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DEVELOPMENTAL CHANGES IN B-50 (GAP-43) IN PRIMARY CULTURES OF CEREBRAL CORTEX: CONTENT AND PHOSPHORYLATION OF B-50

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Abstract—The content and phosphorylation of the neuronal growth-associated protein B-50 (GAP-43) were studied in cultured neocortex as a function of normal development and development in the presence of tetrodotoxin (TTX), a blocker of bioelectric activity (BEA). The observations were correlated with previous morphological findings on neurite outgrowth and B-50 immunolocalization in the same cultures.

In control cultures, the concentration of B-50 reached a maximum at 7 days *in vitro* (DIV) and decreased thereafter, whereas the concentration of neuron specific enolase (NSE), which was used as a neuronal reference marker, rose till 28 DIV and leveled off towards 42 DIV. The degree of basal phosphorylation of B-50 (relative to that of total protein) decreased after the first week *in vitro*. Stimulation of B-50 phosphorylation by phorbol ester also decreased with age *in vitro*, indicating that changes in B-50 phosphorylation were mainly due to changes in protein kinase C (PKC) activity.

The chronic presence of TTX led to a reduced content of B-50 and NSE after 14 DIV. The basal phosphorylation of B-50 was neither affected by acute nor chronic TTX treatment. However, upon stimulation of PKC with phorbol esters, some alterations of B-50 phosphorylation were revealed in cultures grown in TTX. These biochemical observations are in line with the absence of effects of TTX on neurite outgrowth during the first 2 weeks in culture, and later effects of TTX on neuronal survival.

The developmental changes in B-50 concentration and phosphorylation largely correlate with previous morphological observations on axonal outgrowth and growth cone shape in the same cultures. We suggest that B-50 phosphorylation plays an important role in transducing extracellular signals into directed neurite outgrowth.

Key words: B-50 (GAP-43), phosphorylation; protein kinase C, neurite outgrowth, cerebral cortex culture, bioelectric activity, tetrodotoxin.

Formation of specific connections within the developing brain involves neurite outgrowth to the target and the formation of selective synapses. These complex processes are guided by extracellular clues, which, by acting on receptors present in the membrane of growth cones, exert a modulating influence on the intracellular machinery for outgrowth and synaptogenesis.[7,14,16,18,35] A likely candidate for the membrane signal transduction system that conveys directional clues onto the outgrowth mechanism, is the neuron-specific phosphoprotein B-50, also known as GAP-43, pp46, F1, or P57.[4,35] B-50 is a growth-associated protein,[4,35–37] which is most prominently localized in growth cone membranes,[4,10,35] and is strongly expressed during developmental and regenerative neurite outgrowth.[15,36,37] As one of the main substrates for protein kinase C in growth cones,[17,41] it has been suggested that B-50 phosphorylation plays a role in membrane signal transduction by modulating the metabolism of the polyphosphoinositides[41] and the subcellular distribution of calmodulin,[1] in response to external stimuli.[42] Moreover, B-50 has recently been found to affect the binding of GTP to a G_0-protein.[38]

In a preceding paper,[32] we used primary cultures of rat cerebral cortex to demonstrate developmental changes in neurite outgrowth parameters, and effects of chronic addition of tetrodotoxin (TTX), which blocks spontaneously occurring bioelectric activity (BEA) in these cultures. In the present study, we relate these morphological findings to changes in the content and phosphorylation of B-50 in the same cultures grown in the presence or absence of BEA. Since

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Abbreviations: BEA, bioelectric activity; DIV, days *in vitro*; NSE, neuron-specific enolase; PKC, protein kinase C; PAGE, polyacrylamide gel electrophoresis; 4α-PDD, 4α-phorbol-12,13-didecanoate; 4β-PDB; 4×-phorbol-12,13-dibutyrate; PBS, phosphate-buffered saline; Pi, inorganic phosphate; PMSF, phenylmethylsulfonylchloride; SDS, sodium dodecyl sulfate; TTX, tetrodotoxin.

these cultures are only partially neuronal,[30] changes in B-50 content were related to changes in the content of the cytosolic neuron-specific enolase (NSE), which was used as a general neuronal marker, and to total protein and DNA, which reflect changes in all cells. The effects of suppression of BEA and TTX were studied, since this condition has been shown to affect various aspects of neuronal development in culture,[8,19,29–32,34,43,44] as well as the synthesis and/or axonal transport of B-50 *in vivo*.[2,5]

EXPERIMENTAL PROCEDURES

Culturing methods and suppression of BEA

Cultures were established and grown as described in the preceding paper.[32] BEA was chronically suppressed with 0.1 μM TTX starting at 1 or 6 DIV as described in the same paper.

