An inhibition of cyclin-dependent kinases that lengthens, but does not arrest, neuroepithelial cell cycle induces premature neurogenesis

Federico Calegari and Wieland B. Huttner*

Max Planck Institute of Molecular Cell Biology and Genetics, Pfotenhauerstrasse 108, D-01307, Dresden, Germany
*Author for correspondence (e-mail: huttner@mpi-cbg.de)

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Summary

The G1 phase of the cell cycle of neuroepithelial cells, the progenitors of all neurons of the mammalian central nervous system, has been known to lengthen concomitantly with the onset and progression of neurogenesis. We have investigated whether lengthening of the G1 phase of the neuroepithelial cell cycle is a cause, rather than a consequence, of neurogenesis. As an experimental system, we used whole mouse embryo culture, which was found to exactly reproduce the temporal and spatial gradients of the onset of neurogenesis occurring in utero. Olomoucine, a cell-permeable, highly specific inhibitor of cyclin-dependent kinases and G1 progression, was found to significantly lengthen, but not arrest, the cell cycle of neuroepithelial cells when used at 80 μM. This olomoucine treatment induced, in the telencephalic neuroepithelium of embryonic day 9.5 to 10.5 mouse embryos developing in whole embryo culture to embryonic day 10.5, (i) the premature up-regulation of TIS21, a marker identifying neuroepithelial cells that have switched from proliferative to neuron-generating divisions, and (ii) the premature generation of neurons. Our data indicate that lengthening G1 can alone be sufficient to induce neuroepithelial cell differentiation. We propose a model that links the effects of cell fate determinants and asymmetric cell division to the length of the cell cycle.

Key words: Neurogenesis, Olomoucine, TIS21, Whole embryo culture

Introduction

Asymmetric cell division has a fundamental role in the development of multicellular organisms. There are two, principally different, definitions of asymmetric cell division, (i) divisions defined as asymmetric because the daughter cells acquire different fates, and (ii) divisions defined as asymmetric because certain cellular components are distributed unequally between the daughter cells (Horvitz and Herskowitz, 1992). While there are paradigmatic examples showing that an unequal distribution, during mitosis, of cellular components capable of influencing cell fate (cell fate determinants) can, in fact, result in distinct fates of the daughter cells (for a review, see Knoblich, 2001), such unequal distribution does not always have this effect and, conversely, distinct daughter cell fates do not necessarily depend on such unequal distribution. The question arises as to why this is so.

The spatial organization of cells, both with regard to intracellular structure and extracellular environment, is of crucial importance for asymmetric cell division. Thus, asymmetric divisions resulting from an unequal distribution of cell fate determinants during mitosis is based on a polarized intracellular localization of these determinants (Knoblich, 2001). Likewise, distinct daughter cell fates can be brought about by differences in the local environment that daughter cells become exposed to (Horvitz and Herskowitz, 1992).

Besides *spatial* aspects, however, one should also consider *temporal* aspects. For example, it is conceivable that an unequal distribution of cell fate determinants during mitosis may or may not result in distinct daughter cell fates depending on whether or not these cell fate determinants are able to act long enough to differentially influence the daughter cells. Similarly, the length of exposure of two, initially identical, daughter cells to a fate-influencing environment may determine whether or not these adopt distinct fates.

These considerations are particularly relevant for the cell divisions that occur in the neuroepithelium during mammalian embryonic development and that produce the neurons of the central nervous system. At the onset of neurogenesis, a fraction of neuroepithelial (NE) cells shifts from proliferative to neuron-generating divisions (Caviness et al., 1995; McConnell, 1995; Rakic, 1995; Livesey and Cepko, 2001). The latter are thought to be asymmetric, resulting from a polarized intracellular localization of cell fate determinants along the apical-basal axis of NE cells and their unequal distribution to the daughter cells during mitosis (Chenn and McConnell, 1995; Huttner and Brand, 1997; Fishell and Kriegstein, 2003).

Remarkably, however, concomitant with NE cells switching from proliferative to neuron-generating divisions, the length of the G1 phase of their cell cycle increases (Takahashi et al., 1995). Moreover, the first marker shown to be selectively expressed in neuron-generating, but not proliferating, NE cells, TIS21 (Iacopetti et al., 1999), is an inhibitor of G1 progression (Matsuda et al., 2001; Tirone, 2001). These observations suggest some link between the length of the cell cycle, in

particular its G1 phase, and the switch of NE cells from proliferation to neurogenesis. We have investigated the hypothesis that lengthening the cell cycle has a *causal* role in this switch.

Materials and Methods

Whole embryo culture

Embryos were obtained from natural overnight matings of NMRI mice. The following morning, corresponding to E0-E0.5 depending on the exact time of fertilization, was defined as E0.5. Whole embryo culture (WEC) was carried as previously described (Cockroft, 1990; Oback et al., 2000; Osumi and Inoue, 2001; Calegari et al., 2002). Briefly, E9.5 embryos were cultured for 24 hours in the previously described medium (500 μl per embryo, 1 ml per embryo for BrdU incorporation) and gas conditions (Calegari et al., 2002), either in 50 ml tubes on a roller apparatus (Oback et al., 2000) or using an WEC incubator (Ikemoto, Tokyo) (Osumi and Inoue, 2001). Iso-olomoucine and olomoucine (Calbiochem-Novabiochem), whose effects were compared in littermate embryos (see figure legends), were added to the medium from a 50 mM stock in DMSO to a final concentration of 80 μM; BrdU was 50 μM final concentration. To facilitate access of the medium to the neuroepithelium, a hole was made through the yolk sac and the amnion. At the end of WEC, the in vitro development of the embryos was judged by comparison with E10.5 embryos, using established criteria such as heart beat, formation of the telencephalic vesicles, somite number and limb formation. Only E9.5 embryos reaching the developmental stage of E10.5 after 24 hours of WEC were included in this study.

