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[^{3}H]-NITRENDIPINE BINDING IN DEVELOPING DISSOCIATED FETAL MOUSE SPINAL CORD NEURONS

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SUMMARY: The development of [^{3}H]-nitrendipine binding was investigated in spinal cord neurons. Kinetic studies indicated two classes of binding sites which were present throughout development and the dissociation constants (K_d) and B_{max} increased during development. [^{3}H]-nitrendipine binding during development was characterized by a plateau on days 3-5 with maximal binding observed on day 19 after plating.

The role of calcium in neurodevelopment may include effects on neurite extension (23), release of neurotransmitter (17), and the expression of genes for enzymes involved in neurotransmitter synthesis (20). Nitrendipine (NTP), a dihydropyridine, is believed to bind to the voltage-sensitive Ca^{++} channel (6,7). Several studies have traced the ontogenetic development of [^{3}H]-NTP binding in membrane preparations of developing brain and muscle tissue from rat and chick (11,15). In those studies, the number of [^{3}H]-NTP binding sites (B_{max}) increased while the binding affinities (K_d) remained essentially unchanged during development. Each tissue did differ in the general pattern of development.

In this study [^{3}H]-NTP binding was assayed in fetal mouse spinal cord cultures to monitor the development of voltage-sensitive Ca^{++} channels. In order to measure the specificity of NTP binding, nifedipine (NFP), another Ca^{++} channel antagonist, was used to block [^{3}H]-NTP binding. The kinetics of binding were studied to determine if [H^3]-NTP binding site properties changed during development.

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MATERIALS AND METHODS: Spinal cord cells were obtained from 12-14 day old fetal mice and cultured as previously described (16) with the following modifications. Plating density was 0.2 million cells per 16 mm well. Twenty four hours after plating in medium containing 10% fetal calf serum and 10% horse serum, all cultures underwent a complete change to 5% horse serum in defined medium (19).

[^{3}H]-NTP(72 Ci/mmole, New England Nuclear) was used for these binding studies. NFP, a gift from Pfizer, was dissolved in 60% ethanol. Cultures were incubated with increasing [^{3}H]-NTP concentrations, 0.3-200nM, in the presence or absence of 50μM NFP to measure non-specific binding. Specific [^{3}H]-NTP binding was measured by subtracting non-specific binding from the total [^{3}H]-NTP associated with the cultures. Vehicle (1.2% ethanol) alone produced no change in [^{3}H]-NTP binding. Dilutions of NFP and NTP were made with phosphate buffered saline (PBS) at pH 7.3.

Cultures were washed 3 times (1 ml each) with PBS at room temperature. Appropriate dilutions of NFP and TTX were added and the cultures incubated in the dark in a 37°C shaking water bath for 60 minutes. Controls received equivalent volumes of PBS. [^{3}H]-NTP was added to all cultures for an additional 30 minute incubation. Cultures were rinsed 4 times in cold PBS (10°C) and cells were dissolved with 0.2 N NaOH. Aliquots were neutralized with HCl and assayed for bound [^{3}H]-NTP by liquid scintillation spectrometry. The protein content of the culture was determined by the method of Lowry et al., 1951 (12).

^{125}I-tetanus toxin fixation (neuronal surface marker) (14) was performed as previously described (2). In brief, labeled tetanus toxin (2×10^{-10}M) was incubated with cultures for 60 minutes at 37°C. Nonspecific fixation was determined by a 60 min. preincubation with 2×10^{-8}M unlabeled toxin. Cells were washed 4 times with PBS and dissolved with 0.2N NaOH.

RESULTS: Scatchard analysis (Figure 1) of [^{3}H]-NTP binding resulted in non-linear plots throughout development which suggested the presence of two

