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Experimental Neurology 181 (2003) 39–46

Experimental Neurology

www.elsevier.com/locate/yexnr

Regulation of cell survival in the developing thalamus: an in vitro analysis

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Received 17 September 2002; revised 28 November 2002; accepted 4 December 2002

Abstract

There is evidence that developing thalamic cells become dependent for their survival on the integrity of their afferent and/or efferent connections, which may provide required levels of neural activity and/or essential neurotrophic factors. These connections develop in the second half of gestation in mice and, during this time (embryonic days 17–19), isolated thalamic cells either grown as explants or dissociated from each other lose their ability to survive. Here we show that the loss of viability of explants, but not of dissociated cells, is delayed if the cultures are treated with depolarizing stimuli. The survival of dissociated thalamic cells is promoted by culture medium conditioned by thalamic explants grown with depolarizing stimuli, indicating that the effect of depolarization involves trophic factors released by thalamic cells. This survival promoting effect is found prenatally, but not postnatally, and is prevented by the neurotrophin blocker K252a. Culture medium conditioned by cortex also promotes the survival of thalamic cells and this effect does occur postnatally. These findings suggest that diffusible factors, possibly members of the neurotrophin family, and depolarizing stimuli regulate thalamic cell survival before birth, but trophic support from cortex becomes crucial after birth. This culture model may provide a means of investigating the mechanisms of thalamic cell survival during development.

Keywords: Apoptosis; Glutamate; Mouse; Neurotrophin; Thalamus; Trophism

Introduction

The mechanisms that regulate programmed cell death in the developing brain are poorly understood. The thalamus has proved a useful structure in which to study this process. It receives inputs from sensory receptors in the periphery, sends axons to the cerebral cortex, and receives reciprocal corticothalamic projections. Only a minority of its cells are GABAergic (gamma-aminobutyric acid) local circuit neurons (fewer than 1% in most nuclei and no more than 20% in almost all the rest; Arcelli et al., 1997). In the mouse, its connections form during the second half of gestation, with a major phase of development between embryonic day 14 (E14) and birth (reviewed by Ferrer et al., 1992; Lotto and Price, 1995; Yuasa et al., 1994; reviewed by Molnar, 1998; Auladell et al., 2000). The numbers of thalamic neurons undergoing programmed cell death increase several fold from E15 to birth and then by an order of magnitude in the days following birth (reviewed by Finlay and Pallas, 1989; Waite et al., 1992; Spreafico et al., 1995; Alcántara et al., 1997; Lotto et al., 2001). The mechanisms responsible for this increase are not known but studies on the effects of lesions to the sense organs or cerebral cortex have indicated that cell survival is regulated via thalamic afferent and/or efferent connections (Finlay et al., 1986; Cunningham et al., 1987; reviewed by Finlay and Pallas, 1989; Eagleson et al., 1990; Agarwala and Kalil, 1998).

The development of an in vitro system that models the survival requirements of thalamic cells would offer a way of investigating further the mechanisms regulating their survival. Our previous work has indicated that thalamic cells aged E15, whose connections are still immature, survive well when isolated and grown in simple serum-free medium

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doi:10.1016/S0014-4886(03)00025-6

due to neurotrophic-factor-mediated interactions between themselves (Lotto et al., 1997). Thalamic cells from older prenatal and early postnatal brains survive progressively less well in isolation (Magowan and Price, 1996). This may be due to the absence of external influences such as activity and/or trophic support normally obtained from their afferents and/or efferents. Here we tested whether appropriate levels of activity are required for thalamic cell survival in vitro by applying different concentrations of potassium or glutamate to thalamic cells at different ages. Having found a survival-promoting effect of these substances, we tested whether the effect is mediated by diffusible factors released by thalamic cells. We conditioned culture medium with thalamic explants of various ages that had been exposed to different levels of potassium and then tested the trophic effects of these media on dissociated thalamic cells. We also tested the role of cortex conditioned medium on thalamic cell survival. Our new findings suggest that diffusible factors, possibly members of the neurotrophin family, and depolarizing stimuli regulate thalamic cell survival before birth. After birth trophic support from cortex becomes crucial, as indicated by Lotto et al. (2001).

