

Brain Levels of Neuropeptide Y in Experimental Pneumococcal Meningitis

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ABSTRACT

Neuropeptide Y (NPY), which is found in high concentrations in several regions of the brain including nuclei of the brain stem and in nerve fibers surrounding cerebral vessels, has been proposed to play a role in regulating cerebral blood flow (CBF) and systemic vegetative functions. Since CBF is altered during meningitis, we examined whether NPY concentrations changed in various regions of the rabbit brain in response to experimental pneumococcal meningitis. Changes were most pronounced in the medulla, where NPY concentration increased threefold after 48 h of infection. Concomitantly, there was an increase in NPY immunoreactive fibers surrounding small vessels in the dorsolateral medulla, especially in the nucleus tractus solitarius. These results suggest that NPY may play a role in inducing some of the hemodynamic changes seen during pneumococcal meningitis.

Index Entries: Meningitis; neuropeptide Y; neurotransmitter; cerebrospinal fluid; *Streptococcus pneumoniae* infection; nucleus tractus solitarius.

INTRODUCTION

Bacterial meningitis can lead to central nervous system (CNS) injury, but the pathogenesis of this injury is incompletely understood (Schwartz,

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1984). Clinical and animal studies have examined several pathophysiological alterations that may contribute to an adverse outcome. An important change is reduction in cerebral blood flow (CBF), which can potentially cause severe brain ischemia. Some of the events that lead to this decline in CBF have been identified (Paulson et al., 1974; Smith et al., 1987; Pfister et al., 1990). Vascular inflammation resulting in spasm and thrombosis has been documented during meningitis by histopathology and angiography and is likely to lead to focal reductions of CBF (Cairns and Russell, 1946; Gado et al., 1974). Clinical and experimental studies have further documented that autoregulation of CBF is lost during meningitis, making CBF directly dependent on cerebral perfusion pressure (Paulson et al., 1974; Tureen et al., 1990, 1992). Both the occurrence of vasospasm and the loss of autoregulation suggest that the regulation of cerebral vascular tone may be disturbed during meningitis. In addition, systemic consequences of the infection, such as arterial hypotension and hyperventilation, could potentially aggravate impairment of cerebral blood flow during acute meningitis (Täuber et al., 1991; Tureen et al., 1992).

Neuropeptide Y (NPY) is a 36 amino acid peptide found in high concentrations in various regions of the brain, especially brain stem nuclei such as the nucleus tractus solitarius, and in nerve fibers surrounding cerebral vessels (Edvinsson et al., 1983; Chronwall et al., 1985). In larger vessels, NPY has been shown to exert powerful vasoconstrictive properties in vitro and in vivo (Suzuki et al., 1988), a finding that has led to the hypothesis that NPY may contribute to the vasospasm associated with subarachnoid hemorrhage (Uemura et al., 1987; Jackowski et al., 1989). NPY localized in brain stem nuclei may modulate vegetative functions such as systemic blood pressure or respiration (Gray and Morley, 1986). Because of these proposed biological functions of NPY, we hypothesized that some of the systemic or cerebrovascular changes observed during meningitis could be a result of regional changes in NPY concentrations in the brain. In the present study, we used radioimmunoassay and immunocytochemistry to examine NPY concentrations in the brains of rabbits with experimental pneumococcal meningitis. The rabbit model was chosen because data exist regarding changes in cerebral and systemic pathophysiology during various stages of infection (Sears et al., 1974; Täuber et al., 1985, 1991; Tureen et al., 1990, 1992).

MATERIALS AND METHODS

Experimental Strain

A type 3 strain of *Streptococcus pneumoniae* isolated from an adult patient with pneumococcal meningitis was suspended in saline at a concentration of $\sim 10^8$ cfu/mL and stored at -70°C . At the time of use, the

inoculum was thawed and diluted to a concentration of 10^5 cfu/mL in 0.9% saline.

