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Delineating the factors and cellular mechanisms involved in the survival of Cerebellar Granule neurons

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CONFLICT OF INTEREST STATEMENT.

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ABSTRACT

Cerebellar granule neurons (CGNs) constitute the most abundant neuronal population in the mammalian brain. Their postnatal generation and the feasibility to induce their apoptotic death *in vitro* make them an excellent model to study the effect of several neurotransmitters and neurotrophins. Here, we first review which factors are involved in the generation and proliferation of CGNs in the external granule layer (EGL) and in the regulation of their differentiation and migration to internal granule layer (IGL). Special attention was given to the role of several neurotrophins and the NMDA subtype of glutamate receptor. Then, using the paradigm of potassium deprivation in cultured CGNs, we address several extracellular factors that promote the survival of CGNs, with particular emphasis on the cellular mechanisms. The role of specific protein kinases leading to the regulation of transcription factors and recent data involving the small G protein family is also discussed. Finally, the participation of some members of Bcl-2 family and the inhibition of mitochondria-related apoptotic pathway is also considered. Altogether, these studies evidence that CGNs are a key model to understand the development and the survival of neuronal populations.

Keywords:

Cerebellar granule neurons, development, differentiation, neuroprotection, survival pathways

Introduction

During the last decades the cerebellum has been studied in great detail, not only to understand its important role in the control of motor coordination, but also because its laminated structure and limited number of cell types have offered a model system to study neuronal development, differentiation and survival.

Cerebellar granule neurons (CGNs) are the most abundant class of central nervous system neurons. Their progenitors arise prenatally from the rhombic lip, in the boundary of the mesencephalon and metencephalon (mes/met region), to form the external granule layer (EGL). In rodents, the neuroblasts of the EGL proliferate during the first postnatal week. During the second postnatal week, neurons differentiate during their migration through the molecular and Purkinje layers to reach their mature state in the internal granule layer (IGL) by the end of the third postnatal week.[1] During their migration from the EGL towards the IGL, CGNs neurons that fail to receive excitatory inputs from mossy fibres will die by apoptosis.[2,3]

Extracellular factors involved in the proliferation, differentiation and survival of CGNs

The study of the mechanisms and factors involved in CGNs proliferation, differentiation and survival has been facilitated by the possibility to maintain these neurons in culture in the presence of a depolarizing medium. The combined use of CGNs primary cultures together with *in vivo* models has provided a deep knowledge of the main factors sequentially involved in CGNs proliferation in germinal layers and their differentiation and survival during migration to the IGL.

Bone morphogenetic proteins (BMPs) seem to be the main factors for the generation of CGNs progenitors in the rhombic lip. Work by Hatten and co-workers[4] has shown that BMP6, BMP7 and Growth Differentiation Factor 7 induce the expression of *Math1*, a transcription factor required for the specification of CGNs progenitors in the prenatal cerebellum. Moreover, they demonstrated that BMP-treated explants from the mes/met region were able to generate CGNs when transplanted to postnatal cerebellum. CGNs progenitors proliferate postnatally in the EGL. Several factors have been implicated in their proliferation and in the exit from the proliferative state to start differentiation. Numerous reports have shown that Sonic Hedgehog (Shh), secreted by Purkinje cells, is the main factor mediating the proliferation of CGNs progenitors[5], probably by *Nmyc1* activation.[6] However, other factors, such as insulin-like grown factor (IGF) 1 and 2, have also been implicated in the maintenance of the proliferative state of CGNs progenitors. Activation of IGF1 receptor by IGF1 and IGF2 enhances Shh-mediated proliferation of purified CGNs progenitors.[7] The synergy between Shh and IGFs could be related to the fact that *Igf2* expression is modulated by Shh.[8] However, IGF1 and IGF2 are still able to stimulate the proliferation of CGNs progenitors (although to a lesser extend) when Shh signaling is impaired[7] indicating that proliferation of CGNs progenitors in the EGL does not only depend on the presence of pro-mitogenic factors such as Shh, and that other mechanisms are involved. In fact it is known that transition from proliferation to differentiation in the EGL starts even in the continuous presence of Shh. Thus, it should be the presence of other factors (anti-mitogens) that drives CGNs progenitors towards differentiation. For example, IGF binding protein 5 is expressed when progenitors start to proliferate (second postnatal week) and it blocks IGF and Shh-mediated proliferation of purified CGNs progenitors.[7] Another factor that has been reported to induce cell cycle exit in CGNs progenitors is BMP4. The expression levels of BMP4 and Smad1 (an intracellular protein needed for BMP signaling) increase in the EGL during the second postnatal week and BMP4 promotes neuronal cell differentiation in CGNs cultures.[9] Other factors are also known to promote CGNs differentiation such as pituitary adenylate cyclase-activating polypeptide (PACAP), vitronectin, Wnt3 or activation of GPR3 receptors, which shows the complexity of the mechanisms involved in this process at the end of the first postnatal week in the EGL.[10-13]

