This diagram indicates the role of different cell cycle proteins activated after CNS injury and their related endogenous and exogenous inhibitors.

Activation of CDK4 through the binding with cyclin D1 results in its translocation to the nucleus as part of G1 phase. There, the cyclin D1/CDK4 complex phosphorylates Rb, releasing E2F to activate DNA transcription. In late G1 phase, cyclin E and A are up-regulated and bind to CDK2, further phosphorylating Rb and enabling the progression into S phase. At this point, CDK2 is released from cyclin E/A and binds to cyclin B, allowing for the transition into M phase. These various CDKs have both endogenous and exogenous inhibitors acting at various points of the cycle



into the S phase, during which DNA synthesis is associated with the active cyclin A/CDK2 complex [32]. Cyclin A is degraded at the G2/M transition, while the released CDK2 forms a complex with newly synthesized cyclin B. The latter complex is necessary for entry and progression through mitosis [33], and is inactivated in late M by the anaphase-promoting complex [34].

Progression through the cell cycle is strictly controlled, and cells stopped at any of the checkpoints either return to G0 phase or die by apoptosis [35, 36]. For example, depending on the cellular conditions [37], E2F can induce apoptosis in a variety of ways: (1) activation of B- and C-myc genes [38], (2) increased expression of caspases-3, -9 and -8 and Apaf-1 [39], (3) and activation of p53 and p73 [3, 30]. p53 and p73 activate pro-apoptotic Bcl-2 family members, releasing mitochondrial pro-apoptotic proteins such as cytochrome c [39, 40].

Cell cycle inhibitors

Endogenous and exogenous cell cycle inhibitors block the progression of a cell through the cell cycle. There are seven endogenous cell cycle inhibitors, comprising two families that function by binding to the cyclin dependent kinases. The Cip/Kip family functions to broadly and non-specifically block activity of CDKs, and includes p21^{Cip1}, p27^{Kip1}, and p57^{Kip2} [41]. p27 is the most highly expressed

inhibitor in the brain [42]. The Ink4 family, in contrast, is specific for CDK4/6, and includes p16^{Ink4A}, p18^{Ink4C}, and p15^{Ink4B}. These inhibitors are required to maintain cell quiescence, and are up-regulated in response to contact inhibition or anti-mitogenic stimuli, binding to CDK/cyclin complex and preventing activity [41]. They are also up-regulated during development, remaining elevated in the adult brain.

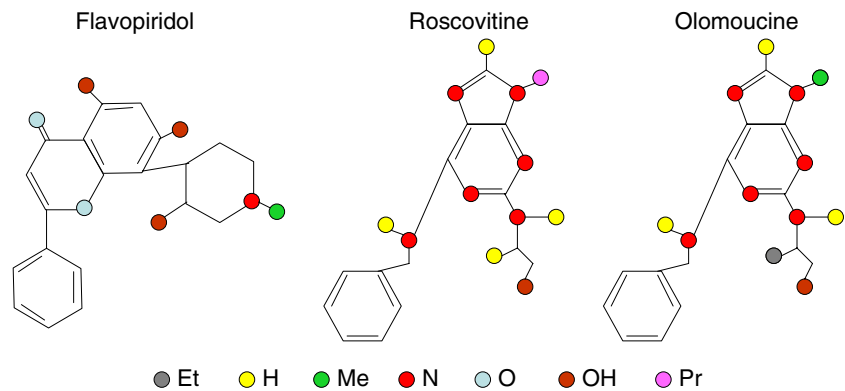
The most commonly used CDK inhibitors include flavopiridol, roscovitine and olomoucine (Figs. 1, 2). Flavopiridol, a semi-synthetic flavonoid derived from the bark of rohitukin [43], inhibits all CDKs, reduces cyclin D1 mRNA transcription, and leads to cell cycle arrest in G1 or at the G2/M transition [44, 45]. Roscovitine, a purine analogue, prevents activation of CDK2 and CDK5 [46]. At higher concentrations, it may inhibit the activity of signal transduction pathway kinases, such as ERK1 and ERK2. Olomoucine (2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine) regulates the activity of CDK2 and CDK5 by competitively binding the ATP-binding site [47].

