Cyclophosphamide cystitis as a model of visceral pain in rats. A survey of hindbrain structures involved in visceroception and nociception using the expression of c-Fos and Krox-24 proteins

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Abstract The evoked expression of the immediate early gene-encoded proteins c-Fos and Krox-24 was used to study activation of hindbrain neurons as a function of the development of cyclophosphamide (CP) cystitis in behaving rats. CP-injected animals received a single dose of 100 mg/kg i.p. under transient volatile anesthesia and survived for 1 to 4 h in order to cover the whole postinjection period during which the disease develops. CP-injected groups included: (1) animals with minor simple chorionic edema, an early characteristic of inflammation (1 h postinjection); (2) animals with well-developed simple chorionic edema (2 h postinjection); (3) animals with mild inflammation (chorionic edema accompanied by epithelial cleavage; 3 h postinjection); and (4) animals with complete inflammation (4 h postinjection). In addition to onset of chorionic edema, the earliest postinjection period also included the general aspects of the nervous reaction consecutive to the injection process (handling, transient volatile anesthesia and postanesthesia awakening, abdominal pinprick, CP-blood circulating effects). Controls included both noninjected animals and saline-injected animals surviving for the same times as CP-injected ones. Quantitative results come from c-Fos expression. It has been shown that: (1) saline injection is a significant stimulus for only nucleus O and central gray pars alpha and nucleus medialis of the dorsal vagal complex; (2) all structures driven by CP injection (nucleus O and central gray pars alpha, locus coeruleus, Barrington’s nucleus and parabrachial area mostly in its ventral and lateral subdivisions, dorsal vagal complex, ventrocaudal portion of lateral bulbar reticular formation) responded vigorously shortly after injection, but only two (dorsal vagal complex, ventrocaudal portion of lateral bulbar reticular formation) showed increased or renewed activity when cystitis completely developed, i.e., when noxious visceral inputs reached highest levels. Regarding the sequential activation of these structures in relation to postinjection time, evidence is given that: (1) a large variety of hindbrain structures are differentially involved in either the general reaction consecutive to the injection process or to various degrees of cystitis; (2) these structures extend from the brain-spinal cord to the pons-mesencephalon transitional junction levels; (3) the two structures most powerfully driven by visceronocceptive inputs are also the most caudal ones, being located at the brain-spinal cord junction level; and (4) the dorsal vagal complex could be the main hindbrain visceral pain center, with three particular subdivisions, the nucleus medialis, nucleus commissuralis, and ventralmost part of area postrema, being involved.

Key words Urinary bladder - Inflammation - Brainstem - Ponto-mesencephalic junction - Rat

Introduction

The study of visceroception and visceronociception has recently become a point of interest for neuroscientists and various models, including mechanical colorectal, uterine or vaginal distension by balloon inflation, intraperitoneal inflammation by local injection of algogens or bladder inflammation by direct application of chemicals, have been proposed (Abelli et al. 1989; Berkley et al. 1995; Birder and de Groat 1992; Lantéri-Minet et al. 1993b; MacMahon and Abel 1987; Menétrey et al. 1989; Nordin et al. 1990; Ness and Gebhart 1988). Recently we reported on a model of cyclophosphamide (CP) cystitis (Lantéri-Minet et al. 1995) that induces mild and controllable bladder injury over short periods of time and that can be used in unanesthetized animals. CP is an antitumor agent, but a limiting aspect of its
therapeutic use is bladder toxicity (Watson and Notley 1973). CP is activated by the hepatic cytochrome P-450 to form inactive circulating metabolites whose renal cleavage will generate toxic byproducts, mostly acrolein (Brock et al. 1981; Cox 1979). It is the prolonged contact of acrolein with the bladder wall during urine accumulation and retention that generates cystitis. The CP cystitis model has some unique features compared with other visceral models. Firstly, the stimulus is a “pure” visceral one confined to one viscus (bladder), since no somatic stimulation is induced either by introducing a stimulation device or from surgical wounds, and no anesthesia is required; secondly, it is the exact replicate of a human disease and its time course can be efficiently monitored through behavioral and histological observations; thirdly, it can be used in behaving animals without departing from ethical rules. This model was successfully used in association with c-Fos and Krox-24 (egr-1, zif-268) expression to demonstrate that, at the spinal level in behaving animals, different degrees of bladder injury, from simple chorionic edema to complete inflammation, result in differential activation of sacral parasympathetic columns, dorsal gray commissure, and superficial layers of the dorsal horn.

