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L1 Neural Cell Adhesion Molecule Is a Survival Factor for Fetal Dopaminergic Neurons

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Cell adhesion molecules play a central role in neural development and are also critically involved in axonal regeneration and synaptic plasticity in the adult nervous system. We investigated whether the neural cell adhesion molecule L1 was capable of stimulating survival and differentiation in the mid-brain dopaminergic neurons which degenerate in Parkinson's disease. Monoclonal L1 antibodies, known to enhance neurite outgrowth, were substrate-coated or added at the time of plating to medium of cultures containing mid-brain dopaminergic neurons from 14-day-old fetal rats. Tritiated dopamine uptake per well and the number of tyrosine hydroxylase-immunopositive neurons increased in a dose-dependent manner with increasing concentrations of L1 antibody, suggesting that L1 acts directly or indirectly as a growth factor for dopaminergic neurons. A monoclonal L1 antibody not enhancing neurite outgrowth was ineffective. The growth-promoting effects of L1 antibodies on dopaminergic neurons in culture did not appear to be mediated by the cAMP-activated protein kinase A pathway, since combined treatment with a phosphodiesterase inhibitor had only additive effects on the L1-induced increase of dopamine uptake, and in addition, antibodies against L1 failed to protect cultures of dopaminergic neurons against the neurotoxin MPP^+, whereas pretreatment with forskolin and phosphodiesterase type-IV inhibitors was strongly protective. J. Neurosci. Res. 53:129–134, 1998. © 1998 Wiley-Liss, Inc.

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INTRODUCTION

The L1 cell adhesion molecule (CAM) is a transmembrane cell-surface glycoprotein of the immunoglobulin superfamily which is differentially involved in homophilic and heterophilic recognition, adhesion, myelination in the peripheral nervous sytem, migration of neuronal cell bodies, neurite outgrowth and fasiculation, and synaptic plasticity (for reviews, see Wong et al., 1995, Hortsch,1996). L1 has an extracellular part consisting of six immunoglobulin-like domains and five fibronectin type III homologous repeats (Moos et al., 1988) which are differentially involved in neurite outgrowth, homophilic and heterophilic binding and signal transduction. These signal transduction events are triggered by binding of the natural ligands to L1 or mimicked by binding of antibodies to L1 at the cell surface which lead to complex cascades of intracellular events involving the non-receptor tyrosine kinases *src*, G-proteins, protein kinase C, cyclic AMP and the tyrosine receptor kinase fibroblast growth factor receptor (Atashi et al., 1992; Beggs et al., 1994; Klinz et al., 1995; Wong et al., 1995; Hall et al., 1996; Schuch et al., 1989; Bixby and Harris, 1991).

Different second messenger pathways are activated by L1 in different cell types. Dorsal root ganglion cells and Schwann cells respond to the addition of purified L1, or a variety of monoclonal and polyclonal antibodies to L1, with increased inositol phosphate turnover and calcium mobilisation (von Bohlen und Halbach et al., 1992), while PC12 cells show a drop in inositol phosphate metabolism (Schuch et al., 1989). Cerebellar neurons respond with a marked increase in cAMP levels, in addition to elevated intracellular calcium and IP3 (von Bohlen und Halbach et al., 1992). These responses to L1 correlate with increased neurite outgrowth in cerebellar neurons and are mediated by the region at the border between the fibronectin type III homologous repeats 2 and 3, since a monoclonal antibody directed at amino acids 818-832 in this region of L1 elicit neurite outgrowth, when substrate-coated and lead to increases in

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intracellular levels of calcium and IP3 (Appel et al., 1995).

Since an elevation of cAMP levels has been found to be a most effective means of improving the survival of dopaminergic neurons, and of protecting them both in vitro and in vivo from the cytotoxic effects of MPTP, a specific toxin for dopaminergic neurons (Hartikka et al., 1992; Hulley et al., 1995), we were interested to see whether L1 would mediate an increase in intracellular cAMP and enhance survival of these neurons. We have used dopamine uptake and cell counts to quantitatively assess the effects of L1 antibodies on survival of dopaminergic neurons, alone or in combination with phosphodiesterase type IV (PDE-IV) inhibitors which prevent the breakdown of cAMP in the cell.