Animal Model

Meningitis was induced using a modification of the model originally described by Dacey and Sande (1974). Twenty-four New Zealand white rabbits (weighing 2–3 kg) were anesthetized with an intramuscular injection of acepromazine, 0.5–1 mg/kg (Promace, Aveco, Fort Dodge, IA), ketamine, 35–50 mg/kg (Ketaset, Bristol Laboratories, Syracuse, NY), and xylazine, 2–5 mg/kg (Gemini, Rugby Laboratories, Rockville, NY). An inoculum consisting of 1×10^5 cfu/mL in 0.3 mL of saline was injected through a 25-g iv administration set (Butterfly, Abbott), which was manually placed through the intact skin into the cisterna magna. Control animals were injected with 0.3 mL of sterile saline. An initial CSF sample was taken at 0 h prior to infection and a second one was taken at either 24, 48, or 72 h. Animals were then sacrificed with a lethal dose of pentobarbital (200 mg/kg) and used either for determination of NPY concentrations in various regions of the brain by radioimmunoassay or for NPY immunocytochemistry.

CSF was examined for bacterial titers by culturing serial 10-fold dilutions on blood agar plates incubated overnight at 37°C in room air containing 5% CO₂. Lactate and glucose concentrations were determined immediately after obtaining the CSF samples on a two channel automatic analyzer (YSI Glucose/Lactate analyzer, ISY Inc., Yellow Springs, OH).

Radioimmunoassay

For radioimmunoassay of NPY, animals were sacrificed at 24, 48, and 72 h of infection by an intravenous pentobarbital overdose (200 mg/kg iv) and the brain was immediately removed *in toto* from the skull and placed on ice. The following brain regions were dissected and immediately frozen in 2M acetic acid: left and right cortex, hippocampus, caudate, hypothalamus, and medulla. Brain samples were kept at –70°C until further processing. Tissue was extracted as previously described (Ferriero and Sagar, 1989). Tissue was thawed, boiled for 10 min, homogenized with a polytron, frozen, and thawed. Aliquots were removed for protein assay, the remainder of the homogenate was dried, and the residue was extracted with hexane/isopropanol and dissolved in 0.01M HCl. Samples were centrifuged and the supernatant was dried in an evacuated centrifuge. The dried residues were redissolved in assay buffer (0.05M sodium phosphate buffer (pH 7.2), 100 mM NaCl, 10 mM EDTA, 0.1% BSA, and 0.02% NaN₃), diluted appropriately, and assayed by a specific double-antibody radioimmunoassay using ¹²⁵I NPY (Bolton-Hunter method, New England Nuclear, Boston, MA) as the radioligand. The primary antiserum was raised in rabbit (Beal et al., 1986). The related peptides avian

pancreatic polypeptide, human pancreatic polypeptide, C-terminal human pancreatic polypeptide showed no crossreactivity at 10^{-7} mol/L; peptide YY showed $< 0.3\%$ crossreactivity in that range. There was no detectable crossreactivity to micromolar concentrations of substance P, somatostatin 14 or 28, oxytocin, angiotensin, arg-vasopressin, neurotensin, bradykinin, secretin, motilin, cholecystokinin octapeptide, thyrotropin-releasing hormone, luteinizing hormone, leu-enkephalin, growth hormone-releasing factor, vasoactive intestinal polypeptide, or alpha-melanocyte-stimulating hormone. Using whole brain extracts, the recovery of added synthetic NPY by RIA was $92 \pm 7\%$. Assay sensitivity was 1 fmol/100 μ L. Interassay variability was measured using standard dilutions of rat whole brain extract and was found to be $< 10\%$.