Measurement of B-50, NSE, protein and DNA

Cultures were harvested in 100 μl ice-cold saline and stored at −80°C. After thawing at 4°C, the tissue was sonicated for 15 sec using an MSE 100 W ultrasonic disintegrator and aliquoted for the different assays. B-50 content was measured by radioimmunoassay.[27] NSE levels were measured by quantitative immunoblotting[31,40] using a rabbit-anti-rat NSE, kindly provided by Dr P. J. Marangos.[21] For the latter assay, aliquots of tissue homogenate were separated by 11% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically blotted onto nitrocellulose. Strips of the blots containing the NSE bands of samples and of a concentration range of reference tissue (21-day-old rat cerebral cortex) were immunostained and quantitated,[40] using a Kontron/IBAS 2000 image analyzer. DNA content was measured according to Brunk *et al.*[9] Protein content was measured using BSA as standard.[20]

Phosphorylation studies

Cultures were rinsed twice with phosphate-free culture medium (at 35.5°C, containing 20 mM Hepes) for 4 min, whereafter they were preincubated for 2 hr with 1 mCi/ml [^{32}P]orthophosphate (carrier free; Amersham) in the same medium. A 2 hr prelabeling period was chosen as this had been found to result in an equilibrated labeled ATP pool. Subsequently the cultures were incubated for 30 min in phosphate-free medium containing active (4β-phorbol-12,13-dibutyrate, 4β-PDB, Sigma) or inactive (4α-phorbol-12,13-didecanoate, 4α-PDD, Sigma) phorbolesters or TTX (0.1 μM). Labeling of the ATP pool remained stable during this 30 min period as checked with high-voltage paper electrophoresis. Similarly, the labeling of total protein and the labeling pattern did not change during this period as seen with TCA precipitation and SDS-PAGE. Following a quick rinse in PBS, the tissue was scraped off into 100 μl Tris buffer (10 mM, pH 7.3), containing 0.1 μM PMSF (phenylmethylsulfonylfluoride, Merck). After the addition of 25 μl 10% SDS, the tissue was boiled for 10 min and stored at −80°C. Phosphatase inhibitors were omitted from the buffer, since in this procedure they affected neither the level nor the pattern of protein phosphorylation in pilot studies. Incorporation of [^{32}P]orthophosphate into total protein and into B-50 were measured after TCA precipitation, and after monospecific (see Fig. 4) and quantitative immunoprecipitation of B-50, respectively.[11]

RESULTS

Developmental profile of DNA, protein, B-50 and NSE in the presence and absence of TTX

In control cultures, DNA content showed a rapid increase starting at 7 DIV, reaching a maximum at 14 DIV, and decreasing more gradually to about 60% of the maximum level at 42 DIV (Fig. 1a). Protein content increased nearly linearly from the beginning, reached its maximum at 21 DIV and declined to about 60% of this level by 42 DIV (Fig. 1b). The content of B-50 also reached a maximum around 21 DIV, showing the highest increase during the first week, and dropped to 50% of its maximum level in the sixth week (Fig. 2a). In contrast, the content of the neuronal reference marker NSE was below the detection limit at 7 DIV, and rose at a constant rate between 7–21 DIV, to reach maximal levels between 21 and 28 DIV, followed by a gradual decrease to 70% towards 6 weeks (Fig. 2b). The B-50 concentration (expressed per μg total protein) was relatively constant throughout development, except for a peak (100% increase)

