Inflammatory pain induces neuronal alterations in NO and JNK dependent manners

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ABSTRACT

Background: Dark neurons are generated in vivo as an acute or delayed consequence of several pathological situations and lesions. The present study was designed to evaluate whether inflammatory pain induces formation of dark neurons in the dorsal horn of rat spinal cord. Since NO and JNK pathway are involved in the mechanisms of pain generation and degenerative neuronal alteration, their roles were also considered.

Methods: Histological procedures were employed for detection of dark neurons following induction of inflammatory pain.

Results: On the fifth day; following daily injections of 5% formalin, numbers of dark neurons increased significantly. Acute and chronic administration of 1% or 2.5% formalin did not induce any remarkable neuronal alteration in the dorsal horn of lumbar spinal cord. Daily intrathecal administration of quercetin (inhibitor of JNK pathway) 100µg/rat, or PTIO (NO scavenger) 30µg/rat before injection of 5% formalin, led to a reliable reduction of formation of dark neurons.

Conclusion: Results indicate that induction of inflammatory pain for longer periods may result in a serious central disorder, and administration of neutralizers or inhibitors of NO and JNK may exert neuroprotective effects.

Key words: Inflammatory pain; Dark neurons; NO; JNK; Quercetin; PTIO.

INTRODUCTION

Studies performed in all neurobiological fields have produced important knowledge about neurodegenerative processes that are responsible for the cell damages and neuronal death. In the histological studies of the central nervous system, it has been known that neurodegeneration could produce quantitative and excitotoxic transsynaptic morphological changes in the neurons. These neurons are characterized by their high affinity for several histological stains which are responsible for their dark appearances and are called 'dark neurons' (1-3). Dark neurons are considered as manifestation of manifold injuries to the neurons which may be generated in vivo as an acute or delayed consequences of several pathological situations and lesions (1-5). As the early histopathological state of neuronal damage, dark neurons develope in the brain just a few hours after the stressful exercises (6).

The morphological changes of dark neurons which is consistent with those of cells undergoing apoptosis, are potentially irreversible and these neurons lack the functional integrity (7, 8). In an electron microscopic study, dark neurons which had been previously identified under light microscope, was confirmed to be clearly degenerative neurons with the intracellular microstructural changes and atypical synaptic appearance (9).

As it has been reported, peripheral nerve injury and hyperalgesia induced by chronic opioid therapy lead to the formation of dark neurons in the superficial dorsal horn (laminae I-II) of rat spinal cord (7). On the basis of reports, subcutaneous injection of formalin as a model of inflammatory pain induces rapid and prolonged hyperalgesia across widespread areas of the body and also activates peripheral nerves leading to the activation of the spinal neurons (10- 11). The aim of the present study was to determine the minimum intensity and duration of inflammatory pain that may results to the formation of dark neurons in the lumbar spinal cord of rat. As it is known, following repetitive noxious stimulation of rat hind paw, a series of molecular events are initiated in the anatomically relevant region of the spinal cord, i.e. the lumbar portion which is

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involved in transmission and control of pain. It has been recently shown in our laboratory that the lumbar portion of the spinal cord is involved in development of tolerance to the morphine analgesia (12).

As known, nitric oxide (NO) a ubiquitous signaling molecule plays a pivotal role in the spinal nociceptive signal processing (13). According to Przewlocka et al., formalin-induced inflammation leads to the increase of NOS-labeled neurons in the dorsal horn of spinal cord (14).

It is also well known that noxious stimulus affects the expression of transcriptional factors such as c-Jun which is induced in the dorsal horn of spinal cord following formalin administration (15). Activation of c-Jun depends to its phosphorylation by c-Jun N-terminal Kinase (JNK), a member of mitogen activated protein kinase family, which is an initiator of apoptosis (16). In fact, JNK/c-Jun signaling is a key component of the cell death machinery (17).

Thus, on the basis participation of NO and JNK pathways in the nociceptive signal processing and neuronal degeneration, their involvement in the probable central consequence of inflammatory pain was also evaluated.

MATERIALS AND METHODS

Experiments were carried out on adult male Wistar rats (weighing 300-350g, about 3.5 months old) from our own breeding facilities. Animals were housed at room temperature with an alternating 12h light/dark cycle and had free access to food pellets and drinking water. Experiments were performed in accordance with the guidelines laid down by the Helsinki Declaration and IASP's guidelines for pain research in animals (18) The study protocols were also approved by the local Ethics Commitee.

