

Overlapping Distributions of Parvalbumin and Orexin Immunoreactivity in Mice Brain Regions Mediating Pain

Distribución Superpuesta de la Inmunorreactividad de la Parvalbúmina y de la Orexina en Regiones del Cerebro de Ratón que Regulan el Dolor

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SUMMARY: The regulatory functions of the orexin system are significantly correlated with pain. Parvalbumin (PV) interneurons, the main subtype of inhibitory gamma-aminobutyric acid (GABA) interneurons, have also been shown to be involved in the regulation of pain. To provide evidence for neural connections between these two types of neurons in pain-related nuclei, a double-label immunohistochemical staining technique was used to reveal the overlap between PV and orexin immunoreactivity in the mouse brain. At the forebrain level, the medial prefrontal cortex (mPFC) mainly consists of the cingulate cortex, area 1 (Cg1), prelimbic cortex (PrL), infralimbic cortex (IL), and dorsal peduncular cortex (DP). These regions all exhibited a similar, clear overlap between orexin-positive fibers and PV-positive neuronal somata and their axons. In the basal forebrain system, specific overlapping patterns were observed in the core of the nucleus accumbens (AcbC) and the shell of the nucleus accumbens (AcbSh). In the diencephalon, close overlap was found between orexinergic fibers and PV-positive neuronal structures in the basolateral amygdala (BLA) and lateral hypothalamic area (LH). In the brainstem, the density of intermingled orexin and PV fibers decreased in the order of the dorsal raphe nucleus (DR) and periaqueductal gray (PAG), with orexin boutons embedded within PV fibers. Additionally, in the ventral tegmental area (VTA) and lateral parabrachial nucleus (LPB), co-localization was observed between PV-positive somata with axons and orexin fibers. The PV and orexinergic systems anatomically converge by jointly targeting brain regions involved in pain regulation. These findings provide key morphological evidence for elucidating the synergistic mechanisms of the two systems in the pathophysiology of pain.

KEY WORDS: Pain; Parvalbumin; Orexin; Immunohistochemistry; Neural Circuit.

INTRODUCTION

Pain, defined by the International Association for the Study of Pain (IASP) as “an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage” (Raja *et al.*, 2020). The neural mechanisms underpinning pain processing involve intricate circuits and a diverse array of neurotransmitter systems distributed throughout the central nervous system (Basbaum *et al.*, 2009; Yam *et al.*, 2018; Karcz *et al.*, 2024). Recent research has increasingly focused on the roles of neuropeptidergic signaling and interneuron systems in the modulation of pain (François *et al.*, 2017; Castro & Bruchas, 2019; Nelson *et al.*, 2023).

Among these, the orexin system, with its cell bodies localized predominantly within the lateral hypothalamus,

has emerged as a key modulator (Peyron *et al.*, 1998; S. Salehi *et al.*, 2020). A substantial body of evidence indicates that orexin plays a crucial role in pain regulation, generally exerting analgesic effects (Razavi & Hossein, 2017). For instance, chemical stimulation of the lateral hypothalamus induces antiallodynic and anti-hyperalgesic effects in models of neuropathic pain, an effect mediated by spinal orexin receptors (Salehi *et al.*, 2020). The system's function, however, is complex and brain-region specific; orexin neurons can exert contrasting effects on itch and pain processing via their projections to the periaqueductal gray (Kaneko *et al.*, 2024). Furthermore, persistent pain and stress are known to activate endogenous, pain-inhibitory pathways that involve orexin (Watanabe *et al.*, 2005).

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In parallel, parvalbumin (PV)-expressing interneurons, a major subclass of GABAergic inhibitory neurons, are critically involved in shaping neural circuit dynamics and have been strongly implicated in the pathophysiology of chronic pain (Liu *et al.*, 2017; Yang *et al.*, 2015). Deficits in PV interneuron function and associated dopamine signaling contribute to anterior cingulate cortex (ACC) dysfunction in chronic pain states (Lançon *et al.*, 2025). In key pain-processing regions like the amygdala, inflammatory pain models can mobilize PV neurons (Nascimento *et al.*, 2020). Recent findings highlight a fundamental role for PV in gating chronic pain through the modulation of inhibitory neuron firing patterns (Qiu *et al.*, 2024). The regulatory influence of PV interneurons extends to cortical areas such as the insular cortex, where their inhibitory output to pyramidal neurons is subject to plasticity that modulates nociception (Kobayashi *et al.*, 2025). The functional contribution of PV neurons is nuanced, as evidenced by findings that perturbing their glutamatergic transmission can actually reduce pain sensitivity (Roccaro-Waldmeyer *et al.*, 2018).

