**β1 integrins activate a MAPK signalling pathway in neural stem cells that contributes to their maintenance**

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**Summary**

The emerging evidence that stem cells develop in specialised niches highlights the potential role of environmental factors in their regulation. Here we examine the role of β1 integrin/extracellular matrix interactions in neural stem cells. We find high levels of β1 integrin expression in the stem-cell containing regions of the embryonic CNS, with associated expression of the laminin α2 chain. Expression levels of laminin α2 are reduced in the postnatal CNS, but a population of cells expressing high levels of β1 remains. Using neurospheres – aggregate cultures, derived from single stem cells, that have a three-dimensional architecture that results in the localisation of the stem cell population around the edge of the sphere – we show directly that β1 integrins are expressed at high levels on neural stem cells and can be used for their selection. MAPK, but not PI3K, signalling is required for neural stem cell maintenance, as assessed by neurosphere formation, and inhibition or genetic ablation of β1 integrin using cre/lox technology reduces the level of MAPK activity. We conclude that integrins are therefore an important part of the signalling mechanisms that control neural stem cell behaviour in specific areas of the CNS.

Supplemental data available online

Key words: Extracellular matrix, Laminin, Fibronectin, Neurosphere, Cre/lox, Stem cell niche, Ventricular zone, Subventricular zone

**Introduction**

Neural stem cells are defined as cells able to undergo self-renewing divisions, and form both neurones and the two classes of glial cell in the CNS, astrocytes and myelin-forming oligodendrocytes. Studies using cell culture at clonal densities have established that these cells are present in both the embryonic (Kilpatrick and Bartlett, 1993) and the adult (Reynolds and Weiss, 1992) mouse forebrain. Although the majority of neuroepithelial cells in early embryonic development have the properties of stem cells, within the postnatal and adult CNS these cells appear to be restricted largely to the subventricular zone (SVZ) adjacent to the lateral ventricles. This zone contains two specific populations that have been suggested to act as neural stem cells: the ependymal cells (Johansson et al., 1999) and a subpopulation of astrocytes capable of giving rise to neuroblasts (Doetsch et al., 1999). A major role for these cells in the rodent is the generation of olfactory neuronal precursors that migrate along the rostral migratory stream. Studies showing increased levels of proliferation within the SVZ following demyelination injuries in adjacent white matter also suggest an important function in the generation of cells for neural repair (Nait-Oumesmar et al., 1999; Decker et al., 2002).

Although a number of the transcription factors that control neural stem cell differentiation into neurones and glia have been identified (Kintner, 2002), the crucial mechanisms that maintain the neural stem population within the SVZ throughout life remain undefined. An important contribution to the maintenance of stem cells in other developmental systems is made by extracellular signals present in specific microenvironments or ‘niches’ (Watt and Hogan, 2000; Spradling et al., 2001). So, for example, BMPs and their analogues have been shown to maintain germline stem cells in mice (Lawson et al., 1999; Ying et al., 2000) and *Drosophila* (Xie and Spradling, 1998). The instructive potential of such extracellular signals for neural stem cells is illustrated by the finding that oligodendrocyte precursor cells can revert back to a stem cell state when exposed to appropriate growth factors (Kondo and Raff, 2000). These experiments highlight the need to identify the ‘niche’ signals within the SVZ and those cell-surface receptors present on neural stem cells that are required for their recognition.

In addition to growth factors, another potentially important class of signals are those provided by extracellular matrix (ECM) molecules recognised by integrin receptors. Integrin signalling pathways are instructive for cell migration, proliferation, differentiation and survival. These pathways interact with those downstream of growth factor receptors, so providing coordinated regulation of cell behaviour by growth factors and the ECM (Yamada and Even-Ram, 2002). Integrins
are heterodimers of two transmembrane chains, α and β. The β1 subunit is widely expressed and can heterodimerize with at least 12 different α subunits, generating integrins with differing ligand specificities (Hynes, 1992). Integrins containing the β1 subunit regulate epidermal stem cell maintenance (Jensen et al., 1999; Zhu et al., 1999; Raghavan et al., 2000). The higher expression of the laminin receptor α6β1 has been implicated in the maintenance of mouse spermatogonial stem cells (Shinohara et al., 1999) and human embryonic stem cells (Xu et al., 2001). Together, these results suggest that integrin signalling may also play a role in neural stem cell maintenance. We have shown previously that neural precursor cell populations containing stem cells express a number of β1 integrins (Jacques et al., 1998). Here we have examined the function of these β1 integrins in neural stem behaviour. We have determined the distribution of β1 integrins and extracellular matrix ligands within the germinal neuroepithelium during CNS development, and have used cell culture assays to demonstrate that MAPK-dependent signalling pathways downstream of these integrins contribute to neural stem cell maintenance.

