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Naftidrofuryl, a putative activator of neuron survival, stimulates the expression of neurofilament heavy subunit in cultivated spinal cord neurons from chicken

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The effect of naftidrofuryl, a drug used in ischemia for its vasodilator properties and its protective effect on neuronal survival, was investigated on the maturation of cultured chicken spinal cord neurons, focusing on the presence of proteins specific for the developing neuronal cytoskeleton. Although no influence of naftidrofuryl on the rate of growth of neurites was observed, the drug enhanced the relative amount of the high molecular weight neurofilament subunit whithout affecting the concentration of a microtubule-associated protein, MAP2. These findings suggest that the effect of naftidrofuryl on cultured spinal cord neurons might involve molecular events directly associated with the induction of a mature cytoskeleton architecture, instead of stimulating undifferentiated neurite growth.

The search for drugs enhancing neuron survival and differentiation is of great importance for improving repair and regeneration of damaged neurons. Naftidrofuryl, a drug used for several years in the management of various brain and peripheral vascular disorders (1-4), was also shown to improve directly the metabolism of cultured neurons (5,6). The molecular mechanisms underlying the neurotrophic effect of naftidrofuryl are not established. However, it was shown earlier that this compound interfers with the serotonin S2 receptors in artery myocytes, and inhibits serotonin-induced artery constriction (7,8). These antiserotonergic property of naftidrofuryl on vascular systems might account for the protective effect of the drug in ischemic disease (3,9,10,11). In addition, naftidrofuryl, which protects neurons in ischemic damage (11), stimulates the intracellular concentration of cyclic AMP in cultured chicken embryonic forebrain neurons (6) and influences directly the neuronal maturation and survival in vitro (5,6). Taken together, these data suggest that naftidrofuryl might influence the recovery (11) or the establishment (5,6) of the axo-dendritic differentiation of immature neurons (12-17). This hypothesis was approached in chicken spinal cord neurons in culture by quantitative analysis of two marker proteins of the differentiation process of neurites into dendrites and axons: the microtubule-associated protein MAP2 specific of mature dendritic microtubules (MT) (15) that is expressed initially in undifferentiated neurites (18) and the neurofilament high molecular weight subunit (NF-H), the

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Abbreviations: Neurofilaments: NF. Neurofilament heavy molecular weight subunit: NF-H. Neurofilament medium molecular weight subunit: NF-M. Neurofilament light molecular weight subunit: NF-L. Microtubules: MT. Microtubule-associated protein 2: MAP2. Tris Buffer Saline: TBS.

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delayed expression of which is indicative of the establisment of a mature axonal neurofilament (NF) network (13,17,19,20).

