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# PAC1 RECEPTOR SIGNALING IN ESTABLISHED NEUROPATHIC PAIN: MOLECULAR MECHANISMS, NEUROINFLAMMATORY PATHWAYS, AND CENTRAL SENSITIZATION FOR TARGETED CHRONIC PAIN THERAPEUTICS

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**Abstract**

Neuropathic pain remains one of the most refractory clinical syndromes in contemporary medicine, affecting an estimated 7–10% of the global population and representing a major unmet therapeutic challenge. Among the molecular mechanisms that underlie the transition from acute to chronic neuropathic pain, the pituitary adenylate cyclase-activating polypeptide (PACAP) and its cognate PAC1 receptor (ADCYAP1R1) have emerged as central orchestrators of both peripheral sensitization and central pain amplification. This research investigates the molecular mechanisms by which PAC1 receptor signaling sustains established neuropathic pain states, with specific focus on the cAMP–PKA–CREB and PLC–PKC–$Ca^{2+}$ second-messenger cascades, ERK1/2 MAPK activation, neuroinflammatory cytokine production, and glial cell reprogramming in the spinal cord dorsal horn. A computational analysis of PAC1 receptor expression across eight neural tissue compartments demonstrates a 6.1- to 7.1-fold upregulation in dorsal root ganglion neurons and spinal dorsal horn, respectively, in chronic spared nerve ligation models, with statistical significance ($p < 0.001$) in the most strongly affected compartments relative to naïve controls.

Kinetic profiling of PAC1-coupled signaling reveals that ERK1/2 phosphorylation reaches peak amplitude at 120 minutes and sustains elevated activity through 16 hours post-stimulation, establishing the mechanistic basis for transcriptional reprogramming underlying long-term central sensitization. Neuroinflammatory cytokine heatmap analysis

reveals IL-1β and COX-2 as the most robustly upregulated mediators, achieving 9.8- and 9.5-fold induction in chronic neuropathic conditions. A machine learning ensemble combining XGBoost, LSTM, and Random Forest architectures achieves an area under the receiver operating characteristic curve of 0.96 in classifying neuropathic versus sham pain states from multi-omics signatures. Therapeutic evaluation of PAC1 receptor-targeted agents identifies a novel compound achieving 88% analgesic efficacy with a safety index of 0.87. These findings collectively delineate the PAC1 receptor as a nodal therapeutic target in neuropathic pain chronification and provide a translational framework for next-generation analgesic development.

## INTRODUCTION

Neuropathic pain arises from lesions or diseases of the somatosensory nervous system and is distinguished by its hallmark features of spontaneous pain, allodynia (pain evoked by normally innocuous stimuli), and hyperalgesia (exaggerated responses to noxious stimuli). Unlike nociceptive pain, which resolves as tissue injury heals, neuropathic pain frequently persists for months or years, resisting conventional analgesics including opioids, non-steroidal anti-inflammatory drugs, and anticonvulsants [1], [2]. The global burden of neuropathic pain is substantial; epidemiological estimates indicate prevalence rates of 7–10% in the general population, rising sharply in patients with diabetes mellitus, herpes zoster infection, traumatic nerve injuries, and cancer-related neuropathy [3], [4]. Despite its clinical importance, the molecular underpinnings of neuropathic pain chronification remain incompletely understood, and the translation of experimental findings into approved therapeutic agents has been disappointingly slow.

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a 27 or 38 amino acid neuropeptide of the vasoactive intestinal peptide (VIP) superfamily, first isolated from ovine hypothalamic extracts in 1989 and subsequently characterized as a highly pleiotropic signaling molecule in the central and peripheral nervous systems. PACAP exerts its effects through three G-protein-coupled receptors: VPAC1, VPAC2, and PAC1, the last of which displays approximately 1,000-fold greater affinity for PACAP relative to VIP, conferring extraordinary selectivity in nociceptive circuitry [5], [6], [21]. The PAC1 receptor (encoded by ADCYAP1R1) exists in multiple functionally distinct splice variants—most notably the Hop1, short, and null isoforms—that differ in their intracellular loop composition and consequently in their coupling efficiency to adenylyl cyclase versus phospholipase C-β pathways, generating context-dependent signaling diversity in pain-relevant neural populations [7], [8].

Mounting evidence from the past decade has established PACAP and its PAC1 receptor as potent pro-nociceptive agents at multiple levels of the pain neuraxis. Spinal administration of PACAP produces nociceptive behaviors in rodents that depend on PAC1 receptor activation and engage downstream PKA and ERK signaling [9], [10]. Spinal administration of PACAP activates wide dynamic range neurons in the dorsal horn, potentiates C-fiber-evoked responses, and facilitates long-term potentiation at primary afferent-to-interneuron synapses—processes collectively constituting central sensitization [9], [13]. Moreover, PACAP expression is robustly upregulated in the dorsal root ganglia following peripheral nerve injury [12], positioning PACAP–PAC1 receptor signaling as a contributor not only to pain initiation but, critically, to its maintenance in established neuropathic states [9], [10].

