Letter to the Editor

Developmentally regulated changes in c-Jun N-terminal kinase signalling determine the apoptotic response of oligodendrocyte lineage cells

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Dear Editor,

Oligodendrocytes (OL), the major myelin-forming cells in the central nervous system (CNS), are highly susceptible to injury in the developing brain with considerable numbers dying by apoptosis in models of neonatal brain injury. In the adult brain, widespread OL loss is associated with demyelinating diseases such as multiple sclerosis. Although c-Jun-N-terminal kinases (JNK) have been implicated in neuronal apoptosis, the role of this subfamily of mitogen-activated protein kinases in OL apoptosis is not well defined. In particular, the differences between highly proliferative OPC and terminally differentiated, postmitotic OL have not been investigated. In many regards, the cell and molecular properties of mature OL are quite distinct from those of dividing OPC.¹ The main objective of this study was to determine the role of JNK signalling in apoptotic cell death, triggered by specific stress signals in OL at defined stages of differentiation. For these studies, we employed the OPC cell line CG-4 that can be propagated as OL precursors and subsequently differentiated by exposure to insulin-like growth factor-1 into mature OL that express galactocerebroside.² We examined JNK signalling in three distinct paradigms of OL apoptosis: exposure to UV irradiation, or treatment with staurosporine (SSP) or with ceramide. Consistent with previous findings,^{3–5} treatment of OPC with selected stress signals dramatically decreased their viability in a time- and dose-dependent manner (Figure 1). In the case of ceramide, our data are in agreement with previous reports that ceramide is a potent inducer of OL apoptosis at any stage of maturation.⁵ In contrast, we observed differentiation-dependent changes in the sensitivity to UV or SSP (Figure 1a and c). Specifically, mature OL were significantly more resistant to UV exposure or SSP-induced apoptosis than OPC. The findings observed by MTT metabolism were also confirmed by replating efficiency and trypan blue exclusion; apoptosis was confirmed by morphological analysis of haematoxylin and eosin-stained cultures (not shown). Our results are consistent with the finding that the maturation process is associated with changes in the vulnerability of OL to apoptotic stimuli.⁶ We next investigated whether the relative insensitivity of mature OL to stress-induced apoptosis was associated with lower levels of JNK activation. Using phospho-specific JNK antibodies to follow JNK activity, we found that the both the basal level of JNK phosphorylation and JNK activation in response to apoptotic stress signals was significantly reduced in differentiated OL (Figure 1d–f). Importantly, this was not due to

developmentally regulated changes in JNK protein levels, which were not altered by OL differentiation, but rather the ability of mature OL to activate JNK signalling. We therefore investigated whether the upstream kinases MKK4 and MKK7 were altered by OL differentiation. While the results with MKK4 mirrored what we observed with JNK activation, MKK7 phosphorylation was as robust in OL as in naïve OPC subjected to UV stress (data not shown). These findings indicate that MKK7 activity is not sufficient to trigger the JNK pathway. Similarly, p38 was equally activated by UV in both undifferentiated OPC and mature OL (data not shown). The question is still open as to why there is reduced JNK activation in mature OL and future studies will focus on the involvement of those kinases upstream of MKK4, activation of specific phosphatases and the role of scaffold proteins for JNK that play an important role in JNK translocation and activation. Although previous studies have documented JNK activation in OL exposed to TNF α , NGF, UV, cytokines or other apoptotic stimuli,^{3,7,8} these did not measure JNK activation at defined stages of OL differentiation nor the relationship between increased JNK activation and OL cell death. The results presented here correlate the differential activation of JNK with changes in OL sensitivity to apoptosis and show that both responses are attenuated with OL maturation. One exception is the response of OL lineage cells to ceramide, where mature OL remain as sensitive as naïve OPC. We found, however, that apoptosis in response to ceramide proceeds in the absence of significant JNK activation, suggesting that alternative apoptotic signal transduction pathways are utilised.

Among the three JNK isoforms (encoded by three different genes), JNK1 and JNK2 are present in most tissues whereas JNK3 is expressed mainly in the CNS. Previous evidence has hinted at the functional diversity of JNK isoforms; JNK1 and JNK2 are thought to be primarily involved in apoptosis during brain development, while JNK3 is thought to have a role in the stress-induced neuronal cell death.⁹ We next investigated changes in the expression and activity of JNK3 in response to apoptotic stress signals during OL maturation. In the absence of a commercially available JNK3 antibody that does not cross-react with JNK1 or JNK2, we adopted an immunodepletion method.¹⁰ While high levels of basal JNK activity have been previously reported in healthy brain tissue,¹¹ our data indicate that in the OL lineage this basal JNK activity is restricted to OPC and is largely due to JNK1 and JNK2 (Figure 2a). Following exposure to the three apoptotic stimuli,

Figure 1 Differential response of OPC and mature OL to stress stimuli. OPC and mature OL were treated with SSP (a) or C2-ceramide (b) for 24 h or exposed to UV-irradiation (c) followed by a 24-h incubation. Cell viability was measured by MTT assay. Results represent the mean $\pm$ S.D. of three separate experiments. The difference between stress-stimuli treated OPC and mature OL was found to be statistically significant at $P < 0.001$ (*Student's *t*-test). In parallel, naïve OPC or mature OL were treated with 100 nM SSP (d) or 10 μ M C2-ceramide (e) for up to 8 h or exposed to UV-irradiation (0–50 J/m²) and incubated for a further 1 h (f). Cell lysates (50 μ g protein) were analysed by immunoblotting for JNK, phospho-JNK, Jun, phospho-Jun and α -tubulin (loading control) expression using specific antibodies (from Cell Signalling Technologies, USA) and detected by enhanced chemiluminescence