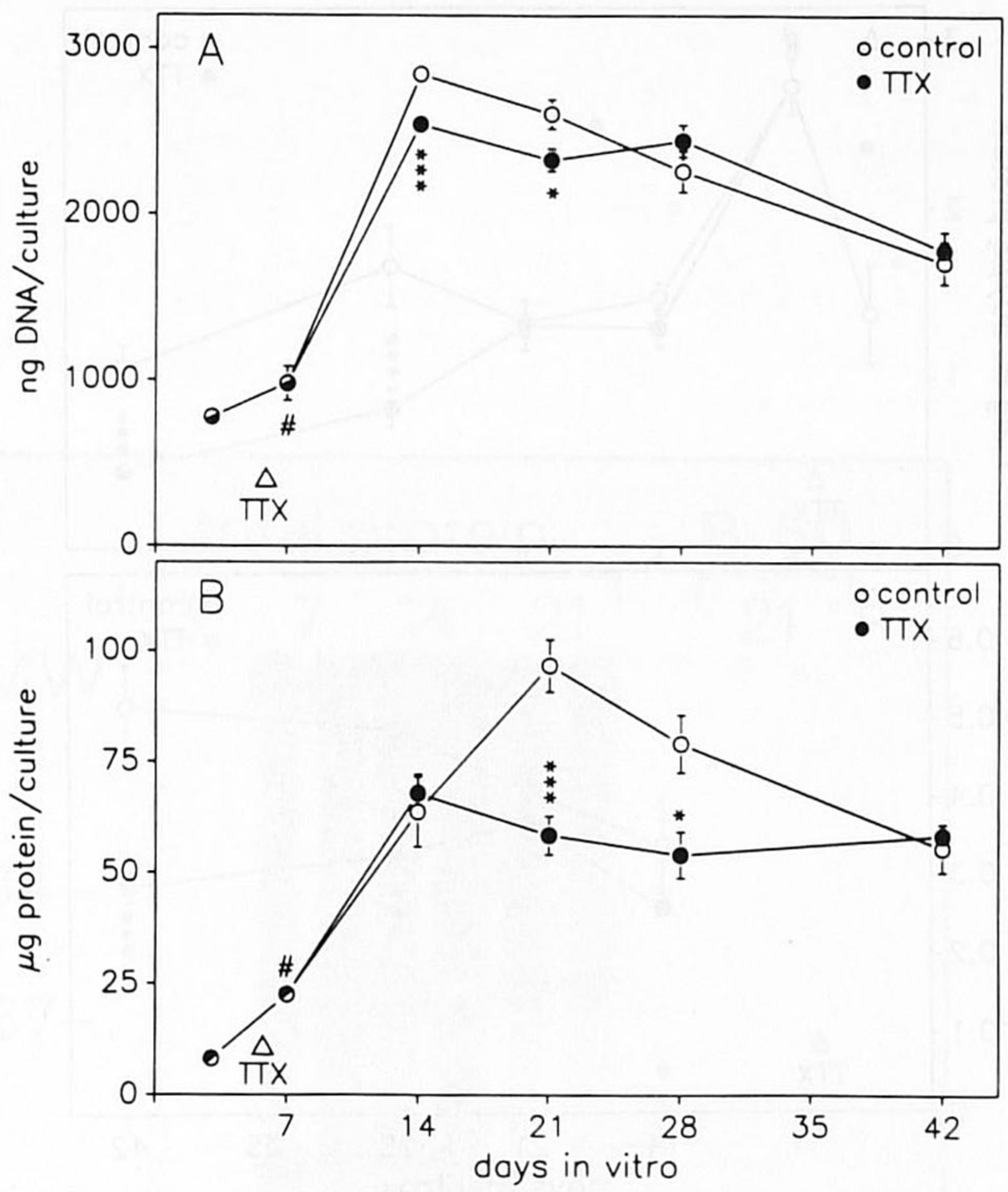

Fig. 1. Content of DNA (A) and protein (B) per culture as a function of age *in vitro* and the presence of TTX. TTX treatment was started at 6 DIV (indicated by arrowhead). Mean values ± S.E.M. Two-sided *t* test between controls and TTX-treated groups of similar age: $^{*}P<0.05$; $^{***}P<0.001$, $n=4/5$; #: TTX value not determined at 7 DIV in this experiment but found similar to the control value in another experiment.

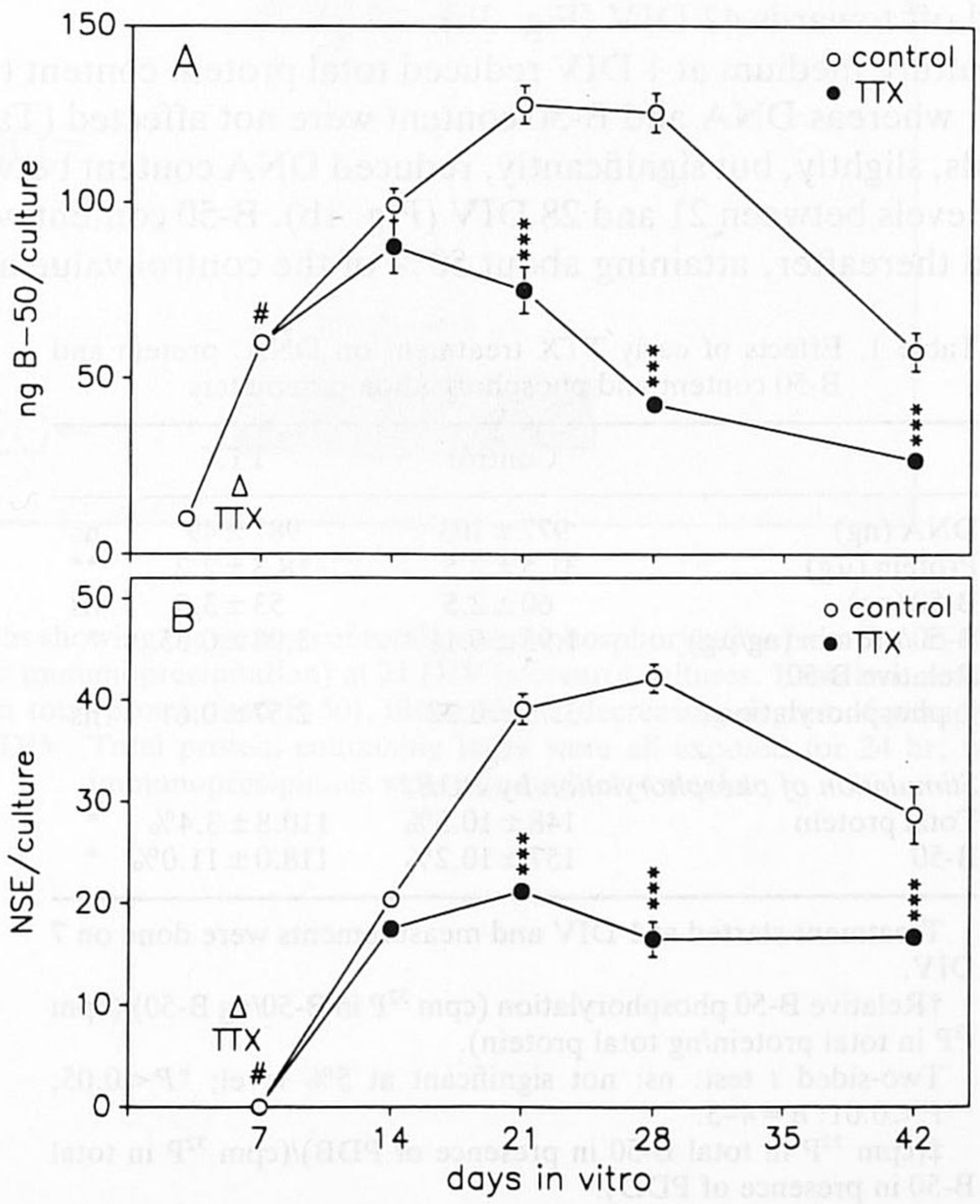

Fig. 2. Content of B-50 (A) and NSE (B) per culture with time *in vitro* in the presence or absence of TTX. NSE is expressed in relative units. See further the legend to Fig. 1.