Materials and methods

Cell culture: 5 days old chick embryos were used. Spinal cord were dissected in culture medium (see below), and cells were dissociated by incubation for 15 min. at 37°C in 0.05% trypsin (Sigma), followed by three passages in Pasteur pipettes with progressive lower diameters. Cells were seeded on poly-L-lysine (Sigma) coated dishes at low density (10^5 cells per $9cm^2$ culture dish) in DMEM (Gibco), 4% fetal calf serum (Gibco), 1% ultroser-G (Gibco), 20 mM glutamine (Sigma). Cultures were incubated at 37°C in a 5% CO_2 atmosphere. Naftidrofuryl was obtained from Lipha Santé (Division Oberval), Lyon, France, by the courtesy of Dr. M. Mosnier.
Neurite length determination: Cells were examined by phase microscopy using a Nikon Diaphot TMD microscope equipped with videocamera and image analyzer (Bit Pad Two Data Tablet, Summagraphics Corp., U.S.A. and Systemes Analytiques, Etanchéité Débit Pression Vision, F.). Neurite length and number in random fields of cultures were mesured on the monitor screen (magnification: 610).
Protein purification: Purified rat brain microtubules were obtained according to (21). NF from bovine spinal cord were purified according to (22). Purification of the NF-H subunit from urea-solubilized NF was made as previously described (23). The pure NF-H molecule was renatured according to (22). Protein concentration was measured according to (24).
SDS-PAGE and immunoblotting: Cell proteins were dissolved in 0.1 ml per dish of Laemmli denaturating buffer (25). Identical samples (40 µl) were analyzed on 6% acrylamide SDS-PAGE according to (25) and transfered under stricIty identical conditions (2 h, 0.8 mA/cm^2 in a semi-dry transfer apparatus, Pharmacia) onto nitrocellulose according to (26). Replicas were saturated with 5% skimmed milk powder in Tris Buffer Saline, pH 7.4 (TBS) for 3 h before incubation with the first antibodies (affinity-purified IgG: polyclonal anti-MAP2 or anti-NF-H) (dilutions 1/1000 in 3% bovine serum albumin, 0.5% polyvinylpyrolidone, Sigma, in TBS). The washing solution contained 0.5% Tween 20 in TBS. The second antibody was an anti-rabbit IgG raised in donkey and coupled with ^{125}I (Amersham) and was used at a concentration of 0.5 µCi/ml in TBS containing 5% skimmed milk powder. An alternative detection method involved peroxidase-coupled anti-rabbit IgG revealed by chemoluminescence with the ECL kit from Amersham. The intensity of the labelled spots on autoradiograms (X-R films 3M, General Electric) of the dryed membranes after extensive washing was measured with an image analyser using identical contrast (Sapphire, Quantel, Cambridge Instruments). Results from three separate experiments were expressed with standard deviation (S.D.) and significant differences were determined by the student t test.
Antibodies: Antibodies against rat brain MAP2 were obtained as previously reported (27). The NF-H polyclonal antibody was obtained by injecting the pure renaturated NF-H subunit to a rabbit through multiple subcutaneous spots on one side (0.1 mg in Freund complete adjuvant), followed by two other injections (on the other side of the animal) of the same amount in incomplete Freund adjuvant within an interval of one month. The specificity of the purified IgG from this serum is shown in figure 3.

Results

The dissociated cells were seeded at low density (see material and methods), allowing the identification of individual neuron and their outgrowing during the first days of culture. Two types of neurons were observed, of different morphological aspects: small cell body likely interneurons and larger cell body likely motoneurons (figure 1A).

Measurements of the average lenght of neurites in cultures after 24h and 48h showed that naftidrofuryl did not significantly stimulate the growth of these extensions at drug concentrations between $10^{-8}M$ and $10^{-5}M$, under the conditions described above (figure 1B). Similarly, the drug did not induce any significant change in the number of outgrowing neurites (not shown).

MAP2, detected by quantitative immunoblotting (figure 2A), is present in significant amounts after 24h of culture, and increased only weakly during the following 24h (figure 3A). The addition of