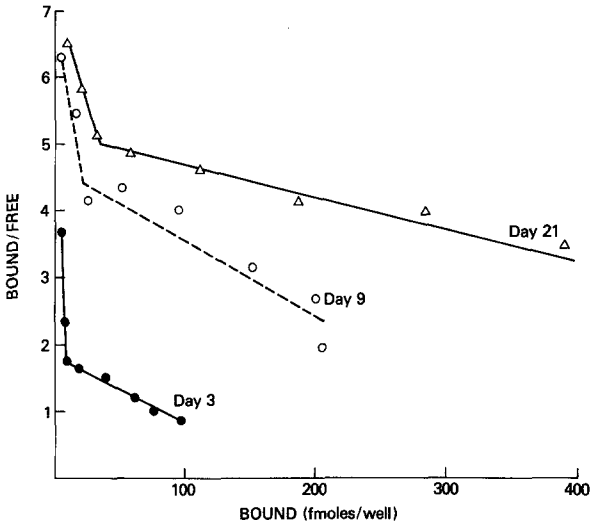

Figure 1. Scatchard analysis of [^{3}H]-NTP binding during development in culture. Nonlinear plots for days 3,9, and 21 suggest two classes of binding sites. Each point is the mean of 4 determinations from two dissections and two Scatchard analysis. The S.E.M. for each point is <10% of the mean. Each well was inoculated with 2 x 10^5 cells. Kinetic parameters for these data are presented in Table 1.

binding sites. Apparent K_d and B_{max} (maximal binding capacity) for both [^{3}H]-NTP binding sites are shown in Table 1. Kinetic parameters were determined with the limiting slopes technique (17). For both sites, values for K_d and B_{max} increased with culture maturation. Between day 3 and day 21, the high- and low-affinity K_d increased as well as the B_{max}. Total protein increased with development in culture from 25 µg/well on day 1 to 100 µg/well on day 21. Specific binding was always greater than 50% and in later development approached 90%. B_{max} values normalized to total protein are also presented. However, previous studies have shown that the protein changes that occurred during development were due predominantly to background cells and not neurons (3).

The results have also been expressed as fmoles of [^{3}H]-NTP bound per cpm ^{125}I-tetanus toxin. This estimates the possible number of binding sites

Table 1

Kinetic Data for Scatchard Analysis of H^3-NTP Binding in Spinal Cord Neurons

	High Affinity Site				Low Affinity Site			
	Kd	Bmax fmoles per			Kd	Bmax fmoles per		
Culture age	nM	Well	Protein µg	I^{125}tetanus cpm	nM	Well	Protein µg	I^{125}tetanus cpm
3	.34	7.0	.277	.005	22	182	14.6	.127
9	.65	8.7	.194	.0035	20	428	8.5	.175
21	1.10	16.0	.160	.0036	50	1050	10.7	.236

Kinetic data for Scatchard analysis (see Figure 1) of H^3-NTP binding in spinal cord neurons. K_d and B_{max} were calculated using the limiting slopes technique.[17]

in relation to the neuronal surface area. The ratio of bound [^{3}H]-NTP to bound ^{125}I-tetanus toxin for the high affinity receptor decreased between day 3 and day 9 and then showed no change with development. In contrast, the low-affinity receptor showed progressive increases in bound [3H]-NTP per neuronal surface throughout development.

The developmental course of [^{3}H]-NTP (10nM) binding is shown in Figure 2. Since there was no pharmacological means for separating the development of the two binding sites, an intermediate concentration of[H^3]-NTP (10nM) was chosen for the study. The curve is biphasic with a plateau between days 3-5. Binding subsequently increased through day 19 in culture. [H^3]-NTP binding increased approximately sixty-fold during development.

DISCUSSION: Channel-related ligands have many potential uses in neuro-developmental research. [^{3}H]-NTP binding was done to characterize this probe for developmental studies in cell culture. Kinetic analysis of

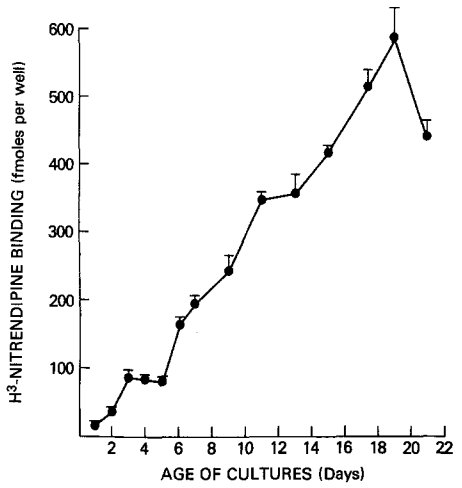

Figure 2. [^{3}H]-NTP binding in developing spinal cord cultures. Each point is the mean of 4 determinations from 3 dissections. The error bar is the S.E.M. Each well was inoculated with 2 x 10^5 cells. Specific binding was determined with 10 nM [^{3}H]-NTP with and without 50 M nifedipine. Protein content ranged from 25 ug/well on day 1 to 100 µg/well on day 21.