Inflammatory pain was induced by single and repeated injections of different concentrations of buffered formalin solution. In acute administration, 50µl of 1%, 2.5%, and 5% formalin was injected unilaterally into the plantar surface of the left or right hind paw in three groups of rats (n=7/group). In daily injections within 4 days, another three groups of rats were selected and subjected to both hind paws injection as follows: intraplantar injections of different concentrations of formalin were performed bilaterally on the day of 1; while on the day of 2, dorsal surfaces of the rat paws were selected for bilateral injections. The same protocol of injection was performed on days 3 and 4, respectively (n=7/group). The saline treated control groups followed the same procedures.

For detection of dark neurons in the dorsal horn of spinal cord; 1.5h and 24h after single injection and 24h after last injection in chronic administration of different concentrations of formalin (hour 120), histological procedures were performed as described before (7, 8), to verify the probable formation of dark neurons. Briefly, animals were anesthetized and perfused transcardially followed by fixative. Twenty-four hours later, lumbar spinal cords were taken out and kept in the fixative overnight. For each rat, two blocks at the L4-L5 levels (about 1-1.5 mm each) were osmicated, then dehydrated and embedded in an epoxy resin. From each block, ten 0.5 µm thick sections were sliced at 50 µm intervals and mounted to gelatin-coated slides. These sections were stained with a dye solution containing 1% toluidine blue and 1% sodium borate and then were cover-slipped for microscopical examination. Finally, six randomly selected sections were viewed for each rat. The same procedures were performed on saline treated control animals.

According to Bennett et al., degenerated neurons of the spinal cord with appearance of dark neurons have three principal characteristics: (i) irregular cellular outlines; (ii) increased chromophilia throughout both nucleoplasm and cytoplasm and for this reason they are called dark neurons; (iii) homogeneously and intensely stained nucleoplasm with almost indiscernible heterochromatin (7). It should be mentioned that sometimes normal neurons of dorsal horn exhibit enhanced cytoplasmic staining, but they do not show intensified nucleoplasmic staining. Glial cells particularly oligodendrocytes may also exhibit chromophilia. However, oligodendrocytes are distinguishable from dark neurons, as they show aggregates of heterochromatin which are seen as clumps under light microscopic examination.

If dark neurons were significantly developed in any group(s), intrathecal (i.t.) injections of PTIO (2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide, Sigma) 20 or 30µg/rat as NO scavenger, and quercetin (3,3′,4′,5,7-pentahydroxyflavone, Sigma) 40 or 100µg/rat as inhibitor of JNK pathway, were administered 10 min before injection of formalin (i.e. totally four i.t. injections of each dose of the drugs, in the case of chronic administration of formalin).

The intrathecally administered drugs were dissolved in 10% DMSO (dimethyl sulfoxide, Sigma) and 90% saline and were delivered in a total volume of 10µL followed by 10µL saline to flush the catheter. A vehicle group was also considered. For intrathecal catheter implantation,

rats were anesthetized with intraperitoneal administration of ketamine (50mg/kg). PE-10 catheters were inserted into the subarachnoid space through an incision at the atlantoccipital membrane. The caudal end of the catheter was gently threaded to the lumbar enlargement and the rostral end was sealed with a steel wire on the top of the skull. The wound was closed with 3-0 silk sutures. Animals were allowed to recover for 1 week before the experiments. Rats exhibiting postsurgical motor deficit were discarded.

Statistical analyses

Two-way ANOVA with subsequent Tukey test was used to determine the differences in numbers of dark neurons between i) the dorsal horn regions, ii) the left and right sides of each region, and iii) the treatment groups. Data are presented as mean ± S.E.M and the level of significance was set at $P<0.05$.

RESULTS

Visual inspections of neurons in the sampled sections of the lumbar spinal cord revealed the development of dark neurons (Figs. 1 and 2), which increased significantly following chronic administration of 5% formalin (Fig. 3, hour 120, $p<0.001$). Dark neurons were predominantly located in laminae I-II and in a much lesser degree in deeper laminae (1.19±0.27 and 0.33±0.14; in laminae III-IV and V-VI, respectively).