Despite compelling evidence for the independent roles of the orexin and PV systems in pain modulation, the anatomical substrate for their potential interaction within pain-related circuits remains largely unexplored. Given that both systems converge in key pain-processing nodes—including medial prefrontal cortex (mPFC) (Mascio *et al.*, 2022; Razmanjani & Reisi, 2022), basolateral amygdala (BLA) (Askari-Zahabi *et al.*, 2022; Butler *et al.*, 2017), lateral hypothalamic area (LH) (Salehi *et al.*, 2020; Wang *et al.*, 2025), periaqueductal gray (PAG) (Razavi & Hosseinzadeh, 2017; Roccaro-Waldmeyer *et al.*, 2018), and dorsal raphe nucleus (DR) (Nascimento *et al.*, 2020; Kang *et al.*, 2021) —investigating their anatomical overlap is a critical step (Peirs & Seal, 2016; Tan & Kuner, 2021). The interplay between the diffuse orexin system and specific, fast-spiking PV interneurons could represent a significant mechanism for state-dependent pain modulation (Xianhui Kang *et al.*, 2021). This interaction may be particularly relevant for understanding the high comorbidity of pain with conditions like sleep disorders and affective states, given orexin's established role in coregulating sleep-pain physiological interplay (Ahmadi-Soleimani *et al.*, 2020).

Therefore, to bridge this knowledge gap, the present study was designed to systematically map the anatomical overlap between orexin and PV immunoreactivity within pain-related nuclei of the mouse brain using a double-label immunohistochemical technique. This morphological investigation aims to provide a foundational basis for understanding how these two distinct neuromodulatory systems may interact functionally to influence pain pathways.

MATERIAL AND METHOD

Experimental Animals. Male SPF inbred C57BL/6J mice (Shanghai SLAC Laboratory Animal Co., Ltd., China), each weighing about 21 - 25 g, were housed in the Laboratory Animal Research Centre of Zhejiang Chinese Medical University and maintained in a room with conditions of an automatically controlled light-dark cycle, appropriate ambient temperature and humidity (lights on at 07:00 and off at 19:00, an ambient temperature of 20 - 25 °C, and a relative humidity of 55 - 60 %). Standard mice were provided ad libitum access to food and water. Every effort was made to minimize the number of animals utilized in this study and potential distress, pain, and discomfort to the mice throughout all experiments.

Ethical consideration. All experimental procedures for animal handling were approved and followed the animal guidelines established by the Laboratory Animal Research Centre of Zhejiang Chinese Medical University, Hangzhou, in Zhejiang province. (Protocol Number: IACUC-20250707-16).

Tissue preparation. Mice (n = 3) were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). Upon confirmation of unresponsiveness to tail- and foot-pinch stimuli, they were transcardially perfused with 0.9 % saline, followed by 4 % paraformaldehyde, both solutions administered at room temperature. Brains were then isolated and post-fixed for 4 hours at 4 °C in the same fixative. Subsequently, brains were transferred sequentially through 10 %, 20 %, and 30 % sucrose in 0.1 M phosphate-buffered saline (PBS) at 4 °C until they sank to the bottom. Four sets of 30-mm coronal sections were prepared using a Leica freezing microtome (Type 820-II), placed in cryoprotectant solution, and stored at -20 °C until immunohistochemical staining.

Immunohistochemical procedures. The first set of stored free-floating tissue sections were rinsed in PBS, washed with 3 % H₂O₂ in PBS for 30 min, and blocked with 10 % goat serum (Cat. No. S-1000, Vector Laboratories, Tucson, AZ, USA) and 0.3 % Triton X-100 in PBS for 1 h at room temperature. The solution was then removed without washing, and sections were subsequently incubated overnight at 4 °C with the primary antiserum. The primary antibodies consisted of a monoclonal mouse anti-orexin (1:1,000, Cat. No. sc-80263, Santa Cruz Biotechnology, Santa Cruz, CA, USA) to identify orexin neurons and a monoclonal rabbit anti-PV (1:1,000, Cat. No. ab181086, Abcam, Cambridge, UK) to identify PV neurons. The sections were then incubated with secondary antibodies — a biotinylated goat anti-mouse IgG (Cat. No. BA-9200, Vector Laboratories, Burlingame, CA, USA) and a

biotinylated goat anti-rabbit IgG (Cat. No. BA-1000, Vector Laboratories) — at room temperature for 2 h, followed by incubation in avidin-biotin-horseradish peroxidase complex (ABC solution; Vectastain Elite ABC Kit, Cat. No. PK-4000, Vector Laboratories). After rinsing with PBS, the sections were visualized with diaminobenzidine (DAB) in the presence of nickel ammonium sulfate. Subsequently, sections were rinsed with PBS, mounted on gelatin-coated slides, air-dried overnight, dehydrated through an ascending alcohol gradient, cleared in xylene, and cover-slipped.