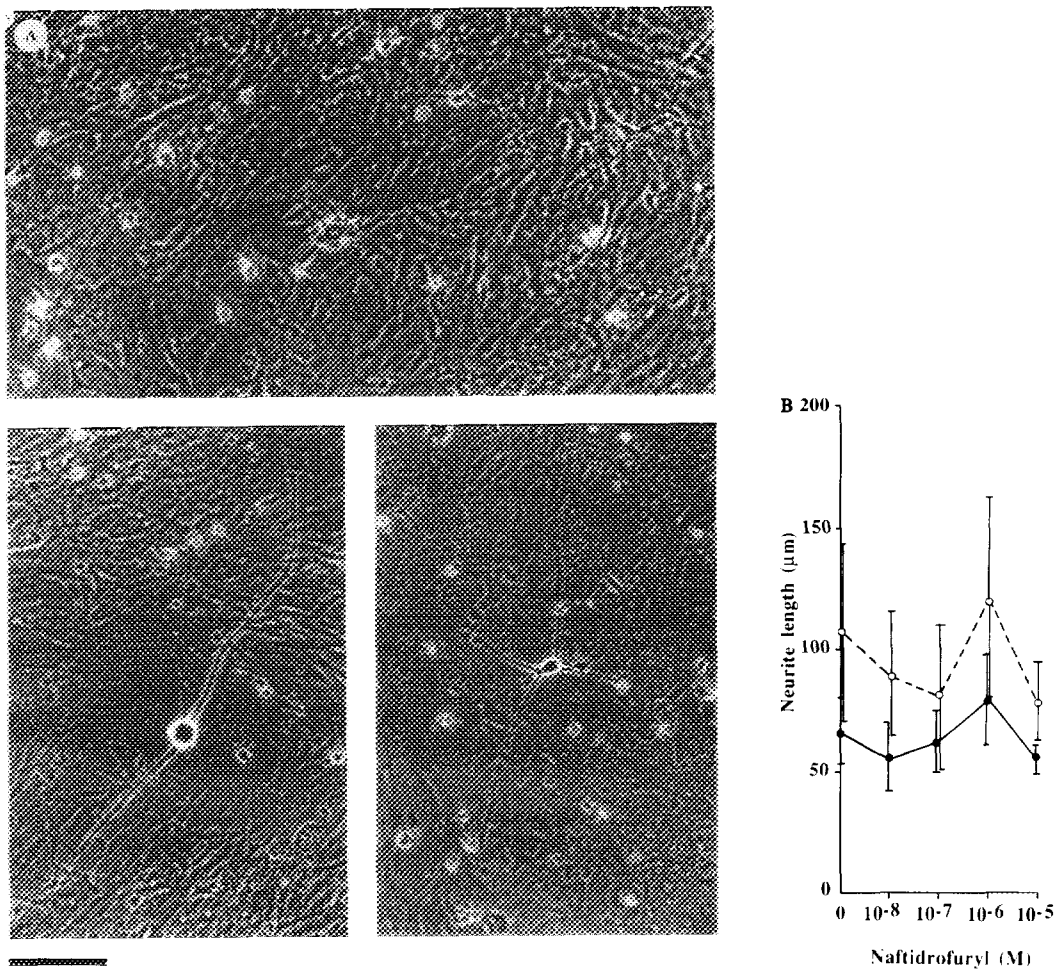

Figure 1. Effect of increasing concentrations of naftidrofuryl on the average neuritic length of chicken spinal cord neurons in culture:
A- Phase contrast light microscopy of standard cultures examined after 48 h. Two types of neurons were regularly present: small cell body neurons (likely interneurons) and large cell body neurons (likely motoneurons). Cells were seeded in low density for the measurements of neuritic lenght of each neuron (see materials and methods). Bar = 50 µm.
B-Average lenght of neurites (+/- S.D.) of neurons after 24 h (●) and 48 h (○) in the presence of increasing concentrations of naftidrofuryl.

naftidrofuryl in the culture medium did not affect the relative amount of the protein in 24h cell cultures. However, an apparent decrease in MAP2 with increasing concentrations of the drug above 10^{-8} M after 48h of culture was noticed in some experiments, such as in figure 3A. The relative amounts of MAP2 estimated by quantitative densitometry of the samples from several experiments, assuming a linear relationship between the intensity of bound labelled-antibody and the amount of antigen analyzed on SDS-PAGE and immunoblotting (figure 2A), did not confirm the statistical significance of this effect (figure 3B).

In contrast, major naftidrofuryl concentration-dependent changes were observed, when analyzing in the same cells the presence of a NF subunit, NF-H, which is considered as a marker for late axonal differentiation (13,20). In figure 4A are shown SDS-PAGE followed by immunoblotting with anti-NF-