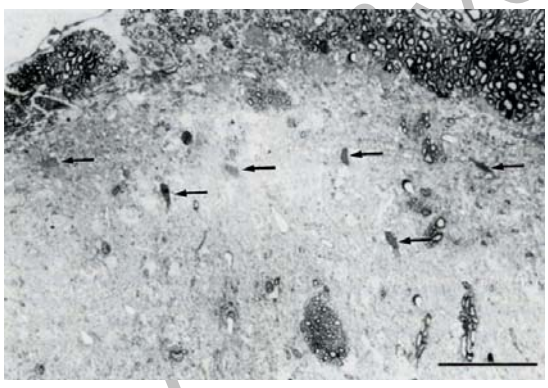

Figure 1. Photomicrographs of dark neurons in toluidine blue-stained semi-thin (0.5 µm) section following induction of inflammatory pain. A portion of the lumbar superficial dorsal horn. Dark neurons (arrows) are observed mostly in laminae I-II, following chronic administration of 5% formalin. The deeper laminae have very few dark neurons. Scale Bar = 50 µm.

Dark neurons were bilaterally distributed and there were no statistical differences between the numbers of dark neurons on the left and right sides of the spinal cord ($P>0.05$). Dark neurons were not developed significantly following single injection of 5% formalin and also after acute and chronic administration of 1% or 2.5% formalin (Fig. 3, $P>0.05$). Pretreatment with PTIO 30µg/i.t./rat or quercetin 100µg/i.t./rat, significantly attenuated formation of dark neurons induced by chronic injections of 5% formalin (Fig. 4, $P<0.05$ and $P<0.01$, respectively). However, pretreatment with PTIO 20µg/i.t./rat, quercetin 40µg/i.t./rat or the vehicle did not have any effect (Fig.4, $P>0.05$).

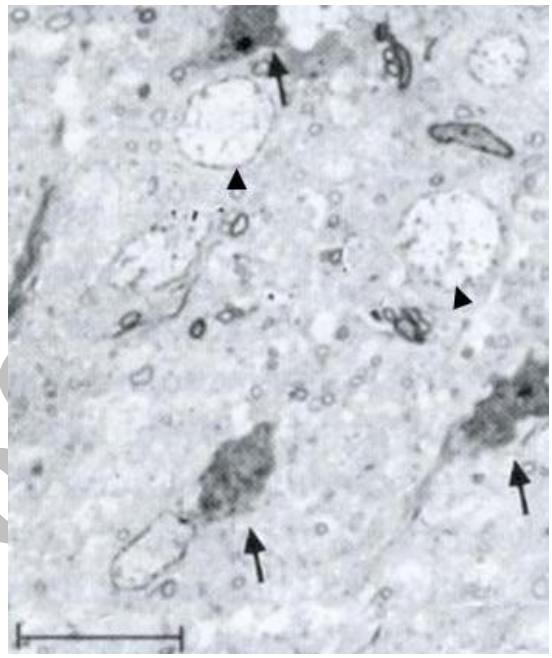

Figure 2. Photomicrographs of dark neurons in toluidine blue-stained semi-thin (0.5 µm) section following induction of inflammatory pain. Dark neurons (arrow) exhibit increased chromophilia throughout both cytoplasm and nucleoplasm with virtually indiscernible heterochromatin. A regular cellular outline is not observed. The nucleus of normal neurons in the dorsal horn (arrow head) can be easily distinguished from those of dark neurons. Scale Bar= 25 µm.

DISCUSSION

The tightly regulated pathophysiological events that may manifest as inflammatory pain, are usually initiated by tissue injury or the presence of foreign materials. In the present study, three concentrations of formalin were used in order to evaluate the consequences of different intensities of inflammatory pain. According to the results, numbers of dark neurons in the superficial laminae I-II of the lumbar spinal cord were significantly increased following chronic administration of 5% formalin (Fig. 3). Exposure to repeated injections of 5% formalin which yields a more intense nociceptive stimulus (19), seems to be involved in the excitotoxic damages to the