Microscopy and Image Processing. Image acquisition was performed with an Olympus IX71 inverted microscope. For consistent anatomical localization across samples, all target

brain regions were mapped against a standardized mouse brain atlas. Low-magnification observations (4× objective) were first conducted to validate section integrity and outline major neuroanatomical structures. High-magnification imaging was then used to precisely document the spatial distribution and morphological features of immunoreactive neurons. All digital photomicrographs were processed in Adobe Photoshop CS2, with adjustments limited to linear brightness and contrast corrections to maintain experimental signal authenticity.

RESULTS

Medial Prefrontal Cortex (mPFC). The mPFC comprises the cingulate cortex (Cg1), prelimbic cortex (PrL), infralimbic cortex (IL), and dorsal peduncular cortex (DP). In Cg1, a low-to-moderate density of varicose orexin fibers with boutons was intermingled with a moderate-to-high density of branching PV fibers, with orexin fibers randomly dispersed among PV immunoreactivity (containing somas and fibers) (Fig. 1A, a1). PrL exhibited a moderate density of medium-sized PV-positive cells and fibers, where varicose orexin fibers with boutons (moderate density) were closely contacted with PV-emitting fibers (Fig. 1A, a2). IL displayed extensive labeling of both orexin and PV immunoreactivity, with a moderate-to-high density of slender varicose orexin fibers with boutons intermixed and contacting PV somas and emitting fibers (Fig. 1A, a3). DP also showed extensive labeling of orexin and PV immunoreactivity, containing a low-to-moderate density of varicose orexin fibers with boutons intermingled with a moderate density of PV-positive somas and branching fibers; notably, numerous slender orexin fibers formed close appositions with the somas and dendrites of PV neurons (Fig. 1A, a4). Overlap of orexin and PV immunoreactivity was evident across all four subregions of the mPFC.

