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SEPARATION OF A- VERSUS C-NOCICEPTIVE INPUTS INTO SPINAL–BRAINSTEM CIRCUITS

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Abstract—This study was designed to determine the organization of nociceptive inputs with different behavioral significance into spinal–brainstem circuits in the rat. Induction of Fos protein was used to localize spinal dorsal horn and hypothalamic neurons activated by noxious heating of the hind paw dorsum at rates known to preferentially activate C- or A-heat nociceptors. This was combined with retrograde transport of cholera toxin subunit B from the dorsolateral/lateral- (DL/L-) or the ventrolateral- (VL-) periaqueductal gray (PAG) in order to map the organization of A- and C-fiber input to spinal–brainstem circuits.

The majority of dorsal horn heat-activated neurons were located in laminae I and II. A significantly larger proportion of C-fiber-activated neurons projected to the VL-PAG ($P<0.05$) compared with its DL/L-sector. In contrast, there was no columnar separation in the projections of A-fiber-activated neurons. However, a significantly greater proportion of A-fiber-activated neurons ($P<0.05$) were retrogradely labeled from the DL/L-PAG, when compared with C-fiber-activated neurons. A large proportion (25–50%) of A- and C-fiber-activated neurons in the lateral spinal nucleus projected to the PAG.

A-fiber-activated neurons were found throughout the rostral hypothalamus but those projecting to the PAG were focused in the lateral area of the anterior hypothalamus (LAAH), from where ~20% projected to the VL-PAG, which was significantly more than to the DL/L PAG ($P<0.05$).

We hypothesize that the organization of A- versus C-fiber inputs to the PAG enables the coordination of coping strategies appropriate to meet the demands imposed by these different noxious stimuli. Hypothalamic-PAG projections activated by A-fiber inputs did not reflect this level of organization and we suggest that this may relate to their role in thermoregulation as opposed to autonomic responses to particular nociceptive inputs.

Key words: Fos, nociception, pain, spinal cord, periaqueductal gray, hypothalamus.

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Abbreviations: AHAd, anterior hypothalamic area dorsal; CAAH, caudal area anterior hypothalamus; CTb, cholera toxin subunit B; DAB, 3,3-diaminobenzidine tetrahydrochloride; DLH, DL-homocysteic acid; DL/L, dorsolateral/lateral; FLI, Fos-like immunoreactivity; LAAH, lateral area anterior hypothalamus; LHA, lateral hypothalamic area; LSN, lateral spinal nucleus; PAG, periaqueductal gray; PB, phosphate buffer; PBS-T, phosphate-buffered saline with Triton X-100; VL, ventrolateral.

Alterations in cardiovascular function evoked by noxious stimuli contribute to protective strategies that enable an individual to avoid, escape or cope with tissue damage in the periphery and, it has long been predicted (Lewis, 1942) that the precise patterns of autonomic change that are evoked by pain arising from cutaneous versus visceral structures would be mediated by distinct central pathways. Many autonomic control centers in the brain stem receive nociceptive inputs (Gauriau and Bernard, 2002; Keay and Bandler, 2002) and the induction of Fos protein has provided detailed information about the functional organization of pathways within the brain that are activated by noxious stimuli arising from different peripheral organs. Much of this work has focused on the organization of nociceptive inputs to the periaqueductal gray (PAG) region of the midbrain.

The PAG can be divided into functional columns that are arranged longitudinally around the aqueduct. Neurons in the different columns are thought to operate during different environmental conditions to mediate either sympathoexcitation and increased motor activity or sympathoinhibition and quiescence. The former pattern of response is coordinated by neurons in the dorsolateral/lateral (DL/L-) PAG, as part of an active coping strategy that operates during states of arousal. In contrast, the latter is mediated by neurons in the ventrolateral (VL-) sector, as part of passive coping strategies that operates during recuperation or after intense exercise (Carrive, 1993; Lovick, 1993; Bandler and Shipley, 1994; Bernard and Bandler, 1998). Nociceptive inputs provide a powerful drive to the PAG and recent work indicates a high degree of columnar organization of these inputs, which is related to their behavioral significance (Bandler et al., 2000); the DL/L-PAG being more concerned with processing afferent information related to brief cutaneous insults and, in contrast, the VL sector with processing deep somatic and visceral pain (Keay and Bandler, 1993; Keay et al., 1994; Clement et al., 1996; Bandler et al., 2000).

