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Research article

The spino–bulbar–cerebellar pathway: Activation of neurons projecting to the lateral reticular nucleus in the rat in response to noxious mechanical stimuli

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HIGHLIGHTS

- Spinoreticualr cells may convey nociceptive information indirectly to the cerebellum.
- Spinoreticular cells were retrogradely labelled from the lateral reticular nucleus.
- 15% of cells expressed pERK in response to pinch applied to a hind paw.
- 60% of pERK – expressing cells possess the NK-1 receptor.
- This pathway may convey nociceptive information to the cerebellum.

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ABSTRACT

It is now well established that the cerebellum receives input from nociceptors which may serve to adjust motor programmes in response to pain and injury. In this study, we investigated the possibility that spinoreticular neurons (SRT) which project to a pre-cerebellar nucleus, the lateral reticular nucleus (LRt), respond to noxious mechanical stimulation. Seven adult male rats received stereotaxic injections of the b subunit of cholera toxin in the LRt. Following a 5 day interval, animals were anesthetised with urethane and a noxious mechanical stimulus was applied to the right hind paw. Animals were fixed by perfusion 5 min following application of the stimulus. Retrogradely labelled SRT neurons of the lumbar spinal cord were examined for immunoreactivity for phosphorylated ERK (pERK) and the neurokinin-1 (NK-1) receptor. Approximately 15% of SRT cells in deep laminae (IV–VII and X) expressed pERK ipsilateral to the site of the stimulus. Around 60% of SRT cells with the NK-1 receptor expressed pERK but 5% of pERK expressing cells were negatively labelled for NK-1. It is concluded that a significant proportion of SRT cells projecting to the LRt respond to noxious mechanical stimuli and that one of the functions of this pathway may be to provide the cerebellum with nociceptive information.

1. Introduction

It is now well established that the cerebellum receives input from nociceptors which may serve to adjust motor programmes in response to pain and injury [21,26]. Nociceptive information is conveyed to the cerebellum via the postsynaptic dorsal column (PSDC) pathway which forms connections with cells of the inferior olivary complex that terminate as climbing fibres on Purkinje cells [8,9]. However, there is also evidence that mossy fibre systems have an involvement in nociceptive processing [30]. The cerebellum also receives information indirectly from the spinal cord via spinoreticular tract (SRT) pathways [1] that form synaptic connections with pre-cerebellar cells of the lateral reticular nucleus (LRt) which ter-

Abbreviations: bVFRT, bilateral ventral flexor reflex tract; CTb, cholera toxin B subunit; DAB, 3,3′-diaminobenzidine; HRP, horseradish peroxidase; IgG, immunoglobulin gamma; LRt, lateral reticular nucleus; NK-1, neurokinin-1; PB, phosphate buffer; PBS, phosphate buffer saline; PBST, phosphate buffer saline containing 0.3% Triton X-100; pERK, phosphorylated extracellular signal regulated kinase; PSDC, postsynaptic dorsal column pathway; SD, standard deviation; SP, substance P; SRT, spinoreticular tract.

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minate within various regions of the cerebellar cortex as mossy fibres [5,29]. It has been known for many years that lesions of the cerebellum reduce the strength of the withdrawal reflex [11] but the mechanisms that underlie this phenomenon are unclear. One of the SRT pathways projecting to the LRt is the bilateral ventral reflex afferent pathway (bVFRT) which projects from all levels of the spinal cord including lumbar segments. This pathway is activated by high-threshold flexor reflex afferents in addition to innocuous modalities [7,18]. It has also been shown that LRt cells respond to noxious mechanical stimuli applied to hind paws [17]; thus, this pathway has the potential to provide the cerebellum with information about the activity of nociceptors. Expression of phosphorylated extracellular signal regulated kinase (pERK) has been used extensively to determine if cells in the dorsal horn of the spinal cord (laminae 1-III) are activated by noxious stimuli [10,13,28]. Many cells that express pERK in response to various noxious stimuli, including pinch, also possess the substance P (SP) neurokinin-1 (NK-1) receptor [24]. The aim of the present study was to determine if SRT neurons, projecting from ventral laminae of the lumbar spinal cord to the LRt, are activated by acute noxious mechanical stimuli; and if so, to determine if such cells possess the NK-1 receptor. In order to achieve these aims, we retrogradely labelled SRT neurons projecting to the LRt from the lumbar spinal cord with the b subunit of cholera toxin (CTb) and investigated them for expression of pERK in response to a noxious mechanical stimulus applied to a hind paw [24,28]. We subsequently examined populations of SRT cells that are immunoreactive for the NK-1 receptor to determine if they also express pERK.

2. Materials and methods

A total of seven adult male Sprague–Dawley rats (Harlan, Bicester, UK) weighing between 250–350 g were used in these experiments. Experiments were approved by the Ethical Review Process Applications Panel of the University of Glasgow and were performed in accordance with the UK animals (Scientific Procedures) Act 1986.