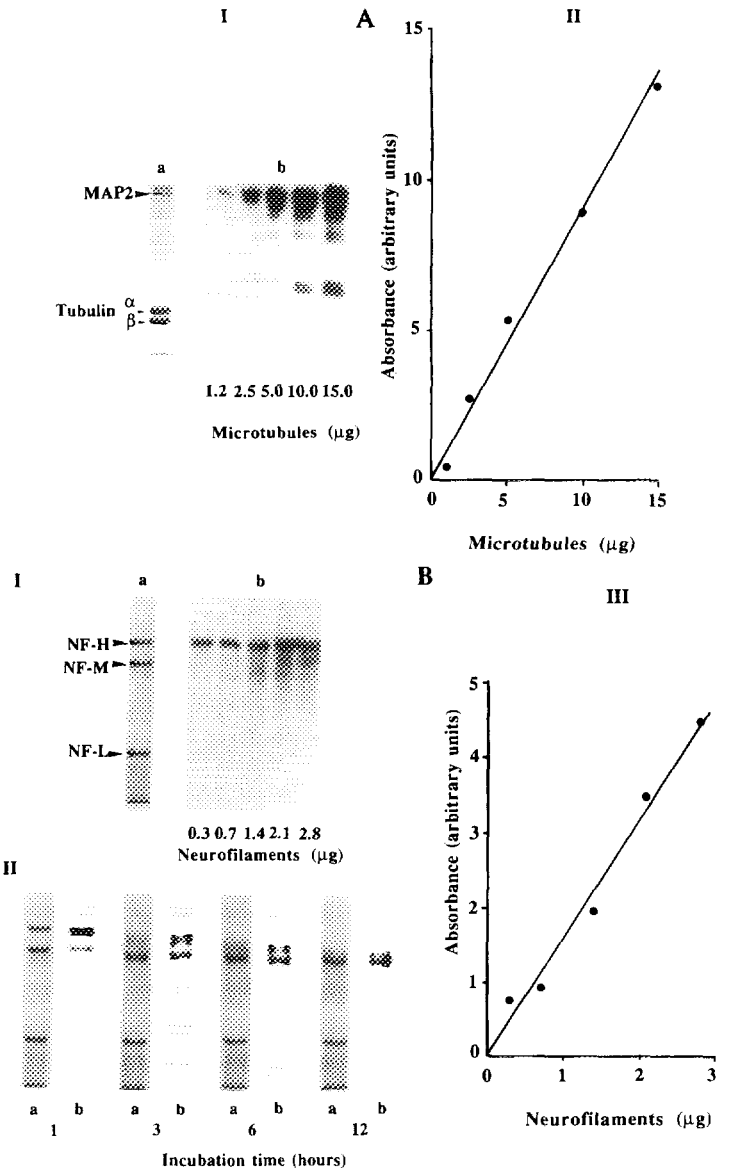

Figure 2. Specificity of NF-H and MAP2 polyclonal antibodies.
A: Characterization of the MAP2 antibody:

I: a: 6% acrylamide SDS-PAGE analysis of 10 μg of purified rat brain MT stained by Commassie Blue. The position of MAP2 (near 10% of total proteins) and tubulin α and β subunits are indicated by arrows. **b:** increasing MT concentrations were analyzed on 6% acrylamide SDS-PAGE and immunoblotting with the MAP2 polyclonal antibody followed by ^{125}I-labelled anti-rabbit IgG. Intact MAP2 is the only molecular specie detected by the antibody, together with minor MAP2-degradation products of smaller size regularly present in MT preparations.

II: Linear relationship between the intensity of the autoradiograms shown in **I-b** and the amount of MT loaded on gels.

B: Characterization of the anti NF-H polyclonal antibody:

I: a: 5 μg purified bovine NF were analyzed on 7.5% SDS-PAGE and stained by Commassie Blue. The positions of the three subunits (NF-H, NF-M, NF-L) are indicated by arrows. **b:** Increasing amount of NF were analyzed by 7.5% SDS-PAGE and immunoblotting with the polyclonal antibody raised against NF-H followed by ^{125}I-labelled anti-rabbit IgG.

II: Effect of in vitro NF dephosphorylation by alkalin phosphatase on the detection of NF subunits by the polyclonal antibody. 1 mg/ml NF were incubated at 35°C for the indicated times in the presence of