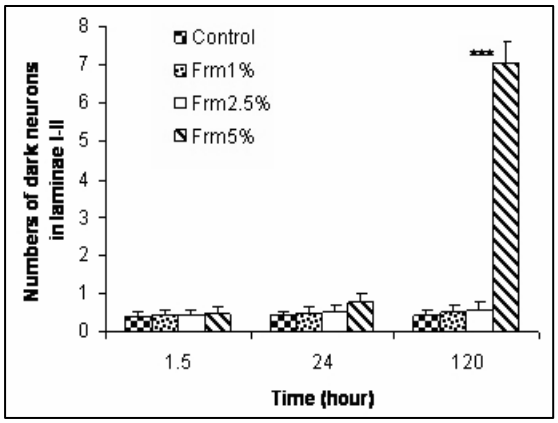

Figure 3. Effects of acute and chronic administration of different concentrations of formalin on the development of dark neurons. As shown, 24h after the last injection of 5% formalin in chronic administration for 4 days (Frm5%, hour 120), a significant formation of dark neuron are induced in the superficial laminae I-II. 1.5 and 24h after acute administration of different concentrations of formalin, and 24h after chronic injections of 1% or 2.5% formalin (hour 120), a significant formation of dark neurons are not observed, as compared to the control. Data represent the mean±S.E.M. ***P<0.001 vs. control and other groups.

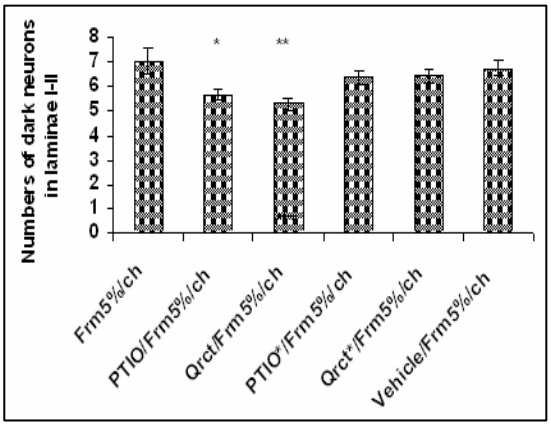

Figure 4. Effects of PTIO and Quercetin on the incidence of dark neurons following chronic injections of 5% formalin (Frm5%/ch). Daily injections of PTIO 30µg/i.t./rat or Quercetin (Qrct) 100µg/i.t./rat, 10 min before injection of 5% formalin (PTIO/Frm5%/ch; Qrct/Frm5%/ch), led to a reliable reduction of formation of dark neurons. Pretreatment with PTIO 20 µg/i.t./rat (PTIO*/Frm5%/ch); quercetin 40 µg/i.t./rat (Qrct*/Frm5%/ch) and the vehicle (vehicle/Frm5%/ch) did not result to any remarkable effect. Data represent the mean ± S.E.M. *P<0.05 ; **P<0.01.

developing neurons. Acute or chronic administration of 1% or 2.5% formalin and single injection of 5% formalin did not induce development of dark neurons (Fig. 3). It seems that occurrence of this histopathological alteration is dependent to the stimulus paradigm and probably other endogenous mechanism(s).

As it was found, the highest frequency of dark neurons occurred in the superficial laminae of the dorsal horn (Fig.1). This topographic distribution indicates that neurons in laminae I-II of the lumbar spinal cord are a main target for formalin-induced excitotoxicity. It also suggests that the loss of function of these neurons may be particularly critical to the spinal cord mechanisms of inflammatory pain. Since dark neurons seem to be inhibitory interneurons (7), their high incidences following induction of inflammatory pain may reflect a loss of inhibition and therefore, persistent imbalance of the excitatory-inhibitory circuitry within the dorsal horn of the lumbar spinal cord. According to Harris et al, apoptosis induces loss of inhibitory systems (16). This, may support the argument of similarity of the signal transduction pathways relating the phenomena of apoptosis and dark neurons.

Pretreatment with PTIO as NO scavenger, led to a significant reduction in inflammatory pain-induced dark neurons in a dose dependent manner (Fig. 4, P<0.05). It is supposed that excessive production of NO trigger intracellular cascades; leading to the excitotoxicity and resultant degenerative neuronal alterations. As it has been reported before, intrathecal pretreatment with benzamide (inhibitor of NO-activated poly (ADP ribose) synthase) and L-NAME (NOS inhibitor), also protected neurons from NO-mediated neurotoxicity (20, 21).

Pretreatment with quercetin also prevented formation of dark neurons in a dose-dependent pattern (Fig. 4, P<0.01). This response indicates the critical role of JNK in the signal transduction pathway related to the inflammatory pain-induced neuronal damage. In another study, amelioration of apoptosis by quercetin in the kidney tubule epithelial cells by has been reported (22).

These results suggest that neutralizers or inhibitors of NO and JNK may exert neuroprotective effects in the toxicities and serious central disorders that may occur following induction of inflammatory pain in longer periods.

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