Differentiation and maturation of CGNs start during the second postnatal week and occur in parallel to their migration from the EGL towards the IGL.[1] During their migration, stimulation of glutamatergic synapses between mossy fibres and CGNs are responsible for their survival.[2,3] It has been reported *in vivo* that activation of NMDA receptors (NMDAR) is necessary for CGNs survival since pharmacological blockade of these receptors produce an increase in the apoptotic rate.[14] Similar requirement has been observed *in vitro*. Cultured CGNs die from apoptosis when potassium chloride (KCl) concentration changes from 25 mM (K25) to 5 mM (K5). The addition of NMDA rescues CGNs from K5-mediated apoptosis.[15,16] Moreover, it has been reported that a 24 hr-exposure to NMDA in immature CGNs cultures (2 DIV) is enough to promote a long-lasting neuroprotective effect since cells survive for up to 8 DIV in K5.[17] Although different signal transduction pathways have been related to the protective effect of NMDA on differentiating CGNs (see below), several studies have shown that NMDA-dependent release of the neurotrophin BDNF is a major factor in CGNs survival. Blocking TrkB receptor activation, by either antagonists or BDNF antibodies, produces an important reduction in NMDA-mediated neuroprotection.[17-19]

CGNs primary cultures have also been used to search other pro-survival factors. Interestingly, some of the factors that are involved in the proliferation of progenitor cells were also related to survival of CGNs during their migration towards the IGL. For example, IGF1 is involved in the proliferation of CGNs progenitors (see above) but it also promotes the survival of CGNs, both *in vitro* and *in vivo*, by down-regulating pro-apoptotic factors such as Bax, Bim and Bad, and up-regulating Bcl-x(L) and Bcl-2.[20-22] Also PACAP, which inhibits the proliferation of progenitors in the EGL[11], acts as a pro-survival factor for CGNs.[23,24] Altogether, it seems that an orchestra of several extracellular factors plays an important, and complementary, role to glutamatergic inputs from mossy fibres in the control of CGNs survival during their migration towards the IGL. Then, we examine by which molecular mechanisms NMDA and the pro-survival factors induce the survival of CGNs in the paradigm of KCl deprivation in cultured CGNs.

Molecular pathways related to the neuroprotection of CGNs

It has been described that specific protein kinase cascades involving phosphoinosite 3-kinase (PI3K)/Akt, extracellular-signal regulated kinase (ERK), protein kinase A (PKA) or calcium/calmodulin kinase IV (CaMKIV) promote the neuroprotective activity of neurotransmitters and pro-survival factors by the increase of transcriptional activity. IGF-1 exerts its neuroprotective effect through the activity of PI3K[25] and the phosphorylation of Akt.[26] The activation of PI3K/Akt decreases the activity of Forkhead transcription factors, favoring the survival role of IGF-1.[22] Other transcription factors, such as the family of Myocite enhanced factor-2, also participate in IGF-1-mediated survival effect.[27]

The requirement of PI3K/Akt pathway was also observed in the neuroprotective effect of NMDA in immature[17] and mature[28, 29] CGNs. But this effect is not restricted to the activation of PI3K/Akt pathway as other protein kinases have also been involved. In mature CGNs, it has been showed that neuroprotection by NMDA is mediated by ERK activation.[29] However, other studies reported that inhibition of ERK pathway does not prevent the neuroprotective activity of NMDA.[18, 28] We recently reported that the survival role of PI3K/Akt and ERK pathways in CGNs depends on the small G proteins acting upstream.[30] We demonstrated that both pathways are involved in NMDA-mediated neuroprotection when they are activated by Ras. In contrast, the stimulation of the ERK pathway by other members of the small G proteins family, such as Rap1, is not sufficient to promote protection. Our results showed that the biological significance of NMDA-mediated activity of PI3K/Akt and ERK pathway depends on which monomeric-G protein is acting upstream. Furthermore, the requirement of Ras to activate these pathways also occurs for other protective factors.[31] Thus, these results support a central role for Ras in the survival of CGNs. NMDA-mediated activation of PI3K/Akt and ERK pathways stimulates the activation of CREB, an important transcription factor for the pro-survival effect of NMDA.[32] As indicated above, the neuroprotective effect of NMDA is