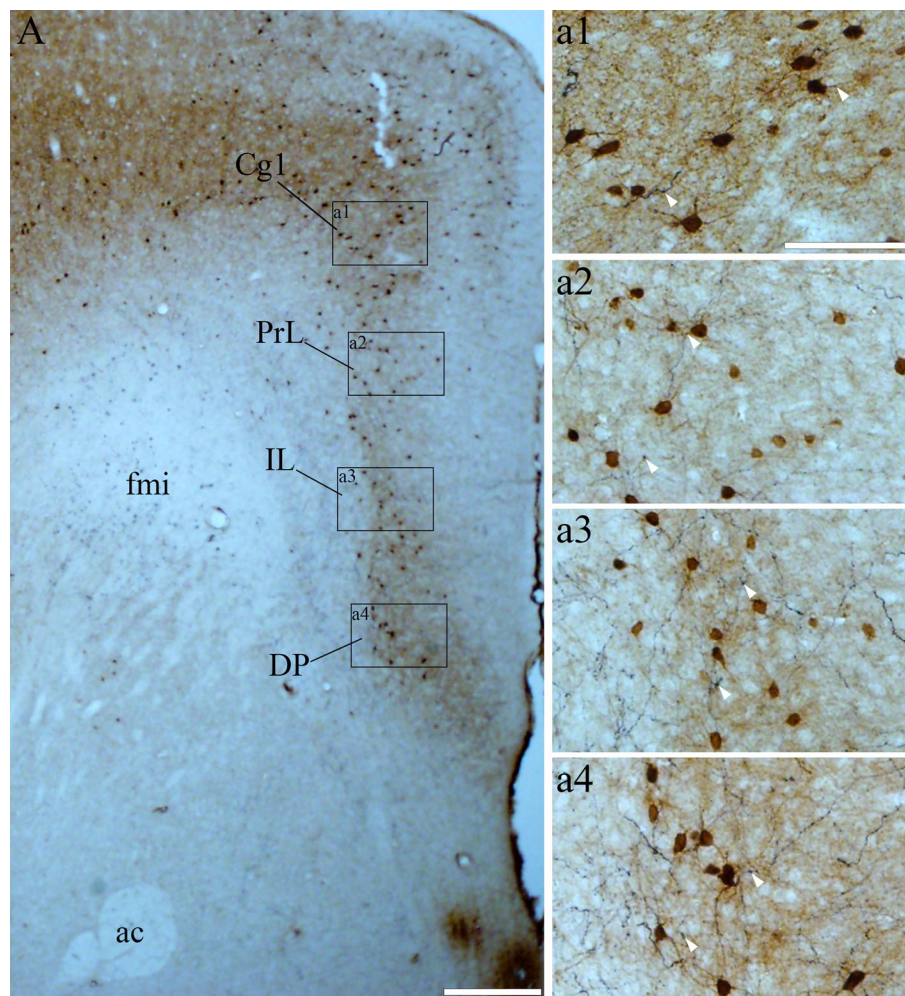


Fig. 1. Brightfield images depicting intermingled orexin and parvalbumin (PV) immunoreactivity in the cingulate cortex, area 1 (Cg1, A, a1), prelimbic cortex (PrL, A, a2), infralimbic cortex (IL, A, a3) and dorsal peduncular cortex (DP, A.a4). a1-a4 are higher-magnification images of the boxed areas depicted in the first photomicrographs in the left column. PV immunoreactive elements stained with monoclonal rabbit anti-PV, appear brown; orexin immunopositive elements stained with monoclonal mouse anti-orexin, appear black. Abbreviations: fmi, forceps minor of the corpus callosum; ac, anterior commissure. Scale bars=200 μ m at the bottom of photomicrograph in A, 100 μ m in a1 (applies to a1-a4).

Nucleus Accumbens (Acb). PV immunoreactivity in the Acb was predominantly localized to somas, axons, and dendrites. Orexin varicose fibers with boutons primarily formed appositions with PV axons or dendrites, while

exhibiting minimal association with PV somas. Notably, overlapping distributions of the two neuronal populations were observed in both the core (AcbC) and shell (AcbSh) regions of the nucleus accumbens (Figs. 2A, a; 2B, b).

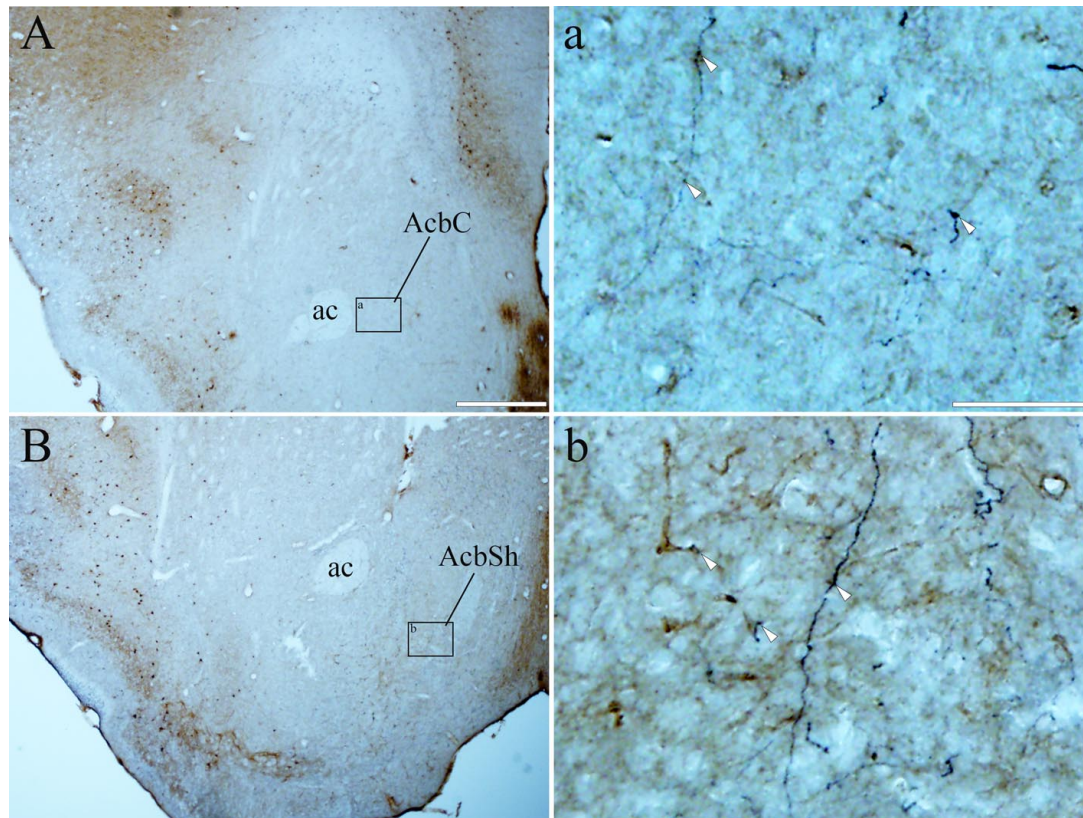


Fig. 2. Brightfield images depicting intermingled orexin and parvalbumin (PV) immunoreactivity in the core of nucleus accumbens (AcbC, A, a), shell of nucleus accumbens (AcbSh, B, b), a-b are higher-magnification images of the boxed areas depicted in the two photomicrographs in the left column. PV immunoreactive elements stained with monoclonal rabbit anti-PV, appear brown; orexin immunopositive elements stained with monoclonal mouse anti-orexin, appear black. Abbreviation: ac, anterior commissure. Scale bars=500 μ m at the bottom of photomicrograph in the left column (applies to A-B), 100 μ m in a (applies to a-b).