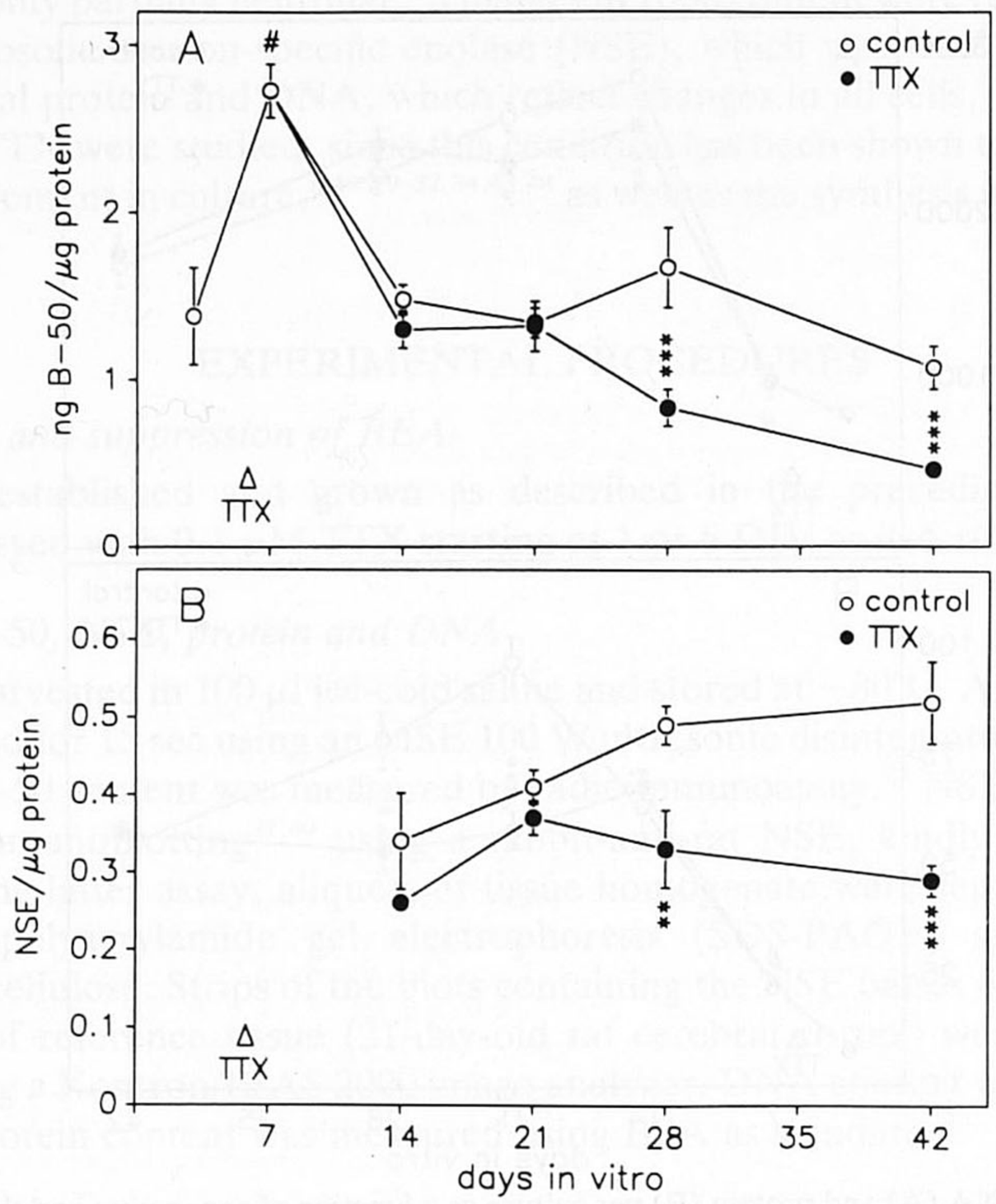

Fig. 3. Concentration of B-50 (A) and NSE (B) relative to total protein as a function of age *in vitro* and the presence of TTX. NSE is expressed in relative units. See further the legend to Fig. 1.

around 7 DIV (Fig. 3a), whereas the NSE concentration gradually increased between 14 and 28 DIV, and then leveled off towards 42 DIV (Fig. 3b).

TTX added to the culture medium at 1 DIV reduced total protein content to about 60% of the control level at 7 DIV, whereas DNA and B-50 content were not affected (Table 1). Addition of TTX for 6 DIV onwards, slightly, but significantly, reduced DNA content between 14 and 21 DIV (Fig. 1a), and protein levels between 21 and 28 DIV (Fig. 1b). B-50 content was unaffected up to 14 DIV, but decreased thereafter, attaining about 50% of the control value at 42 DIV (Fig. 2a),

Table 1. Effects of early TTX treatment on DNA, protein and B-50 content and phosphorylation parameters

	Control	TTX	
DNA (ng)	977 ± 103	987 ± 49	ns
Protein (μg)	31.5 ± 2.5	18.3 ± 2.7	**
B-50 (ng)	60 ± 2.5	53 ± 3.5	ns
B-50/protein (ng/μg)	1.95 ± 0.11	3.08 ± 0.35	*
Relative B-50 phosphorylation†	3.71 ± 0.32	2.57 ± 0.61	ns
Stimulation of phosphorylation by PDB‡			
Total protein	148 ± 10.5%	110.8 ± 3.4%	*
B-50	157 ± 10.2%	118.0 ± 11.0%	*

Treatment started at 1 DIV and measurements were done on 7 DIV.