Materials and methods

Culture methods

C3H and Balb/C mice were obtained from overnight matings and the day of the vaginal plug was deemed E1. Tissues were dissected between E13 and postnatal day 2 (P2). Dorsal thalamic and posterior cortical explants, as well as explants from other regions (cerebellum, tectum, heart, and liver), were obtained as described by Rennie et al. (1994). Explants of thalamus were cut to a consistent size ($500 \times 500 \times 350$ μm); this involved trimming tissue from the medical and lateral edges at the older ages. Explants of cortex were 350 μm thick and 500 μm wide in the tangential direction and contained the full thickness of the cortex. All other explants were $500 \times 500 \times 350$ μm. To test the effects of potassium concentration and to generate conditioned media, explants were put onto collagen-coated microporous membranes (40 explants per membrane) suspended over wells (Transwell-COL, Costar, UK) filled with 500 μl serum-free culture medium containing different levels of potassium (Yamamoto et al., 1989; Molnar and Blakemore, 1991). They were cultured at 37°C with 5% CO_2 in serum-free medium (Rennie et al., 1994) for 24 h; the inserts carrying the explants were removed and conditioned culture media were harvested and added to dissociated thalamic cells. Cultures testing the effects of glutamate on thalamic explants lasted 3 days. Thalamic and cortical explants were fixed in 4% paraformaldehyde, wax-embedded, cut at 10 μm, and stained with the nuclear dye bisbenzimide (5 μg/ml). For dissociated cell cultures, E15 or P2 thalamic cells were dissociated enzymatically as described by Lotto et al. (1997) and plated at high (4000 cells/mm^2) density. For E15 cells, after 24 h in culture in serum-free medium at 37°C with 5% CO_2, 80 μl of culture medium was replaced by normal culture medium or conditioned medium, depending on the experiment. P2 thalamic cells were cultured for 24 h with unconditioned medium or with medium conditioned with P2 thalamic or P2 cortical explants. Viability was assessed after 5 days in vitro by staining with bisbenzimide and propidium iodide. Some cultures were reacted for terminal deoxytransferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) as described by Gavrieli et al. (1992). The exact design of experiments using these culture methods was as follows.

Effects of potassium concentration on explant survival

E13, E15, E17, E19, and P2 thalamic explants and E19 and P2 cortical explants were used. Some were cultured with no potassium added to that present in control medium; others were cultured with an extra 0.5, 5, or 50 mM potassium.

Effects of glutamate concentration on thalamic explant survival

E13, E15, E17, and E19 thalamic explants were cultured, some with no glutamate additional to that in control medium and others with an extra 0.005, 0.05, 0.5, or 5 mM or 10 μM NMDA (previous work has shown that similar concentrations of NMDA activate NMDA receptors when bath-applied to brain slices in vitro; Glitsch and Marty, 1999). In further experiments we increased the glutamate concentration surrounding E17 thalamic explants by 0.05 mM, the age and dose at which we found the greatest effect, and the specific NMDA receptor antagonist 2-amino-5-phosphonovalerate (APV) was added to give final APV concentrations of 0.5, 5, 50, or 500 μM.

Effects of thalamic and cortical conditioned medium on thalamic cells

We tested the survival-promoting effects of adding normal medium containing an additional 0.5, 5, or 50 mM potassium or conditioned medium obtained from thalamic or cortical explants cultured with these concentrations of potassium to dissociated E15 thalamic cells. K252a was added to some cultures at 100, 500, and 1000 nM. These doses are known to have effects in blocking specifically the neurotrophin receptors (Tapley et al., 1992). We also tested the effects of adding P2 thalamic or cortical conditioned medium or control medium to dissociated P2 thalamic cells.