Histochemistry

Immunohistochemistry on 10 μm cryosections of paraformaldehyde-fixed embryos was performed as described previously (Calegari et al., 2002), except that PBS containing 0.2% gelatine, 0.3% Triton X-100, 300 mM NaCl was used throughout. For BrdU detection, after permeabilization and prior to quenching, sections were treated for 30 minutes with 2 M HCl at 37°C. Affinity-purified K4 antibody against TIS21 (Iacopetti et al., 1999) and commercially available monoclonal antibodies against MAP2, β-III-tubulin and BrdU (Sigma) were used. Immunofluorescence was quantitated as described previously (Calegari et al., 2002), and the values for iso-olomoucine and olomoucine were expressed relative to the respective background value (adjacent mesenchyme), which was subtracted. For the determination of apoptosis, the TUNEL reaction was carried out on 10 μm crysections using the in situ cell death detection kit according to the manufacturer's instructions (Roche Diagnostics). TUNEL-positive NE cells were expressed as percentage of the total number of NE cells present in a defined volume of telencephalic neuroepithelium, NE cell number being deduced from the average NE cell size (see Results).

Cumulative BrdU labeling and calculation of cell cycle parameters

BrdU at 50 μM final concentration was added to E9.5 littermate embryos grown for 3 hours in WEC in the presence of 80 μM iso-olomoucine or olomoucine, and WEC was continued for various times followed by fixing the embryos and immunohistochemistry, as described above. The proportion of BrdU-stained nuclei in the neuroepithelium observed after the various times of BrdU labeling was used to calculate, by linear regression analysis, the rate of S-phase entry of NE cells over time. The resulting data were then used to determine the time of BrdU labeling needed to reach the maximum value of BrdU-stained nuclei, which corresponds to the total cell cycle length minus the length of S phase (Tc-Ts) (Nowakowski et al., 1989; Alexiades and Cepko, 1996).

Results

From E9.5 to E10.5, the onset of neurogenesis in vivo and in whole embryo culture is indistinguishable

We used mouse embryos developing in whole embryo culture (WEC) to investigate the possible role of cell cycle length in the switch of NE cells from proliferation to neurogenesis. It has previously been shown that, from the early somite to the organogenetic stage, development in WEC is indistinguishable from that in utero when assessed by parameters such as somite number, size and shape of developing organs, and limb formation (Cockroft, 1990). However, to our knowledge, the switch of NE cells from proliferative to neuron-generating divisions, which is known to occur in a defined temporal and spatial pattern (Nornes and Carry, 1978; Gardette et al., 1982), has not been analyzed in WEC. We therefore compared the caudal-to-rostral gradient of neurogenesis in E9.5 mouse embryos developed for a further 24 hours in WEC (E9.5+24h) and embryos developed in utero until E9.5 and E10.5. For this purpose, we analyzed sagittal cryosections through the neural tube by double immunofluorescence for MAP2, a marker of neurons, and TIS21, a marker of neuron-generating NE cells (Iacopetti et al., 1999). The TIS21 mRNA is specifically detected in neuron-generating NE cells but not in proliferating NE cells nor in neurons, whereas immunoreactivity for the TIS21 protein is observed not only in neuron-generating NE cells but also in newborn neurons which apparently inherit it from their progenitors.

In the ventral mesencephalon at E9.5, we observed a caudal-to-rostral gradient of TIS21 immunoreactivity (Fig. 1a), which was strongest towards the apical side of the neuroepithelium (Fig. 1, arrowheads). At this stage, no MAP2 immunoreactivity was yet detectable (Fig. 1a′), whereas for both E10.5 (Fig. 1b′) and E9.5+24h WEC (Fig. 1c′) embryos, a similar onset and pattern of MAP2 expression were observed, indicative of the formation of a layer of newborn neurons (Fig. 1, arrows). Likewise, for both E10.5 (Fig. 1b) and E9.5+24h WEC (Fig. 1c) embryos, TIS21 immunoreactivity was not only detected in the ventricular zone but also found to be accumulated in the neuronal layer basal to it, consistent with the inheritance of the TIS21 protein by newborn neurons from neuron-generating NE cells (Iacopetti et al., 1999).

In the rostral telencephalon, which initiates neurogenesis by about one day later than the ventral mesencephalon, no TIS21 expression was yet detected at E9.5 (Fig. 1d), whereas a similar onset of TIS21 immunoreactivity in the neuroepithelium was found for both E10.5 (Fig. 1e) and E9.5+24h WEC (Fig. 1f) embryos. At the latter stage, the level of TIS21 immunoreactivity was still low, but yet another day later in development, at E11.5 when the first neuronal layer appears in this region of the telencephalon (Caviness et al., 1995), expression massively increased and TIS21-immunoreactive cells were detected in both the ventricular zone and the neuronal layer (not shown). Consistent with this delay in the onset of neurogenesis in the rostral telencephalon, MAP2 immunoreactivity was undetectable not only in E9.5 (Fig. 1d′), but also in E10.5 (Fig. 1e′) as well as E9.5+24h WEC (Fig. 1f′) embryos.

At all stages of development analyzed, both in utero and in WEC-developed embryos, MAP2 was always found to be expressed in cells that were also immunoreactive for β-III-