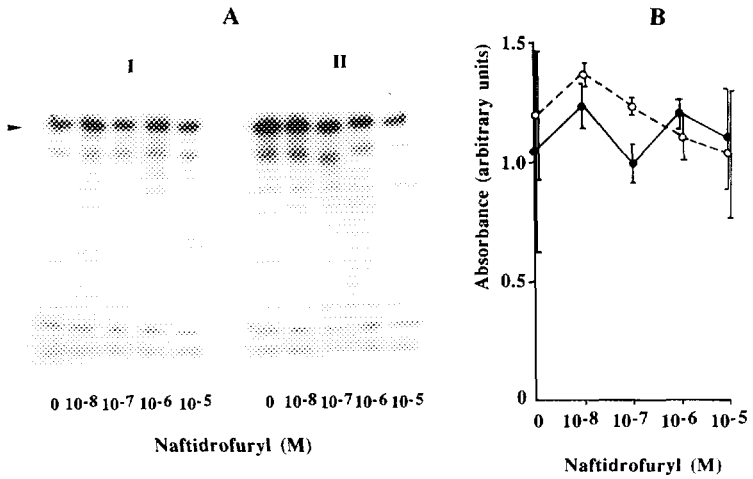

Figure 3. Relative quantification of MAP2 in neurons cultivated with increasing concentrations of naftidrofuryl by SDS-PAGE and immunoblotting:
A: Identical amounts of total cell lysates cultivated for 24h (**I**) and 48h (**II**) in the presence of increasing concentrations of naftidrofuryl were analyzed on 6% acrylamide SDS-PAGE and immunoblotting with MAP2 antibodies followed by ^{125}I-labelled anti-rabbit IgG. MAP2 (arrowhead) and several minor degradation products were present in all extracts. A decrease in the relative amount of MAP2 with increasing naftidrofuryl concentrations was frequently observed after 48h of culture.
B: Measure of the intensity of autoradiograms from immunoblots incubated with anti-MAP2 and ^{125}I-labelled anti-rabbit IgG from three separate experiments (+/- S.D.) of cells cultivated for 24h (●) and 48h (○) in the presence of increasing concentrations of naftidrofuryl. Only the 280 kDa intact MAP2 molecule (arrowhead in A) was taken into consideration for quantification.

H antibodies of the same samples from cultures (after 24h and 48h) examined in figure 3A for their content in MAP2. Traces of NF-H were found in cells cultivated for 24h, the protein being strongly expressed between 24h and 48h (figure 4A). Quantification of NF-H by immunoblotting (figure 2B) was performed on three independent experiments (figure 4B). The addition of increasing concentrations of naftidrofuryl in the culture medium induced a drug-dependent increase in NF-H (figure 4). This effect was already detectable for the highest concentration (10^{-5}M) of naftifrofuryl after 24 h of culture, and was consistent in 48 h cell cultures, with a concentration for half-maximum stimulation close to 10^{-7}M naftidrofuryl (figure 4B).

Although individual variability of the intensity of immunoblotting spots of MAP2 and NF-H detected in samples from cells incubated with increasing concentrations of naftidrofuryl were recorded (as shown by standard deviation bars in figures 3 and 4), the individual ratios between intensities

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alkalin phosphatase (20 U/ml) and protease inhibitors (see materials and methods). The reaction was stopped by mixing with Laemmli denaturation sample buffer and identical amounts of proteins (5 µg) were resolved by 7.5% acrylamide SDS-PAGE and stained with Commassie Blue (**a**) or analyzed by immunoblotting with the polyclonal antibody followed by peroxidase-conjugated anti-rabbit IgG revealed by chemoluminescence (**b**). Dephosphorylation induces a higher electrophoretic mobility of both NF-H and NF-M. Dephosphorylation of NF-H does not abolish its detection by the antibody, but induces a lower reaction against the highly dephosphorylated molecules (6 and 12 h incubation). NF-M is not detected by the antibody in native NF (**I-b**), but NF-M dephosphorylation-dependent epitopes are revealed by dephosphorylation and increase with time (**IIb**).
III: Linear relationship between the amount of NF resolved on SDS-PAGE and the intensity of autoradiograms of immunoblots shown in **I-b**.