Effects of withdrawing or heating cortical conditioned medium on thalamic cells

In some cultures of E15 thalamic cells, E19 cortical conditioned medium or normal medium was added after

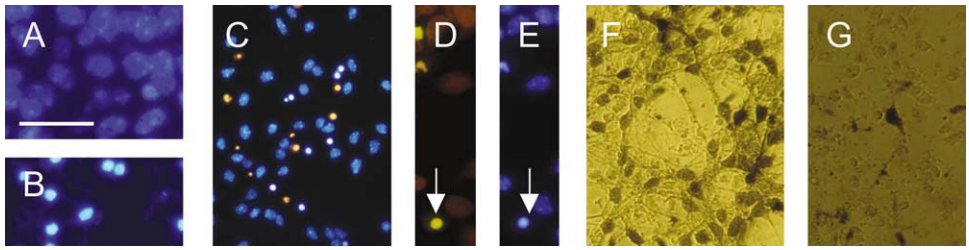

Fig. 1. Photomicrographs illustrating the appearances of cells from (A,B) explants and (C–G) dissociated cultures. (A) Bisbenzimide stained section of an E15 thalamic explant, showing diffusely stained healthy nuclei. (B) Bisbenzimide stained section of an E19 thalamic explant (no added potassium) showing dead cells with dense chromatin condensation, a feature associated with apoptosis (Kerr et al., 1972). (C) A culture of dissociated thalamic cells showing vital staining with bisbenzimide (blue) and propidium iodide (orange). The nuclei of many cells are healthy; those of others show dense chromatin condensation and some of these cells contained propidium iodide, indicating disruption of their cell membranes (Rennie et al., 1994), a feature associated with late-stage apoptosis. (D) TUNEL-positive cells (yellow, e.g., arrow) in a culture of E15 thalamic cells. (E) Same field of view as in D showing staining with bisbenzimide; the TUNEL-positive cells have large dense chromatin condensations (arrow). (F) A culture of E15 dissociated thalamic cells after 5 days in cortical conditioned medium, stained for GABA: most cells are healthy, only a small proportion are GABAergic (dark brown). (G) A culture of E15 dissociated thalamic cells after 5 days in normal medium, stained for GABA: most cells are dead, only one healthy cell with processes is seen and it is GABAergic. Scale bar: 10 μm in A and B; 40 μm in C,F, and G; 20 μm in D and E.

24 h and then changed for normal medium after 2 or 4 days in vitro. In other cultures of E15 thalamic cells, E19 cortical conditioned medium that had been heated to 85°C for 30 min was added after 24 h.

Immunohistochemistry for GABA

Dissociated thalamic cells were fixed in 4% paraformaldehyde for 15 min. They were reacted with affinity-purified (polyclonal) rabbit anti-GABA antibody (1:1000, Sigma, UK; 4°C overnight), a biotinylated anti-rabbit secondary antibody (4°C overnight), avidin and biotin complex (ABC kit, Vector, UK; 30 min), and 3,3′-diaminobenzidine. For negative controls, the primary antibody was either omitted or preadsorbed with GABA (1:100, mol/mol).

Analysis

In both explants and dissociated cultures, the numbers of cells that were living or dead were counted (with the observer blind to the nature of the culture conditions) in a series of randomly placed grid squares on a fluorescence microscope and the percentages of living and dead cells were obtained. Data for several repeats of each condition were combined and compared with Student's *t* test.

Results

Fig. 1 illustrates the appearances of live and dead cells in explants (Fig. 1A,B) and dissociated cultures (Fig. 1C–E). Dead cells showed dense chromatin condensation characteristic of apoptosis (Kerr et al., 1972); those that were in the late stages of apoptosis allowed propidium iodide to enter the cell body (Fig. 1C). Cells that were identified as apoptotic with bisbenzimide were also TUNEL positive (Fig. 1D,E); having confirmed this, we did not use TUNEL in all experiments.

Effects of potassium and glutamate concentrations on explant survival

The proportions of cells surviving in thalamic explants aged E13–P2 that had been cultured without additional potassium or glutamate decreased sharply with age (black bars, Fig. 2A,B). Before E17, most survived, whereas after E17 most died; at E17, around 50% of cells survived. Raising potassium concentrations by 0.5 or 5 mM had little or no effect on survival of E13 and E15 explants, but caused a large increase in survival of E17 and E19 explants (Fig. 2A; $P < 0.01$). Raising potassium concentrations by 50 mM decreased survival of E13–E19 explants; raising potassium concentrations by 0.5–50 mM had no effect on P2 explants (Fig. 2A). Raising glutamate levels by 0.05 mM increased the survival of E17 thalamic explants ($P < 0.01$) but had no effect at other ages (Fig. 2B). Higher levels of glutamate decreased the viability of E13–E17 explants; increasing glutamate levels had no effect on the viability of E19 explants (Fig. 2B). Adding NMDA to E17 explants had a similar effect to the addition of 0.05 mM glutamate, increasing their viability to 81 ± 9% (SEM) ($P < 0.01$; $n = 6$ explants). Adding APV decreased the viability of E17 explants grown with an additional 0.05 mM glutamate to 49 ± 6% with 0.5 μM APV, 45 ± 2% with 5 μM APV, 28 ± 7% with 50 μM APV, and 3 ± 3% with 500 μM APV ($n = 3$ explants and $P < 0.05$ in all cases).