JNK3 formed a substantial part of all stress-induced JNK activity in OPC (Figure 2a and c). In sharp contrast, JNK3 activation was negligible in mature OL triggered to undergo apoptosis, although JNK3 protein levels remained constant. These results are consistent with the activation of JNK3 as a specific stress response in the brain. In agreement with our findings, it has been reported that exposure of cerebellar neurons to environmental stress selectively activates JNK3 in the presence of constitutive JNK1 activity.¹² In rat OPC, JNK1 and JNK3 have been shown to mediate NGF-dependent apoptosis via activation of Rac GTPase and the low-affinity neurotrophin receptor p75.¹⁰ Finally, two recent reports have demonstrated a specific involvement of JNK3 in the TNF α -induced death of adult human OL¹³ and in the ischaemic destruction of hippocampal neurons¹⁴ although a caveat to these last two studies is the use of a JNK3 antibody that cross-reacts with JNK1 and JNK2.

Our findings reinforce the strong link between JNK activation and phosphorylation of the transcription factor

Figure 2 Modulation of JNK3 expression and activity during OL maturation. Naïve OPC or mature OL were stimulated with 100 nM SSP or 10 μ M C2-ceramide for 1 h or exposed to UV (25 J/m²) then incubated for a further 1 h. JNK1 and JNK2 were immunodepleted from cell extracts using specific antibodies and then the remaining JNK3 activity in the supernatant determined by GST-Jun kinase assay kit (from Cell Signalling Technologies, USA) detected by densitometric analysis (a). Quantitative analysis of JNK3 activity was determined by scanning densitometry and the results plotted as the mean ($\pm$ S.D.) of three independent experiments (c). Depletion of JNK1 and JNK2 was confirmed by immunoblotting using a monoclonal antibody that recognises both JNK1 and JNK2 (from Pharmingen, USA) (a). Specific JNK3 protein expression was determined by immunoblotting from the immunodepleted cell lysates using a JNK3 antibody (Upstate Technologies, USA) with α -tubulin expression used as a loading control (b). To determine the role of JNK in stress-induced apoptosis, naïve OPC were pretreated for 1 h in the presence or absence of D-JNKI (5 μ M). Cell monolayers were subsequently exposed to 50 nM SSP, or 10 μ M C2-ceramide, or 25 J/m² UV irradiation and incubated for a further 1 h after which JNK activity was determined or for a further 24 h after which cell viability was assayed by MTT. Results represent the mean ($\pm$ S.D.) of three separate experiments and are expressed relative to the effect of UV-induced JNK activity or to cell survival in untreated naïve OPC, defined as the maximum response. The difference between stress-stimuli treated OPC in the presence or absence of D-JNKI was found to be statistically significant at $P < 0.001$ (**) or $P < 0.01$ (*) by Student's *t*-test

c-Jun, which has been implicated in neuronal cell death.¹⁵ In naïve OPC, we found that the kinetics of c-Jun phosphorylation correlated closely with JNK activation and apoptosis (Figure 1d and f). In contrast, in ceramide-induced apoptosis of mature OL, we found only low levels of phospho-Jun protein and the absence of JNK activity, suggesting that this pathway is not involved in the ceramide stress response (Figure 1e). This is in accord with a previous report that dominant-negative c-Jun does not attenuate ceramide-induced OL cell death.¹⁶ To determine whether the JNK pathway is required for apoptosis of OL lineage cells, we employed D-JNKI, a pharmacological inhibitor of JNK activity based on the binding domain of c-Jun interactive protein-1. D-JNKI is highly cell-permeable and acts as a decoy substrate, potently and

selectively inhibiting all JNK isoforms.¹⁷ While D-JNKI effectively blocked both JNK activity and c-Jun phosphorylation in naïve OPC exposed to UV, it only partially protected against apoptosis while there was no reduction in SSP toxicity (Figure 2d). It has been similarly shown that mutation of the JNK phosphorylation sites in c-Jun leads to partial protection against UV-induced cell death.¹⁸ One possibility for this apparent paradox may be that some JNK family members may be important for cell survival and so blocking all JNK isoforms may result in cell death, depending on the balance of the signalling pathways. Therefore, the selective inhibition of JNK3 may be more relevant to reducing OL or neuronal cell death following injury. We are currently adopting an RNAi approach to investigate the effects of selectively reducing JNK3 expression.

In conclusion, we have shown that JNK activation is differentially regulated in response to apoptotic stress stimuli at defined stages of OL maturation. While the changes in JNK activation correlate well with the changes in OL sensitivity to UV and SSP, this is not the case in ceramide-induced apoptosis. Significantly, while we found that JNK3 activation forms a substantial part of the OPC stress response, the presence of the JNK3 protein in mature OL points to a developmental difference in the upstream activators. Further studies are required to identify the upstream signals that distinguish the survival responses of OPC and mature OL.

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