**Materials and methods**

**Reagents and antibodies**

FGF2 was obtained from PeProtech, EGF from Calbiochem and B27 supplement from Gibco Life Technologies. All pharmacological signalling pathway inhibitors were obtained from Sigma (wortmannin) or Calbiochem (LY294002, PD98059, AG1478 and U0126/4). Monoclonal anti-integrin β1 antibodies were obtained from Pharmingen (Ha2/5) and from Chemicon (MB1.2). Polyclonal antisera against β1 were obtained from Chemicon or, for the double-labelling experiments with anti-nestin, as a kind gift from Professor Charles Streuli (Manchester). Antibodies against the EGFR receptor were obtained from Santa Cruz and Upstate Biotechnology. MAPK and phosphorylated MAPK antibodies were purchased from New England Biolabs, and the Akt and Phospho-Akt (serine 473) were from Cell Signalling Technology. The following antibodies were used for immunohistochemistry: monoclonal anti-Nestin (Pharmingen) and anti-β3 tubulin (Sigma); and polyclonal anti-Fibronectin (Dako), anti-GFAP (Dako), anti-laminin 1 (Sigma), anti-laminin α2 chain (Santa Cruz) rabbit antiserum and anti-phospho-histone H3 rabbit antiserum (Upstate Biotech). All fluorescent secondary antibodies were obtained from Jackson Immunochemicals. TUNEL was performed using the Apoptag kit from Intergen.

**Neurosphere culture preparation**

Primary cultures were prepared from newborn rats and mice (postnatal day 0-2; P0-P2) (Jacques et al., 1998). Briefly, spheres of neural precursors were grown in EGF or FGF2 (20 ng/ml) from dissociated P0-P2 rat forebrain in DMEM/Hams-F12 supplemented with B27 (Shinohara et al., 1999) and human embryonic stem cells (Xu et al., 2001). Together, these results suggest that integrin signalling may also play a role in neural stem cell maintenance. We have shown previously that neural precursor cell populations containing stem cells express a number of β1 integrins (Jacques et al., 1998). Here we have examined the function of these β1 integrins in neural stem behaviour. We have determined the distribution of β1 integrins and extracellular matrix ligands within the germinal neuroepithelium during CNS development, and have used cell culture assays to demonstrate that MAPK-dependent signalling pathways downstream of these integrins contribute to neural stem cell maintenance.

**Western blots**

For protein expression analysis neurospheres were lysed in lysis buffer (10 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl buffer and 1% Triton X-100) containing proteases and phosphatase inhibitors (5 μg/ml leupeptin, 2 μg/ml aprotinin, 2 mM PMSF, 1 μg/ml pepstatin, 2 mM sodium fluoride, 2 mM sodium vanadate; all from Sigma). The supernatant was clarified by centrifugation at 16,000 g for 20 minutes at 4°C. Protein concentrations in the supernatant were determined using a Bio-Rad protein assay, with BSA as a standard. For western blotting, 20 μg of protein was loaded in each condition. Proteins were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes (Hybond-C, Pharmacia). Membranes were blocked in 10% non-fat dry milk in Tris-buffered saline (TBS) for 1 hour at room temperature. Blots were then incubated with the primary antibodies overnight at 4°C in milk-TBS containing 0.1% Tween-20 (TBS-T), followed by a 2 hour incubation with the appropriate secondary peroxidase-conjugated antibody (Amersham). To visualize the immunoreactive proteins the ECL kit was used, following the manufacturer’s instructions (Amersham).

**Fluorescence activated cell sorting (FACS) of neural stem cells**

Neurospheres were dissociated and incubated with the FITC-conjugated Ha2/5 monoclonal antibody (Pharmingen) in PBS at 4°C for 30 minutes; this antibody was used previously to study β1 expression levels in fetal liver cells (Suzuki et al., 2000). Control cells were incubated in PBS only. The cells were then counter-stained with propidium iodide (5 μg/ml, Sigma) for live/dead discrimination and sorted through a MoFlow (Cytomation). Non-FITC fluorescent cells were excluded from the sorting. Amongst the fluorescent cells (of either EGF- or FGF2-grown neurospheres) we defined two groups: a group of very strongly fluorescent cells and a group of cells with intermediate levels of labelling. The same number of events was then sorted from each population. Five thousand cells were deposited in each well of a 24-well plate. The cells isolated by FACS were then grown for a week in the appropriate medium and the number of secondary neurospheres formed for each condition was counted. These experiments were done five times for EGF- and four times for FGF2-grown neurospheres. Statistical significance was determined using Student’s t-test.

**Serial dilution assays**

Neurospheres were completely dissociated, and 4000 cells were re-suspended in 400 μl of culture media with EGF or FGF2. Dilution assays were set up on 96-well plates (Iwaki) as follows: 200 μl of the initial 400 μl containing the 4000 cells was transferred to the adjacent well, which already contained 200 μl of media. From this second well another 200 μl was collected and transferred. This operation was repeated for all the wells in each line creating a range of cell concentrations from 2000 (first well) to 1-2 cells (twelfth well). Each individual experiment was performed in triplicate, with the exception of the experiments using U0126/4 which were performed in duplicate. Serial dilutions were set up in the presence of the EGF receptor inhibitor AG1478 (20 μM), MAPK inhibitors (PD98059, 50 μM; U0126/4, 100 μM), the p38 MAPK inhibitor SB203580 (50 μM) and PI3K inhibitors (wortmannin, 50 nM; LY294002, 2.5 μM). After one week, the newly formed neurospheres were counted in each well and plotted against the initial number of cells per well. The resulting slope of the line was used to compare the different experimental conditions. Statistical analysis of the slopes of the regression lines was performed using Student’s t-test.