Cell cycle proteins in vitro

Neurons

Cell cycle proteins play a major role in models of neuronal damage and death in vitro. For example, kainic acid, which

Fig. 2 The chemical structures of exogenous cell cycle inhibitors. Flavopiridol, roscovitine, and olomoucine are a small sample of available CDK inhibitors



induces excitotoxic cell death in cerebellar granule neurons, up-regulates caspase-3 and -9 as well as the cell cycle proteins cyclin D, cyclin E, PCNA and E2F1 [11]. Kainic acid also increases BrdU uptake into neurons after injury, suggesting DNA replication. Other models of neuronal apoptosis associated with caspase activation, such as serum withdrawal, also show increased cyclin D1 protein expression [48].

Following KCl withdrawal from cerebellar neurons, there is increased activity of the cyclin dependent kinases, increased expression of cyclin E, and translocation of cyclin D and CDK4/6 to the nucleus prior to neuronal apoptosis [7]. These changes are accompanied by an increase in Rb phosphorylation.

Inhibition of p27 by siRNA in primary neuronal cultures also increases Rb phosphorylation, as well as neuronal cell death [42]. p27 siRNA decreases p27 expression within 24 h after transfection, and is accompanied by an increase in BrdU uptake into neurons. Neuronal death induced by p27 reduction is nearly completely inhibited by olomoucine [42].

Six hours of hypoxia *in vitro* significantly increases PCNA expression associated with apoptosis [49]; olomoucine completely inhibits this neuronal cell death. Etoposide (50 μ M) application also causes neuronal apoptosis, as measured by an increase in cleaved caspase-3 reactivity [1] and LDH release [10]; this is blocked by flavopiridol, roscovitine and olomoucine (Figs. 1, 2). In this model, flavopiridol is the most potent, having optimal effects at 10 μ M, whereas the other drugs were only effective at doses above 100 μ M. These cell cycle inhibitors also provide neuroprotection following KCl withdrawal from cerebellar neurons [7]. Flavopiridol inhibits cyclin D1 expression and translocation to the nucleus and up-regulates p27 expression [1, 7, 10] while decreasing phosphorylation of Rb [50]. Additionally, flavopiridol pre-treatment decreases LDH release from neurons treated with kainic acid and blocks the uptake of

BrdU into damaged neurons [11]. Flavopiridol is also protective in a colchicine model of neuronal apoptosis, reducing release of cytochrome c from mitochondria [51].

The mechanism by which DNA damage or neuronal injury causes up-regulation or activation of cell cycle proteins is unknown. However, studies in other cell lines, such as fibroblasts or kidney cells, show that the activation of CDK4 with cyclin D1 is necessary for progression to apoptosis [48]. Further, reduction of CDK4 expression in neuronal cultures is protective against hypoxia-induced neuronal apoptosis [52]. However, increased cyclin D1 expression is not necessary for apoptosis; rather, increased CDK4 activity may be sufficient [50]. In primary neuronal cultures treated with camptothecin, increased CDK4 activity is the first response, accompanied by an increase in Rb phosphorylation, and followed by caspase-3 cleavage and neuronal apoptosis [50]. In the latter model, the actual amount of cyclin D1 and CDK4 protein did not increase as the neurons underwent damage and death. Therefore, the release of E2F by Rb phosphorylation appears to be the signal driving the cell into apoptosis, as mutating Rb to prevent phosphorylation blocks neuronal apoptosis, while over-expression of E2F induces it [50]. It is important to emphasize that the activation or expression of cell cycle proteins does not necessarily cause ‘normal’ re-entry into the cell cycle; rather, the expression and activity are aberrant, occurring out of sequence. This is clearly demonstrated by Padmanabhan et al. [7], in which cyclin E-related kinase activity was out of sequence with CDK4/6 activity after KCl withdrawal.

As many of the cells involved in cell cycle-mediated apoptosis also demonstrate cleavage of caspase-3 [1, 10], it is hypothesized that this model of neuronal apoptosis is caspase-mediated. Studies demonstrating increases in cell cycle proteins also show increases in DEVD-AFC cleaving activity, further supporting caspases as the mediator of such neuronal apoptosis [7].