Following studies at the spinal level, we have now used this model to study c-Fos and Krox-24 activation of hindbrain neurons as a function of the development of cystitis. The expression of these immediate early genes (IEGs), which couple extracellular signals and long-term genomic responses by modulating the transcription rate of secondary target genes, is known to be, among others, one possible indicator of cellular activity (see references in Morgan and Curran 1991; and Piechaczyk and Blanchard 1994). c-Fos and Krox-24 belong to two separate superfamilies of transcription factors (for review see Bravo 1990), the differential transcriptional properties of which, in terms of threshold activation, make them complementary if the aim is to monitor multiple aspects of brain processing (Gass et al. 1993; Herdegen et al. 1991; Lantéri-Minet et al. 1993a, b; 1994; Mack and Mack 1992; Worley et al. 1993). Effects from cystitis-related inputs are compared with the general aspects of the nervous reaction consecutive to saline injection. Part of these results have been presented in abstract form (Bon et al. 1994).

Materials and methods

Experiments were performed on 50 male Sprague-Dawley rats (Centre d’Elevage Charles Rivers, France) housed in individual cages with sawdust bedding, given food and water ad libidum, and kept in an animal house at a constant temperature of 22°C with a 12-h alternating light-dark cycle. The experiments were performed on naturally behaving awake animals and conformed to Principles of laboratory animal care (NIH publication no. 80-23, revised 1985). Behavioral observation, bladder examination, and detection of IEG-encoded protein expression were conducted in parallel. Most animals used in this study were from a study devoted to spinal levels of protein expression (Lantéri-Minet et al. 1995).

Stimulated and control animals

Drug-injected animals (n=18) received a single dose of CP (100 mg/kg i.p. in 1 ml saline) under transient volatile anesthesia (mixture one-third O2, two-thirds N2O, 2% halothane) using an anesthetic box into which the animals could enter freely. Animals behaved freely between the CP injection, recovery from volatile anesthesia, and death. Animals survived for 1 to 4 h in order to cover the entire survival time over which the disease develops. The shortest survival times were used to assess the general aspects of the nervous reaction consecutive to the injection process ( handling, transient volatile anesthesia, abdominal injection, period of hyperactivity that accompanies postanesthesia recovery, CP-blood circulating effects) and onset of chorionic edema; the longest survival times were used to follow the progressive development of bladder impairment, from simple chorionic edema to complete inflammation (Lantéri-Minet et al. 1995), thus with progressive increase in nociceptive inputs. Survival times longer than 4 h were not considered because of ethical guidelines (Naquet 1993) and to avoid pronounced necrosis of the mucosal epithelium.

Controls consisted of freely behaving noninjected (n=5) or sham (n=12, 3 per survival time from 1 to 4 h) animals. Control animals were used to assess basal expression. Sham animals were treated like drug-injected animals except for substitution of CP with saline and were used to assess long-term effects of the experimental paradigm. Differential staining between sham-evoked versus baseline expressions provides information on the effects from the injection process. Differential staining between CP-evoked versus sham-evoked and baseline expressions gauges the effects of specifically related CP inputs (possible blood circulating effects; genesis of cystitis).

Tissue processing

Animals were killed by intracardiac perfusion under deep anesthesia (1 ml i.p. of pentobarbital) using 200 ml of 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 400 ml of 4% paraformaldehyde in PBS. Macrosopic observation of abdominal peritoneum at the injection site never revealed signs of local irritation. Both the neuraxis and several visceral tissues (bladder, prostate, seminal vesicles, pelvic peritoneum) were removed, but only the latter were postfixed (10% formalin for 3 days). Nervous tissue was cryoprotected overnight in phosphate-buffered saline (PBS and 0.3% Triton X-100) for 30 min then in the primary antibody solution at 4°C before cutting. This perfusion protocol was the most efficient in preserving both types of immunoreactivities, as it has been observed that prolonged postfixation reduced the sensitivity of the immunohistochemical procedure.

Visceral tissue

Visceral tissues were embedded in paraffin then cut on a microtome in 5-μm-thick sections. Sections were mounted, cleared in toluene, hydrated in alcohol, stained with hematoxylin and eosin, and safran. After final dehydration the sections were coverslipped with Eukitt. Histological examination of the bladder was used to estimate the severity of lesions and to enable selection of groups of animals to be fully analyzed.

Nervous tissue

Frozen serial transverse sections (40 μm thick) were collected in PBS to be processed immunohistochemically as free-floating sections. Sections were incubated in 10% normal goat serum in PBST (PBS and 0.3% Triton X-100) for 30 min then in the primary antibody for 2 days at 4°C. Both antibodies were polyclonal and generated in rabbits. c-Fos antibody was the kind gift of Dr. Hunt (MRC Centre, Cambridge, UK). The properties of this antibody, which is directed against a synthetic peptide sequence (2–17) unique to the N-terminal portion of the protein, have been previously reported (Hunt et al. 1987). Krox-24 antibody (S88 sc 110)