**Conditional knock-out of β1 in neurospheres**

Neurospheres were prepared from P0 mice bred to have either the floxed β1/null or floxed β1/wild-type (wt) phenotype (by crossing mice carrying the floxed allele with those heterozygous for a null allele of β1), and were grown in both EGF and FGF2 (20 ng/ml). The floxed β1 allele was generated as previously described (Brakebusch et al., 2000). After 10 days in culture, the neurospheres were dissociated, cells were infected with an adenoviral vector expressing cre recombinase (Kalamardies et al., 2002) (a kind gift from Dr Marco Giovannini, INSERM U434, Paris) and then replated in the same growth factors. The culture medium was changed after 3 days to medium without adenosine. Recombination was confirmed by the expression of β-galactosidase in the newly-formed neurospheres, as excision of the β1 gene activates a lacZ reporter.
gene in the floxed allele. The number of entirely blue neurospheres formed by the floxed β1/null was then compared with the number of entirely blue neurospheres made by the control floxed β1/wt for an equal number of plated cells (3000 cells/well of a 24-well plate). Neurospheres in which excision had not occurred or was incomplete (as evidenced by cells not expressing β-galactosidase) were not included in the assay. For the experiments analysing MAPK phosphorylation, neurospheres generated from floxed β1/null or floxed β1/wt cells were passaged at least ten times and then incubated overnight in the EGF receptor inhibitor AG1478 (20 μM), in the presence of 20 ng/ml EGF and FGF2. The spheres were then lysed as above and western blot analysis performed using MAPK and phosphorylated MAPK antibodies, as described above. Equal loading was confirmed by protein quantification. The gels were scanned and quantified using the NIH Image 1.62 software, with the ratio of the band intensities of total MAPK and phosphorylated MAPK calculated for each neurosphere cell line, with and without the inhibitor. Three cell lines of each genotype were analysed. All samples were run on a single gel and processed simultaneously, with the total MAPK analysis performed by stripping the membrane after the P-MAPK analysis. Student’s t-test was used to compare the ratio of band intensities following exposure to AG1478 in both of the two genotypes.

**Immunohistochemistry and cell counts**

For proliferation assays BrdU was added to the culture media (10-20 μmol/l) 30 minutes, 3 hours or 24 hours before fixation. Neurospheres and neonatal or embryonic brain tissue were then fixed in 2-4% paraformaldehyde in PBS (phosphate buffered saline). The samples were then transferred to a 25% solution of sucrose (w/v) and left overnight at 4°C. Cryostat sections (14 μm) were prepared from imbedded brains or neurospheres (TissueTek-Sakura) and used for immunohistochemistry. Sections were blocked in PBS (0.1% Triton X-100) containing normal goat serum and incubated overnight with the appropriate antibodies at 4°C. After incubation with the appropriate secondary antibodies and counter-staining with DAPI, pictures were acquired using a Zeiss fluorescence microscope equipped with a Hamamatsu Orca camera. Images were processed using Open Lab (Improvision).

**Results**

**β1 integrin and ECM expression in germinal neuroepithelium**

Previous studies have shown that stem cells in human skin can be identified by their high levels of β1 integrin expression, as compared with the transit-amplifying keratinocyte precursor cell population (Jensen et al., 1999). To determine whether neural stem cells also express high levels of β1 integrin, we examined the ventricular (germinal) region in embryonic and new-born mouse and rat (Fig. 1). At E12.5 in the mouse (Fig. 1A,B,E), β1 and α6 integrins were expressed throughout this region, with the highest levels immediately adjacent to the ventricle. Similar results were seen in the embryonic rat (data not shown). In postnatal animals, however, only a subpopulation of cells was strongly labelled by antibodies against β1 integrin (Fig. 1L,O). These cells were only present in the lateral wall of the ventricle, and no labelling was seen in the medial wall (Fig. 1O). The cells had an elongated morphology perpendicular to the ventricular surface. Experiments using antibodies against phosphorylated histone-H3 showed mitotic cells within the region expressing high levels of β1 in the E12.5 embryonic mouse ventricular zone (Fig. 1C), with double-labelling showing β1 expression directly on these cells (Fig. 1D-G). Double-labelling

![Image](image-url)
experiments also showed that β1-expressing cells in the pre- and postnatal ventricular zone expressed the stem and precursor cell marker nestin (Fig. 1H–K,L,N), but not the polysialated isoform of NCAM (PSA-NCAM) seen on precursor cells (not shown), from which we conclude that at least some of these β1-expressing cells are neural stem cells.