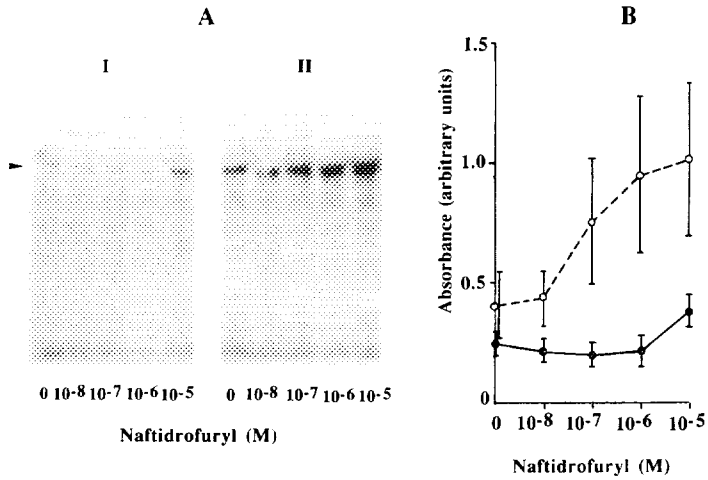

Figure 4. Relative quantification of NF-H in neurons cultivated with increasing concentrations of naftidrofuryl by SDS-PAGE and immunoblotting:
A: Identical amounts of total cell lysates cultivated for 24h (**I**) and 48h (**II**) in the presence of increasing concentrations of naftidrofuryl were analyzed on 6% acrylamide SDS-PAGE and immunoblotting with NF-H antibody followed by ^{125}I-labelled anti-rabbit IgG.
B: Measure of the intensity (+/- S.D.) of autoradiograms from immunoblots incubated with NF-H antibody and ^{125}I-labelled anti-rabbit IgG from the same three separate experiments tested in figure 3 for MAP2. The average (low) amount of NF-H increased in control (0) and treated cells between 24h (●) and 48h (o) of culture. Increasing naftidrofuryl concentrations determined a concentration-dependent increase in the amount of detected NF-H between 10^{-8}M et 10^{-6}M after 48 h ($p < 0.02$ between values in control samples and cells incubated with 10^{-5}M naftidrofuryl).

associated with the two molecules measured for each culture sample were found significantly modified at concentration of naftidrofuryl above 10^{-8}M, and after 48h of culture (figure 5). A net relative decrease in the ratio between MAP2 and NF-H in the same cells occured after 10^{-8}M naftidrofuryl and reached a significant plateau value (50% decrease) at 10^{-5}M (figure 5).

Discussion

The present observations are consistent with previous evidence that naftidrofuryl affects directly neuronal physiology (5,6,11). However, Hauw et al (5) showed that a narrow concentration window (between 10^{-8} and 10^{-7}M naftidrofuryl) determined the stimulation of neurites number and length in cultured rat spinal root ganglia cells, with no effect at 10^{-6}M. That no significant modification occurred in the neurite length and number within the same concentration range of naftidrofuryl in our experiments, although a possible weak stimulation of neuritic growth at 10^{-6}M naftidrofuryl could not be fully exclued (figure 1), is possibly the consequence of a distinct differentiation pattern among neurons subtypes. Our results suggest however that naftidrofuryl affects the molecular composition of the neuritic cytoskeleton of chicken spinal cord neurons in vitro in a drug-concentration-dependent manner (figures 3-5), rather than inducing an elongation process of neurites (figure 1), thus suggesting that naftidrofuryl could possibly influence their maturation into dendrites and axons.

The molecular mechanisms of the maturation process of neuronal extensions was shown to follow several critical steps from undifferentiated neurites containing labile MT (28) which are

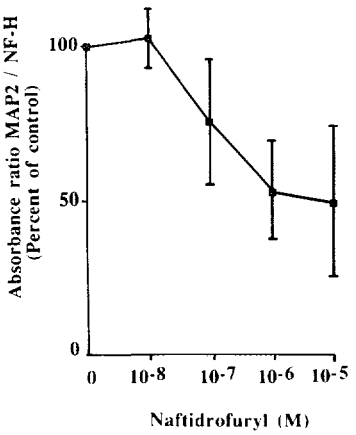

Figure 5. Naftidrofuryl concentration-dependent change in the ratio between MAP2 and NF-H in the same cell cultures:
The ratios between intensities of autoradiograms of MAP2 and NF-H (detected by immunoblotting in figures 3 and 4) measured for each sample of cell cultures incubated for 48h in the presence of increasing concentrations of naftidrofuryl were expressed as percentage of the same ratio found in the extracts from control cultures (100%). Data from three experiments were analyzed and mean ratio values (+/- S.D.) are represented. A significant decrease (50%) in the MAP2/NF-H ratio is induced by naftidrofuryl concentrations above 10^{-8}M and reach a plateau between 10^{-6}M and 10^{-5}M ($p < 0.01$ between values in samples incubated respectively with 10^{-8}M and 10^{-5}M naftidrofuryl).