Most cells in E19 and P2 cortical explants survived without additional potassium and increasing potassium concentrations by 0.5 or 5 mM had no effect (Fig. 3); most cells died when the concentration was increased by 50 mM (unfilled bars in Fig. 3).

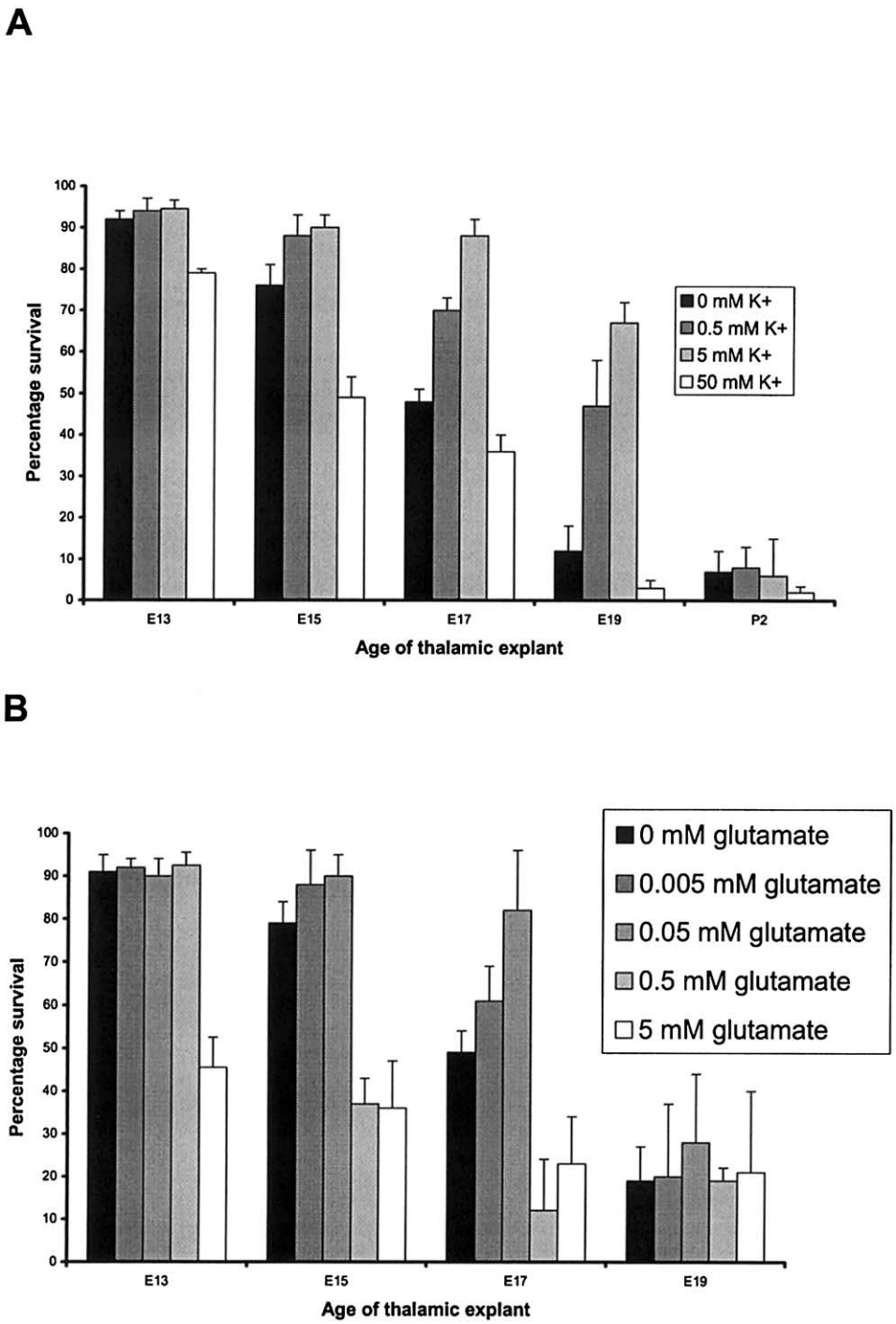

Fig. 2. Histograms show average percentages (± SEMs; $n = 4–7$ explants per data point) of surviving cells in explants of E13–P2 thalamus cultured (A) with 0–50 mM additional potassium or (B) with 0–5 mM additional glutamate.

Effects of conditioned medium on dissociated thalamic cells

When E15 dissociated thalamic cells were cultured for 5 days in normal (unconditioned) medium, most died (filled bar on the left in Fig. 4). An additional 0.5 or 5 mM potassium did not increase their survival; an additional 50 mM potassium reduced their survival to almost zero (Fig. 4). Culturing in