Astrocytes and Microglia

Whereas aberrant cell cycle activation causes apoptosis in post-mitotic cells [53], it induces proliferation in mitotic cells such as astrocytes and microglia [54]. Cell cycle related proteins are up-regulated following stimulation in astrocytes and microglia. PCNA protein expression is increased in microglia stimulated with lipopolysaccharide (Fig. 3), a component of bacterial cell walls that stimulates the toll-like receptor 4 on microglia to induce activation (unpublished results). It is also up-regulated in astrocytes following hypoxia [55]. Application of the cytokine GM-CSF to a microglial cell line culture also increases cyclin expression and BrdU uptake, which can be reversed by cytokine withdrawal [56].

Cell cycle proteins also play a role in density dependent inhibition of cell proliferation. As the density of the mouse microglial cell line GMI-M6-3 increases, p27 expression is increased, while levels of cyclin A decrease [56]. However, no change in cyclin D1, cyclin E or CDK4 is detected. In contrast, using a different cell line, rat GMI-R1, cyclin D1 has been shown to be density-dependent [57]; it is unclear what role the species specificity plays in this response. Similarly, cyclin D1 and cyclin A are density-dependent in astrocytes; cyclin D1 is down-regulated by contact inhibition, and p27 expression increases [58]. At the same time phosphorylation of Rb is found in growing astrocyte cultures, and lost in dense cultures.

Expression of the CDK inhibitors p27 and p21 has also been studied in microglial cultures. GM-CSF application to a microglial cell line decreases the expression of p27 and p21, and over-expression of p27 decreases microglial BrdU uptake [56].

Administration of cell cycle inhibitors, including flavopiridol, roscovitine and olomoucine, inhibits microglial and astrocyte proliferation [1, 10, 55]. In astrocytes, it appears that flavopiridol is acting at the same stage of the cell cycle, inhibiting a progression of the cell cycle to S phase, thus blocking DNA replication [10]. Moreover, application of roscovitine to primary rat microglial cell cultures significantly inhibits microglial proliferation in response to LPS and TNF α stimulation (Fig. 4). Cell cycle inhibition

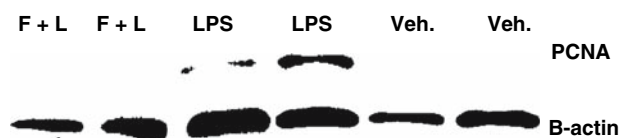


Fig. 3 Proliferating cell nuclear antigen (PCNA), a marker of cell cycle activation, Western blot. PCNA is detected 24 h after LPS stimulation of purified microglial cultures, while it is not seen in vehicle (Veh.) cultures. Pre-treatment of these cells with the cell cycle inhibitor flavopiridol (F) blocks this increase in PCNA

appears to not only inhibit microglial proliferation, but also activity, blocking nitric oxide (NO) production 24 h after stimulation (Fig. 4). In addition, flavopiridol blocks COX-2 activity in cortical mixed cultures [59].

Cell cycle proteins in vivo

In brain regions such as hippocampus, piriform cortex and amygdala, cyclin D1 expression is normally low [60]. In non-injured cortex, cyclin D1 is co-expressed in MAP2 positive cells, demonstrating its presence in neurons [6, 61]. Expression of the CDKIs, such as p27, is high in the normal cortex [58]. In non-neuronal cells, such as microglia and astrocytes, expression of cell cycle proteins is not found in normal, non-injured tissue [5].

Traumatic brain and spinal cord injury

Traumatic brain (TBI) and spinal cord injury (SCI) cause a subacute neuronal loss in the lesion periphery in the days following the initial injury. Many of these neurons undergo apoptosis as a result of excitotoxic-or inflammation-induced injury in the surrounding tissue, and much of this apoptosis is related to the up-regulation of cell cycle proteins [2, 6, 62].

Microarray analysis shows a marked up-regulation of cell cycle-related genes, including cyclin D1 and CDK4 at various time points after either TBI [10, 63] or SCI [64, 65] (Fig. 5). Confirmation of these microarray findings with immunohistochemistry demonstrates up-regulation of these cell cycle proteins in neurons and glia. Cell cycle proteins in neurons are often associated with the expression of cleaved caspase-3, a marker of caspase-mediated apoptosis [10]. After brain trauma, 13% of neurons show an increase in cyclin D1 expression, and 75% of those are also positive for cleaved caspase-3 [10].