Basolateral amygdala (BLA). Within the BLA, a moderate-to-high density of PV immunoreactivity, which mainly contained somas, emitting slender axons and branching dendrites, was dispersed over the BLA. This was intermingled with a low-to-moderate concentration of varicose orexin fibers and tiny boutons. Orexin fibers were in apparent contact with PV fibers (Fig. 3A, a).

Lateral hypothalamic area (LH). The LH exhibited dense distributions of both PV and orexin neurons, with well-defined neuronal morphology. Extensive overlap between the two populations was observed. Close appositions were frequently detected between somas, thick axons of varying lengths, and elongated arborized dendrites labeled for PV and orexin immunoreactivity (Fig. 3B, b).

Periaqueductal gray (PAG). Numerous varicose orexin fibers with abundant boutons were observed in the PAG. These were intermingled with a moderate-to-high density of branching PV fine fibers, and many orexin boutons were closely associated with PV immunoreactive processes (Fig. 4A, a1).

Ventral tegmental area (VTA). The VTA contained a low density of PV neurons, characterized by somas and fine axons. Orexin innervation was sparse, consisting of thin fibers and tiny boutons. Overlap between orexin and PV immunoreactivity was minimal; occasional close appositions were observed between orexin fibers or boutons and a few slender PV axons (Fig. 4A, a2).

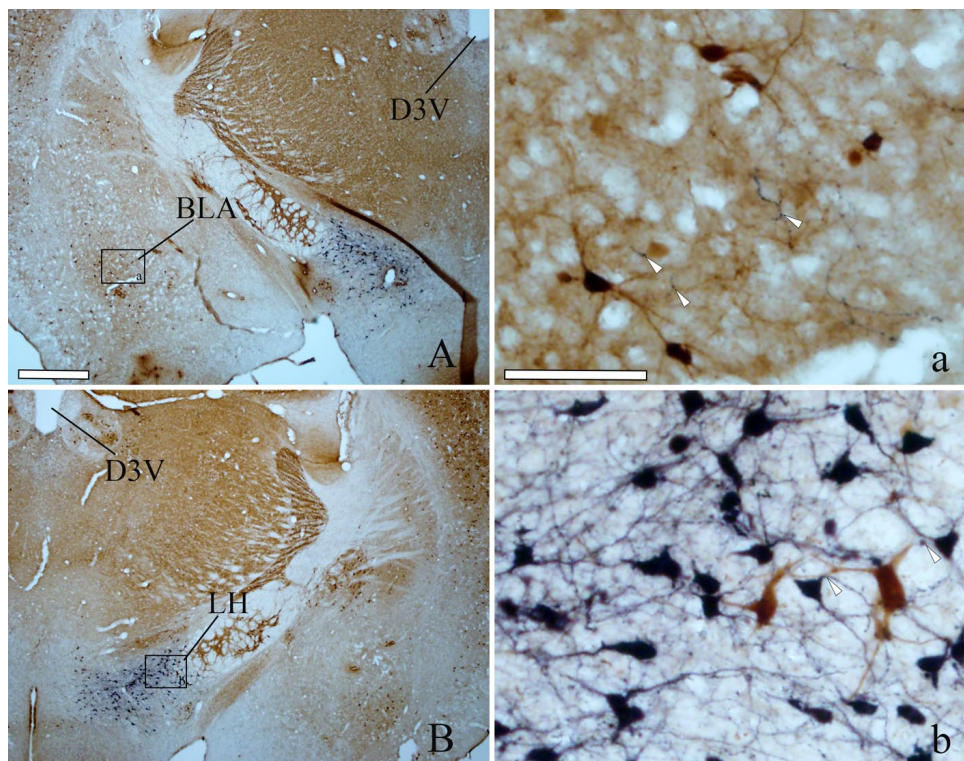


Fig. 3. Brightfield images depicting intermingled orexin and parvalbumin (PV) immunoreactivity in the basolateral amygdaloid nucleus (BLA, A, a), lateral hypothalamic area (LH, B, b). a-b are higher-magnification images of the boxed areas depicted in the two photomicrographs in the left column. PV immunoreactive elements stained with monoclonal rabbit anti-PV, appear brown; orexin immunopositive elements stained with monoclonal mouse anti-orexin, appear black. Abbreviations: D3V, dorsal 3rd ventricle. Scale bars=200 μ m at the bottom of photomicrograph in the right column (applies to A-B), 100 mm in a (applies to a-b).2