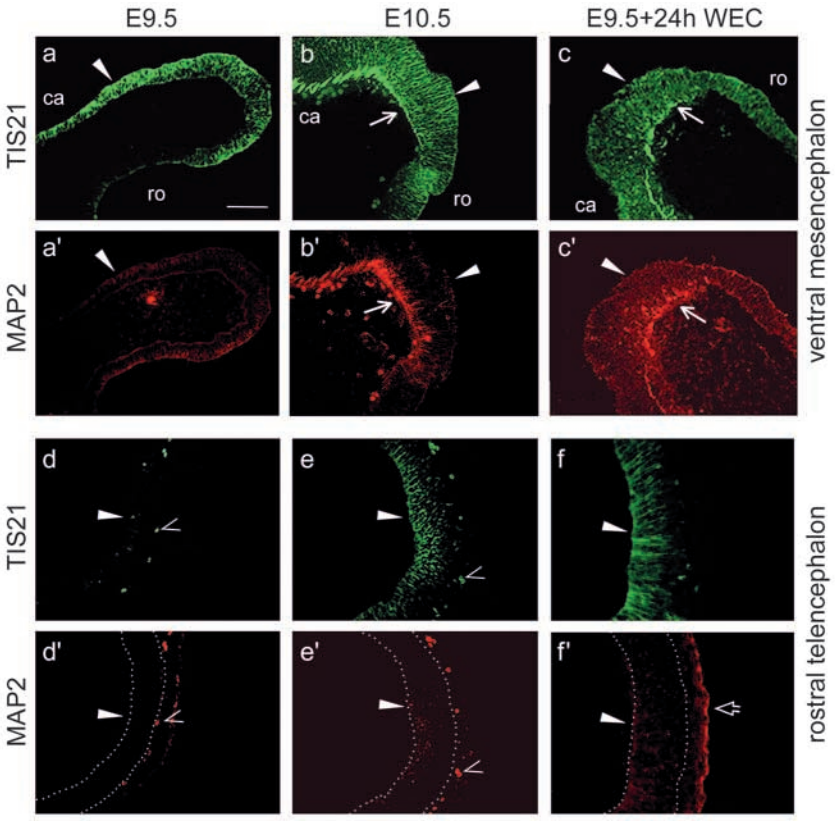

Fig. 1. WEC faithfully reproduces neurogenesis in vivo. Double immunofluorescence for TIS21 (a-f) and MAP2 (a′-f′) on sagittal cryosections through the ventral mesencephalon (a-c′) and rostral telencephalon (d-f′) of embryos developed in utero up to E9.5 (E9.5) or E10.5 (E10.5) or in utero to E9.5 followed by WEC for 24 hours (E9.5+24h WEC). Note the caudal (ca) to rostral (ro) gradient of neurogenesis in the ventral mesencephalon. Filled arrowheads, apical (ventricular) side of the neuroepithelium; arrows, neuronal layer; open arrowheads, auto-fluorescent blood cells (absent in embryos developed in WEC because of the loss of blood cells after opening of the yolk sac); open arrow, reaction of the anti-mouse secondary antibody with the skin, presumably with adsorbed mouse immunoglobulins present in the mouse serum used for WEC. Dotted lines delineate the boundaries of the neuroepithelium. Scale bar: 100 μm.

tubulin, another marker of neurons (Lee et al., 1990) (data not shown). Essentially all of the cells in the neuronal layer(s) and, rarely, cells in the ventricular zone (presumably newborn migrating neurons), showed MAP2 and β-III-tubulin immunoreactivity. The latter observation indicates that MAP2, like β-III-tubulin, is an early marker of neurogenesis.

Taken together, our data show that, from E9.5 to E10.5, WEC does not delay, or prematurely induce, neurogenesis and that the migration of the newborn neurons from the ventricular to the neuronal layers is not inhibited by the present culture conditions.

Olomoucine increases NE cell cycle length in WEC

In order to lengthen the G1 phase of NE cells, we explored the use of the cell-permeable cell cycle inhibitor olomoucine. Olomoucine, a well-characterized anti-mitotic agent, is a highly specific CDK inhibitor that exerts its action through binding to the ATP pocket of CDK2 when complexed to cyclin E or A, and of CDK1 when complexed to cyclin B (IC_{50} values in all three cases are 7 μM with purified proteins), thereby inhibiting the G1 to S and G2 to M transition, respectively (Vesely et al., 1994; Abraham et al., 1995; Schutte et al., 1997). In cell culture, the G1 to S and G2 to M transitions have previously been found to be blocked in all cells by addition of 200 μM olomoucine (Machiels et al., 1996; Schutte et al., 1997), but only partially inhibited at lower concentrations such as 50 μM (Schutte et al., 1997). We therefore investigated whether olomoucine at a moderate concentration, i.e. 80 μM, would be able to lengthen, but not block, the cell cycle of NE cells in WEC. Iso-olomoucine, the inactive isomer of olomoucine, was used as control.

To obtain a first clue, we examined the effect of olomoucine on the growth of embryos by measuring the increase in their size (crown-to-rump, post-fixation) from E9.5 (22-26 somites, about 2.5 mm) to E10.5 (30-35 somites, about 3.75 mm) (Kaufman, 1992) (Fig. 2A). The size of E9.5 embryos, developed for 24 hours in WEC either without addition (3.74±0.14 mm) or with 80 μM iso-olomoucine (referred to as E9.5+WEC/iso-olo embryos, 3.61±0.13 mm), did not significantly differ from that of E10.5 embryos developed in utero. In contrast, approximately 40% reduction in growth (3.27±0.11 mm) was observed for E9.5 embryos developed for 24 hours in WEC in the presence of 80 μM olomoucine (referred to as E9.5+WEC/olo embryos). This reduction in growth was not associated with any obvious changes in parameters of development such as somite number, limb formation or maturation of the heart (data not shown).

The reduced growth of olomoucine-treated embryos, besides reflecting effects of the CDK inhibitor on cell cycle parameters (see below), could (at least in part) be due to increased apoptosis, which has been shown to be caused by olomoucine at concentrations of 200 μM (Machiels et al., 1996; Schutte et al., 1997). To examine this, we compared the proportion of apoptotic cells in E9.5+WEC/olo embryos with that in E9.5+WEC/iso-olo embryos and E10.5 embryos developed in utero. In these and all following experiments, exposure to iso-olomoucine or olomoucine was examined in littermate embryos (see figure legends). TUNEL analysis of the telencephalic neuroepithelium revealed a very similar degree of apoptosis in this tissue under the three conditions, with an average of 0.4-0.6% of the cells showing TUNEL-positive nuclei. This value is in the range of apoptosis reported for the ventricular zone of the developing neocortex of E14-E19 embryos (0.3-0.9%) (Thomaidou et al., 1997). Within the telencephalic neuroepithelium, certain regions contained a much greater proportion of apoptotic cells, such as the region shown in Fig. 2B,a,b. Yet, also here no significant difference in apopotosis was observed between E9.5+WEC/iso-olo embryos (Fig. 2B,a) and E9.5+WEC/olo embryos (Fig. 2B,b), both being indistinguishable from E10.5 embryos developed in utero (data not shown). These results show that neither WEC

nor 80 μM olomoucine increase apoptosis in the E9.5-E10.5 neuroepithelium. In addition, no significant increase in apoptosis by olomoucine was observed in any of the other tissues of the developing embryo examined (not shown), eliminating the possibility that the reduction in growth of olomoucine-treated embryos was due to apoptosis.