To determine whether extracellular matrix ligands for β1 integrins were present in the stem cell-containing regions we examined the expression of laminins and fibronectin by immunohistochemistry. Laminin distribution was determined using a polyclonal antibody against all three chains of laminin immunohistochemistry. Laminin distribution was determined using a polyclonal antibody against all three chains of laminin 1-expressing cells in the pre-

β1 integrin and ECM expression in sectioned neurospheres
The location of high-β1-expressing cells reported above corresponds to that described for neural stem cells within the SVZ of the postnatal CNS, as detected by cell proliferation studies (Craig et al., 1996; Morshhead et al., 1998). To determine the relationship between β1 expression and neural stem cells in more detail, we used a three-dimensional culture technique in which neural cells are plated at clonal density on non-adherent substrates in the presence of FGF2 and/or EGF.

Fig. 2. Expression of fibronectin, laminin 1 (L1) and laminin α2 chain (Lα2) in the rat VZ/SVZ. In the rat at E15.5, fibronectin is expressed in a speckled pattern throughout the developing cortex (A), L1 is found around the blood vessels and on the pial surface (B) and laminin α2 shows a condensation in the VZ (C; arrowheads). In the P2 rat brain, fibronectin can be found (D) in the VZ/SVZ, whereas L1 is present only around blood vessels (E) and Lα2 levels are extremely low (F and inset). L1, Lα2 and fibronectin are shown in red, and DAPI-counterstained nuclei are shown in green. All sections are coronal. Scale bar: 20 μm.

In these cultures some of the cells form spheres (neurospheres) which, when dissociated and replated at clonal density, will form new spheres. Following each such passage, cells within the spheres can differentiate into neurones, astrocytes or oligodendrocytes, confirming that the sphere-forming cells represent neural stem cells (Reynolds and Weiss, 1992; Reynolds and Weiss, 1996). Each sphere therefore represents a mixture of stem and committed precursor cells that arise from a single stem cell. We reasoned that these spheres were likely to develop a three dimensional architecture in which stem and precursor cells separate, as seen in the intact embryonic CNS. If so, and if cells expressing high levels of β1 integrin represent a stem cell population, then it follows that these cells will be spatially distinct from those expressing markers of differentiation and will be present in areas of the sphere expressing stem cell markers.

To test these predictions we cut cryostat sections of intact neurospheres and immunostained them with anti-β1 integrin antibodies. These experiments showed that, for spheres prepared from either rat or mouse, cells expressing high levels of β1 were present only on the edge of the sphere (Fig. 3A,B). We then compared this distribution with that of a stem and early precursor cell marker (the intermediate filament protein nestin, Fig. 3B,C) and with that of markers of committed precursor cells for neurones (β3 tubulin, Fig. 3F) and astrocytes (GFAP, Fig. 3D,E). Nestin+ cells were present around the edge of the sphere within the population expressing high levels of β1 integrin (Fig. 3B), whereas the differentiation markers were mainly present inside the sphere (Fig. 3D-F), demonstrating the separation of stem cell and committed precursor populations.

An important feature of the architecture of the intact CNS is that the proliferating stem cell population is distinct from the newly-differentiated post-mitotic neurones present in more superficial regions of the cortex. This is illustrated by a separation of phosphorylated histone H3 or BrdU labelling (both of which identify dividing cells) and β3 tubulin expression (a marker of neuronal differentiation) in the embryonic ventricular region (as shown in Fig. 3G-I). To determine whether the same distinction was also present in neurospheres, the spheres were exposed to short pulses (30 minutes) of BrdU before fixation and sectioning. BrdU incorporation was seen only in cells at the edge of the sphere. These cells did not express markers of neuronal differentiation (Fig. 3K), which were present only in central regions of the sphere, but did express the EGF receptor (Fig. 3L), present on neural stem cells grown in FGF (Lilien and Raphael, 2000). With longer pulses of BrdU (3 and 24 hours) labelled cells were seen further towards the centre of the sphere (not shown).
Integrin β1 function in neural stem cells and the labelling index increased from 12% with a 30-minute exposure to BrdU, respectively. Interestingly the figures of 12 and 24% for the 30-minute and 3-hour exposure to BrdU are in keeping with the figure of 18.5% established for in vivo BrdU incorporation in the VZ/SVZ of E14 embryos exposed to BrdU for 35-40 minutes (Miyata et al., 2001). The appearance of labelled cells within the centre of the sphere is consistent with the differentiation of precursors that have arisen from dividing stem cells as the sphere expands by division of cells around the edge. Division of cells at the periphery of the neurospheres was also detected using antibodies against phosphorylated histone H3 (data not shown), confirming the results obtained with BrdU.

Just as in the intact developing CNS, we found that laminin α2 was present in the region containing the cells that express high levels of β1 (Fig. 4A,D). Laminin 1, by contrast, was expressed in the central regions of the sphere (Fig. 4C), and fibronectin was diffusely localised in a speckled pattern (not shown) similar to that seen in the intact CNS. Together, these observations reveal a distribution of the different molecules within the sphere that mirrors that seen in the intact CNS; the three-dimensional structure of the spheres is summarised in Fig. 4B.