On the other hand, p27 is down-regulated after TBI, whereas p21 remains unchanged [10]. In contrast, Findings by Kobori et al. [66] and Katano et al. [67] show an increase in p21 from 2 to 24 h after TBI. p21 may also play a role after SCI, as local application of p21 following dorsal hemisection in the spinal cord significantly improves both motor function and increases the number of axons extending beyond the lesion site [68].

Traumatic injury to the adult CNS also rapidly activates resident astrocytes, leading to glial scarring [69]. Astrocytes appear to isolate the damaged region by increasing the expression of a structural protein, glial fibrillary acidic protein (GFAP), migrating from the bordering undamaged tissue towards the injured site, and entangling their processes [70]. Moreover, astrocytes produce a class of molecules known as extracellular

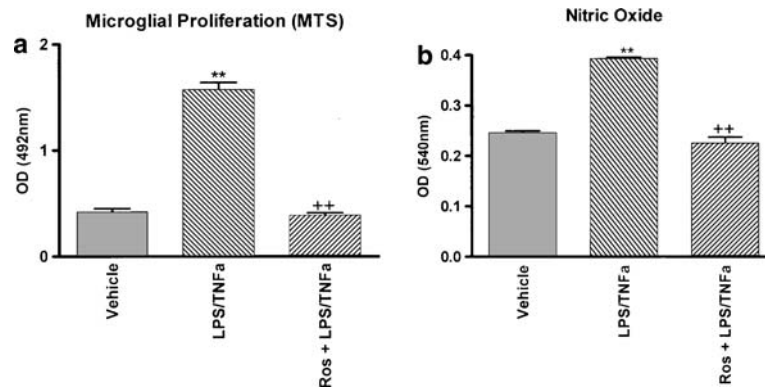


Fig. 4 Response of microglia to LPS and roscovitine. LPS and TNF α result in an increase in both microglial proliferation (a; as measured by a MTS assay) and nitric oxide (b) synthesis. However, 1-h pretreatment of purified microglial cells with roscovitine (100 μ M)

resulted in a complete loss of this LPS-induced increase in proliferation and activity. ** $P < 0.001$ in comparison to vehicle. ++ $P < 0.001$ in comparison to LPS/TNF α (One-way ANOVA). Bars represent mean \pm SEM

matrix (ECM) molecules with distinct inhibitory effects on axonal regeneration [71]. In contrast, it has been suggested that the glial scar may also contribute to the blood-brain barrier repair, prevent excessive inflammatory responses, and limit cellular degeneration [72]. Nevertheless, considerable evidence indicates that preventing and/or reducing the inhibitory environment of the glial scar provides a better environment for neurons to regenerate [73].

After TBI, both microglia and astrocytes show increases in cyclin D1 by 24 h [10]. Additionally, proliferation of microglia in response to a cortical stab wound, as measured by BrdU uptake, is inhibited by over-expression of p27 [56]. However, in this model, the microglia do not return to a ramified, resting form; rather, they remain in an amoeboid ‘activated’ state, although the level of their activation was not measured.

After TBI, flavopiridol reduces the number of neurons with increased cyclin D1 expression by over 50% [10]. It also decreases the expression of cell cycle proteins in mi-

croglia and astrocytes, as well as the amount of GFAP and OX-42 staining after TBI [1]. Moreover, flavopiridol treatment significantly reduces brain lesion volume and improves functional recovery by 21-days post-injury [1]. Olomoucine also improves function and decreases lesion volume when administered after SCI [74]. Further, olomoucine significantly reduces the number of TUNEL positive neurons at 3 days after SCI, while decreasing astrocyte proliferation by 7 days.

Preliminary work on cyclin D1 knockout mice shows that lack of this cell cycle protein improves recovery after SCI (unpublished results; Fig. 6). Tissue was obtained from wild-type and cyclin D1 knockout mice at 72 h after moderate SCI. Lesion size, obtained from at least 10 randomly selected sections spanning the 10 mm centered around the lesion site and assessed using the Cavalieri method [75], shows a decrease in knockout mice. Further, cyclin D1 knockout mice demonstrate a greater recovery of movement of the hindlimbs by 72 h post-injury than their wild-type littermates (Fig. 6).