The reduced growth of embryos treated with 80 μM olomoucine could also reflect a decrease in cell size. We addressed this issue with regard to NE cells. For both E9.5+WEC/iso-olo embryos and E9.5+WEC/olo embryos, we determined, in 10 μm thick sections, the number of cell nuclei that were contained in an area of the telencephalic neuroepithelium corresponding to 10,000 μm^2 (i.e. 100 pl), by counting three lots of at least 500 DAPI-stained nuclei each. This revealed that, on average, 100 pl of neuroepithelium of iso-olomoucine- and olomoucine-treated embryos contained 133±21 and 143±24 cell nuclei (SD, *n*=3), respectively, which (ignoring extracellular space) would correspond to a mean NE cell volume of 0.75±0.14 pl and 0.70±0.13 pl. Assuming a spherical NE cell shape, these values would correspond to a mean NE cell radius of 5.64±0.32 μm and 5.52±0.28 μm for iso-olomoucine- and olomoucine-treated embryos, respectively. Hence, treatment of E9.5 embryos for 24 hours with 80 μM olomoucine does not have a significant effect on NE cell size.

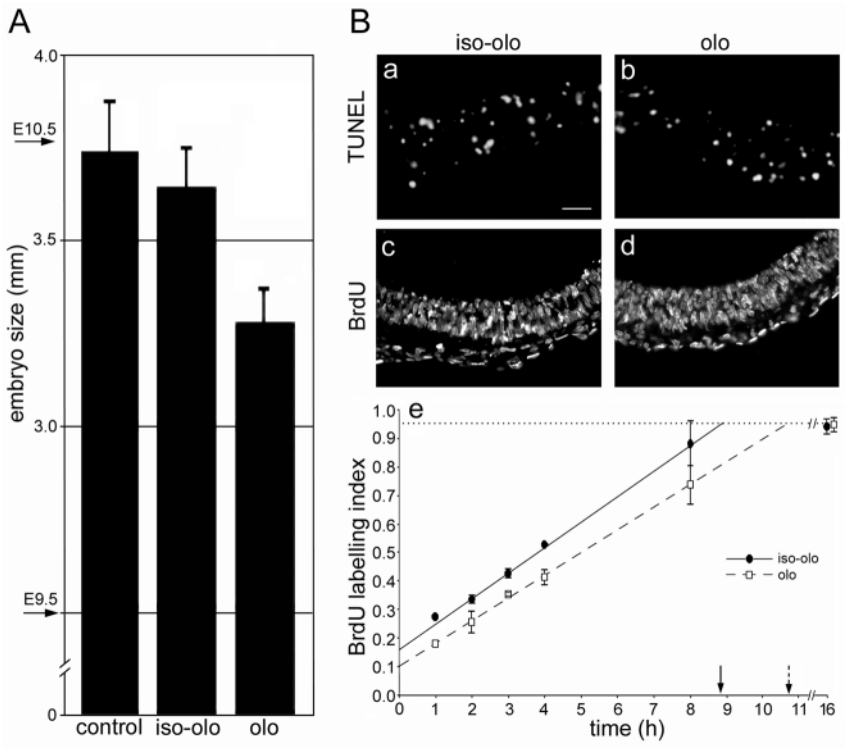

Fig. 2. Olomoucine induces lengthening of the cell cycle in WEC. (A) Size (crown to rump) of E9.5 embryos developed for 24 hours in WEC in the absence (control) or presence of 80 μM iso-olomoucine (iso-olo) or olomoucine (olo) followed by fixation. Arrows indicate the reported (Kaufman, 1992) size of fixed embryos with 22-26 pairs of somites (E9.5) and 30-35 pairs of somites (E10.25-10.5). Bars represent the s.d.; control, *n*=5; iso-olo, *n*=8 and olo, *n*=7; olo vs. iso-olo $P<0.0001$. (B) Analyses of NE cells in the rostral telencephalon of E9.5 littermate embryos developed in WEC in the presence of 80 μM iso-olomoucine (iso-olo) or olomoucine (olo). (a,b) TUNEL staining after 24 hours WEC. (c,d) BrdU immunoreactivity after 19 hours WEC, with 50 μM BrdU added at 3 hours. (a-d) Scale bar: 50 μm. (e) Cumulative BrdU labeling, with 50 μM BrdU added at 3 hours (time=0) and WEC being continued for the indicated times. The BrdU labeling index indicates the proportion of DAPI-stained cell nuclei in the neuroepithelium that were stained for BrdU (all nuclei stained for BrdU=1.0). For each time point, one littermate embryo each was analyzed for iso-olo (filled circles) and olo (open squares); data are the mean of two independent litters (except for the 1 hour time point); bars indicate the variation of the duplicate values from the mean; correlation coefficients are $r^2=0.995$ and $r^2=0.998$ for iso-olo and olo, respectively. The dotted line indicates the BrdU labeling index observed after 16 hours of BrdU labeling, which was the same for iso-olo and olo (for clarity, the filled circle and open square symbols at the 16 hours time point are placed next to each other). The extrapolated intercept of the iso-olo and olo best-fit lines with the dotted line is indicated by the solid and dashed arrow on the abscissa, respectively, and provides an estimate of the sum of the lengths of the G2, M, plus G1 phases (plus the fraction of S phase required to detect BrdU incorporation).