†Relative B-50 phosphorylation (cpm ^{32}P in B-50/ng B-50)/(cpm ^{32}P in total protein/ng total protein).

Two-sided *t* test: ns: not significant at 5% level; $^{*}P<0.05$; $^{**}P<0.01$; $n=4$–5.

‡(cpm ^{32}P in total B-50 in presence of PDB)/(cpm ^{32}P in total B-50 in presence of PDD).

PDB: 4×-phorbol 12,13-dibutyrate (1 μM); PDD: 4α-phorbol 12,13-didecanoate (1 μM).

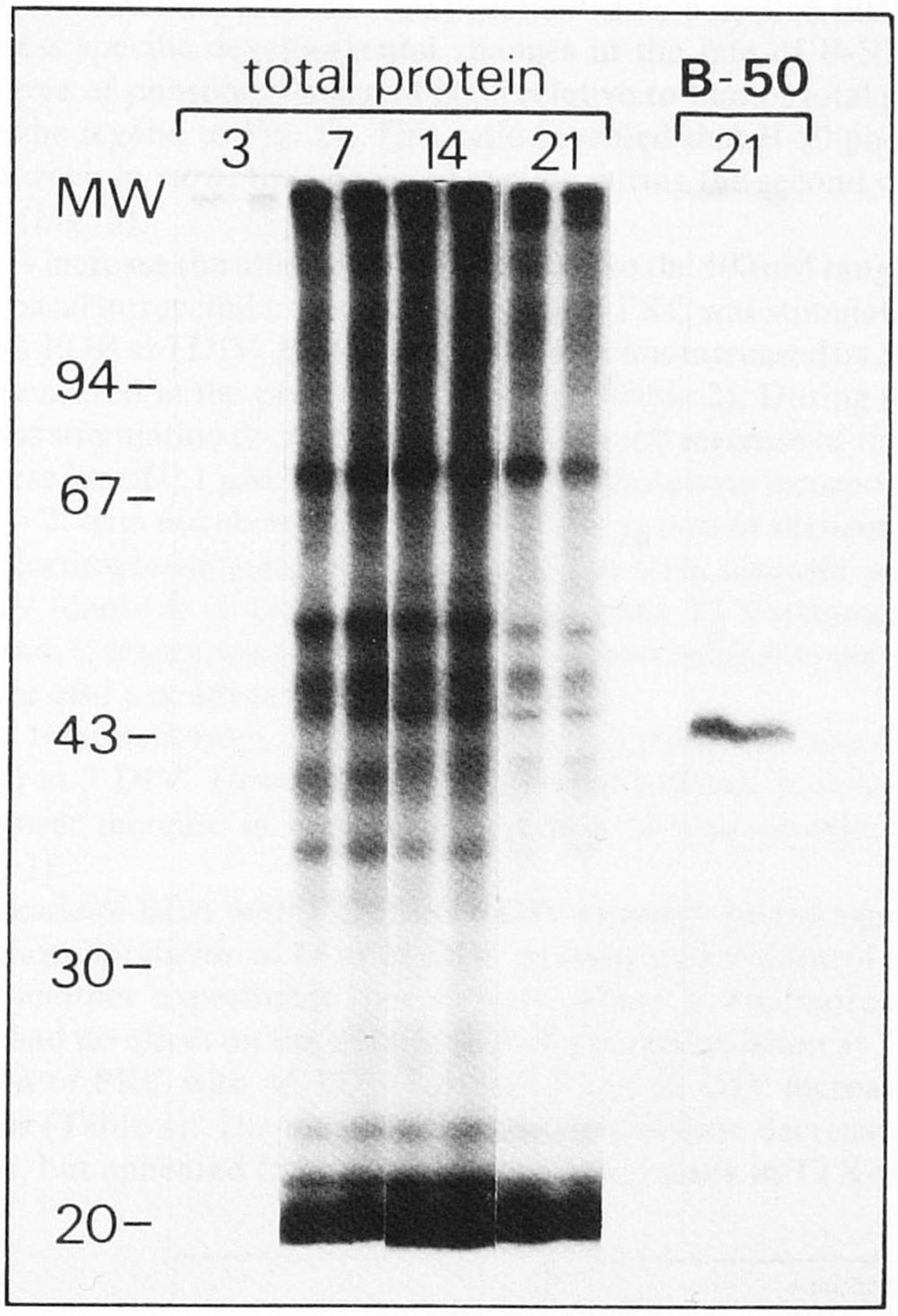

Fig. 4. Autoradiographs showing the pattern of total protein phosphorylation from 3 to 21 DIV and B-50 phosphorylation (after immunoprecipitation) at 21 DIV in control cultures. Each lane contains 1/6th of the total culture (both total protein and B-50), illustrating a decreasing degree of phosphorylation of total protein after 7 DIV. Total protein containing lanes were all exposed for 24 hr, whereas B-50 immunoprecipitates were exposed for 4 weeks.

NSE levels showed the same effect as seen in the pattern of total protein, i.e. it remained at the 14 DIV control level up to 42 DIV (Fig. 3a). Like B-50 levels, NSE levels at 42 DIV were about half of the 42 DIV control levels. The concentrations of B-50 and NSE relative to total protein did not differ between chronically silenced and active cultures up to 21 DIV, but thereafter were lower than in the controls (Fig. 3a and 3b).