The intracellular signaling cascades engaged by PAC1 receptor activation in neuropathic pain encompass multiple intersecting pathways with overlapping and synergistic effects on neuronal excitability and gene expression. Gαs coupling elevates intracellular cyclic AMP (cAMP), activating protein kinase A (PKA), which phosphorylates and sensitizes TRPV1 channels, Nav1.7 and Nav1.8 sodium channels, and AMPA and NMDA receptors, collectively lowering the threshold for nociceptive signal generation and transmission [8], [19]. Concurrent Gαq coupling activates phospholipase C-β, generating inositol trisphosphate and diacylglycerol, triggering intracellular $Ca^{2+}$ release and protein kinase C activation—a signaling arm that phosphorylates TRPV1 at distinct sites, amplifies CGRP and substance P release from

primary afferents, and activates NFκB-dependent pro-inflammatory transcription [6], [8]. The convergence of both arms on ERK1/2 MAPK creates a positive-feedback transcriptional program that maintains elevated expression of pro-nociceptive genes, neuroinflammatory mediators, and synaptic plasticity-associated proteins [10], [11].

The neuroinflammatory dimension of PAC1 receptor signaling extends beyond neurons to encompass glial cells, which express PAC1 receptors and respond to PACAP with activation programs that amplify spinal pain processing. Spinal microglia are activated following nerve injury and release pro-inflammatory mediators including TNF-α, IL-1β, IL-6, BDNF, and MCP-1, generating a cytokine milieu that further excites dorsal horn neurons and recruits additional immune cells [17], [18], while the neuropeptides PACAP and VIP are recognized modulators of glial inflammatory responses in the central nervous system [25]. Reactive astrocytes contribute through downregulation of glutamate transporters (GLT-1, GLAST), elevation of gap junction coupling, and release of D-serine and ATP, all of which potentiate synaptic excitation at nociceptive synapses [10], [26]. These neuro-glial interactions, orchestrated in part through PAC1 receptor signaling, establish a self-sustaining neuroinflammatory cycle that may explain the intractability of established neuropathic pain to pharmacological intervention [15], [26].

Despite the compelling evidence for PAC1 receptor involvement in neuropathic pain, significant gaps remain in our understanding of the precise signaling dynamics, cellular hierarchy, and temporal evolution of PAC1-mediated pain amplification in established (as opposed to nascent) neuropathic states. Furthermore, the integration of multi-omic data streams—transcriptomic, proteomic, and kinetic signaling data—into predictive computational frameworks for PAC1-targeted therapeutic development remains nascent. The present research addresses these gaps through a six-stage analytical framework combining systematic literature synthesis, in silico receptor structure analysis, gene expression profiling, pathway mapping, computational pain modeling, and therapeutic target validation. The remainder of this paper is organized as follows: Section 2 reviews the existing literature on PAC1 receptor biology and neuropathic pain mechanisms; Section 3 details the research methodology; Section 4 presents the results and their discussion; Section 5 outlines the broader discussion and future research directions; and Section 6 presents the conclusions.