If stem cells are present around the edge of the neurosphere, then changes in growth factor receptor expression normally seen in these cells in vivo should also be observed in this region. To test this prediction, we took advantage of the observation that FGF2 promotes the upregulation of the EGF receptor in neural stem cells (Lillien and Raphael, 2000). In confirmation of these results, when spheres grown in EGF alone were sectioned and immunolabelled with anti-EGF receptor antibodies, they showed low but distinct labelling at the edges of the spheres in the region shown above to contain the EGF receptor-expressing cells (Fig. 5A). However, spheres grown in FGF2 showed much higher levels of EGF receptor expression at the edge of the sphere (Fig. 5B), once again in the region containing the high-β1-expressing cells. We also
obtained increased levels of EGF receptor expression in FGF2 treated spheres when analysed by western blotting (Fig. 5C). Taken together, we conclude from these results that neurospheres do have a distinct three-dimensional architecture with the stem cell population around the edge, in a region containing laminin α2 and β1 integrin-expressing cells, as observed in the intact developing CNS, whereas the more differentiated cells lie within the central regions of the sphere.

**Neurosphere formation by cells expressing high or intermediate levels of β1 integrin**

If the cells around the edge of the neurospheres expressing high levels of β1 are neural stem cells, then we would predict that these cells will be more likely to form neurospheres [which derive from a single stem cell in clonal density cultures (Reynolds and Weiss, 1996)] than cells expressing lower levels of β1 integrin. To test this predicted relationship between β1-expressing cells and neurosphere formation, we performed immunofluorescent labelling experiments and FACS analysis on dissociated neurosphere cells grown in either FGF2 or EGF. As expected from the immunofluorescent pattern seen in the sectioned neurospheres, antibody labelling of cells dissociated from neurospheres revealed the presence of a small subpopulation of cells expressing higher levels of β1 integrin (Fig. 6A,B). The FACS profiles confirmed the presence of cells expressing high levels of β1, and also revealed that β1 expression levels were higher in the EGF-grown spheres than those grown in FGF2 (also shown by western blotting of neurosphere lysates, as shown in Fig. 5). To examine neurosphere formation potential, we selected a defined number of cells from the population comprising the 5% of cells with the greatest levels of expression (boxes 1 and 3 in Fig. 6C and D, respectively) and carried out neurosphere-forming assays to measure the number of stem cells by plating the cells at low density (4000 cells/cm²). For comparison, the same number of cells were selected from the population expressing intermediate levels of β1 (boxes 2 and 4 in Fig. 6C and D, respectively) and were grown in each condition. There were no significant differences in the viability of the cell populations immediately after sorting, as assessed by trypan blue exclusion (EGF: high β1 expression 5.6±3.7%; intermediate β1 expression, 7.55±2.15%. FGF: 6.35±0.75% and 7.25±2.55%, respectively). However significantly more spheres formed from the high β1-expressing population after one week in culture in either growth factor (Fig. 6E), confirming that cells in this population have a higher probability of behaving like neural stem cells than cells expressing intermediate levels of β1 integrin. Both groups, however, showed a higher potential to form spheres than unselected cell populations also containing cells expressing low levels of β1 integrin. In two separate experiments in which we compared the sphere forming potential of high-β1-expressing and intermediate-β1-expressing cells with the whole (ungated) population (after exclusion of non-viable cells as described in Materials and methods, and as illustrated in the supplementary figure, see Fig. S1 at http:dev.biologists.org/ supplemental), we found that in EGF high-β1-expressing cells formed 12 times more spheres than the ungated group. Likewise, EGF-grown intermediate-β1-expressing cells generated four times more secondary spheres than the ungated group. For FGF-grown spheres, high-β1-expressing cells generated five times more spheres than the ungated population.
Integrin β1 function in neural stem cells

...and the intermediate-β1-expressing cells generated 3.4 times more neurospheres than the ungated population.