Given the lack of effect of olomoucine treatment on apoptosis and cell size, we investigated its influence on the cell cycle of NE cells. We first examined, by quantifying BrdU incorporation, whether 80 μM olomoucine would block the cell cycle in (perhaps only a portion of) NE cells. At E11, the average length of the cell cycle of NE cells in the rostral telencephalon is about 8 hours (Takahashi et al., 1995). Hence, to determine the proportion of NE cells progressing through the cell cycle in the presence of 80 μM olomoucine, E9.5 embryos were developed in WEC for 19 hours in the presence of 80 μM iso-olomoucine or olomoucine, with 50 μM BrdU being added after 3 hours of WEC. Subsequent immunocytochemistry of the rostral telencephalon revealed that for both iso-olomoucine- and olomoucine-treated embryos, 95±2% of all cell nuclei in the neuroepithelium (detected by DAPI staining) had incorporated BrdU (Fig. 2B,c,d, dotted line and 16 hours symbols in e). Given the length of BrdU labeling (16 hours) and the fact that NE cells are not synchronized with regard to the various phases of the cell cycle, these data indicate that 80 μM olomoucine in WEC does not block cell cycle progression of NE cells, specifically G1 to S- and G2 to M-phase transition (the known site of action of olomoucine in the cell cycle), not even in a portion of cells.

Since 80 μM olomoucine in WEC did not (i) increase apoptosis, (ii) decrease cell size or (iii) block the cell cycle, the reduction in the growth of the embryo was presumably caused by reduction in the rate of cell proliferation, i.e. a lengthening of the cell cycle. To directly investigate this for the neuroepithelium, we subjected E9.5 embryos in WEC to cumulative BrdU labeling, which reveals the successive entry of NE cells into S-phase over time and provides information about the length of the cell cycle (Nowakowski et al., 1989; Alexiades and Cepko, 1996). Littermate embryos were allowed to develop in the presence of 80 μM iso-olomoucine or olomoucine, with 50 μM BrdU being added after 3 hours and cumulative BrdU labeling being performed for various times, up to 16 hours (Fig. 2B,e). For control,

E9.5+WEC/iso-olo embryos (Fig. 2B,e, solid line), this revealed that the sum of G2+M+G1 [T_C–T_S (Nowakowski et al., 1989)] was 8.8 hours (Fig. 2B,e, solid arrow), whereas for E9.5+WEC/olo embryos, we observed a significant reduction in the rate of accumulation of BrdU-labeled nuclei (Fig. 2B, panel e, dashed line), with T_C–T_S being increased by ≈2 hours to 10.7 hours (Fig. 2B,e, dashed arrow).

Olomoucine induces a premature switch of NE cells from proliferative to neuron-generating divisions

We next investigated whether olomoucine influenced the switch of NE cells from proliferative to neuron-generating divisions. For this purpose, we compared littermate E9.5+WEC/iso-olo embryos and E9.5+WEC/olo embryos with regard to the expression of TIS21, focussing on the rostral telencephalon in which TIS21 expression begins at E10.5 (see Fig. 1d-f). E9.5+WEC/iso-olo embryos showed a low level of TIS21 expression in the rostral telencephalon (Fig. 3a, white dotted line), as expected for this developmental stage (see Fig. 1,e and f). Remarkably however, in all of the E9.5+WEC/olo embryos examined (see Table 1), we observed premature accumulation of TIS21 immunoreactivity in NE cells of the rostral telencephalon (Fig. 3b, white dotted line) to levels normally observed at a later stage of development, i.e. E11.5. Premature TIS21 expression in NE cells was not seen throughout the rostral telencephalon but was observed most frequently near the ventral and dorsal telencephalic-mesencephalic boundaries (Fig. 3b, white arrowheads). A quantitative analysis of the olomoucine-induced increase in TIS21 immunoreactivity in NE cells, as compared to iso-olomoucine-treated littermate control embryos, is shown for three independent experiments in Fig. 3c-e.

Table 1. Effect of olomoucine on the appearance of TIS21 and MAP2 immunoreactivity in the developing mouse telencephalon

	TIS21-positive cells		MAP2-positive cells	
Treatment	Experiments	Embryos	Experiments	Embryos
Iso-olomoucine	1/6	1/8	0/5	0/7
Olomoucine	6/6	7/7	3/5	3/6

Littermate E9.5 embryos were developed for 24 hours in WEC in the presence of 80 μM iso-olomoucine or olomoucine. Frozen sections were analyzed for TIS21 and MAP2 immunoreactive cells in the neuroepithelium and neuronal layer (Figs 3 and 4) by double immunofluorescence. Data were collected from six independent experiments, one of which was only subjected to TIS21 analysis. Values indicate the number of experiments and the number of embryos in which immunoreactive cells were observed.

Olomoucine induces premature neurogenesis

The same littermate E9.5+WEC/iso-olo embryos and E9.5+WEC/olo embryos that were analyzed for TIS21 expression were also used to investigate the expression of MAP2, a marker of post-mitotic neurons. Consistent with the

