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Thermal Stress-Induced Expression of CB1 Cannabinoid Receptors in the Rat Rostral Pons

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Endocannabinoids are a family of biologically active lipids in the brain that mediate the psychoactive effects of cannabis by two distinct receptors, cannabinoid receptor type I (CB1) and type II (CB2). It has been shown that the CB1 is the major cannabinoid receptor in the brain and is highly expressed in areas that are involved in pain modulation. There is also recent evidence that its activation reduces nociceptive processing in acute and chronic animal pain models. In this study, we demonstrate that thermal stress induces the expression of CB1 cannabinoid receptors in certain brainstem regions associated with pain sensation in the rat rostral pons, using light microscopic immunohistochemistry with a subtype-specific antibody. In particular, we were able to reveal that the pontine gray matter, the locus coeruleus, the parabrachial nuclear complex and the pontine raphe nuclei show high levels of CB1 cannabinoid receptors with a possible role in specific brain functions, such as nociception. The present results suggest that in addition to their known expression in excitatory glutamatergic and inhibitory GABAergic neuronal subpopulations, the CB1 receptors are also present in a subset of noradrenergic, cholinergic and serotonergic neurons in the rat rostral pons. It can be inferred that CB1 receptors may have divergent roles in nociceptive processing in a broad brainstem area, depending on the exact neurotransmitter system they modulate.

Key words: cannabinoid receptors, endocannabinoids, locus coeruleus, parabrachial nuclear complex, periventricular gray, pontine raphe nuclei, rat.

Introduction

The endocannabinoid system is a recently discovered neuromodulatory system implicated in a multitude of physiological and pathophysiological functions by influencing the activity of diverse neurotransmitter systems, including GABA, noradrenaline, dopamine, glutamate and acetylcholine [9]. This system consists of endogeneous ligands, i.e. endocannabinoids, the enzymes for their biosynthesis and degradation, and their specific cannabinoid receptors [15, 18].

Endogenous cannabinoids (endocannabinoids) are a group of neuromodulatory lipids that mediate the psychoactive effects of cannabis and participate in a variety of physiological processes including appetite, pain sensation and memory [6]. They exert their physiological actions by at least two G protein-coupled receptors that inhibit adenylate cyclase activity. To date, two receptor subtypes, the cannabinoid receptor type 1

(CB1) and type 2 (CB2), have been described with regard to their primary structure, ligand-binding properties, and signal transduction systems [10, 14].

The CB1 is the major cannabinoid receptor in the brain while CB2 cannabinoid receptors are widely distributed in the peripheral nervous and immune systems [2, 8, 18]. Specifically, CB1 receptors are highly expressed in rat brain areas that are involved in pain modulation, including the periaqueductal gray [20, 22] and the dorsal horn of the spinal cord [7] whereas the brainstem exhibits low levels of CB1 receptor activity [18, 20]. Recent studies have also shown that their activation reduces nociceptive processing in acute and chronic animal pain models [reviewed in 17]. Our study was thus designed to test whether thermal stress induces the expression of CB1 receptors in other brainstem regions associated with pain sensation in the rat brain.

Materials and Methods

The experiments were carried out on adult Wistar rats of both sexes, weighing 250-300 g. The animals were divided into three experimental groups of 3 rats: rats after thermal stress exposure, ones treated with the cannabinoid receptor agonist anandamide, and untreated controls. Thermal stress nociception was induced using the hot plate test as previously described by Bocheva et al. [3]. One group of rats (n = 3) received subcutaneous injections of anandamide (10 mg/kg b.w.) dissolved in physiological saline. All the procedures were performed according to a standard protocol established by the Bioethical Commission of the Biomedical Research at the Institute of Neurobiology of the Bulgarian Academy of Sciences. All efforts were made to minimize the number of animals used and their suffering.