**A MAPK signalling pathway is essential to neural stem cell maintenance**

The demonstration that EGF receptor expression and β1 integrin co-distribute at the edge of the neurospheres raises the possibility that shared downstream signalling molecules might be important in the regulation of neural stem cell behaviour. One candidate is MAPK, shown to play a role in the maintenance of the human skin stem cells (Zhu et al., 1999). To determine whether MAPK is activated in neural stem cells, we immunolabelled neurosphere sections with an antibody against phosphorylated MAPK. Bright labelling was seen only
at the edge of the sphere with cells grown in either FGF2 or EGF alone (Fig. 7A). Activated MAPK was also observed by western blotting lysates prepared from the neurospheres (not shown, but see Fig. 8). In order to establish the role of MAPK signalling in neural stem cell maintenance we used pharmacological inhibitors. To quantify the effects of these inhibitors we used serial dilutions assays rather than plating cells at low density. In these assays, a fixed number of cells was progressively diluted in adjacent wells of a 96-well plate, and the number of spheres that formed in each well was counted in the presence of inhibitors of p42,44 MAPK, p38 MAPK, PI3K or the EGF receptor. The very low plating density in the high dilution wells ensures that spheres represent growth from a single cell and not an aggregation of cells. As shown in Fig. 7, log-log plots of the data from individual experiments confirmed that, for all dilutions at which one or more spheres form, the relationship between the number of cells plated and the number of spheres formed was constant, as evidenced by the straight line in the graph. We conclude from this that the ability of the stem cells to form spheres is independent of plating density and that regression lines calculated from the linear plots accurately reflect cell behaviour at all densities. The validity of the regression lines to represent the data from these assays is confirmed by R² values of >0.85 for all experimental conditions. When neurospheres were dissociated and cells were replated in the serial dilution assay in the presence of the MAPK p42,44 pathway inhibitor PD98059, and grown in either FGF2 or EGF, a reduction in the number of cells able to form new neurospheres was seen, as evidenced by the reduced slope shown in Fig. 7E,F. For cells grown in EGF alone the mean slope of the regression lines from three experiments was 0.028±0.002 (mean±s.e.m., R²=0.94, n=11), as compared with 0.006±0.001(R²=0.855) for cells grown in EGF and PD98059 (P<0.001, n=6). For cells grown in FGF the values were 0.05±0.01 (R²=0.99) and 0.016±0.011 (R²=0.99), respectively (P<0.05, n=5). For cells grown in EGF, the EGF receptor inhibitor decreased sphere formation (0.01±0.003, R²=0.94, P<0.001, n=6) but this inhibitor had a much smaller, although still significant, effect on cells grown in FGF (0.03±0.005, R²=0.95, P<0.05, n=3). Inhibitors of PI3K (wortmannin, LY294002) or p38 MAPK (SB203580) had no effect on sphere formation (Fig. 7). A significant reduction was also observed in separate experiments when the cells were exposed to another MAPK p42,44 pathway inhibitor, U0126 (EGF, 0.05±0.021; EGF + U0126, 0.024±0.012; n=2, P<0.05), whereas the control U0124 compound had no effect (Fig. 7). The results with the PI3K inhibitors suggest that the reduction in sphere formation seen in the presence of MAPK inhibition does not simply reflect perturbation of signalling pathways required for proliferation and sphere formation, as the increased proliferation seen in neurospheres derived from PTEN/+ mice demonstrates a role for PI3K in neural stem/precursor cell proliferation (Groszer et al., 2001).

**β1 integrins activate MAPK in neural stem cells**

Having established (1) that β1 integrins are expressed at a high level on neural stem cells, (2) that at least one extracellular ligand (laminin α2 chain containing laminin) is present in the stem cell-containing regions of the developing CNS, and (3) that MAPK signalling is required for stem cell maintenance,
we next investigated whether β1 integrin signalling activated MAPK in neural stem cells. To do this, we used blocking antibodies and genetic techniques. For the blocking antibody studies, we compared the intensity of the phosphorylated MAPK band in western blotting experiments with or without a monoclonal anti-β1 integrin-blocking antibody present in the neurosphere culture medium. These experiments showed a partial inhibition of the phosphorylation of MAPK in spheres grown in both FGF2 and EGF (Fig. 8A), demonstrating a role for β1 integrins in MAPK signalling.

For the genetic studies, we used cre/lox technology to remove the β1 gene from neurosphere cells and determine the effect on MAPK activation. This technology has been used previously to excise β1 integrin efficiently from chondrocytes (Iba et al., 2000). Mice containing a floxed β1 allele (Fassler and Meyer, 1995; Brakebusch et al., 2000) generated by homologous recombination were bred with heterozygous β1-null mice to generate animals in which the second allele was either β1-null or wild type. Neurosphere cultures were prepared from these animals and grown in EGF and FGF2. The spheres were dissociated, replated at a density of 10 cells/μl and exposed to an adenoviral vector expressing cre recombinase. Excision of the floxed β1 gene was confirmed by the activation of a reporter lacZ gene inserted downstream of the β1 allele, which is activated only following excision of the β1 sequence (Brakebusch et al., 2000; Potocnik et al., 2000). Spheres derived from stem cells in which excision had occurred could be identified by the presence of β1-galactosidase staining in all cells. The reduction in β1 integrin following excision was confirmed in three ways. First, neurospheres exposed to the adenoviral vector but not yet dissociated and replated were sectioned as above and immunolabelled with anti-β1 antibodies. Second, lysates from treated and control spheres were used in western blotting experiments. Third, FACS analysis was performed to detect changes in the level of β1 expression. As shown in Fig. 8, following gene excision we observed a reduction in β1 integrin immunolabelling around the edge of the spheres (Fig. 8B–E), a reduction in β1-labelling levels in the FACS analysis (Fig. 8F,G) and a reduction in the intensity of the β1 integrin band, as revealed by western blotting (Fig. 8H). Having confirmed the excision procedure, we next performed western blotting experiments using antibodies against phosphorylated MAPK. These showed a reduction in MAPK activation in primary neurospheres exposed to the cre-expressing adenovirus (Fig. 8I). However, subsequent passages of the β1-excised spheres showed normal levels of activated MAPK (Fig. 8J, Fig. 9), even though the presence of β1-galactosidase in these spheres and a shift to the left in the FACS analysis of β1 expression confirmed that these spheres derived from cells in which gene excision had occurred (data not shown). We also counted the number of neurospheres that formed when these cells were plated at low density. No differences were seen in the number of spheres in these assays, or when secondary and subsequent passaging of the spheres was performed.