METHODS

Cell culture, dopamine uptake and treatment with MPP^+ have been described (Hartikka et al., 1992). Primary cultures containing dopaminergic neurons were prepared from the ventral mesencephalon of 14-day-old fetal rats and plated in 24 well plates. A culture well usually contained 6×10^5 cells/well, of which 3–5% were dopaminergic. There are no available methods for isolating pure cultures of dopaminergic neurons from mesencephalic tissue. Duplicate cultures were prepared for dopamine uptake or tyrosine hydroxylase staining. Dopamine uptake was measured using tritiated dopamine at concentrations of 50 nM (sp. act. 45 Ci/mmol, New England Nuclear, Boston, MA). The survival of dopaminergic neurons in the culture was assayed by counting tyrosine hydroxylase (TH) immunopositive neurons, stained with a monoclonal antibody to TH (Boehringer Mannheim, Mannheim, Germany).

The monoclonal L1 antibodies 327 and 557.B6 (referred to as 557) were added to the culture medium on the day of plating at concentrations of 7.5–120 μg/ml and left for 3 days. In addition to treatment of the culture medium with antibodies, they were also substrate coated prior to plating of the cells, as described (Appel et al., 1993). In experiments with MPP^+ (active metabolite of methyl-phenyl-tetrahydropyridine, MPTP), antibodies or cAMP-elevating agents were added to the culture medium of 5-day-old cultures, followed by 1 μM MPP^+ on day 6 and cells were fixed on day 8 for immunocytochemistry. The PDE type IV inhibitor NQ-A (1-(3-carbomethoxyphenyl)-3-benzyl-quinazoline-2,4-dione) was synthesised at Novartis Ltd, Basel according to a published protocol (Lowe et al., 1991) and forskolin and MPP^+ were from Sigma Ltd. (Buchs, Switzerland).

RESULTS

Both immunoglobulin-like domains and fibronectin homologous repeats of the L1 glycoprotein have been shown to promote neurite outgrowth and participate in short-term cell adhesion with varying efficacy (Appel et al., 1993; Holm et al., 1995). Furthermore, a monoclonal antibody (557) reacting with a short peptide connecting the fibronectin type III repeats 2 and 3 is as effective as the purified L1 molecule itself in increasing both intracellular calcium levels and inositol phosphate turnover, and in promoting neurite outgrowth (Appel et al., 1995). We therefore used antibody 557 to evoke an intracellular response in dopaminergic neurons and stringently controlled its effects by using another antibody against L1 (327), which does not generate changes in second messengers nor enhance neurite outgrowth. We have compared the effects of these antibodies, both as substrates and as culture medium additives, on survival of primary mid-brain cultures containing 3–5% embryonic rat dopaminergic neurons.

Cultures from embryonic rat mid-brain exhibit a dramatic post-plating loss of dopaminergic neurons, with up to 50% of cells dying in the first three days of culture, followed by a more gradual decrease to around 80% cell loss by 13 days after plating (Hartikka et al., 1992). Therefore, in the experiments described here, cultures were treated with potential survival factors at the time of plating and then left for three days. At this point cultures were either fixed for immunocytochemistry or dopamine uptake was performed. The antibody 557 improved tritiated dopamine uptake by dopaminergic neurons (a measure of dopamine metabolism) at concentrations of 60 μg/ml whether used to coat the culture well or when added to the culture medium at the time of plating (not shown). Therefore most subsequent experiments were done using antibodies as culture medium additives and all the results presented here are from this treatment method. A dose response curve was made using concentrations ranging from 7.5–120 μg/ml and dopamine uptake/well increased in a dose-dependent manner, even at 7.5 μg/ml, when a slight increase was observed (Fig. 1). The control monoclonal L1 antibody, 327, which reacts with a conformational epitope on the sixth Ig-like domain of L1 (Appel et al.,1995) had no effect on dopamine uptake in the concentration range 7.5–120 μg/ml (result for 60 μg/ml shown in Fig. 1).