Immunocytochemistry

For immunocytochemistry of NPY neurons, two control animals and three animals infected for 48 h were sacrificed by iv pentobarbital and immediately perfused through the left cardiac ventricle with saline, followed by 4% paraformaldehyde (PFA) dissolved in phosphate buffered saline (PBS), pH 7.4. The brains were removed and postfixed overnight in 4% PFA in PBS. Fifty micron sections of the brainstem were cut on a Vibratome Series 1000 (Pelco) and suspended in 0.1M phosphate buffer. Sections were washed with phosphate buffer, and incubated in 2% normal goat serum with 0.5% BSA in phosphate buffer for 2 h. The sections were treated with 1% hydrogen peroxide in phosphate buffer to reduce endogenous peroxidase. The sections were incubated for 24 h in a solution containing primary antiserum (Beal et al., 1986) to NPY diluted 1:1000. The sites of antibody binding for the immunocytochemistry were visualized with the avidin-biotin-peroxidase method (Vectastain ABC kit, Vector Labs, Burlingame, CA). Diaminobenzidine (Sigma) was used as the chromogen. Sections were examined and photographed with bright field optics. The specificity of this immunocytochemical procedure was demonstrated by abolition of fiber and cell body staining when primary antiserum was omitted or when synthetic NPY (Peninsula Labs, Torrance, CA) at a concentration of 10 ng/mL was included during incubation with NPY antiserum.

Statistical Analysis

Results are presented as mean \pm SD. Comparison between groups was performed by one way analysis of variance, followed in the case of significant differences by Student's *t*-test adjusted for multiple comparisons by the Bonferoni correction. In some groups data was not normally distributed and nonparametric tests were used as well. Results obtained by parametric and nonparametric tests were identical.

Table 1
CSF Bacterial Titers and Chemical Changes
in Rabbits with *S. pneumoniae* Meningitis^a

Time, h	N	Titer, log ₁₀ cfu/mL	Lactate, mmol/L	Glucose, mg/dL
Control	4	sterile ^b	2.1±0.9 ^b	108.0±13.1 ^b
24	5	6.9±1.2	10.4±7.1	56.0±28.9
48	7	4.8±1.0	5.6±1.2	64.8±12.5
72	3	5.4±2.5	8.0±3.3	50.3±19.0

^aData is expressed as mean±SD

^b $p < 0.05$ between controls and infected animals for all three parameters

RESULTS

Characterization of Meningitis

A previously well characterized strain of *S. pneumoniae* type 3 was used in this study that produces relatively mild meningitis during the first 2–3 d after intracisternal inoculation (Täuber et al., 1991). Because of this slow course of the disease, most animals showed few clinical signs of meningitis even after 72 h of infection. The presence of meningitis was documented by positive CSF cultures for *S. pneumoniae* and by significantly increased CSF lactate concentrations ($p < 0.05$) and decreased CSF glucose concentrations ($p < 0.05$) in all infected animals (Table 1). There was no statistically significant difference for any of the CSF parameters between the groups of animals infected for either 1, 2, or 3 d, and the somewhat higher titers and CSF lactate concentrations in the group of animals infected for 24 h was a result of one very sick animal with high CSF bacterial titers and pronounced CSF chemical changes (Table 1).

Neuropeptide Y Concentrations in Various Brain Regions

Brain levels of NPY showed different patterns of changes in the brain regions examined. The most clear changes were seen in the medulla of animals infected for 48 h, where concentrations of NPY increased about threefold ($p < 0.001$), while they were similar to control values 24 h after infection and returned to that level in animals infected for 72 h (Table 2). In the hippocampus, there was a clear trend to reduced NPY levels after 72 h of infection ($p < 0.02$ for direct comparison between control and infected animals at 72 h), while the hypothalamus showed the opposite trend with increased levels seen in animals infected at 72 h ($p < 0.05$). In these two regions, we found the largest scatter at the 48-h time-point, since some animals had NPY levels that were still in the baseline range,

Table 2
 NPY Concentrations in Brain Regions of Rabbits
 with *S. pneumoniae* Meningitis of Various Duration