We reasoned that an alternative signalling pathway could be responsible for the lack of any sustained effect of β1 excision on the levels of activated MAPK. As the EGF receptor is known to activate MAPK (Schlessinger, 2000) in neural stem cells (Learish et al., 2000), and because we observed reciprocity between the levels of β1 and EGF receptor (Fig. 5) in neurospheres, it is possible that upregulated growth factor signalling via MAPK can compensate for the loss of β1. To test this hypothesis we added the EGF receptor inhibitor AG1478 to loxβ1/null and loxβ1/wt neurospheres grown in EGF and FGF after exposure to the adenoviral cre vector. We predicted that this would expose upregulation of EGF signalling in response to the loss of β1, by revealing a greater decrease in MAPK activation in the β1-excised cells than in cells expressing β1 following EGF receptor inhibition. We therefore established three neurosphere cell lines from different loxβ1/null and loxβ1/wt mice.

Each line was passaged at least ten times following cre-mediated excision, by which time MAPK levels had returned to the levels seen in the β1-expressing spheres (Fig. 8I). After an overnight exposure to the inhibitor, levels of total MAPK and phosphorylated MAPK (P-MAPK) were analysed by western blotting (Fig. 9A,B). A marked decrease in P-MAPK was observed in the β1-excised cells, in contrast to the β1-expressing cells (Fig. 9A,B). Analysis of all six lines showed a significant reduction in the P-MAPK/total MAPK ratio in the β1-excised cells (1.05±0.08 to 0.84±0.01 in the presence of AG1478, P=0.005), but no significant reduction in the β1-expressing cells (1.22±0.15 to 1.23±0.22 in the presence of AG1478), consistent with an increased dependence of MAPK activation on EGF signalling in the β1-deficient cells, and suggesting that upregulation of growth factor signalling contributes to the compensation for the loss of β1 integrin. The lack of viability and absence of any sphere formation when cells were grown at high dilutions in the presence of the EGF receptor inhibitor (Fig. 7D) prevents the comparison of the β1-expressing and β1-deficient cells grown in EGF required to analyse whether this compensatory upregulation is associated with an increased dependence on EGF signalling for maintenance. However maintenance in the presence of FGF2 was not altered by the addition of the inhibitor to β1-excised cells (data not shown), showing that self-renewal in response to other growth factors was not reduced.

**Fig. 9.** (A) Western blot of lysates from floxed β1/wt (right lane) and floxed β1/null (left two lanes) neurospheres after more than ten passages, showing that MAPK is phosphorylated equally in both groups. (B) Western blot of lysates from floxed β1/wt (right) and floxed β1/null (left) neurospheres (the same cultures as in A) exposed to AG1478 (20 μM), an inhibitor of the EGF receptor. This panel shows that MAPK phosphorylation is reduced to a greater extent in the null spheres (floxed β1/null) than in the floxed β1/wt in the presence of the inhibitor. AG. AG1478. WT represents floxed β1/wt and Null represents floxed β1/null spheres following exposure to cre recombinase.
Discussion

In the present work we have made the following principal observations. First, laminins and fibronectin are expressed in different locations of the embryonic and postnatal germlinal neuroepithelium. The distribution of laminin α2 present in laminins 2, 4 and 12 corresponds to the region immediately adjacent to the ventricle (the ventricular and subventricular zones) containing the neural stem cells. Second, β1 integrin is also highly expressed by cells in the ventricular region of the embryonic CNS, and by a subpopulation of cells in this region of the postnatal CNS. Third, we have demonstrated a three-dimensional architecture of neurospheres with cells expressing nestin and the EGF receptor, both molecules present in neural stem cells, being found at the outside edge of the sphere. Both laminin α2 and β1 integrin are highly expressed by cells in this region, with other ECM molecules present within the sphere rather than around the edge. Fourth, neurosphere cells expressing higher levels of β1 can be selected by FACS and will generate relatively more neurospheres than cells expressing lower levels of the integrin, so demonstrating stem cell properties. Fifth, we have shown, by using pharmacological inhibitors, that MAPK is required for neural stem cell self-renewal, as assessed by neurosphere generation. Finally, we have shown by antibody-blocking and genetic techniques that β1 integrin signalling activates MAPK, although the reduction observed in the blocking experiments is not sustained and is associated with a compensatory increase in growth factor signalling. Together these results indicate an important role for β1 integrin/extracellular matrix molecule interactions in the regulation of neural stem behaviour.