For the immunohistochemical experiments, the rats were deeply anesthetized and transcardially perfused first with 0.05 M phosphate-buffered saline (PBS), pH 7.4, followed by 4% paraformaldehyde (PFA) in 0.01 M phosphate buffer (PB), pH 7.4. The brain was dissected out, sliced at the level of the rostral pons and postfixed in the same fixative overnight at 4ºC. Thereafter, the tissues were embedded in paraffin and cut into 5 μm thick sections. The samples were then deparaffinized with xylene and ethanol, and subsequently processed for avidin-biotin-horseradish peroxidase complex (ABC) immunohistochemistry using an ImmunoCruz™ goat ABC Staining System (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Briefly, the sections were treated with hydrogen peroxide (1% in absolute methanol; 30 min) to inactivate endogenous peroxidase and the background staining was blocked with 1.5% normal goat serum (NGS) in PBS for 1 hour. Between the separate steps, the sections were rinsed with cold PBS/Triton X-100. Afterwards, they were incubated with a polyclonal goat anti-CB1 receptor antibody (diluted 1:500, Santa Gruz Biotechnology) overnight at 4 °C in a humid chamber, followed by biotinylated donkey anti-goat IgG (Santa Gruz Biotechnology, 1:500) for 2 h at room temperature, and lastly the AB enzyme reagent was applied for 30 min at room temperature. Finally, the peroxidase activity was visualized by 1-3 drops of peroxidase substrate using diaminobenzidine as a chromogen. After the immunoreaction, the sections were dehydrated in ethanols, cleared in xylene and coverslipped with Entellan (Merck, Darmstadt, Germany). The slides were observed and photographed with a Nikon research microscope equipped with a digital camera DXM1200c. The brainstem structures were identified according to the rat brain atlas of Paxinos and Watson [13].

The specificity of the immunostaining was controlled by omission of the primary antiserum from the incubation medium or its replacement with PBS. No immunoreactivity was detected in either case.

Results

The immunohistochemical experiments demonstrated staining for CB1 in the thin layer of cells in the dorsal pontine tegmentum called the central pontine gray. The cell bodies and proximal processes of the neurons were strongly immunostained (**Fig. 1A**), in comparison with these in the untreated control rats. Many intensely stained neurons were also observed in the locus coeruleus and the subcoeruleus nucleus (**Fig. 1B**). In addition, moderately stained neurons were found in the pontine parabrachial nuclear complex, comprising the medial parabrachial nucleus and lateral parabrachial nucleus (**Fig. 1C**). A number of neurons in the raphe nuclei of the pontine reticular formation