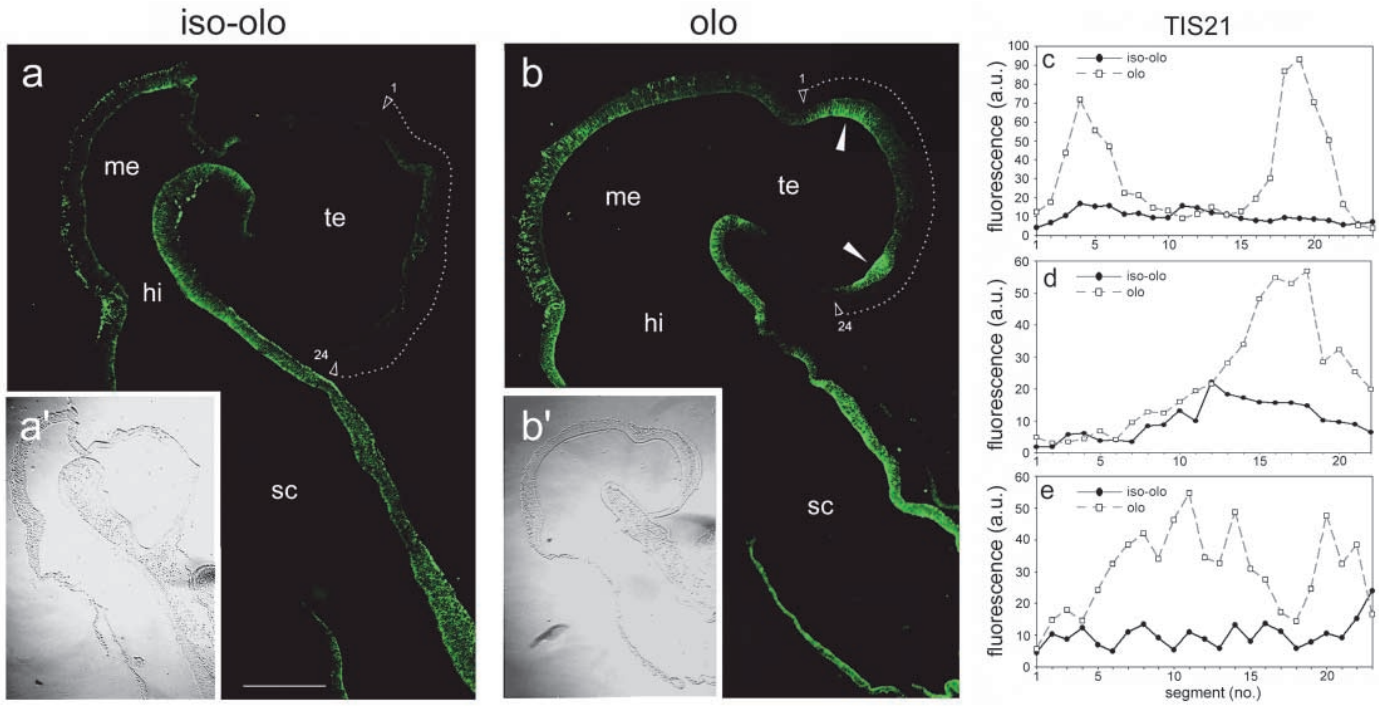

Fig. 3. Olomoucine increases the proportion of neuron-generating NE cells. (a,b) Composite showing TIS21 immunofluorescence in sagittal cryosections through the spinal cord (sc), hindbrain (hi), mesencephalon (me) and telencephalon (te) of E9.5 littermate embryos developed for 24 hours in WEC in the presence of 80 μM iso-olomoucine (iso-olo, a) or olomoucine (olo, b). (a′,b′) Corresponding phase contrast images shown at lower magnification. Filled arrowheads in b indicate areas of the telencephalon prematurely expressing TIS21. Scale bar: 400 μm. (c) Quantitation of TIS21 immunoreactivity. The region of the telencephalon indicated by the dotted lines between the open arrowheads numbered 1 and 24 in a and b was divided into 24 segments of similar area, and the fluorescence in each area was quantitated; a.u., arbitrary units. Filled circles, iso-olo; open squares, olo. Graphs in d and e show a similar quantitation of the TIS21 immunoreactivity shown in Fig. 4a and b (segments 1-22 corresponding to bottom-to-top) and Fig. 4c and d (segments 1-23 corresponding to top-to-bottom), respectively.

results for E10.5 embryos (Fig. 1e′) and E9.5 embryos developed for 24 hours in WEC without any drug (Fig. 1f′), E9.5+WEC/iso-olo embryos did not show MAP2 expression in the telencephalon (Fig. 4a′,c′). However, premature expression of MAP2 was found in three out of six E9.5+WEC/olo embryos (Table 1), and this expression was always confined to regions of the telencephalon prematurely overexpressing TIS21 (Fig. 4b,d). The extent of premature neurogenesis in E9.5+WEC/olo embryos, as judged from the number of MAP2-positive cells, varied between different experiments. The two most extreme cases are shown in Fig. 4b′, where only a few MAP2-positive cells were detected in the ventricular zone (presumably newborn migrating neurons), and Fig. 4d′, where a substantial number of MAP2-positive cells at the basal side of the ventricular zone were observed, consistent with the premature formation of a neuronal layer. We believe that this variability, and the finding that only half of the olomoucine-treated embryos showed premature generation of neurons by the end of the 24-hour WEC period, reflect the variation, between the various litters, in the actual time point of fertilization, given that fertilization could occur at any time in a 12 hours period (see Discussion).

Discussion

The interplay between cell fate determination and cell cycle regulation has received increasing attention recently (Ohnuma et al., 2001; Bally-Cuif and Hammerschmidt, 2003; Cremisi et al., 2003). The prevailing view has been that a given cell differentiation program may influence, as one of its many target processes, the cell cycle (Ohnuma et al., 2001; Cremisi et al., 2003). The converse, however, i.e. that a cell differentiation program may be influenced by cell cycle regulators, has also been shown (Ohnuma et al., 2001; Cremisi et al., 2003).

The present data make two novel points with regard to cell cycle regulators influencing cell fate determination. First, inhibition of cyclin-dependent kinases (CDK1, CDK2) by the highly specific inhibitor olomoucine (Vesely et al., 1994; Abraham et al., 1995; Schutte et al., 1997) induces, in the developing mouse embryo, a premature switch of NE cells from proliferative to neuron-generating divisions and, consequently, premature neurogenesis. Previously, CDK inhibitors known to slow-down or arrest G1-S transition have been shown to be able to influence neural cell fate determination (Ohnuma et al., 2001; Cremisi et al., 2003). However, as paradigmatically shown for the cyclin-dependent kinase inhibitor $p27^{Kip1}$ (Ohnuma et al., 1999; Vernon et al., 2003), such influence on cell fate determination has been demonstrated to be independent of inhibition of cell cycle progression (Cremisi et al., 2003). In contrast to $p27^{Kip1}$, the only known targets of olomoucine are the cyclin-dependent kinases themselves. Hence, our data, which are consistent with the previous observation that olomoucine induces neuronal differentiation of PC12 cells in vitro (Dobashi et al., 2000), provide direct evidence that inhibition of CDK1 and CDK2 can alone be sufficient to influence mammalian cell fate in vivo, i.e. to induce neurogenesis. As discussed below, this effect is most likely related to the length of the cell cycle, given that the only established function of CDK1 and CDK2 is to control the cell cycle, but it is worth noting that in the *Drosophila* embryo, CDK2 function has also been implicated in the localization of cell fate determinants (Tio et al., 2001).