Time, h	N	Cortex	Caudate	Hippocampus	Hypothalamus	Medulla
control	4	6.5 ± 4.1 ^a	7.8 ± 1.3	33.0 ± 11.8 ^c	10.0 ± 6.8 ^d	3.8 ± 1.3
24	5	17.6 ± 15.2	20.4 ± 19.5	51.2 ± 36.1	14.0 ± 5.1	3.8 ± 0.8
48	7	9.3 ± 4.2	13.9 ± 16.5	27.6 ± 37.0	41.1 ± 54.0	12.9 ± 5.6 ^b
72	3	3.4 ± 0.8	11.3 ± 5.6	5.2 ± 2.8 ^c	57.3 ± 34.4 ^d	3.5 ± 2.4

^ang NPY/mg protein expressed as $X \pm SD$

^b $p < 0.001$ for 48 h vs all other groups

^c $p < 0.02$ for pairwise comparison between control and 72 h

^d $p < 0.05$ for pairwise comparison between control and 72 h

while others had levels similar to those found more frequently after 72 h of infection. NPY levels in the cortex and in the caudate did not show any significant changes over time.

Immunocytochemistry for NPY in the Medulla

Immunocytochemical analysis was performed based on the results with the radioimmunoassay that indicated the most significant increase in NPY concentrations in the medulla of rabbits infected for 48 h. In the dorsolateral medulla, NPY immunoreactive cell bodies and fibers were seen around blood vessels in noninfected brains (Fig. 1A). In infected animals, the immunostaining was increased around blood vessels (Fig. 1B) but was especially prominent in many nerve fibers in that region. In particular, there was increased immunostaining of fibers in the nucleus tractus solitarius bilaterally in the infected animals (Figs. 2A,B). This increased fiber reactivity was seen homogeneously throughout the extent of the nucleus tractus solitarius. When compared to the control brain (Fig. 2C) there appeared to be much less cell body staining and much more fiber staining.

DISCUSSION

In this rabbit model of experimental pneumococcal meningitis, we found a significant increase in NPY concentration in the medulla of animals infected for 48 h. Immunocytochemistry confirmed the significant increase of NPY in the medulla and localized the NPY-like immunoreactivity more specifically to the nucleus tractus solitarius and surrounding blood vessels in the dorsolateral medulla. After 48 h, increasing NPY concentrations were also found in the hypothalamus, whereas hippocampal NPY concentrations appeared to decrease.

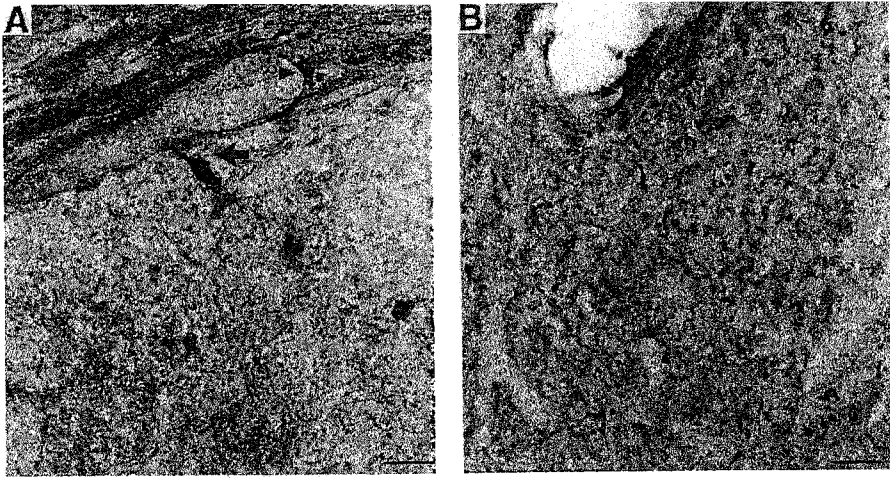


Fig. 1. (A) Photomicrograph of a coronal section through the NTS in a noninfected rabbit showing the normal NPY-LI cell and processes abutting a blood vessel (arrow). Occasional fiber staining is seen (arrowhead). Scale bar = 20 μ . (B) A coronal section through the NTS of a rabbit infected for 48 h with *S. pneumoniae*. Note the increased NPY-LI in neuronal fibers. NPY-LI is also seen around the blood vessel wall (arrowhead). Scale bar = 20 μ .