How might β1/ECM interactions affect stem cell behaviour? Increased adhesion to niche ECM components could ensure that the cell is held in the niche and so exposed to other extracellular cues that instruct stem cell maintenance. One example of such extracellular cues is provided by the Drosophila protein Upd, which is secreted by hub cells in the testis and contributes to the maintenance of germline stem cells by activation of the JAK/STAT signalling pathway (Kiger et al., 2001). Upd binds to the extracellular matrix (Harrison et al., 1998) and previous studies using embryonic mosaics to examine the role of Upd in segmentation have noted that upd mutant cells could behave normally only when immediately adjacent to wild-type cells (Gergen and Wieschaus, 1986), suggesting a limited range of diffusion as a result of tethering to the matrix. Within the testis germ cell niche, the matrix could limit the range of Upd diffusion from the hub cell ensuring that only adjacent germline stem cells held in the niche by appropriate adhesion molecules respond to this signal.

In addition to a role in simply holding the stem cells within a niche, our results point to a direct signalling role for β1 integrins in neural stem cell regulation, as we have shown that self-renewal, or maintenance, in neural stem cells is partly regulated by β1 integrins and growth factors through a MAPK signalling pathway. A similar role for MAPK in stem cell maintenance has been previously described in human epidermal stem cells (Zhu et al., 1999). The interaction between integrin and growth factor signalling, well described in other cell types (Lee and Streuli, 1999; Renshaw et al., 1999; Byzova et al., 2000; Yamada and Even-Ram, 2002), provides a mechanism by which neural stem cell behaviour can be regulated by local ECM molecules, as well as by longer range growth factor signals. Our results indicate a novel integrative role of MAPK that may be important in allowing populations of stem cells to change behaviour during development and repair in response to changes in the growth factor environment, while at the same time allowing individual stem cells to be finely tuned by their immediate microenvironment. Such signalling mechanisms with the potential for compensation between integrin and growth factor signalling pathways may also be operative in other stem cell types; for example, studies of haematopoietic stem cells in mice chimeric for β1 null cells show that β1 integrin is not required for stem cell maintenance, but is necessary for the homing of these cells to the liver (Hirsch et al., 1996).

The conclusion that β1 integrins regulate neural stem cell behaviour within the CNS niche leads us to question the nature of the ligands recognised by these integrins. One important group of candidates are the laminins, which are recognised by at least four β1 integrins, α1β1, α3β1, α6β1 and α7β1. α6β1 expression has previously been shown in other stem cell systems (Shinohara et al., 1999, Xu et al., 2001), and in RT-PCR experiments we have found all four alpha subunits to be expressed in neural stem cells (J. Moore and Cff.-C., unpublished). Laminins are expressed from the earliest stages of development, and have been shown to maintain human ES cells in an undifferentiated state (Xu et al., 2001). Proximity to a basement membrane is a feature of epithelial and germ cell niches, and transplantation experiments using reconstitution of spermatogenesis as an assay reveal that stem cells can be enriched from testis cell populations by selecting either for laminin binding or for expression of β1 or α6 integrin subunits (Shinohara et al., 1999). In this paper, we have described the expression of the laminin α2 chain in the developing germininal zone and also around the edge of the neurospheres in the regions containing the stem cell populations. Laminins containing this chain therefore provide a potential β1 integrin ligand for neural stem cells that is likely, by analogy with the ES cell and spermatogonial stem cell systems, to play a role in the control of neural stem cell behaviour. Furthermore, changes in the expression levels of laminin α2 provide an additional mechanism for the regulation of neural stem cell behaviour. We observe a decline in laminin α2 expression in the postnatal brain that may play an instructive role in the reduced level of proliferation within the germinal neuroepithelium at that time, as laminin can increase the proliferation of neuroepithelial cells (Drago et al., 1991a). Equally, the increase in laminin chain mRNAs seen within neuroepithelial cells exposed to bFGF may contribute to the mitogenic effects of the growth factor (Drago et al., 1991a; Drago et al., 1991b).

The importance of extrinsic signals in the regulation of stem cell behaviour is emphasized by the argument that, “rather than referring to a discrete cellular entity, a stem cell most accurately refers to a biological function” (Blau et al., 2001). Our results point to a complex interplay between integrin and growth factor signals in the genial neuroepithelium being important regulators of this function, with MAPK being a key integrative signalling molecule. For future studies examining the developmental significance and therapeutic potential of these interactions, our work highlights the value of the analysis of neurosphere structure. The morphological observations described above suggest that neurospheres derived from postnatal brains can be used as a model for the
developing (midgestation) neuroepithelium. These spheres develop a three-dimensional structure that is remarkably similar in ECM composition and distribution of cellular phenotypes to the developing CNS. In particular the edge of the neurosphere is a complex niche, easily accessible and amenable to acute biochemical manipulation and analysis using inhibitors and blocking antibodies that will greatly facilitate further work.

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References


A representative experiment showing the FACS profiles and gates used to compare the sphere-forming potential of cells grown in EGF (A) or FGF (C) and sorted for high β1 expression (gate R2) or intermediate β1 expression (gate R3) with unsorted cells grown in EGF (B) or FGF (D) from which a single gate (high propidium iodide (PI)-RS) was used to remove dead cells. See text for results.