progressively transformed into specialized axonal processes lacking MAP2, and dendrites where the MAP2 molecule is restricted (14-16). The further maturation of neuronal processes is associated with the progressive stabilization and local molecular specialization of MT (12,28), together with the appearance of NF networks in axons. The dense reticulation between NF in axons is reported to be based on the late appearance of the NF-H subunit (13,17,19,20), which is thought to participate directly in NF cross-bridges, presumably modulated by phosphorylation of the molecule (29,30). Thus, the late expression of NF-H reflects likely the establishment of a mature axonal cytoskeleton. The present study is in accordance with this widely accepted scheme, describing a 24h delayed expression of NF-H with regard to that of MAP2 (figures 3,4), and the finding that NF-H expression is selectively increased by low concentrations of naftidrofuryl after 48h (figure 4) further support the hypothesis that naftidrofuryl affects the spatio-temporal differentiation pattern of neuronal extensions.

The present data do not exclude the possibility that both an increase in amounts of the NF-H molecule and a change in its phosphorylation state might be simultaneoulsy modified by increasing concentrations of naftidrofuryl in the culture medium. The polyclonal antibody described here is directed against a mixture of phosphorylation-dependent and independent epitopes of the NF-H molecule (figure 2B-II). However, immunoblotting analysis of NF samples incubated for increasing periods of time with alkalin phosphatase showed that an increasing immunoreactivity was associated with the NF-M subunit, suggesting that the antibody recognized dephosphorylation-dependent epitopes of the NF-M molecule, in addition to NF-H phosphorylation-dependent and independent epitopes. The fact that no change was observed in the apparent electrophoretic migration of NF-H (apparent molecular weight 200 kDa in figure 4A similar to that of the native phosphorylated molecule in purified NF, figure 2B) and that no other molecular specie was detected by the antibody in culture extracts (figure 4A), is a strong indication for the existence of phosphorylated NF-H isoforms in all cell fractions tested.

The question of the nature of the cellular mechanisms by which naftidrofuryl influenced the relative modification of MT and NF components is not addressed by the present study. From previous reports on the vasodilator effects of the drug (2,5,31), and the evidence that naftidrofuryl inhibits S2-serotoninergic receptors of the vascular smooth muscle (5), one can presumably assume that similar serotoninergic receptors, present on neurons (32,33), could be involved in the effect of the drug on neurons. However, it was shown that S2 serotoninergic receptors involve phospholipase C pathways and do not affect the activity of the adenylate kinase, in contrast with the other types of serotonin receptors (33). Instead, evidence that naftidrofuryl could determine a drug concentration-dependent increase in cAMP in cultured neurons from chicken forebrain (6) may argue for the interaction of naftidrofuryl with a type of neuronal receptor other than S2 (32,33), and suggest also that such receptor should be functionally linked to adenylate kinase. Thus, the exact nature of the neuronal membrane receptor(s) involved in the effects of naftidrofuryl on neurons in culture reported by several authors remains to be determined more precisely. This could be of particular importance with regards to the search for neurotransmittors and other natural factors influencing NF expression. Following this line of reasonning, the available data on the effects of naftidrofuryl on neurons allow the hypothesis that the stimulation of NF-H expression observed in the present work might be the consequence of a cascade of events consecutive to the interaction of naftidrofuryl with its membranous receptor, since major changes in NF-H were observed after 48 hours of culture in the presence of the drug (figure 4). Whether or not the induction of NF-H molecule involves cAMP responsive elements remains to be analyzed.

In conclusion, the present results bring evidence that naftidrofuryl affects differently the neuritogenesis of spinal cord neurons in culture (figure 1) and that of other types of neurons previously studied (5,6). This suggests that the drug might stimulate the differentiation program of each neuronal subtype, which could account for the apparent survival effect on neurons of the drug in situ (2,11).

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