The observed changes in brain NPY concentrations in this infection of the central nervous system are of interest due to their potential relationship to the local and systemic consequences of meningitis. Although the normal function of NPY remains poorly defined, recent studies have indicated that this neuropeptide may have very profound effects on both the cerebral circulation as well as systemic vegetative functions (Gray and Morley, 1986; Suzuki et al., 1988). Cerebral blood flow and systemic circulation and respiration show marked changes in humans with meningitis and in animal models, including the rabbit model caused by *S. pneumoniae* that was used in the present study (Sears et al., 1974; Tureen et al., 1990; Täuber et al., 1991). The mechanisms for these changes are incompletely understood. With regard to alterations of CBF, direct effects of the infection and inflammation on cerebral vessels are generally held responsible, presumably either because they cause vasculitis with associated thrombosis and vasospasm, or because they induce the release of vasoactive substances, such as prostaglandins (Mustafa et al., 1989). In the case of the most prominent systemic changes, hypotension is generally attributed to complications of the disseminated infection, which lead to dehydration and septic shock, whereas hyperventilation may be a compensatory effort induced by the CSF lactic acidosis resulting from brain anaerobic metabolism (Sears et al., 1974; Tureen et al., 1992). However, conclusive proof of the causal role of these mechanisms for the observed pathophysiological alterations is lacking and alternative explanations are certainly conceivable.