In order to determine whether triggering of L1 at the cell surface might increase dopamine uptake by elevating intracellular cAMP levels, the three day treatment of cells with antibody was combined with exposure to phosphodiesterase inhibitor NQ-A, which blocks the breakdown of cAMP (Lowe et al., 1991). While the phosphodiesterase inhibitor NQ-A caused a slight elevation of dopamine uptake at 0.5 μM concentration as previously reported (Fig. 1; Hulley et al., 1995), this increase in uptake was additive to the effects seen with the 557 antibody, rather

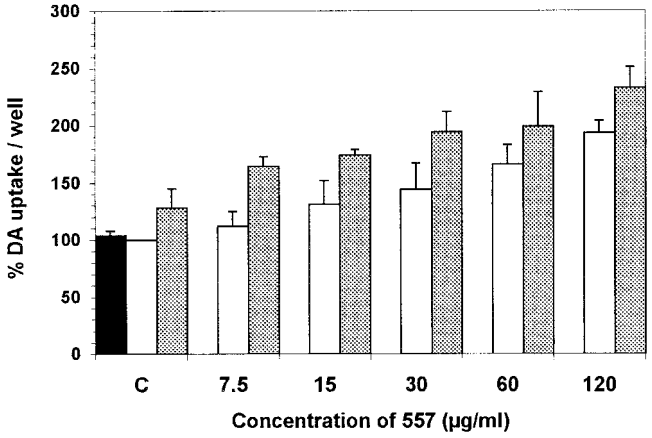

Fig. 1. Dose-dependent increase of tritiated dopamine uptake in 3-day-old primary cultures of embryonic rat ventral mesencephalon. Cultures were treated with 60 µg/ml of antibody 327 (black bar), or the indicated concentrations of antibody 557 without (open bars), and with (stippled bars), 0.5 µM phosphodiesterase type-IV inhibitor NQ-A, on day of plating and [^{3}H]dopamine uptake was assayed 3 days later. Results from 4 independent experiments (each with 4 wells/dose point) are expressed as a percentage ± SEM, with untreated controls designated 100%. 100% corresponds to 4298 ± 455, 4637 ± 453, 3055 ± 39, 4528 ± 495 cpm/well in experiments 1, 2, 3 and 4 respectively.

than synergistic (Fig. 1). This suggests that the two substances are exerting their effects through separate signal transduction pathways, since an exponential increase in dopamine uptake is seen when forskolin and any PDE-IV inhibitor are combined, both substances acting to elevate cAMP (Hulley et al., 1995).

Dopamine uptake can be elevated in two ways, by an increase in the number of dopaminergic neurons or by an increase in the number of uptake transporter sites per neuron which takes place during differentiation. In order to establish which of these accounted for the L1 antibody-induced elevation of dopamine uptake, duplicate cultures were treated with 557 antibody for three days. Of the duplicate cultures, half were processed for immunocytochemistry using tyrosine hydroxylase antibodies and dopamine uptake measurements were performed on the other half. Figure 2 shows that the number of tyrosine hydroxylase immunopositive neurons increased with increasing concentrations of L1 antibody, 557 (Fig. 2A), and that this correlated well with dopamine uptake (Fig. 2B). The dopamine uptake per neuron remained unchanged (Fig. 2C). This indicates that the L1-induced increase in dopamine uptake was caused by an increase in the number of surviving cells and not simply by an enhanced differentiation of existing cells or an increase in uptake mechanisms. The control antibody 327 had no significant effect on either cell number or dopamine uptake (not shown).