E19 thalamic conditioned medium (Fig. 4: E19TCM) generated with 0–5 mM potassium increased survival ($P < 0.01$) (conditioned medium generated with an additional 50 mM potassium was ineffective). The greatest increase was when the thalamic conditioned medium had been generated with additional potassium and K252a blocked this effect in a dose-dependent manner (Fig. 5). Culturing in P2 thalamic conditioned medium (Fig. 4:

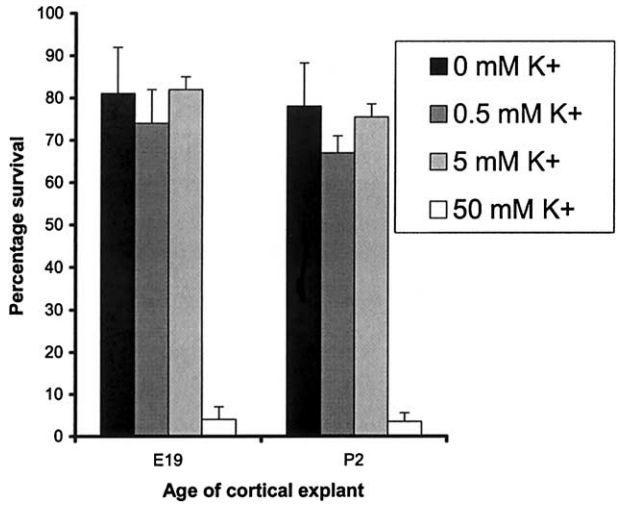

Fig. 3. Histograms show average percentages (± SEMs; $n = 4$–5 explants per data point) of surviving cells in explants of E19 and P2 cortex cultured with 0–50 mM additional potassium.

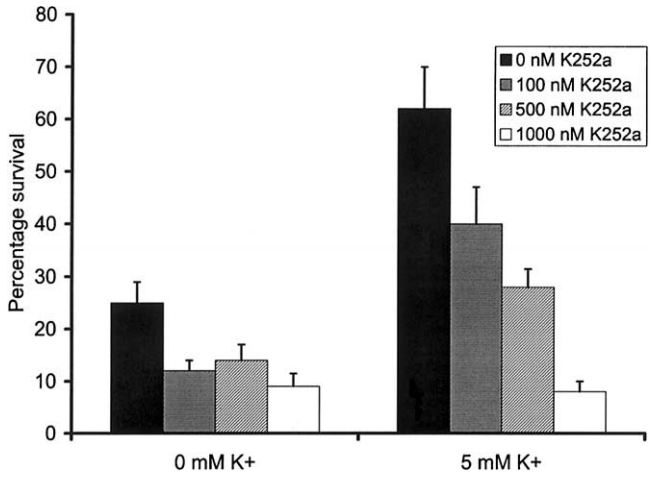

Fig. 5. Histogram shows the blocking effects of K252a on the potassium-induced survival of E19 thalamic cells (shown in Fig. 4, E19TCM). Values are means (± SEMs; $n = 4$ cultures each).