Phosphorylation of B-50 during development and effects of TTX

During development, the incorporation of ^{32}Pi into B-50, expressed per ng B-50, showed a qualitatively similar profile as the incorporation of ^{32}Pi into total protein expressed per ng total protein: both showed a maximum at 7 DIV, followed by a decline till 21 DIV (total protein, see Fig. 4). To assess specific developmental changes in the fate of B-50 phosphorylation, we expressed the degree of phosphorylation of B-50 relative to that of total protein (by calculating the ratio shown in the legend to Fig. 5). This ratio revealed that B-50 phosphorylation was highest during the first week *in vitro*, then declined rapidly during the second week and stabilized during the third week (Fig. 5).

Phorbol esters increase the affinity of PKC for Ca^{2+} to the 100 nM range, resulting in a full activation of PKC at basal intracellular Ca^{2+} levels.[25] When PKC was stimulated with 1 μM of the active phorbol ester 4β-PDB at 7 DIV, B-50 phosphorylation was increased by 50% over basal phosphorylation levels (measured in the presence of 4α-PDD; Table 2). During the following 2 weeks, the magnitude of this stimulation decreased to a nonsignificant increase of 15% at 21 DIV (Table 2).

The acute presence of 0.1 μM TTX, did not affect phosphate incorporation into B-50 in 17-day-old cultures ($n = 2$; data not shown). Extension of the period of silencing to 2.5 hr by adding TTX during the [^{32}P]orthophosphate-prelabeling period as well, also did not affect B-50 phosphorylation at 23 DIV (controls: $1.42 \pm 0.03 \times 10^6$ versus acute TTX treatment: $1.45 \pm 0.17 \times 10^6$ cpm/culture; $n = 4$ and 5, respectively). Moreover, in both experiments phosphate incorporation into total protein was also not affected by TTX treatment.

Chronic TTX treatment from 1 DIV onwards did not affect the basal degree of B-50 phosphorylation (Table 1) at 7 DIV. However, in TTX-treated cultures, activation of PKC with 4β-PDB resulted in a lower increase in the phosphorylation of total protein and B-50 than in control cultures (Table 1).

Chronic blockade of BEA with TTX from 6 DIV onwards did not significantly affect the degree of basal B-50 phosphorylation at 14 or 21 DIV, as compared to control cultures (Fig. 5). This was reproduced in another experiment (not shown), where it was moreover found that the TTX treatment also had no effect on the degree of B-50 phosphorylation at 7 DIV, i.e. after 24 h (Fig. 5:#). Activation of PKC with 4β-PDB between 7 and 21 DIV increased B-50 phosphorylation over basal levels (Table 2). The magnitude of this stimulation decreased with development as in control cultures, but appeared to be retarded by about 1 week in TTX-treated cultures (Table 2).

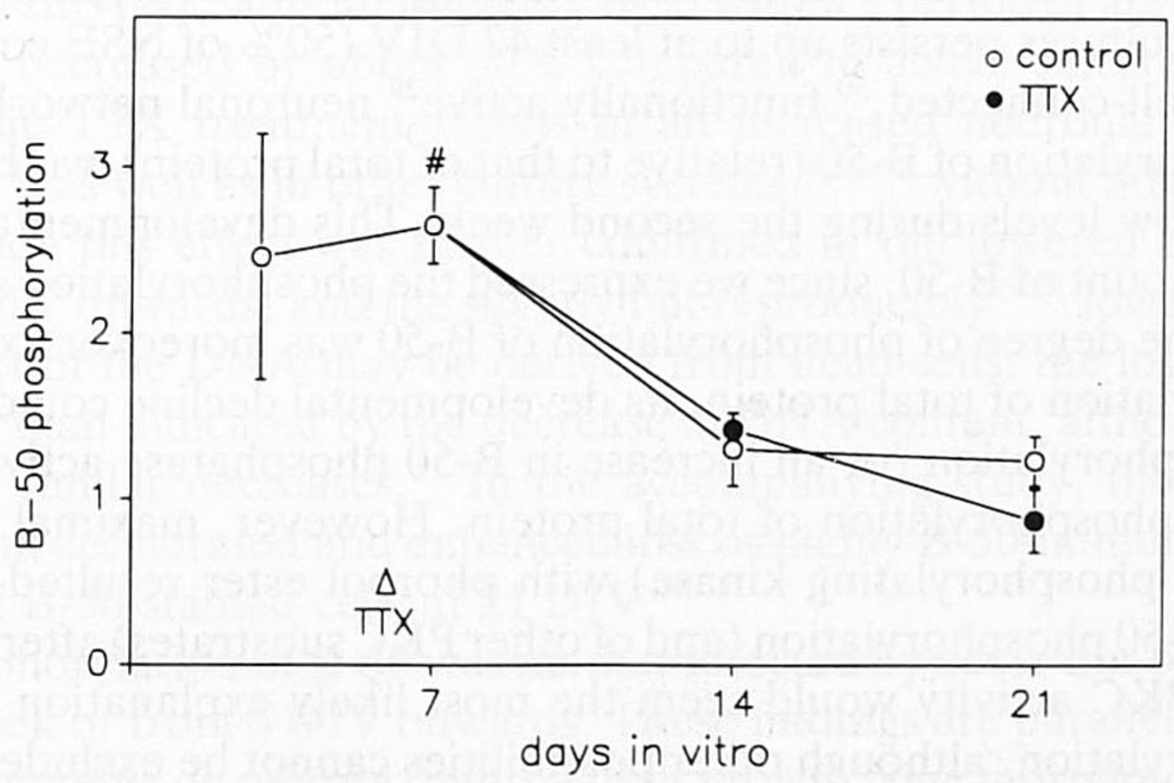

Fig. 5. Changes in the degree of phosphorylation of B-50 in culture with age and in the presence or absence of BEA. B-50 phosphorylation was expressed relative to total protein phosphorylation by calculating the ratio ($cpm_{in\ B\text{-}50}$/ng B-50)/($cpm_{in\ total\ protein}$/ng protein). A Mann–Witney U test revealed a significant decrease of the degree of B-50 phosphorylation with age ($P < 0.05$) for the control cultures. See further the legend to Fig. 1.