The anterior hypothalamus, an important brain region for the integration of autonomic functions, is thought to exert its effects, at least in part, after a relay in particular columns of the PAG (Rizvi et al., 1996; Semenenko and Lumb, 1999). It is of considerable interest therefore, that hypothalamic-PAG projection neurons that are activated by either noxious visceral or by noxious somatic stimuli constitute largely separate populations of cells that innervate the VL- or DL/L- columns of the PAG respectively (Snowball et al., 2000; Parry et al., 2002).

Visceral nerves contain a relatively high proportion of C-fiber nociceptors (Cervero and Tattersall, 1987) and, as

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a consequence, visceral pain may be expected to be conveyed principally by activity in C-fibers, in contrast to cutaneous pain that may result from both C- and A-nociceptor activation. As such, differential activation of, or organization of hypothalamic projections to, the DL/L- and VL-columns of the PAG might be more directly related to the type of nociceptive afferent activated rather than the location of the particular end organ. The finding that hypothalamic neurons driven by C-fiber activation project predominantly to the VL-PAG (Lumb et al., 2002) supports this hypothesis.

The hypothesis to be tested in the current study was that nociceptive inputs of different behavioral significance target the PAG in a functionally relevant manner that reflects their roles in triggering different patterns of autonomic response. To test this hypothesis, experiments were designed to determine (i) the columnar organization of spino-PAG projections activated by C- versus A-nociceptive inputs and (ii) A-nociceptive input to hypothalamic neurons and the columnar organization of their projections to the PAG.

EXPERIMENTAL PROCEDURES

All procedures involving experimental animals were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines. All experiments conformed to international guidelines on the ethical use of animals. Every effort was made to minimize animal suffering and to reduce the number of animals used.

Injection of retrograde tracer

Experiments were carried out on male Wistar rats (250–300 g). Anesthesia was induced (60–70 mg kg^{-1}, i.p.) and maintained (17–20 mg kg^{-1}, i.p.) with sodium pentobarbitone (Sigma-Aldrich, Dorset, UK) at a level at which there was an absence of withdrawal reflexes to minor noxious stimuli. Arterial blood pressure was monitored via the carotid artery and body temperature was maintained at approximately 37 °C. A small area of the cortex was exposed just rostral to lambda. Under stereotaxic guidance, a DL-homocysteic acid (DLH, 0.05 M; Sigma-Aldrich) –filled glass micropipette was attached to a pressure injection system and positioned in the PAG between 7.4 and 7.6 mm caudal to bregma, at a depth between 4.0 and 5.0 mm below the cortical surface. DLH at 30–50 nl was injected and the evoked blood pressure response recorded. Once a 'depressor' or a 'pressor' site had been located the coordinates of the site were noted and the pipette was removed from the brain. Cholera toxin subunit B (CTb 1%; List Biological Laboratories Inc., Campbell, CA, USA) was drawn up into the micropipette and the pipette repositioned at the same location. CTb (100–200 nl) was pressure-injected into the PAG over approximately 10 min and the pipette left in place for a further 15 min before being withdrawn. A total of 14 animals were injected with CTb at pressor sites and another 16 at depressor sites.

The skin overlying the skull was sutured, the arterial cannula was removed and the carotid artery ligated. The rats were allowed to recover and left for 5–7 days to enable retrograde axonal transport of the cholera toxin.