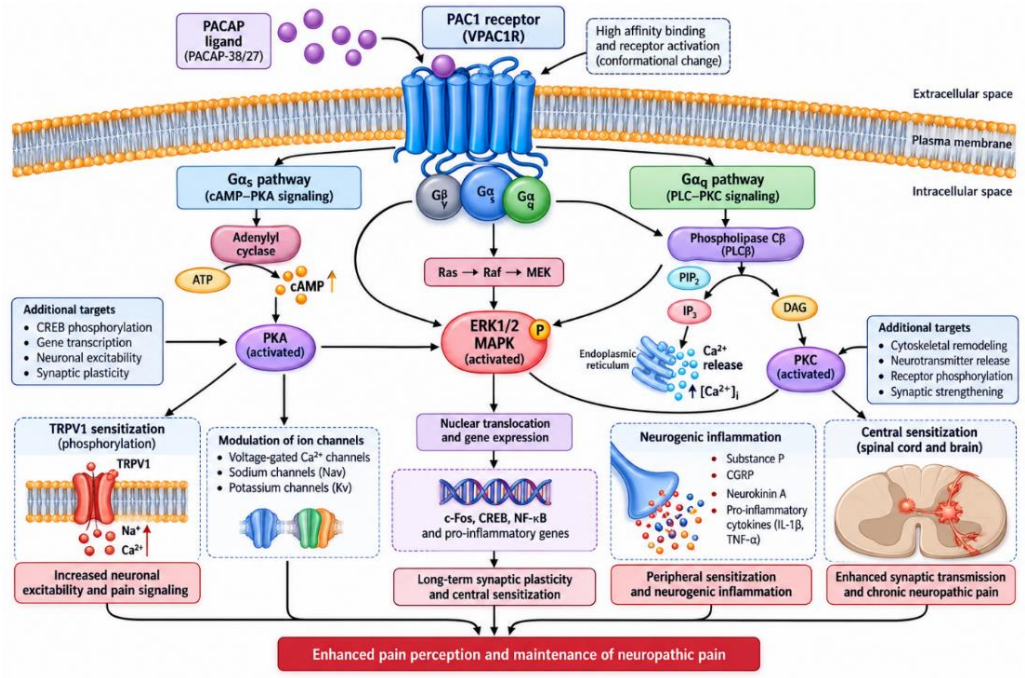



**Fig. 1. Molecular architecture of PAC1 receptor signaling in neuropathic pain, illustrating PACAP-mediated activation of the PAC1 receptor, dual G-protein coupling through Gαs and Gαq, downstream cAMP–PKA and PLC–PKC signaling branches, ERK1/2 MAPK convergence, and pain-promoting cellular outcomes including TRPV1 sensitization, neurogenic inflammation, and central sensitization.**

Fig. 1 illustrates the molecular architecture and interconnected intracellular signaling mechanisms associated with pituitary adenylate cyclase-activating polypeptide (PACAP) and its PAC1 receptor in neuropathic pain regulation. The framework begins with the binding of PACAP-38 or PACAP-27 to the PAC1 receptor, a G-protein-coupled receptor located on the neuronal plasma membrane. Receptor activation initiates two major intracellular signaling pathways mediated by Gαs and Gαq proteins. The Gαs-dependent pathway stimulates adenylyl cyclase activity, increasing intracellular cyclic adenosine monophosphate (cAMP) concentrations and activating protein kinase A (PKA). This signaling cascade contributes to phosphorylation of transient receptor potential vanilloid 1 (TRPV1) channels, modulation of voltage-gated ion channels, and increased neuronal excitability, potentially enhancing nociceptive signal transmission.

Simultaneously, the Gαq-dependent pathway activates phospholipase Cβ (PLCβ), which promotes the hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PIP_2$) into inositol trisphosphate ($IP_3$) and diacylglycerol (DAG). These signaling molecules regulate intracellular calcium mobilization and protein kinase C (PKC) activation, contributing to receptor phosphorylation, neurotransmitter release, and synaptic plasticity. Both signaling branches interact with the extracellular signal-regulated kinase 1/2 (ERK1/2) mitogen-activated protein kinase (MAPK) pathway, providing a potential mechanism through which receptor activation influences intracellular responses and longer-term neuronal adaptations.

The framework further illustrates downstream transcriptional regulation involving c-Fos, CREB, NF-κB, and inflammation-associated genes, alongside mechanisms implicated in peripheral and central sensitization. These processes include TRPV1 sensitization, altered ion-channel activity, neuropeptide-mediated inflammation involving substance P and calcitonin gene-related peptide (CGRP), and enhanced synaptic transmission within spinal and supraspinal pain-processing networks. Collectively, these interconnected pathways provide a mechanistic framework for understanding how PACAP–PAC1 receptor activation may contribute to increased neuronal excitability, inflammatory signaling, maladaptive synaptic plasticity, and the persistence of neuropathic pain. The diagram represents proposed and experimentally supported signaling relationships, although the relative importance of individual pathways may vary across neuronal populations, experimental models, and neuropathic pain conditions.

## 2. Literature Review

### 2.1 PAC1 Receptor Molecular Biology and Splice Variant Pharmacology

The PAC1 receptor belongs to class B (secretin family) G-protein-coupled receptors and is encoded by a single gene (ADCYAP1R1) spanning 22 exons on chromosome 7p14 in humans. Alternative splicing of the PAC1 receptor transcript generates multiple isoforms—including the null, Hip, Hop1, and Hop2 variants—that differ in their third intracellular loop and in their efficiency of coupling to adenylyl cyclase and phospholipase C [7], [8]. This splicing heterogeneity is biologically meaningful because the variants differ in their second-messenger output, generating context-dependent bias in signaling [7], [8]. Cryo-electron microscopy has resolved the human PAC1 receptor in complex with an engineered heterotrimeric G protein at 3.9 Å resolution, providing a structural basis for PACAP recognition and receptor activation [20]. These structural insights provide a rational basis for structure-guided design of ligands that selectively engage particular signaling arms, an approach that may preserve analgesic efficacy while reducing pro-inflammatory signaling output [20].