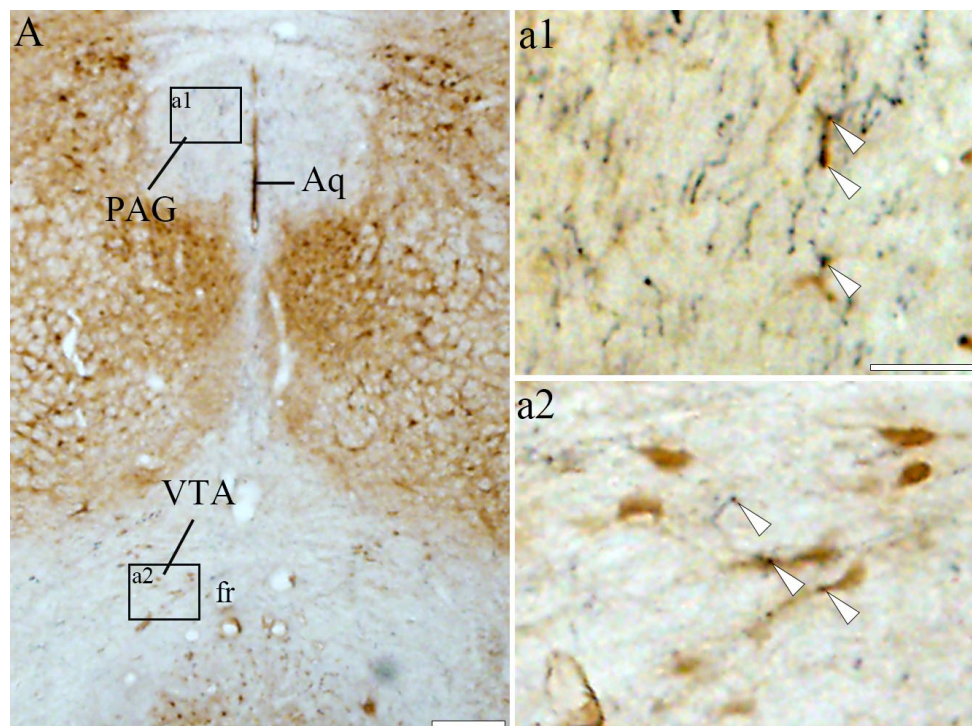


Fig. 4. Brightfield images depicting intermingled orexin and parvalbumin (PV) fibers in the periaqueductal gray (PAG, A, a1) and ventral tegmental area (VTA, A, a2). a1-a2 are higher-magnification images of the boxed areas depicted in the two photomicrographs in the left column. PV immunoreactive elements stained with monoclonal rabbit anti-PV, appear brown; orexin immunopositive elements stained with monoclonal mouse anti-orexin, appear black. Abbreviations: Aq, aqueduct; fr, fasciculus retroflexus. Scale bars=200 μ m at the bottom of photomicrograph in A, 100 μ m in a (applies to a1-a2).

Dorsal raphe nucleus (DR). The DR was innervated by long, thick orexin fibers bearing numerous boutons. These were intermingled with a moderate-to-high density of PV slender fibers, and many orexin boutons were closely associated with branching PV immunoreactive processes (Fig. 5A, a1).

Lateral parabrachial nucleus (LPB). Both orexin and PV fibers exhibited moderate densities in the LPB. Numerous varicose orexin fibers with abundant boutons were intermixed with slender PV fibers, and many small orexin boutons were closely associated with PV immunoreactive elements (Fig. 5A, a2).