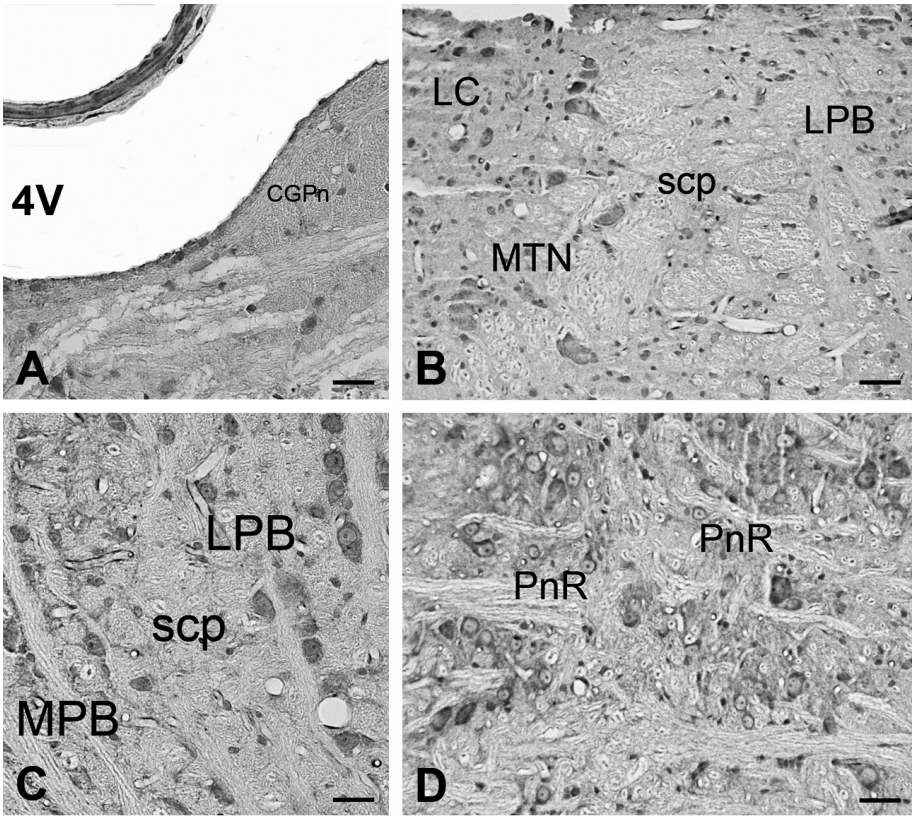

Fig. 1. Expression of CB1 cannabinoid receptors in the rat rostral pons. (**A**) Immunohistochemical staining for CB1 in the central pontine gray (CGPn). Note that both perikarya and proximal processes of cholinergic neurons around the fourth ventricle (4V) are intensely immunostained. (**B**) Low-magnification photomicrograph of the pons demonstrating CB1 immunoreactivity in the locus coeruleus (LC) and adjacent nuclei. The majority of noradrenergic cerulean neurons and their proximal axonal profiles are strongly CB1-immunostained. (**C**) Higher magnification of the parabrachial nuclear complex showing that neurons in both medial parabrachial nucleus (MPB) and lateral parabrachial nucleus (LPB) express CB1 immunoreactivity. (**D**) Serotonergic reticular neurons in the pontine raphe nucleus (PnR) also exhibit strong CB1 immunostaining. MTN, mesencephalic trigeminal nucleus, scp, superior cerebellar peduncle. Scale bars = 200 μm (**A**); 100 μm (**B, D**); 50 μm (**C**).

and, in particular, the pontine raphe nucleus and inferior central nucleus were CB1-immunopositive as well.

Discussion

It is well known that the endocannabinoids bind to type I cannabinoid receptors, located at the presynaptic level, and exert important control upon some neuronal functions by modulating the release of several neurotransmitters [1, 4, 16]. Previous investigations have focused on the distribution of CB1 receptors in rat brainstem regions such as the periaqueductal gray traditionally associated with pain transmission in relation with opioids [22]. It has also been revealed that the CB1 cannabinoid receptors are expressed solely in the gray matter around the fourth ventricle under normal conditions in rats [18, 20]. Here we report the contribution of certain rostral pons-located nuclei and their possible role for pain inhibition by administration of cannabinoids. The present data demonstrate that mild thermal stress induces high expression of CB1 cannabinoid receptors in other rat brainstem regions associated with pain sensation such as the pontine gray matter, locus coeruleus, parabrachial nuclear complex and pontine raphe nuclei.

Furthermore, the presence of CB1 receptors in the rodent brain has been verified in certain glutamatergic, GABAergic, dopaminergic and adrenergic neurons [5, 11, 21] and recently in septohippocampal cholinergic [12] and raphal serotonergic neurons in mice [9]. Our results provide immunohistochemical evidence that CB1 receptors are also present in a subset of noradrenergic, cholinergic and serotonergic neurons in the rat rostral pons. In particular, the central gray of the pons, known as the cholinergic Ch6 cell group, is the site of origin of the descending pain control pathway that relays to the raphe nuclei of the rostral medulla and caudal pons. In turn, the raphe nuclei of the pontine reticular formation, the pontine raphe nucleus and the inferior central nucleus which are serotonin-containing (B5 and B8 cell groups, respectively) are implied in the regulation of anxiety and depression. On the other hand, the locus coeruleus is considered the most important pain-related nucleus in the pons and the major noradrenergic nucleus in brain (cell group A6). It is believed that its activation by noxious stimuli leads to inhibition of perceived pain [19]. Last but not least, our findings also indicate that noradrenergic neurons in the rat pontine parabrachial nuclear complex which usually contain endogenous opioids and their receptors possibly plays important roles in pain modulation via activation of CB1 receptors, thus implying probable functional interactions between cannabinoids and opioids within the complex.

Conclusion

Our data support the existence of an extended brainstem cannabinoid system that possibly has a role relevant for nociception. It can be inferred that CB1 receptors may have divergent functions in nociceptive processing in this broad area, depending on the exact neurotransmitter system they modulate.

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