2.1. Animal surgery

Stereotaxic surgery was performed on animals induced and maintained under general anaesthesia with 2–4% isoflurane in oxygen. Rats were placed in a stereotaxic frame and a small burr hole was made in the skull. Glass micropipettes (tip diameter of 20 μm) were filled with CTb (Sigma–Aldrich, Co., UK; 1% in sterile distilled water) which was pressure injected (200 nl) into the left LRt at inter-aural co-ordinates (−4.8 mm antero-posterior; +1.8 mm medio-lateral; −0.4 mm dorso-ventral) [22]. Pipettes were left in place for 5 min to prevent any backflow of the tracer. On conclusion of the surgery, the scalp was sutured and the animals were placed in an incubator to aid recovery. All animals were given subcutaneous injections of Rimadyl 0.1 ml/100 g (Carprofen 50 mg/ml, Pfizer, Dumfries, UK) postoperatively. Within a two hour period, the animals recovered and started to drink and explore.

2.2. Noxious stimulation, fixation and tissue preparation

Following a five day interval after surgery, animals were deeply anesthetised (tested by absence of the corneal reflex) with urethane (10%; 3–5 ml i.p.; 1.25 g/kg). A noxious mechanical stimulus was applied with a Kocher's forceps with toothed jaws, by pinching the skin on the dorsal and plantar aspects of the right hind paw for 2 min (contralateral to the LRt injection site). The stimulus was applied at five selected sites of the foot pad as well as the dorsum of the foot with each site having ∼20 s of stimulation. No withdrawal reflex was observed during the application of noxious stimuli as animals were in a state of deep anaesthesia. After an interval of 5 min following the last stimulus, animals were perfused transcardially via the left ventricle with 25–50 ml of mammalian ringer's solution followed by 1000 ml of 4% freshly depolymerised formaldehyde (Sigma–Aldrich, Co., UK) in 0.1 M PB (Sigma–Aldrich, Co., UK). The brain and mid-lumbar spinal cord segments were removed and left overnight in the same fixative at 4 °C with 30% sucrose added for brain. The brainstem was removed and rinsed in 0.1 M PB sucrose and 100 μm thick coronal sections were cut with a freezing microtome for histological examination of injection sites. Lumbar 3–5 segments were identified on the basis of their dorsal roots and rinsed in 0.1 M PB. Transverse spinal cord sections (50 μm) were cut using a vibrating microtome (Leica VT1200, Leica Microsystems, UK). Both brain and spinal cord sections were placed in an aqueous solution of 50% ethanol for 30 min to enhance antibody penetration.

2.3. Identification of injection sites

CTb injection sites were visualized using 3,3′-diaminobenzidine (DAB) as a chromogen. Brain sections were incubated in goat anti-CTb (1:50,000; List Quadratech, USA) for two days at 4 °C, rinsed in PBS (phosphate buffer saline) and placed overnight in biotinylated anti-goat IgG (immunoglobulin,1:5000; Jackson Immunoresearch, USA) for 3 h at room temperature. These sections were then rinsed again in PBS, incubated in avidin-horseradish peroxidise (1:1000; Sigma–Aldrich, UK) for 1 h and hydrogen peroxide and DAB were applied for 15 min to reveal the chromogen. Sections were then mounted onto gelatine-coated slides. Photographs of injection sites were captured digitally with AxioVision 4.8 software (Axiocam, Carl Zeiss, Inc., Germany). Injection sites were confirmed by projecting digital images onto brainstem diagrams from the stereotaxic rat brain atlas of Paxinos and Watson [22] and drawing them.

2.4. Immunocytochemistry

To determine if SRT neurons are activated by noxious stimuli, sections (L3–L5) from all seven animals were reacted with solutions of primary antibodies for 48 h to identify CTb and pERK (goat anti-CTb, 1:5000, List, Campbell, USA and mouse anti-pERK, 1:500, Cell signalling/Millipore, UK). Subsequently they were incubated in secondary antibodies coupled to fluorophores for 3 h (donkey anti-goat IgG, coupled to rhodamine red, 1:100, Jackson Immunoresearch, West Grove, USA and donkey anti-mouse IgG coupled to Alexa 488, 1:500, Molecular probes, Eugene, USA) and mounted on glass slides with anti-fade medium, (Vectashield; Vector Laboratories, Peterborough, UK). A further series of experiments was conducted on sections from four of the animals to determine whether SRT cells that express pERK also possess the NK-1 receptor. The procedure was the same as described above except that an additional primary antibody for NK-1 (1:10, 000, Rabbit, Sigma, USA) was added to the anti-CTb and anti-pERK mixture and an anti-rabbit secondary antibody coupled to Dylight 649 (donkey anti-rabbit, 1:500, Molecular probes, Eugene, USA) was added to the secondary antibody mixture.

2.5. Image acquisition and analysis

Three sections per segment (L3–L5) were selected randomly from each group and entire sections were systematically scanned with a confocal microscope (LSM 710, Zeiss, Germany) at a magnification of ×10 (1.4 zoom with an increment of 2 μm) to obtain a complete series of images in order to make a montage. Image stacks were analysed with Neurolucida for Confocal (Micro Bright Field Inc., Colchester, VT, USA). Retrogradely labelled cells in three zones of the lumbar cord contralateral to the LRt injection site; deep dorsal