P2TCM) generated with 0–5 mM extra potassium caused only a small increase in survival ($P < 0.05$) (conditioned medium generated with an additional 50 mM potassium was ineffective). E19 and P2 cortical conditioned medium were both equally effective in promoting survival when prepared with 0–5 mM ($P < 0.01$), but not with 50 mM additional potassium (Fig. 4: E19CCM and P2CCM).

The ability of the E19 thalamic conditioned medium prepared with 0.5 or 5 mM additional potassium to stimulate a large increase in dissociated thalamic cell survival (Fig. 4: E19TCM) is similar to the survival enhancing effect of similar doses of potassium on E19 thalamic explants (Fig. 2A). P2 thalamic conditioned medium stimulated a small increase in dissociated thalamic cell survival (Fig. 4: P2TCM; $P < 0.05$), although the use of additional potassium did not enhance the effect, reflecting the lack of an effect of additional potassium on P2 thalamic explants (Fig. 2A). We tested whether the P2 thalamic conditioned medium was more effective on dissociated P2 thalamic cells, rather than on E15 thalamic cells cultured for 5 days, even though this seemed unlikely given that so few cells survived in isolated P2 thalamic explants (Fig. 2A). As predicted, only 4 ± 1% ($n = 3$) of P2 thalamic cells survived with P2 thalamic conditioned medium. Also, we confirmed that addition of P2 cortical conditioned medium to P2 dissociated thalamic cells increased their survival to 44 ± 4% ($n = 3$; $P < 0.01$).

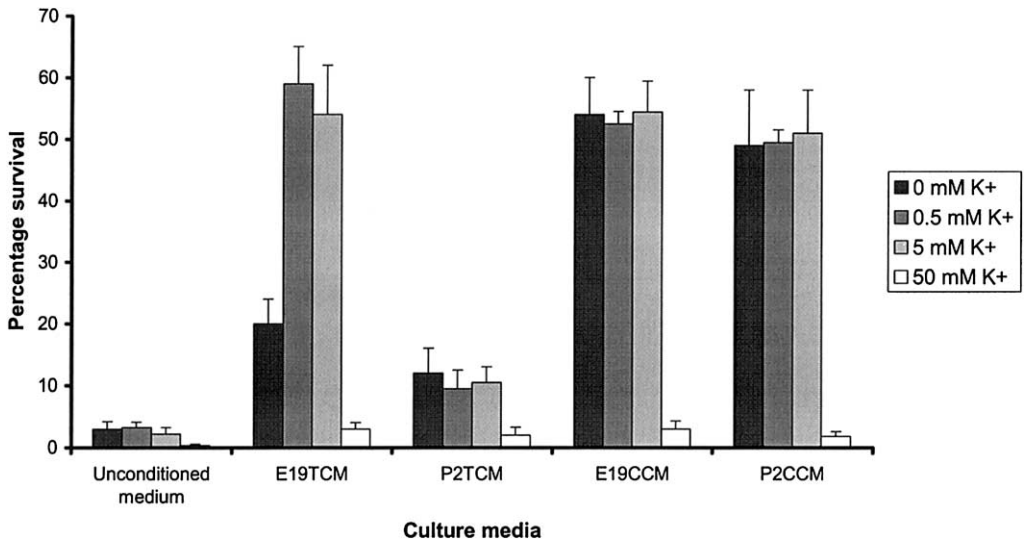

Fig. 4. Histograms show average percentages (± SEMs; $n = 4$ cultures per data point) of E15 thalamic cells surviving in dissociated culture after 5 days. The medium was either unconditioned or conditioned with E19 or P2 thalamic explants (E19TCM and P2TCM) or E19 or P2 cortical explants (E19CCM and P2CCM). Potassium (0–50 mM) was added to the unconditioned or conditioned medium.