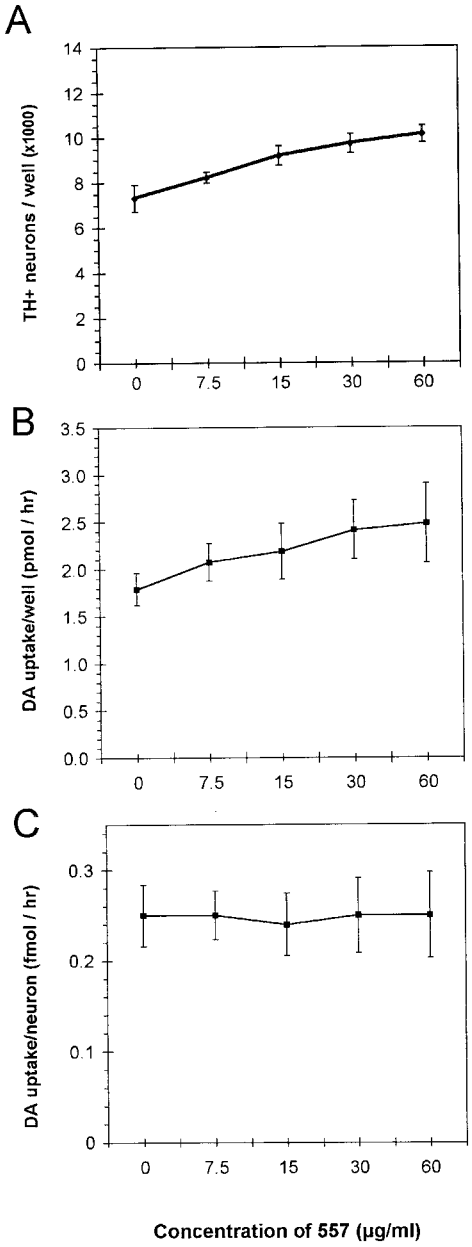

Fig. 2. Correlation between dopamine (DA) uptake and survival of dopaminergic neurons in 3-day-old primary cultures of embryonic rat ventral mesencephalon plated in the presence of increasing amounts of anti-L1 antibody, 557. **A:** Number of TH^+ neurons/well. **B:** [^{3}H] dopamine uptake/well. **C:** [^{3}H] dopamine uptake per TH^+ neuron. This is one representative example of 3 separate experiments, $n = 1$, ±SD (4 wells/dose point).

Dopaminergic neurons can be protected from MPP^+-induced neurotoxicity by treating 5 day old cultures with cAMP analogues or forskolin for one day before exposure to MPP^+ (Hartikka et al., 1992). A similar result was achieved here using a combination of forskolin and

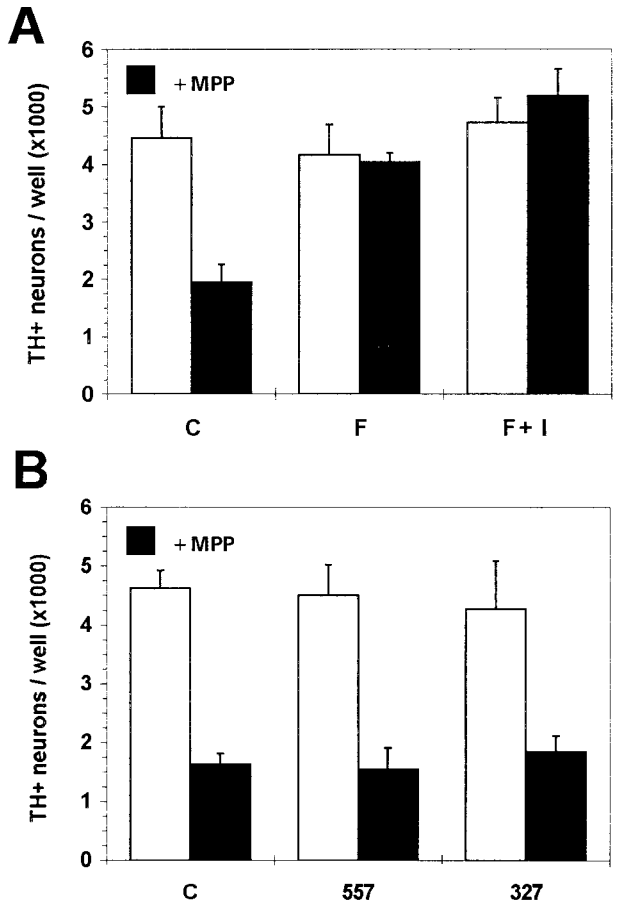

Fig. 3. Influence of cAMP elevators and antibodies against L1 on survival of dopaminergic neurons in the presence of MPP^+ neurotoxin (black bars). Five day old cultures were treated as indicated with **(A)** 0.5 µM forskolin alone, or combined with 1 µM phosphodiesterase type-IV inhibitor, NQ-A and **(B)** antibodies 557 or 327 (120µM each). On day 6, 1µM MPP^+ was applied and 48 hours later, the cells were stained with a tyrosine hydroxylase antibody and counted. C = untreated control, F = forskolin, F + I = forskolin in combination with PDE-IV inhibitor, NQ-A. Values are given ± SD, n = 4 wells/point. Experiments were repeated 4–6 times each.