Table 2. Effects of stimulation of PKC with phorbol esters on B-50 phosphorylation in cultures grown in the presence or absence of TTX as a function of culture age

Age		Control			TTX		
		PDD	PDB	Increase	PDD	PDB	Increase
7 DIV	Mean	4552	6866	51%	ND	ND	
	S.E.M.	274	294	***			
	n	4	5				
14 DIV	Mean	1618	2114	31%	1417	2114	49%
	S.E.M.	65	66	***	80	93	**
	n	5	5		4	5	
21 DIV	Mean	590	679	15%	356	488	37%
	S.E.M.	91	23	ns	30	25	**
	n	4	5		5	5	

PDD: 4α-phorbol-12,13-didecanoate (1 μM); PDB: 4β-phorbol-12,13-dibutyrate (1 μM); cpm in total B-50/culture. Increase: (cpm ^{32}P in B-50 in presence of PDB)/(cpm ^{32}P in B-50 in presence of PDD) × 100%. Two-sided *t* test: ns: not significant at 5% level, $^{**}P<0.01$, $^{***}P<0.001$; ND: not determined in the same experiment; however, in another culture series the increase in B-50 phosphorylation by PDB was the same as in 7 DIV control cultures. Note: no comparison was made between controls and TTX-treated cultures, since the differences in total B-50 phosphorylation between these conditions were due to different B-50 contents, and disappeared after correction for B-50 contents (Fig. 5).

DISCUSSION

B-50 parameters during normal development in culture

The concentration of B-50 relative to total protein peaked at 7 DIV and preceded the increase in NSE, a cytosolic neuronal marker protein.[21] This indicates a high initial rate of B-50 synthesis in comparison to NSE and most other proteins in culture, which closely matches a peak in B-50 synthesis observed in rat cerebral cortex *in situ* between 3 and 7 days postnatally.[15] Moreover, it coincides with a peak in axonal elongation rate in these cultures around 8 DIV, and falls in the middle of the period characterized by neurite outgrowth,[32] in agreement with a role for B-50 in neurite outgrowth.[36,37] In short-term cerebral cortex cultures, B-50 synthesis has been found to be correlated with neurite extension.[28]

After 28 DIV the content of B-50 decreased in our long-term cultures, in contrast with another study, where B-50 levels in spinal cord cultures decreased within the first week.[39] In our cultures, this decrease probably reflects the loss of highly immunoreactive peripheral axons after 21 DIV[32] and a partial neuronal cell death,[30,31] which is also responsible for the decreased contents of NSE, DNA and protein after the third week. In spite of these regressive phenomena, a substantial part of the neurons in these cultures persists up to at least 42 DIV (50% of NSE content remaining at 42 DIV), sustaining a well-connected,[30] functionally active[29] neuronal network up to that age.

The degree of phosphorylation of B-50 (relative to that of total protein) was high during the first week, and declined to low levels during the second week. This developmental decline does not reflect changes in the amount of B-50, since we expressed the phosphorylation state relative to the content of B-50. Since the degree of phosphorylation of B-50 was moreover expressed relative to the degree of phosphorylation of total protein, its developmental decline could either result from a decrease in B-50 phosphorylation (or an increase in B-50 phosphatase activity) or from an increase in the degree of phosphorylation of total protein. However, maximal activation of PKC (which is the only B-50 phosphorylating kinase) with phorbol ester resulted in a progressively lower enhancement of B-50 phosphorylation (and of other PKC substrates) after 7 DIV. Therefore, at present, decreasing PKC activity would seem the most likely explanation for the decreasing degree of B-50 phosphorylation, although other possibilities cannot be excluded. Interestingly, in hippocampal synaptosomes, the γ-subtype of PKC shows a peak in its activity around postnatal day 14, and declines to negligible values thereafter, whereas α- and β-PKC show a continuous rise.[25] Thus, it is possible that this PKC subtype is responsible for B-50 phosphorylation in our cultures.