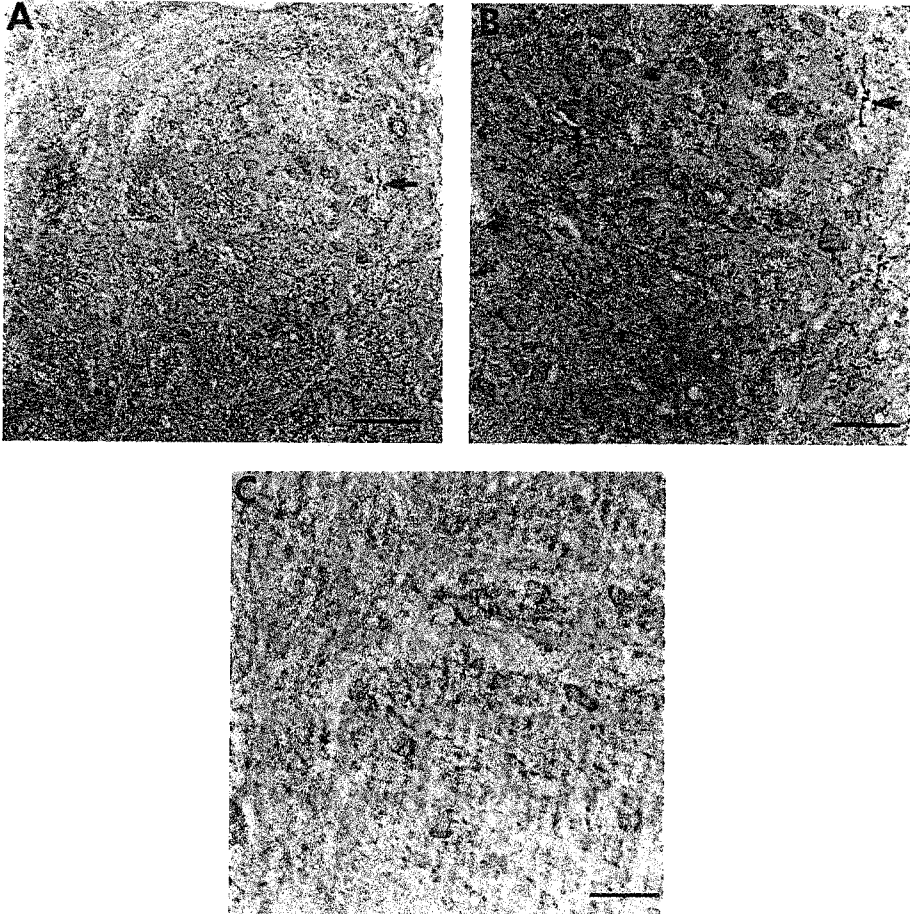


Fig. 2. (A) Low power photomicrograph through the dorsal medulla in the region of the nucleus tractus solitarius showing NPY-immunoreactive fibers coursing through the nucleus (arrow). Scale bar = 80 μ . (B) Higher magnification through the same region demonstrating the NPY-immunoreactive process seen in A. Scale bar = 40 μ . (C) Section through the same region of noninfected medulla showing very little NPY-immunoreactivity. Scale bar = 40 μ .

The possibility that alterations of neurotransmitter production and turnover may play a significant role for either the CNS or systemic changes during meningitis has received little attention. A study in infant rats with meningitis documented an increase in forebrain norepinephrine and dopamine levels at the time of acute infection and evidence of persistent perturbation of monoamine neuronal transmission in adult rats surviving the disease (Konkol et al., 1987). These authors hypothesized that the neurotransmitter changes may explain certain neurological sequelae, such as motor hyperactivity, that are observed following meningitis. In

the present study, we observed alterations of the neurotransmitter NPY during acute meningitis with a prominent regional increase in the medulla. The changes in NPY and norepinephrine may be connected, since in the central nervous system of rats, the two classes of neurotransmitters coexist in some groups of cells of the brain stem, especially in the dorsolateral part of the nucleus of the solitary tract (Härfstrand et al., 1987). A high density of NPY-immunoreactive nerve cell bodies are found in the dorsal nuclei of the nucleus tractus solitarius where the baroreceptor afferents terminate (Härfstrand et al., 1987). There is evidence for coexistence of phenylethanolamine-*N*-methyltransferase (PNMT) immunoreactivity in these perikarya (Everitt et al., 1984). In addition, in the dorsal medulla of the rat, the catecholamine terminal networks are paralleled by NPY-immunoreactive fibers (Hunt et al., 1981). Intracisternal injection of NPY in the rat causes prolonged hypotension and bradycardia (Fuxe et al., 1983; Härfstrand, 1986). This biological effect of NPY together with its increased concentrations in the region of the nucleus tractus solitarius support the involvement of NPY in mediating the cardiovascular effects seen during meningitis.

In our model of meningitis, changes in NPY concentrations in various regions of the brain were relatively slow to occur, even though the inflammation was well developed within the first 24 h. This is in contrast to an animal model of head trauma in the rat, where impact led to an acute and transient decrease in regional cerebral blood flow within minutes that was associated with an increase in NPY concentrations (McIntosh and Ferriero, 1992). Apparently, the development of stimuli responsible for changes in NPY concentrations during infection of the CNS requires a longer time-course. Although the observed changes of NPY immunoreactivity may be a result of alterations in release, synthesis, degradation, or processing of the peptide, our finding of increased fiber staining in the medulla indicates that at least one component involves increased release into the processes of NPY-producing neurons.

The precise role of NPY in meningitis remains to be elucidated. Clearly the topographical localization of the transmitter in the medulla suggests a possible effect on systemic vegetative function, such as blood pressure or respiration. NPY concentrations may also change in other regions of the brain during meningitis, but the consequences of these changes are presently unclear. Further studies to examine the association between sequelae of meningitis and changes in neurotransmitters, such as NPY, are needed.

SUMMARY

NPY-LI was found to be increased in the medulla of rabbits infected with *S. pneumoniae* at 48 h after the onset of infection. These levels were threefold elevated by radioimmunoassay of medullary tissue extracts and

corresponded to an increase in NPY-LI in fibers of the nucleus tractus solitarius by immunocytochemical analysis of fixed tissue sections. These results may explain some of the hemodynamic changes seen during meningeal infection, especially the hypotension associated with severe infection.

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