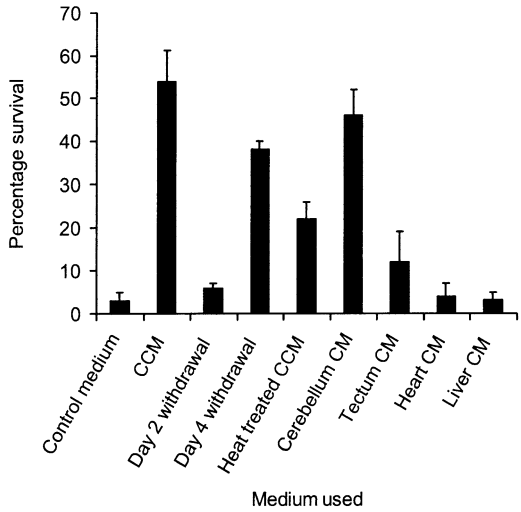

Fig. 6. Histograms show average percentages (± SEMs) of E15 thalamic cells surviving in dissociated culture after 5 days. Medium was unconditioned (control; $n = 10$ cultures); was conditioned with E19 cortical explants (CCM; $n = 10$ cultures); had E19 cortical conditioned medium added and then withdrawn after 2 or 4 days ($n = 10$ cultures in each case); was conditioned with heat-treated E19 cortical conditioned medium ($n = 5$ cultures); was conditioned with E19 cerebellum, tectum, heart, or liver ($n = 10$ cultures in each case).

In experiments testing the effects of cortical conditioned medium on E15 thalamic cells cultured for 5 days, most (68% on average) of the few live cells remaining in unconditioned medium were GABAergic whereas most (91% on average) cells surviving in conditioned medium were not GABAergic (Fig. 1F,G). This indicates that it is the non-GABAergic thalamic projection neurons that are most affected by the absence of trophic support from cortex.

We tested the effects of withdrawing cortical conditioned medium from E15 thalamic cells cultured for 5 days, either after 2 or after 4 days in vitro. Following withdrawal after 2 days, survival at 5 days was no different from that without cortical conditioned medium (Fig. 6).

Following withdrawal after 4 days, survival at 5 days was lower than when cortical conditioned medium was present throughout but higher than without cortical conditioned medium (Fig. 6). Heat treating cortical conditioned medium roughly halved its effectiveness in promoting survival (Fig. 6; $P < 0.01$). Survival of thalamic cells was also obtained with medium conditioned by some other tissues: cerebellum was very effective ($P < 0.01$), tectum was effective ($P < 0.05$) but less so, and heart and liver were not effective (Fig. 6).

Discussion

When E13–P2 thalamic explants were isolated and cultured, their ability to survive decreased with their age. Up to the time of birth, it was possible to maintain the viability of most cells in the explants provided that the concentrations of potassium or glutamate were optimized, but this was not the case after birth. Previous work has shown that depolarizing agents such as glutamate and KCl enhance the survival of other central neurons in vitro (Ghosh et al., 1994; Cohen-Cory et al., 1991; Meyer-Franke et al., 1995). Our results indicate that these trophic effects become important in the thalamus at the time when its afferent and efferent connections are becoming established and active (reviewed by Ferrer et al., 1992; Lotto and Price, 1995; Yuasa et al., 1994; reviewed by Molnar, 1998; Auladell et al., 2000). Additional glutamate was only effective at E17 while additional potassium was effective at both E17 and E19; our evidence is that, at E17, activation of NMDA receptors mediates the response to additional glutamate and is essential for the viability of thalamic cells. There is considerable evidence implicating glutamate as a neurotransmitter in the thalamus and functional NMDA receptors are present throughout the embryonic forebrain (Rossi and Slater, 1993; Sillito et al., 1990; Scharfman et al., 1990). Work on other parts of the nervous system has shown that, in addition to its role in neural transmission, glutamate can be either trophic or neurotoxic depending on its concentration (Ikonomidou et al., 1999; reviewed by Lipton and Kater, 1989; Llado et al., 1999). An additional requirement for nonglutamatergic signaling may become increasingly important as the thalamus develops toward birth, which may explain the lack of effect of glutamate concentrations at E19. Our findings indicate that this culture system may mimic many of the survival requirements of developing thalamic cells in vivo, where intact thalamic afferents and targets are required for the survival of a normal complement of thalamic cells (Finlay et al., 1986; Cunningham et al., 1987; reviewed by Finlay and Pallas, 1989; Eagleson et al., 1990; Agarwala and Kalil, 1998). This offers a model system that may aid with the identification of molecules required for thalamic survival.