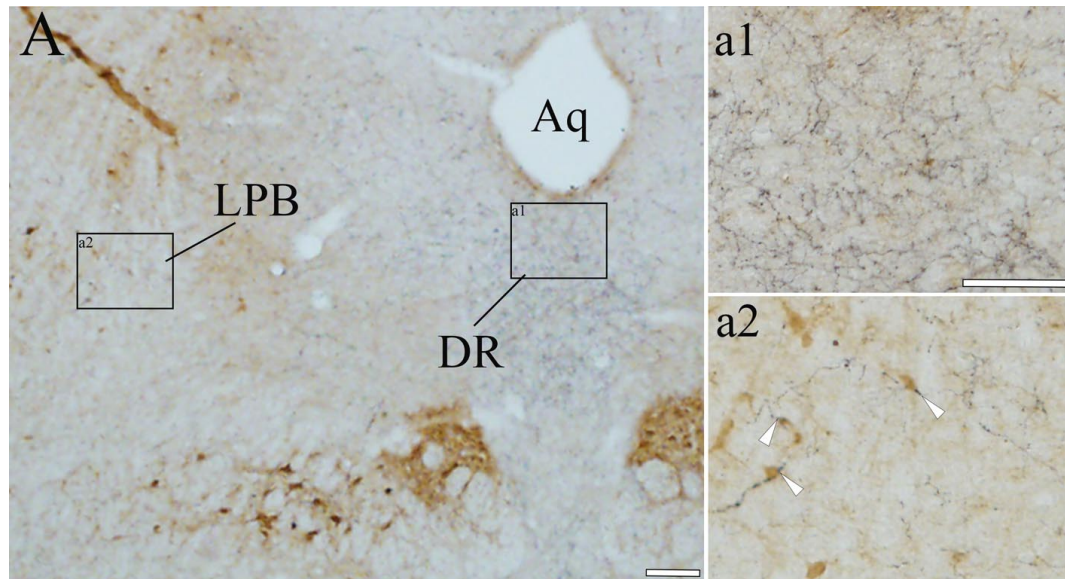


Fig. 5. Brightfield images depicting intermingled orexin and parvalbumin (PV) fibers in the dorsal raphe nucleus (DR, A, a1) and lateral parabrachial nucleus (LPB, A, a2), a1-a2 are higher-magnification images of the boxed areas depicted in the two photomicrographs in the left column. PV immunoreactive elements stained with monoclonal rabbit anti-PV, appear brown; orexin immunopositive elements stained with monoclonal mouse anti-orexin, appear black. Abbreviations: Aq, aqueduct. Scale bars=500 mm at the bottom of photomicrograph in A, 100 mm in a1 (applies to a1-a2)

DISCUSSION

The present study employed a double-label immunohistochemical technique to detect overlapping distributions of orexin and PV immunoreactivity in pain-related nuclei in the mice. Although orexin-containing neurons represent a relatively small population specifically localized within the hypothalamus, their projections are widely distributed throughout the rat brain (Kang *et al.*, 2021), including the pain-associated nuclei described above. Our results regarding the distribution of orexin immunoreactivity in several pain-related nuclei in the mouse show a striking similarity with previous studies (Nambu *et al.*, 1999). As for the distribution of PV immunoreactivity, our findings are consistent with earlier reports across multiple brain regions (Hontanilla *et al.*, 1998; Roccaro-Waldmeyer *et al.*, 2018). In the mPFC and basal forebrain areas including the VTA, PV-immunoreactive neurons have been characterized (McKenna *et al.*, 2013). Within the basal ganglia and ventral striatum, including the Acb, PV distribution has been mapped (Hontanilla *et al.*, 1998). PV-

immunoreactive neurons have been reported to be recruited in both the amygdala and the DR during temporomandibular inflammatory pain (Nascimento *et al.*, 2020). Furthermore, in the PAG, as well as in brainstem regions such as the DR and LPB, the distribution of PV and other calcium-binding proteins has been described (Schwaller *et al.*, 2002). The distributions of PV immunoreactivity in our findings are consistent with the above studies.

Moreover, overlapping distributions of orexin and PV immunoreactivity in different pain-related nuclei in the mouse were observed through a double-label immunohistochemical technique in the present study (Fig. 1-5). At the forebrain level, the mPFC mainly consists of the Cg1, PrL, IL, and dorsal DP. These regions all exhibited a similar, clear overlap between orexin-positive fibers and PV-positive neuronal somata and their axons. In the basal forebrain system, specific overlapping patterns were observed in the AcbC and AcbSh. In the diencephalon, close

overlap was found between orexinergic fibers and PV-positive neuronal structures in the BLA and LH. In the brainstem, the density of intermingled orexin and PV fibers decreased in the order of the DR and PAG, with orexin boutons embedded within PV fibers. Additionally, in VTA and LPB, co-localization was observed between PV-positive somata with axons and orexin fibers. The neuroanatomical overlap between orexinergic elements and PV immunoreactivity at multiple sites of pain-related nuclei suggests that their functional co-actions are likely to be involved in modulating pain processing.