PDE-IV inhibitor NQ-A (Fig. 3A). The number of surviving dopaminergic neurons was assessed on Day 8 of culture. When cultures were treated according to the same schedule with 120 µg/ml concentrations of the antibodies 557 and 327, there was no protection from MPP^+-induced neurotoxicity (Fig 3B). Concentrations of 30 µg/ml and 60 µg/ml 557 antibody were also tested with no protective effect (not shown). In untreated control cultures, there is a relatively slight decrease in dopaminergic cell number between Days 5 and 8 of culture (Hartikka et al., 1992), hence the selection of this treatment period.

DISCUSSION

Antibody 557, directed against the junction between repeats 2 and 3 of the fibronectin type III domain of the L1 neural cell adhesion molecule, promotes the survival of fetal dopaminergic neurons either when added acutely to the culture medium or as a coated substrate, as shown by an increase in both dopamine uptake and cell number. This correlates with a previous report that antibody 557 causes a pronounced second messenger response and increased neurite outgrowth in small cerebellar neurons (Appel et al., 1995). There is increasing evidence that ligand binding alone does not automatically cause a cellular response, and that not all domains of L1 are involved in signal transduction (Appel et al., 1995; Holm et al., 1995). The control antibody, 327, is directed against an immunoglobulin-like repeat which is unable to activate signal transduction in cerebellar neurons, and this proved to be true also for dopaminergic neurons.

The neural cell adhesion molecules, L1 and N-CAM, were first shown to influence second messenger systems when triggered with specific antibodies at the surface of PC12 cells (Schuch et al., 1989). These antibodies or the isolated molecules themselves have been further shown to elicit cell type-specific responses that can be modulated by the substrate on which the cells are maintained (von Bohlen und Halbach et al., 1992). Depending on the cell type, treatment with antibodies or CAMs triggers L1 or N-CAM connected pathways, resulting in up- or down-regulation of inositol phosphate turnover, by raising intracellular Ca^{2+} levels, or by an increase or decrease of intracellular pH. Elevation of intracellular cAMP in response to L1 and its antibodies has only been reported in cerebellar neurons (von Bohlen und Halbach et al., 1992). In dopaminergic neurons it does not seem as if cAMP is responsible for the improved survival observed with L1 triggering, since phosphodiesterase inhibitors do not potentiate this response in an exponential manner. Presumably another, as yet uncharacterised pathway is involved.

L1 has been shown to signal via the FGF receptor in experiments where the neurite outgrowth response to L1 was completely blocked by specific antibodies for the FGF receptor (Doherty et al., 1995), and in an FGF-receptor independent manner, by activation of *src*-kinase (Ignelzi et al., 1994). Basic FGF has previously been reported to have beneficial effects on the survival of dopaminergic neurons in vitro and in vivo (Otto and Unsicker, 1990; Date et al., 1993; Chadi et al., 1993). This may be an indirect effect, since when astrocytes are activated with bFGF, they produce a factor or factors which strongly promotes the differentiation of mid-brain dopaminergic neurons in culture (Gaul and Lübbert, 1992). Further evidence for an indirect effect comes from a recent report by Hou et al. (1997) which shows that

bFGF stimulates glia to produce glutathione, and that glutathione is the central factor by which bFGF protects dopaminergic neurons from 6-hydroxydopamine toxicity. Furthermore, the neuro-protective and neurotrophic effects of bFGF in vitro are abolished by inhibition of cell proliferation, which implicates glia rather than neurons (Knüsel et al., 1990; Engele and Bohn, 1991; Park and Mytilineou, 1992; Hou et al., 1997). We have found that while bFGF enhances dopamine uptake, it does not improve the survival of dopaminergic neurons in vitro, nor is it able to protect them from MPP^+ toxicity under culture conditions where cAMP was strongly protective (Hartikka et al., 1992). This suggests that L1 might not be acting on neuron survival through the same pathway as bFGF on neurite outgrowth promotion, as suggested by Williams et al. (1994), since we see a clear improvement in the number of surviving dopaminergic neurons upon triggering of L1. Since the bFGF receptor is not tyrosine phosphorylated upon binding of *N*-CAM antibodies (Beggs et al., 1997), it would be interesting to assess tyrosine phosphorylation status of the bFGF receptor after L1 antibody binding.