mediated by the release of BDNF.[33] We recently described that the interaction of CREB with the *Bdnf* promoter is enhanced by potassium depolarization, whereas it is not improved by NMDA treatment.[19] We demonstrated that the stimulation of NMDAR triggers the CREB-dependent *Nurr1* activation, which results in BDNF up-regulation. Moreover, we have characterized *Nurr1* as a key factor in NMDA-dependent survival of CGNs. Other factors mediating the survival of CGNs are related to the activation of ERK-CREB pathway. Interestingly, BMP-6 has been described to promote CGNs survival in culture through the stimulation of MEK-ERK-CREB pathway.[34] Thus, differential pathway activation by BMP-6 could be related to its function promoting the formation of CGNs progenitors (Smad-dependent) or the survival of CGNs (Smad-independent) during cerebellum development.

PACAP exerts a potent neuroprotective effect on cultured CGNs.[35, 37] More recently, it has shown in mice deficient for PAC1, the high-affinity PACAP receptor, that endogenous PACAP is crucial for the survival of CGNs.[38] The neuroprotective effect of PACAP is mediated through the activation of ERK [24] and the cAMP-dependent PKA[39,40]. The increased activity of these pathways leads to the stimulation of prosurvival gene expression, such as *c-fos* or *Bcl-2*.[41,42] In addition, it has been described that PACAP mediates its neuroprotective effect, in part, by inhibition of delayed rectifier K(+) current (I(K)) via cAMP/PKA transduction pathway.[43,44] Moreover, the survival effect of PACAP could be mediated by others pathways. It has been demonstrated that PACAP promotes the increase of intracellular calcium from intracellular stores and calcium influx through calcium channels.[45,46] The mobilization of calcium allows the activation of CaMKIV, and then the increase of CREB activity.[47,48] More recently, it has been shown that PACAP-mediated protective effect is also through the release of tissue plasminogen activator.[49]

The pivotal role of the Bcl-2 family proteins and the mithocondrial pathway in the apoptosis of CGNs has been extensively documented. Bax and other members of the Bcl-2 family are sufficient to promote apoptosis of CGNs.[50,51] The reduction of these proteins is an important step in the neuroprotection of CGNs. It has been shown that neuroprotection mediated by trophic factors is associated with the reduction of Bax, Bad and Bim levels.[21,22] On the other hand, the increased expression of anti-apoptotic members of Bcl-2 family has been related to the neuroprotective effect of IGF-1, PACAP, NMDA and others.[21,42,52] The regulation of the Bcl-2 members allows a reduction of the apoptotic mithocondrial activity, reducing cytochrome c release[52,53] and the activation of caspases.[17,21,54,55] The inhibition of other apoptotic pathways is also an important step in the survival of CGNs. For instance, the inhibition of JNK prevents the apoptotic death of CGNs[17], and the long-lasting neuroprotective effect of NMDA was also related to the inhibition of JNK and the phosphorylation of c-jun.[52] Taken together, the survival of CGNs mediated by NMDAR and neurotrophin receptors results in the stimulation of common survival mechanisms and suppression of apoptotic pathways.

Conclusion

The data presented here support that CGNs are a key model to decipher which factors and cellular mechanisms underlie neuronal development and survival. Recent publications unraveled the action of numerous neurotrophic factors and stimulation of NMDAR in promoting the activity of CGNs at postnatally stages. Moreover, significant works revealed the similarity between these two, not only in the promotion of the survival, but also in terms of the signaling pathways that they activate in CGNs.