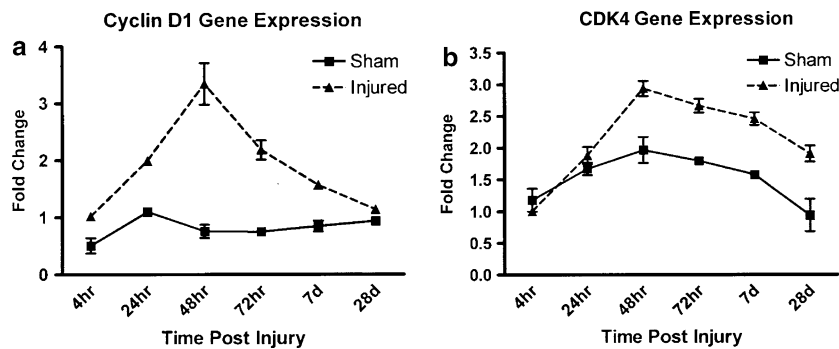


Fig. 5 Cell cycle protein mRNA expression after moderate SCI. Cyclin D1 (a) and CDK4 (b) are both significantly up-regulated by 24–48 h after SCI (dashed line) in comparison to sham controls (solid line). This up-regulation persists for at least 7 days post-injury, and

disappears by 28 days. Individual measurements are indicated by boxes, averages are indicated by circles. X-axis = days post-injury. Y-axis = fold increase. Only values above 1.5-fold are considered significant

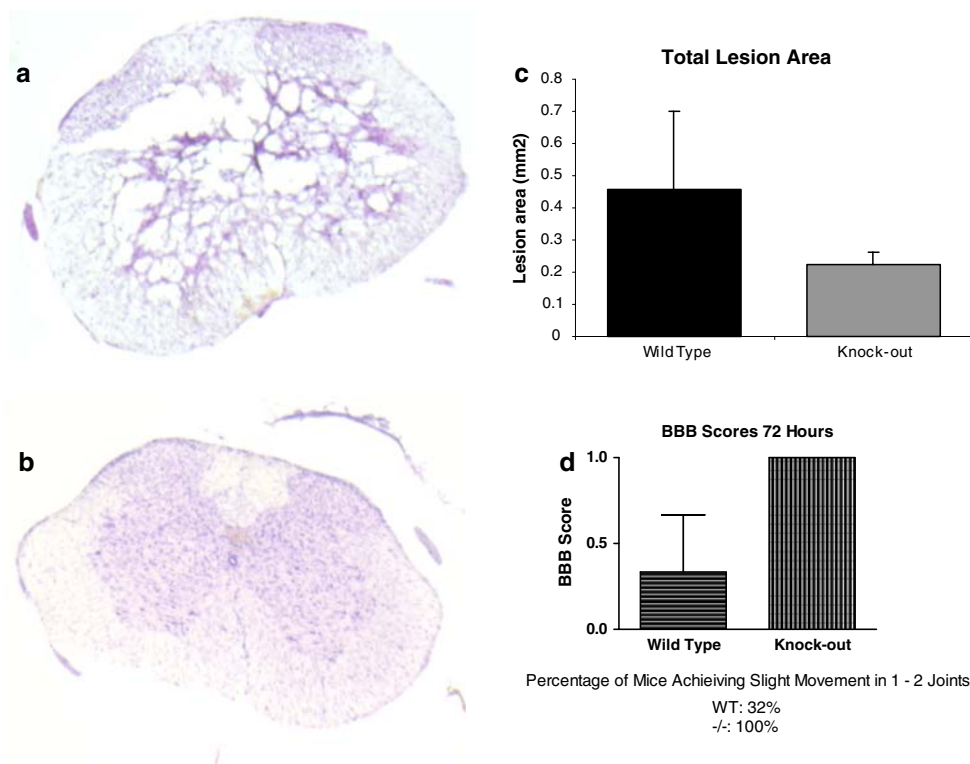


Fig. 6 Histological and functional assessment at 3 days after moderate SCI in wild type and cyclin D1 knockout mice. Cresyl violet stained transverse spinal cord sections at 2 mm rostral to the lesion epicenter demonstrate a maintenance of gray and white matter architecture in cyclin D1 knockout mice (**b**), while wild type animals showed signs of tissue loss and disrupted gray and white matter viability. Quantitation of the lesion area within the 10 mm segment centered around the lesion epicenter demonstrated a reduction in area