Second, the concentration of the CDK inhibitor was chosen such that it lengthened, but did not arrest, the NE cell cycle. In previous studies, irrespective of the finding that molecules such as $p27^{Kip1}$ function beyond cell cycle regulation (Ohnuma et al., 1999; Vernon et al., 2003), any influence of a cell cycle regulator on cell fate determination was attributed to cell cycle *arrest* rather than cell cycle *lengthening* (e.g. Durand et al.,

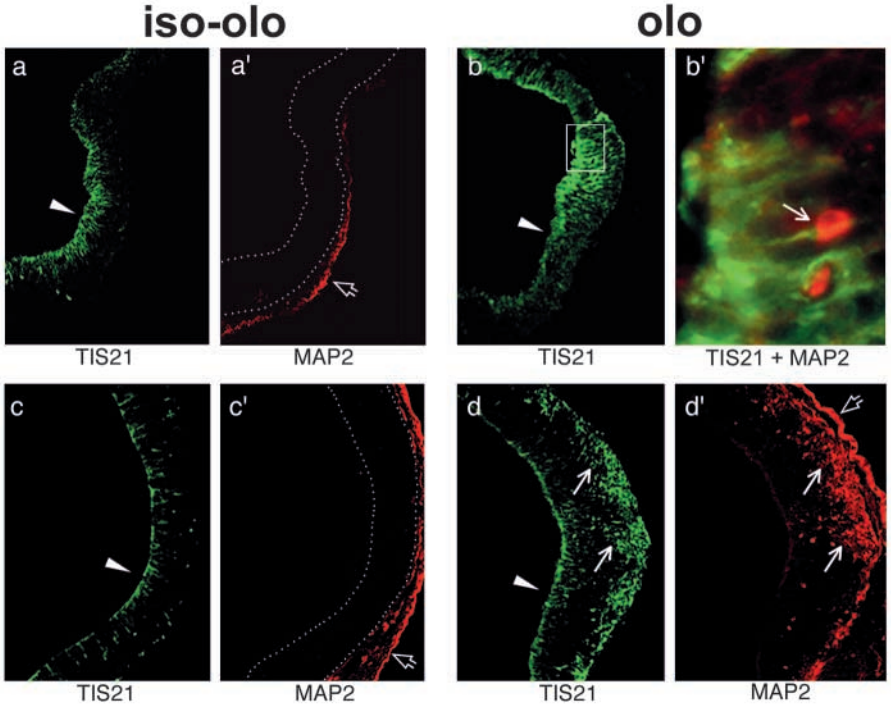

Fig. 4. Olomoucine induces premature neurogenesis. Double immunofluorescence for TIS21 (a-d, b′, green) and MAP2 (a′-d′, red) on sagittal cryosections through the rostral telencephalon of littermate (a vs. b, c vs. d) E9.5 embryos developed for 24 hours in WEC in the presence of 80 μM iso-olomoucine (iso-olo; a, a′ and c, c′) or olomoucine (olo; b, b′ and d, d′). Arrowheads, apical (ventricular) side of neuroepithelium. Arrows indicate prematurely generated neurons. The white box in b indicates the area shown at higher magnification in b′. b′ and d′ show different degrees of premature neurogenesis upon olomoucine treatment, with few (b′) and many (d) neurons being generated. Open arrows in a′, c′ and d′ indicate reaction of the anti-mouse secondary antibody with the skin, presumably with adsorbed mouse immunoglobulins present in the mouse serum used for WEC. Dotted lines in a′ and c′ delineate the boundaries of the neuroepithelium.

1998; Durand and Raff, 2000). The olomoucine-induced lengthening of the NE cell cycle observed in the present study presumably affected primarily G1, given that (i) olomoucine has little, if any, effect on the length of S and M phase and specifically increases the length of G1 and G2 (Vesely et al., 1994; Abraham et al., 1995; Schutte et al., 1997) and (ii) in NE cells G1 is significantly longer than G2 (Takahashi et al., 1995). It is interesting to note that it is the G1 phase of the NE cell cycle that selectively lengthens as NE cells switch from proliferative to neuron-generating divisions (Takahashi et al., 1995). Our data therefore suggest that lengthening the G1 phase of the NE cell cycle can alone be sufficient to induce neurogenesis. In other words, lengthening the G1 phase of the NE cell cycle appears to be a cause, rather than consequence, of their differentiation.

If so, one might expect that agents that shorten the G1 phase of the NE cell cycle should prevent their differentiation. In fact, observations consistent with this, describing the effects of extracellular factors on NE cell cycle kinetics and the type of NE cell division in vitro, have recently been reported (Dehay et al., 2001; Lukaszewicz et al., 2002). Specifically, in contrast to neurotrophin 3, which lengthens G1 and promotes differentiative divisions, basic fibroblast growth factor has been found to shorten G1 and to prevent such divisions, promoting instead proliferative divisions (Lukaszewicz et al., 2002). Furthermore, mice lacking $p27^{Kip1}$, which has been shown to lengthen G1 in NE cells (Mitsuhashi et al., 2001), show increased proliferation of adult neural stem cells (Doetsch et al., 2002).

How may lengthening the G1 phase of NE cells cause neurogenesis? In Fig. 5, we propose a model whose key feature is that whether or not a cell fate determinant will actually produce an effect on cell fate depends on the length of *time* during the cell cycle this determinant can act (see legend for details). Thus, a certain length of time will be required for a cell fate determinant (i) to be produced to the appropriate level, (ii) to reach the relevant site within the cell, and/or (iii) to generate sufficient product, such that a change in cell fate will occur. A (relatively speaking) short cell cycle, or short period of the critical phase of the cell cycle, may not provide enough time for these processes to become effective, whereas a longer one will. If we assume, for example, that NE cells constitutively produce a cell fate determinant capable of inducing their switch from proliferative to neuron-generating divisions, NE cells would nonetheless not switch to neurogenesis as long as their cell cycle is too fast for this determinant to become effective, but would do so upon lengthening of their cell cycle.