[^{3}H]-NTP binding sites showed a developmental increase in the number of [^{3}H]-NTP binding sites and decrease in affinity.

Channel ligand binding may be influenced both by the type of preparation and/or by the temperature at which the assay is conducted. Janis and Scriabine have pointed out that binding discrepancies may be due to temperature effects or to the use of membrane preparation versus cell culture (10). Intact cells may be preferred for Ca^{++} channel ligand studies since Ca^{++} channels are reportedly not functional in isolated membrane vesicles (17). High and low affinity binding sites for [H^3]-HTP have been described in adult guinea-pig heart homogenates (1). Incubation of the homogenates was at 37°C; the high affinity K_d was reported to be 0.3-0.4 nM and the low affinity Kd approximately 70 nM . These values correspond to those for older cultures (day 21) in our studies. Two sites have also been reported in developing cultured chick myocardium incubated for 60 minutes

at 37°C; the high affinity K_d was .15nM and the low affinity Kd was 19nM (13). The low affinity Kd corresponded to the IC50 for inhibition of muscle contraction. These values correspond to our values for younger cultures (days 3 & 9). Ontogenetic studies in muscle and brain tissue from rat and chick only report the high affinity binding site (11,15). These studies were conducted at 25°C and do not report the increasing trends in K_d with development as seen in the present study. The changes in K_d and B_{max} seen in this study may also be due to [^{3}H]-NTP uptake in living cells. The detection of the low affinity site may be temperature dependent. Temperature effects on NTP binding in the neuronal cultures are yet to be determined.

The comparision of fmoles of [^{3}H]-NTP to [^{125}I]-tetanus toxin binding may reflect the number of binding sites in relation to neuronal surface. Previous studies (2) have shown that the number of ^{125}I-tetanus toxin binding sites increases during development whereas the number of neurons is decreasing. In addition, extensive increases in the complexity and number of neuronal processes has been shown to occur during development in culture. These data suggest that the number of tetanus toxin binding sites per neuron increases because of the growth of processes. Such an increase in neuronal surface correlates best with the increase in the number of low-affinity sites. However, this intrepretion must remain tentative since is not known if the expression of tetanus binding sites or the [^{3}H]-NTP binding sites per neuron changes during development. However, this ratio does demonstrate a difference between the high and low affinity receptors with respect to development. It is not clear which receptor is linked to the neurophysiological function of the calcium channel, although several studies have suggested that it is not the high affinity site (4,9).

Saxitoxin and scorpion toxin are specific ligands available for labeling the voltage-sensitive Na^+ channel (4). However, these two ligands bind at different sites. Saxitoxin binds at a site which inhibits ion flux. Scorpion toxin binds at a site which is involved in channel inactivation.

[^{3}H]-saxitoxin binding peaked 10 days before [^{3}H]-NTP, while scorpion toxin peaked 2 days after [^{3}H]-NTP (22). Impulse-dependent neuronal survival correlated with the development of spontaneous electrical activity and a period of major increase in [^{3}H]-saxitoxin binding. Developmental increases in [^{3}H]-NTP binding sites did show initiation of a phase of site proliferation at the same time (days 5-7) that cells in this culture electrical activity-dependent survival. Heyer, et al., 1981, reported that action potentials in spinal cord neurons showed both Ca^{++} and Na^{+} components (8). Ion channel ligand binding studies discussed here show a similarity in the developmental course of NTP binding, saxitoxin binding and electrical activity. However, electrophysiologic studies in this culture system demonstrated these initial action potentials to be TTX-sensitive (21). There is no evidence to support the existence of action potentials in immature neurons which exclusely utilize Ca^{++} as the inward current.

[^{3}H]-NTP binding in developing spinal cord cultures presumably reflect the development of Ca^{++} channels. Electrophysiologic studies are needed to confirm the action of NTP on the Ca^{++} channel, and to determine the function represented by each of the binding sites.

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