One of our main aims was to test whether increased thalamic viability with added potassium requires or is mediated by the release of diffusible trophic factors. We found that medium in which late prenatal (E19) thalamic explants were cultured with optimal doses of potassium was able to promote the survival of most dissociated thalamic cells grown from E15 for 5 days, by which time almost all of these cells would have died in normal (unconditioned) medium. Since the addition of potassium to dissociated thalamic cells in normal medium was insufficient to promote their viability, this suggests that the thalamic explants whose viability was promoted by optimal doses of potassium released diffusible factors that enhanced the viability of thalamic cells. The results of adding K252a to the cultures suggested that these factors may have been one or more members of the neurotrophin family. The reason that additional potassium alone was unable to promote the survival of dissociated thalamic cells, whereas it did promote the survival of E17 and E19 thalamic explants, may have been (1) because normal contacts between thalamic cells must be maintained for them to generate trophic factors or

(2) because the numbers of cells in the dissociated cultures are insufficient to generate high enough levels of trophic factors in the medium. In other systems, the trophic effect of potassium is thought to act by inducing release of neurotrophic factors (Ghosh et al., 1994). Both in vitro and in vivo, neurotrophin production and secretion are regulated by electrical and synaptic activity (Castrén et al., 1992; Lindholm et al., 1994; Schoups et al., 1995; Blochl and Thoenen, 1995, 1996; Goodman et al., 1996). This may also be the case in our experiments.

The vast majority of P2 thalamic cells in isolated cultured explants died. They could not be rescued by adding potassium and medium conditioned by P2 thalamus had little or no effect on dissociated thalamic cells. The only way that we were able to enhance the viability of dissociated P2 thalamic cells was by culturing them in medium conditioned by cortex. We found that both prenatal and early postnatal cortical explants survived well when isolated in culture; this might reflect the fact that cortex matures later than thalamus and that less well-developed areas may have less stringent requirements for survival. Our results indicated that these cortical explants generated diffusible trophic factors that could enhance the survival of thalamic cells, including those aged P2.

We suggest that both prenatal and postnatal thalamic cells require trophic support but, whereas prenatally that support is derived from the thalamus itself, being enhanced by neural activity in the days before birth, postnatally the essential source of trophic molecules becomes the cerebral cortex. The fact that, in vivo, many thalamic cells die when the cortex is lesioned postnatally also suggests that the postnatal thalamus depends on trophic support from the cortex (Cunningham et al., 1987; Eagleson et al., 1990). Our results indicated that cortex-derived factors are unlikely to be specific to cortex, since medium conditioned by other neural tissues (cerebellum and tectum) also had trophic effects on thalamic cells, although for thalamic cells cortex would be their major source in vivo.

The increasing dependency of thalamic cells on an appropriate level of depolarization and later on trophic factors from the cortex indicates that the viability of these cells becomes increasingly dependent on influences from outside the thalamus itself. One interesting question concerns the mechanisms by which thalamic cells alter their trophic requirements as they develop. It is possible that programmed changes in the expression of genes that regulate apoptosis, for example, bcl-2 (anti-apoptotic) and possibly bax (pro-apoptotic) (Pettman and Henderson 1998), increase the propensity of thalamic cells to undergo programmed cell death as they age. For example, bcl-2 production declines with age in most CNS neurons (reviewed by Holm and Isacson, 1999). The CNS expresses bcl-2 during mid to late gestation and expression is downregulated at the time of birth, persisting in regions with late differentiation such as the dentate gyrus (Merry et al., 1994). Changes in the bax/bcl-2 expression ratio correspond with the timing of neuronal death in the rat cortex and thalamus (Mooney and Miller, 1999). Furthermore, bcl-2 has a neuroprotective effect against neurotrophin deprivation in many types of neurons and changes in its levels may control the timing of programmed cell death (Allsopp et al., 1993; reviewed by Holm and Isacson, 1999). Changes in the expression of genes such as bcl-2 may reduce the intrinsic ability of thalamic cells to suppress their apoptotic pathways and may increase their reliance on survival enhancing biochemical pathways that are activated by extracellular stimulation. The manipulation of expression of such genes in the culture system described here offers one way of testing such hypotheses.

Acknowledgments

This work was supported by The Wellcome Trust, a Thai Government Scholarship to P.A., and a Wellcome Trust Prize Studentship to G.A.

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