Why, then, did only half of the olomoucine-treated embryos show premature generation of neurons although all showed premature up-regulation of TIS21 in NE cells, i.e. a switch towards neurogenesis? The answer almost certainly lies in the length of WEC, which was conducted for 24 hours, a time period during which mouse embryo development occurs in a highly reliable and reproducible manner, in contrast to longer culture periods. A 24-hour WEC allows for two complete NE cell cycles and thus was long enough for olomoucine-induced G1 lengthening to influence NE cell fate such that, in the subsequent NE cell cycle, TIS21 was up-regulated and NE cells switched to neuron-generating divisions, but was borderline in length to observe the outcome of the latter divisions, i.e. the generation of neurons. Hence, given that fertilization could have occurred at any time point within a 12 hours period and that the embryos subjected to WEC therefore ranged in developmental stage from E9.0 to E9.5, one would expect to detect prematurely generated neurons only in about half of the olomoucine-treated embryos.

A further, important aspect of our model is that it provides a possible explanation for whether or not an unequal distribution of cell fate determinants upon mitosis will result in asymmetric daughter cell fate (for details, see legend to Fig. 5). Thus, only if a cell fate determinant is allowed to act for an appropriate length of time, will an unequal distribution of this

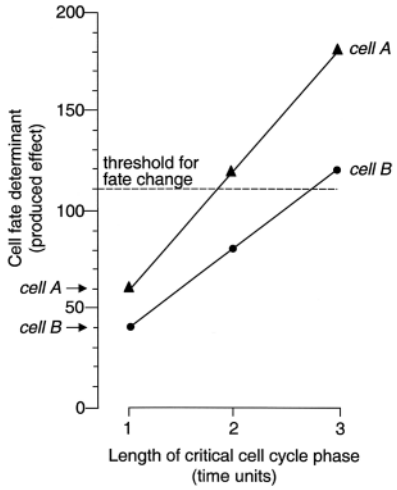

Fig. 5. Cell cycle length, asymmetric cell division and cell fate determination – a model. In our model, a molecule capable of inducing a cell fate change (cell fate determinant) is assumed to be distributed unequally upon mitosis, e.g. with daughter cell A receiving 60% and daughter cell B 40% of this determinant (arrows). For a cell fate change to occur, the effect of the cell fate determinant has to reach a threshold (dashed line), i.e. the cell fate determinant needs to act for a certain length of time (for example, a transcription factor activating transcription until a certain amount of gene product has been produced). Given the unequal amounts of cell fate determinant in cells A and B, this threshold will be reached by neither cell A nor cell B after one unit of time, by cell A but not cell B after two units of time, and by both cell A and cell B after three units of time. If the cell fate determinant would, for example, be able to induce the switch of NE cells from a proliferative to a neuron-generating division, neither NE cell would switch to neurogenesis if the length of the critical phase of the cell cycle corresponded to one time unit, NE cell A but not NE cell B would switch upon doubling this phase, and both NE cells would switch upon tripling it. Hence, an unequal distribution of a cell fate determinant upon mitosis will only lead to an asymmetric daughter cell fate if the length of the cell cycle is appropriate. Moreover, if early in G1, the cell fate determinant were to drive the production of a molecule that causes cell cycle arrest upon reaching the threshold, depending on the length of G1 either none, one or both of the daughter cells would acquire a post-mitotic state (i.e. turning G1 into G0), as is characteristic of neurons. Note that the basic principle of the model, i.e. the dependence of cell fate on time, holds true for any cell, irrespective of asymmetric division. Moreover, the model can be adopted to two cells being exposed to different concentrations of an extracellular factor affecting cell fate, or to two cells being exposed to the same concentration of such a factor for different times.

determinant result in asymmetric daughter cell fate. Our model is not only consistent with the observation that concomitant with the progressive lengthening of the NE cell cycle during embryonic development, an increasing proportion of NE cells switch to neurogenesis (Caviness et al., 1995; Takahashi et al., 1995; Takahashi et al., 1996). It also would explain (for details, see legend to Fig. 5) why at the onset of neurogenesis, only one of the NE cell daughters becomes a post-mitotic neuron (asymmetric division) (McConnell, 1995), whereas as neurogenesis proceeds and the NE cell cycle lengthens further (Caviness et al., 1995; Takahashi et al., 1995; Takahashi et al., 1996), both daughter cells become post-mitotic (symmetric, differentiating division) (McConnell, 1995).

At a superficial glance, the 'cell cycle length model' proposed in Fig. 5 may seem to resemble the 'intrinsic timer model' proposed previously to explain the role of p27 in the differentiation of oligodendrocyte precursors (Durand and Raff, 2000) and of retinal progenitor cells towards Müller cells (Ohnuma et al., 1999), although our model is, in fact, distinct in several key aspects. First, the 'intrinsic timer model' attributes significance to the gradual accumulation of p27 in progenitor cells over several cell cycles (Ohnuma et al., 1999; Cremisi et al., 2003), whereas the present 'cell cycle length model' considers the consequences of time within a single NE cell cycle. Second, in the 'intrinsic timer model', cell fate determination is linked to cell cycle arrest (Durand et al., 1998; Ohnuma et al., 1999; Durand and Raff, 2000; Cremisi et al., 2003), whereas in our model, cell cycle arrest as a characteristic feature of differentiation is seen as the consequence of an increase in cell cycle length. Third, in contrast to the 'intrinsic timer model' and other previous models, our 'cell cycle length model' provides a mechanistic explanation for the hitherto unresolved discrepancy between an unequal distribution of cell fate determinants and asymmetric cell fate (see above).

In the present study, TIS21, the expression of which in NE cells distinguishes neuron-generating from proliferating cells (Iacopetti et al., 1999), has been used as a marker to identify NE cells that have switched to neuron-generating divisions. Remarkably, TIS21 has been shown to be an inhibitor of G1 progression in vitro (Matsuda et al., 2001; Tirone, 2001). Given our results, which indicate that lengthening the G1 phase of the NE cell cycle can alone be sufficient to induce neurogenesis, we find it likely that the expression of TIS21 in NE cells in vivo has a direct functional role in promoting neurogenesis.

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