Treatment of mid-brain cultures with the L1 antibody 557, at concentrations which clearly improve cell survival in culture, failed to protect against MPP^+ toxicity. This might tie in with our findings that the L1 antibody does not appear to generate a significant cAMP response in combination with a PDE type IV inhibitor (indirectly assessed by measuring cAMP-responsive dopamine uptake). Combined treatment of cultures with PDE-IV inhibitor and L1 antibody caused an additive increase of dopamine uptake, indicating that two separate pathways are being activated. An exponential increase results when PDE-IV inhibitor is combined with forskolin (Hulley et al., 1995), since both substances act on cAMP metabolism, and this combination of substances effectively protects against MPP^+ toxicity. While L1 strongly promoted neuronal survival during the plating of primary cultures, it was not able to protect dopaminergic neurons from MPP^+ toxicity at later stages in culture at the concentrations tested. However, cultures were only treated with antibody for one day prior to MPP^+ exposure, these being the conditions which work with cAMP elevating agents, and it is possible that a longer pretreatment might prove more effective.

The mechanisms by which the L1 antibody 557 promotes the survival of dopaminergic neurons in culture are unclear, but are likely to involve the complex signaling cascades that are emerging for other cell types in the prevention of cell death. In conclusion, recognition of an appropriate cellular environment appears to be very important for neuronal survival and function, both in the embryonic and adult nervous systems, and cell recognition molecules undoubtedly mediate this process.

REFERENCES

Appel F, Holm J, Conscience JF, Schachner M (1993): Several extracellular domains of the neural cell adhesion molecule L1 are involved in neurite outgrowth and cell body adhesion. J Neurosci, 13:4764–4775.

Appel F, Holm J, Conscience JF, von Bohlen und Halbach F, Faissner A, James P, Schachner M (1995): Identification of the border between fibronectin type III homologous repeats 2 and 3 of the neural cell adhesion molecule L1 as a neurite outgrowth promoting and signal transducing domain. J Neurobiol 28:297–312.

Atashi JR, Klinz SG, Ingraham CA, Matten WT, Schachner M, Maness PF (1992): Neural cell adhesion molecules modulate tyrosine phosphorylation of tubulin in nerve growth cone membranes. Neuron 8:831–842.

Beggs HE, Baragona SC, Hemperly JJ, Maness PF (1997): NCAM140 interacts with the focal adhesion kinase p125fak and the *src*-related tyrosine kinase p59fyn. J Biol Chem 272:8310–8319.

Beggs HE, Soriano P, Maness PF (1994): NCAM-dependent neurite outgrowth is inhibited in neurons from *Fyn*-minus mice. J Cell Biol, 127:825–833.

Bixby JL, Harris WA (1991): Molecular mechanisms of axonal growth and guidance. Annu Rev Cell Biol 7:117–159.

Chadi G, Moller A, Rosen L, Janson AM, Agnati LA, Goldstein M, Ogren SO, Pettersson RF, Fuxe K (1993): Protective actions of human recombinant basic fibroblast growth factor on MPTP-lesioned nigrostriatal dopamine neurons after intraventricular infusion. Exp Brain Res 97:145–158.

Date I, Yoshimoto Y, Imaoka T, Miyoshi Y, Gohda Y, Furuta T, Asari S, Ohmoto T (1993): Enhanced recovery of the nigrostriatal dopaminergic system in MPTP-treated mice following intrastriatal injection of basic fibroblast growth factor in relation to ageing. Brain Res 621:150–154.

Doherty P, Williams E, Walsh SH (1995): A soluble chimeric form of the L1 glycoprotein stimulates neurite outgrowth. Neuron 14:57–66.

Engele J, Bohn MC (1991): The neurotrophic effects of fibroblast growth factors on dopaminergic neurons in vitro are mediated by mesencephalic glia. J Neurosci 11:3070–3078.