in knockout animals (**c**). Assessment of function at 3 days post-injury in wild type and knockout mice using the BBB locomotor score shows improvement in the knockout mice (**d**). Of the three animals in this preliminary study, 100% of the knockout mice achieved some functional movement (BBB score = 1) by day 3, whereas only 1 of the wild-type mice had any hindlimb movement at this point. Bars represent mean \pm SEM

Ischemia

Following transient brain ischemia, there is an increase in BrdU uptake into adult neurons [76], accompanied by positive TUNEL staining [77], indicating aberrant cell cycle reentry by hippocampal neurons.

Cyclin D1 and CDK4 are both expressed in association with apoptosis after transient spinal cord ischemia [78]. However, after global brain ischemia cyclin D1 and CDK4 are not increased above normal values [4, 61, 76]. However, elevated levels of CDK5 were found after ischemia [76]. Importantly, CDK5 was found to be essential for cell cycle control; knockout of CDK5 resulted in continuous BrdU labeling of post-mitotic neurons [79].

Cyclin D1 levels remain constant over the 7 days after reperfusion [61], perhaps because increased microglial expression is offset by decreased neuronal expression (due to neuronal death). In this model, only microglia are found to express nuclear cyclin D1 positive staining. On the other hand, this study used MAP-2 staining as a

neuronal marker, noting that MAP-2 staining decreases over time. The authors also note that there are nuclear cyclin D1 positive cells that fail to label with a microglial marker, allowing for the possibility of MAP-2 negative, cyclin D1 positive neurons in the cortex prior to neuronal apoptosis. In contrast, Kato et al. [5] showed a slight increase in cyclin D1 expression in damaged hippocampal neurons after global ischemia, as well as in microglia.

Regardless, administration of either CDK inhibitors or dominant negative CDK4/5 results in a reduction of neuronal apoptosis [31]. Further, knockout cyclin D1 diminishes the proliferation of astrocytes after focal ischemia, as does application of olomoucine [55]. Dominant negative knock-down of CDK4 also reduces neuronal death after global ischemia; however, knock-down of CDK2 or 5 failed to result in neuroprotection [52]. Interestingly, when using a focal ischemia model, hypothesized to induce excitotoxic neuronal death, knockout of CDK5 was neuroprotective [52].

Other models

Using an animal model of excitotoxic cell death, Ino et al. [60] demonstrated an up-regulation of both CDK4 and cyclin D1 in neurons of the amygdala, hippocampus and piriform cortex after application of kainic acid, as well as nuclear translocation of these proteins. However, no CDK4 staining is apparent in cells actively undergoing apoptosis and TUNEL positive, although addition of anti-sense oligonucleotides against CDK4 or cyclin D1 after kainic acid treatment effectively blocks the excitotoxic neuronal death, demonstrating the necessity of both CDK4 and cyclin D1 in this model.

Microglia also show increased cyclin D1 and CDK4 translocation into the nucleus after excitotoxic neuronal death [60]. It is unclear, however, whether this is due to signals from dying neurons or from kainic acid activity on microglia directly.

Summary

Considerable data indicate that cell cycle proteins can cause both proliferation of mitotic cells and apoptosis of post-mitotic cells. There are numerous examples showing that activation of the cyclin D1/CDK4 complex and phosphorylation of Rb can lead to caspase-dependent apoptosis in neurons, although it is debated as to whether these proteins must be up-regulated as well. Whether there is increased protein expression of cyclin D1, for example, may be dependent on the injury model and species. Regardless, many studies demonstrate that inhibition of cell cycle activity in post-mitotic neurons prevents neuronal cell death, whereas such inhibition in mitotic astrocytes and microglia reduces scar formation and inflammation. Thus, cell cycle inhibition can serve to decrease post-traumatic or post-ischemic injury through multiple mechanisms.

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