The phosphorylation of B-50 in growth cones is influenced by external stimuli such as depolarization and muscarinic activation.[42] On the other hand, B-50 phosphorylation affects the metabolism of inositol phosphates,[41] and may modulate the subcellular localization of calmodulin.[1] Moreover, B-50 has recently been shown to affect the binding of GTP to a G_0protein.[38] B-50 may thus be a key element in the regulation of growth cone functioning through its phosphorylation by PKC in response to certain environmental stimuli. The prominent localization of B-50 in filopodia,[32] which are regarded to be the sensors of growth cones for external stimuli and to mediate their guidance,[7,14,22] would be in line with this notion. As the expression of B-50 has been shown to induce filopodia,[45] a function of B-50 might be to regulate changes in growth cone shape, possibly through its degree of phosphorylation. In the preceding paper,[32] we found B-50 predominantly in growth cones and distal axons from 3 till 21 DIV. During this period growth cones decreased in size and complexity, and lost their filopodia after 7 DIV. By 21 DIV, growth cones were either completely lost, or transformed into presynaptic elements. During the first week, when the degree of B-50 phosphorylation was maximal, growth cones showed a 'complex' shape, which has been related to a high level of growth cone 'decision making'.[6,26] Combining these observations, we hypothetize that the phosphorylation of B-50 may function as part of a signal transduction system that conveys extracellular cues onto the growth cone cytoskeleton (in particular the microfilament system[32]), to mediate directed outgrowth. A role for B-50 phosphorylation in axonal elongation as such would seem less clear. Although after 7 DIV B-50 phosphorylation and axonal elongation rate declined in parallel, at 3 DIV a low axonal elongation rate was accompanied by a high degree of B-50 phosphorylation. However, the low net elongation rate during the first week could result from a high incidence of growth cone retractions,[32] which might actually reflect growth cone 'decision making'. Clearly, more detailed and quantitative investigations are required to relate B-50 phosphorylation to directed neurite outgrowth.

Effects of TTX treatment on B-50 content and phosphorylation

In vivo chronic TTX treatment has been found to reduce the amount of newly synthesized B-50 in regenerating nerve around 30 days, but not at 14 days after the nerve was damaged.[2,5] In our cultures, possibly similar effects of TTX were observed. Chronic TTX treatment during the first week did not affect the content of B-50, although total protein was reduced (Table 1). The latter was not due to effects on cell survival, as total DNA (and cell metabolism; unpublished observations) was not affected. Thus, during the first week TTX treatment apparently affected protein synthesis, while sparing B-50 synthesis. In the previous study these effects were parallelled by a decreased cell size without effects on neurite outgrowth (see also below). Later TTX treatment, started at 6 DIV, neither affected the content of B-50 nor total protein at 14 DIV, but did lower the content of DNA. Thus, early and later TTX treatment appeared to have a differential effect on cell growth and survival, without affecting B-50 within a period of about 1 week. After 14 DIV, B-50 content was decreased by about 50% compared to active cultures. Previous studies have shown that chronic TTX treatment results in an increased neuronal cell death from 14 DIV onwards in our,[29–31] as well as in other culture systems,[8,19,34] without affecting cell proliferation.[31] In the present study this effect was further confirmed in the lowered content of NSE and total protein from 21 DIV onwards, and the slightly but reproducibly[29,31] lower DNA content at 14 and 21 DIV. Since part of the DNA may be derived from dead cells, the loss of cells may actually be somewhat greater than indicated by the decrease in DNA content, although measurements of cell metabolism show similar decreases.[31] In the accompanying study, the effects on B-50 content were reflected in an accelerated and enhanced loss of highly B-50-immunostained axons and a decreased density of B-50-stained cells at 21 DIV.[32]

The basal phosphorylation of B-50 was neither affected by acute nor by chronic TTX treatment during the first week or from 6 DIV onwards. These findings are parallelled by the absence of any effect of these treatments on neurite outgrowth or growth cone morphology in the previous study. However, upon activation of PKC with phorbol ester, both early and later TTX treatment showed alterations in B-50 phosphorylation, apparently resulting from changes in PKC levels. Early TTX treatment reduced the phorbol ester-induced stimulation of the phosphorylation of B-50 and other PKC substrates. Since BEA only gradually appears at the end of the first week,[29] this

alteration may not be related to blocking BEA, but rather to an unknown side-effect of TTX, which also affected protein synthesis. Chronic TTX treatment started at 6 DIV seemed to retard the developmental decline in the phorbol ester-induced increase in B-50 phosphorylation by 1 week. Since in control cultures the level of bioelectric activity increases from 6 DIV onwards, the latter effect suggests that bioelectric activity could be partially responsible for the downregulation of PKC activity in our cultures. Possibly, low-level activation of PKC through neuronal network activity could over a longer time period result in a downregulation of PKC, comparable to that induced by phorbol ester in a short time period.[33] Taken together, it would seem that chronic TTX treatment affects the levels of PKC in such a way that basal levels of cellular activation do not affect B-50 phosphorylation, and that only upon stimulation an altered B-50 phosphorylation is revealed. If B-50 phosphorylation is indeed regulating growth cone shape and axonal outgrowth, this could explain why in the preceding study[32] no effects of TTX on these parameters were found. However, effects of TTX on these parameters should then be revealed upon activation of PKC (with phorbol ester, depolarization or muscarinic activity).[42] Previously, it was found that chronic TTX treatment in cerebral cortex cultures may retard synaptogenesis by about a week, alter synaptic ultrastructure,[43] and to arrest certain aspects of electrophysiological maturation at a young culture age.[29] If, and how, these phenomena are related to the changes in B-50 phosphorylation is not clear.

Conclusions

In the present and preceding study, developmental changes in the content and phosphorylation of B-50 were found to correlate with changes in axonal outgrowth rate and growth cone morphology. Suppression of BEA with TTX did not affect basal B-50 phosphorylation, in line with the absence of effects on axonal elongation and growth cone morphology. Decreases in the content of B-50 and other markers occurred only after 14 DIV and appeared primarily due to effects of TTX on neuronal survival. Our results support the GAP hypothesis,[35,37] and moreover suggest a link between B-50 phosphorylation and axon outgrowth, in which membrane signal transduction systems acting through PKC and B-50 modulate axonal outgrowth, possibly via microfilament-dependent changes in growth cone morphology.

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