Peripheral stimulation

Rats previously injected with retrograde tracer at pressor ($n=14$) or depressor ($n=16$) sites were re-anesthetized (sodium pentobarbitone, 60–70 mg kg^{-1} i.p.) before removal from their home cage. Anesthesia was monitored by assessing corneal reflexes and maintained with supplementary doses of sodium pentobarbitone (17–20 mg kg^{-1}, i.p.) as required. After 2 h, slow (2.5 °C s^{-1}; starting temperature 30 °C; cutoff 55 °C; ramp duration ~60 s; $n=13$) or fast (7.5 °C s^{-1}; starting temperature 30 °C; cutoff 57 °C; ramp duration ~6 s; $n=17$) rates of skin heating were applied to the dorsal surface of the hind paw ipsilateral to the injection site in the PAG (six times per animal, with an 8 min gap between heating ramps), to preferentially activate C- or A-heat nociceptors respectively (Yeomans and Proudfit, 1996; McMullan et al., 2004; Leith et al., 2007).

The rats were maintained under anesthesia for a further 2 h, to allow time for the expression of Fos protein. They were then given a lethal dose of pentobarbitone (100 mg kg^{-1} i.p.) and perfused transcardially with 0.1 M phosphate buffer (PB) followed by 4% paraformaldehyde in 0.1 M PB. The brains were removed, post-fixed overnight in 4% paraformaldehyde then transferred to 25% sucrose PB solution for 24–48 h.

Control animals

Four rats were maintained, unstimulated, under anesthetic for a total of 4 h and then perfused with 4% paraformaldehyde as above. Of these, three had been injected previously with retrograde tracer.

Tissue processing

In order to localize the CTb injection site, two series of 50 μm transverse sections were cut through the PAG using a freezing microtome. One series was mounted onto gelatin/chrome alum–coated microscope slides and counterstained with Neutral Red to aid orientation and the other immunostained for CTb as described by Clement et al. (2000), but using as secondary antibody a biotinylated anti-sheep immunoglobulin (Sigma-Aldrich). Sections were then washed in several changes of PB for at least 30 min.

Double immunostaining

Sections (40 μm) of spinal cord lumbar segments L3–L5 were cut on a freezing microtome and free-floating sections processed for Fos-like immunoreactivity (FLI) and for the presence of the retrograde tracer CTb.

Three series of 40 μm transverse sections were cut through the hypothalamus. One series was mounted and counterstained with Neutral Red, one series was immunostained for Fos and CTb and the third was kept in buffer in reserve.

Free-floating hypothalamic sections were double stained for FLI and CTb immunoreactive retrograde cells. After three washes in phosphate buffered saline containing 0.1% Triton X-100 (PBS-T), the sections were incubated in a polyclonal rabbit anti-Fos antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:5000 in 0.1 M PB containing 10% newborn calf serum, 1% normal goat serum and 0.1% Triton) for 48–72 h at 4 °C. The sections were washed again in three changes of PBS-T and then incubated in biotinylated anti-rabbit immunoglobulin (Dako Ltd., Ely, UK; 1:500 in PBS-T) for 1–2 h at room temperature and then washed as before and transferred to extravidin-peroxidase (Sigma-Aldrich; 1:1000 in PBS-T) for 1–2 h. Sections were washed again in three changes of PB. The peroxidase was then visualized using 3,3-diaminobenzidine tetrahydrochloride (DAB; 0.015%; Sigma-Aldrich) with nickel intensification and glucose oxidase to generate the hydrogen peroxide. The sections were washed again in PB and transferred to antibody to CTb (Quadratech Ltd., Surrey, UK; 1:20,000) for 24–48 h. After further washing in PB, sections were transferred to anti-sheep immunoglobulin (1:500 in PB, as above), and left for 1–2 h after which they were washed and transferred to extravidin peroxidase as before. The peroxidase was visualized using DAB with glucose oxidase to generate the hydrogen peroxide but without nickel intensification.