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Figure legends

Figure 1: Extracellular factors involved in the proliferation, differentiation and protection of CGNs at postnatal stages. Main factors introduced in the text involved in the proliferation (prolifer.), differentiation (differ.) and protection (antiapop.) of CGNs at different postnatal stages, from the external granule layer (EGL) to the internal granule layer (IGL). Dashed arrow indicates the migration of CGNs from EGL to IGL. In brackets, the action of glutamate is through the stimulation of NMDA receptor (NMDAR). ML indicates molecular layer, PL, Purkinje layer and WM, white matter.

Figure 2: Cellular mechanisms involved in the neuroprotection of CGNs. We introduced the signaling pathways involved in the survival of CGNs promoted by some neurotrophins, neuropeptides and the NMDAR stimulation in the paradigm of potassium deprivation. Bold arrows indicate activation and dashed arrows denote inhibition.

Figure 1
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FIGURE 1

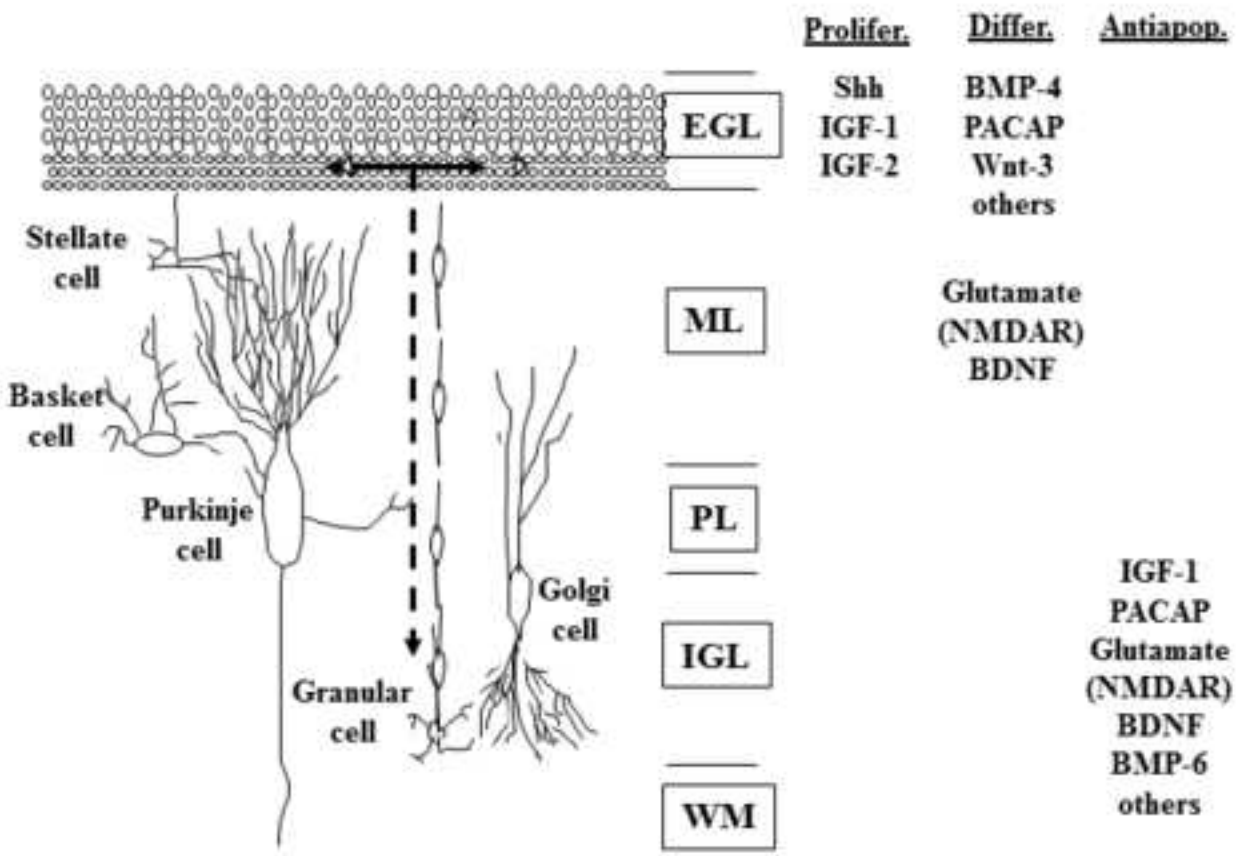

Figure 2
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FIGURE 2