The regulatory functions of the orexin system are significantly correlated with pain (Razavi & Hossein, 2017). For instance, chemical stimulation of the lateral hypothalamus induces antiallodynic and anti-hyperalgesic effects in models of neuropathic pain, an effect mediated by spinal orexin receptors (Salehi *et al.*, 2020). Furthermore, persistent pain and stress are known to activate endogenous, pain-inhibitory pathways that involve orexin (Watanabe *et al.*, 2005). PV-expressing interneurons, a major subclass of GABAergic inhibitory neurons, are critically involved in shaping neural circuit dynamics and have been strongly implicated in the pathophysiology of chronic pain (Qiu *et al.*, 2024; Lançon *et al.*, 2025). The functional contribution of PV neurons is nuanced, as evidenced by findings that perturbing their glutamatergic transmission can reduce pain sensitivity (Roccaro-Waldmeyer *et al.*, 2018). Recent findings highlight a fundamental role for PV in gating chronic pain through the modulation of inhibitory neuron firing patterns (Qiu *et al.*, 2024). In key pain-processing regions like the amygdala, inflammatory pain models can mobilize PV neurons (Nascimento *et al.*, 2020), and deficits in PV interneuron function contribute to ACC dysfunction in chronic pain states (Lançon *et al.*, 2025). The regulatory influence of PV interneurons extends to cortical areas such as the insular cortex, where their inhibitory output to pyramidal neurons is subject to plasticity that modulates nociception (Kobayashi *et al.*, 2025).

The interplay between the diffuse orexin system and specific, fast-spiking PV interneurons could represent a significant mechanism for state-dependent pain modulation (Kang *et al.*, 2021). This interaction may be particularly relevant for understanding the high comorbidity of pain with conditions like sleep disorders and affective states, given orexin's established role in coregulating sleep-pain physiological interplay (Ahmadi-Soleimani *et al.*, 2020). The anatomical convergence observed in the present study within critical nodes of the pain matrix, including the mPFC, Acb, BLA, LH, PAG, VTA, DR, and LPB, provides a morphological substrate for such functional interactions. For example, the convergence of orexin and parvalbumin systems in the VTA and Acb is notable given the demonstrated role of the VTA-

Acb glutamatergic projection in the regulation of pain and pain-related anxiety. (Abdul *et al.*, 2022). Similarly, the overlap in the PAG is significant as the PAG is one important supraspinal site of orexin pain modulation (Razavi & Hosseinzadeh, 2017), and orexin neurons in the LH project to the PAG to exert contrasting effects on itch and pain processing (Kaneko *et al.*, 2024).

In conclusion, the PV and orexinergic systems anatomically converge by jointly targeting brain regions involved in pain regulation. This finding provides key morphological evidence for elucidating the synergistic or antagonistic mechanisms of the two systems in the pathophysiology of pain. The precise nature of their functional interaction—whether synergistic, antagonistic, or modulatory—within these overlapping circuits warrants further investigation using functional and circuit-specific manipulations.

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RESUMEN: Las funciones reguladoras del sistema de la orexina están correlacionadas significativamente con el dolor. Se ha demostrado que las interneuronas de parvalbúmina (PV) —el subtipo principal de interneuronas inhibitorias de ácido gamma-aminobutírico (GABA)— también participan en la regulación del dolor. Para aportar evidencia sobre las conexiones neuronales entre estos dos tipos de neuronas en núcleos relacionados con el dolor, se utilizó una técnica de tinción inmunohistoquímica de doble marcaje para revelar la superposición entre la inmunorreactividad a la PV y a la orexina en el cerebro del ratón. A nivel del prosencéfalo, la corteza prefrontal medial (mPFC) comprende principalmente la corteza cingulada área 1 (Cg1), la corteza prelímbica (PrL), la corteza infralímbica (IL) y la corteza peduncular dorsal (DP). Todas estas regiones mostraron una superposición clara y similar entre las fibras positivas para orexina y los somas neuronales positivos para PV junto con sus axones. En el sistema del prosencéfalo basal, se observaron patrones específicos de superposición en el núcleo (core) del núcleo accumbens (AcbC) y en la periferia (shell) del núcleo accumbens (AcbSh). En el diencéfalo, se halló una estrecha superposición entre las fibras orexinérgicas y las estructuras neuronales positivas para PV en la amígdala basolateral (BLA) y el área hipotalámica lateral (LH). En el tronco encefálico, la densidad de fibras entremezcladas de orexina y PV disminuyó en el orden: núcleo del rafe dorsal (DR) y sustancia gris periacueductal (PAG), observándose botones de orexina inmersos entre las fibras de PV. Además, en el área tegmental ventral (VTA) y el núcleo parabraquial lateral (LPB), se observó colocalización entre somas positivos para PV (con sus axones) y fibras de orexina. Los sistemas de PV y orexinérgico convergen anatómicamente al proyectarse conjuntamente hacia regiones cerebrales relacionadas con la regulación del dolor. Estos hallazgos proporcionan evidencia morfológica clave para dilucidar los mecanismos sinérgicos de ambos sistemas en la fisiopatología del dolor.

PALABRAS CLAVE: Dolor; Parvalbúmina; Orexina; Inmunohistoquímica; Circuito neuronal.

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