Gaul G, Lübbert H (1992): Cortical astrocytes activated by basic fibroblast growth factor secrete molecules that stimulate differentiation of mesencephalon dopaminergic neurons. Proc R Soc Lond [Biol] 249:57–63.

Hall H, Walsh FS, Doherty P (1996): Review: A role for the FGF receptor in the axonal growth response stimulated by cell adhesion molecules? Cell Adhes Commun 3:441–450.

Hartikka J, Staufenbiel M, Lübbert H (1992): Cyclic AMP, but not basic FGF, increases the in vitro survival of mesencephalic dopaminergic neurons and protects them from MPP^+-induced degeneration. J Neurosci Res 32:190–201.

Holm J, Appel F, Schachner M (1995): Several extracellular domains of the neural cell adhesion molecule L1 are involved in homophilic interactions. J Neurosci Res 42:9–20.

Hortsch M (1996): The L1 family of neural cell adhesion molecules: old proteins performing new tricks. Neuron 17:587–593.

Hou J-GG, Cohen G, Mytilineou C (1997): Basic fibroblast growth factor stimulation of glial cells protects dopamine neurons from 6-hydroxydopamine toxicity: involvement of the glutathione system. J Neurochem 69:76–83.

Hulley P, Hartikka J, Abdel'Al S, Engels P, Buerki H-R, Wiederhold K-H, Müller T, Kelly P, Lowe D, Lübbert H (1995): Inhibitors of type IV phosphodiesterases reduce the toxicity of MPTP on substantia nigra neurons in vivo. Eur J Neurosci 7:2431–2440.

Ignelzi MA, Miller DR, Soriano P, Maness PF (1994): Impaired neurite outgrowth of *src*-minus cerebellar neurons on the cell adhesion molecule L1. Neuron 12:873–884.

Klinz SG, Schachner M, Maness PF (1995): L1 and *N*-CAM antibodies trigger protein phosphatase activity in growth-cone enriched membranes. J Neurochem 65:84–95.

Knüsel B, Michel PP, Schwaber JS, Hefti F (1990): Selective and non-selective stimulation of central cholinergic and dopaminergic development in vitro by nerve growth factor, basic fibroblast growth factor, epidermal growth factor, insulin and the insulin-like growth factors I and II. J Neurosci 10:558–570.

Lowe JA, Archer RL, Chapin DS, Cheng JB, Helweg D, Johnson JL, Koe BK, Lebel LA, Moore PF, Nielsen JA, Russo LL, Shirley JT (1991): Structure-activity relationship of quinazolinedione inhibitors of calcium-independent phosphodiesterase. J Med Chem 34:624–628.

Moos M, Tacke R, Scherer H, Teplow D, Früh K, Schachner M (1988): Neural cell adhesion molecule L1 as a member of the immunoglobulin superfamily with binding domains similar to fibronectin. Nature 334:701–703.

Otto D, Unsicker K (1990): Basic FGF reverses chemical and morphological deficits in the nigrostriatal system of MPTP-treated mice. J Neurosci 10:1912–1921.

Park TH, Mytilineou C (1992): Protection from 1-methyl-4-phenylpyridinium (MPP^+) toxicity and stimulation of regrowth of $MPP(^+)$-damaged dopaminergic fibers by treatment of mesencephalic cultures with EGF and basic FGF. Brain Res 599:83–97.

Schuch U, Lohse MJ, Schachner M (1989): Neural cell adhesion molecules influence second messenger systems. Neuron 3: 13–20.

von Bohlen und Halbach F, Taylor J, Schachner M (1992): Cell type-specific effects of the neural adhesion molecules L1 and N-CAM on diverse second messenger systems. Eur J Neurosci 4:896–909.

Williams EJ, Furness J, Walsh FS, Doherty P (1994): Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, *N*-CAM, and *N*-cadherin. Neuron 13:583–594.

Williams EJ, Furness, Walsh FS, Doherty P (1994): Characterisation of the second messenger pathway underlying neurite outgrowth stimulated by FGF. Development 120:1685–1693.

Wong EV, Kenwrick S, Willems P, Lemmon V (1995): Mutations in the cell adhesion molecule L1 cause mental retardation. Trends Neurosci 18:168–172.