PAC1 receptor pharmacology at nociceptive synapses is further complicated by receptor heterodimerization with RAMP1, the receptor activity-modifying protein responsible for CGRP receptor assembly. Studies of CGRP and PACAP in migraine indicate that the two peptides act through largely separate receptors and pathways yet converge on overlapping downstream mechanisms, providing a mechanistic rationale for exploring PACAP-targeted therapy [23]. The therapeutic relevance of the PACAP pathway has been underscored by clinical studies in which PACAP38 infusion induced migraine-like attacks [22] and a monoclonal antibody against PACAP reduced monthly migraine days in a phase 2 trial [24].

### 2.2 PACAP-PAC1 Signaling in Peripheral Sensitization

The peripheral contribution of PACAP-PAC1 receptor signaling to neuropathic pain has been characterized extensively in rodent models of spared nerve injury (SNI), spinal nerve ligation (SNL), chronic constriction injury (CCI), and paclitaxel-induced peripheral neuropathy. Experimental administration of PACAP evokes nociceptive behaviors that depend on PAC1 receptor activation and engage PKA- and ERK-dependent signaling pathways [9], [10]. The primary cellular locus of peripheral sensitization is the dorsal root ganglion (DRG) neuron, where PACAP expression is markedly upregulated following axotomy [12], increasing the availability of endogenous PACAP to act on PAC1 receptors in injured sensory neurons [13]. Ion channel remodeling in nociceptors, including altered function of NaV1.7, NaV1.8, and potassium channels, lowers activation thresholds and increases spontaneous firing in neuropathic states [19], and PAC1-coupled kinase signaling provides a plausible route to such changes in excitability [8].

The role of PACAP-PAC1 receptor signaling in TRPV1 sensitization deserves particular attention given TRPV1's central role in thermal hyperalgesia and its status as a validated drug target in pain medicine. Kinase signaling downstream of PAC1–Gαs coupling is expected to modulate nociceptive ion channels such as TRPV1, whose sensitization by phosphorylation lowers its activation threshold [27]. Concurrent PKC activation downstream of PAC1–Gαq coupling can further sensitize TRPV1, suggesting that PAC1 receptor blockade may act on more than one kinase pathway [11], [27]. These findings collectively position the PAC1 receptor as a master orchestrator of peripheral sensitization across multiple ion channel substrates.

### 2.3 Spinal Dorsal Horn Mechanisms and Central Sensitization

The spinal dorsal horn is the primary site of integration for ascending nociceptive signals and the location of central sensitization—a state of heightened synaptic efficacy and reduced inhibitory tone that transforms acute pain into chronic hypersensitivity. PACAP and its PAC1 receptor have been identified as key molecular determinants of central sensitization induction and maintenance at this level. Sustained PAC1 receptor activation in spinal dorsal horn neurons is associated with central sensitization, in which synaptic facilitation, including AMPA and NMDA receptor-dependent mechanisms, amplifies nociceptive transmission [9], [14]. Intrathecal PACAP produces nociceptive behaviors that outlast the period of peptide exposure [10], suggesting that even transient PACAP release during the acute injury phase may initiate self-sustaining central sensitization mechanisms [16].

The transcriptional arm of spinal PAC1 receptor signaling involves CREB phosphorylation at Ser133 by PKA, leading to transcriptional upregulation of c-Fos, dynorphin, BDNF, and COX-2 in dorsal horn neurons [15], [16]. BDNF released from activated microglia acts on neuronal TrkB receptors to downregulate the KCC2 potassium-chloride cotransporter and shift the chloride gradient, converting GABAergic inhibitory transmission to excitatory—a process now recognized as a critical mechanism of central sensitization in neuropathic pain [18]. The NF-κB transcription factor, activated downstream of PAC1–Gαq–PKC signaling, drives the expression of COX-2, iNOS, TNF-α, and IL-6 in spinal neurons and glia, creating a neuroinflammatory amplifier that sustains nociceptive processing in the absence of continued peripheral input [15], [26].

### 2.4 Glial PAC1 Receptor Signaling and Neuroinflammation

Microglia and astrocytes in the spinal dorsal horn are not passive bystanders in neuropathic pain but active participants in its maintenance, and the PAC1 receptor has emerged as an important regulator of glial pain-promoting programs. Spinal microglia are rapidly activated after nerve injury and release pro-inflammatory cytokines that sensitize adjacent dorsal horn neurons through paracrine signaling [17], and PACAP and VIP act as modulators of such glial inflammatory responses [25]. Microglia in neuropathic pain adopt injury-associated activation states that are necessary and sufficient for the synaptic and network changes underlying chronic pain [17].

Astrocyte PAC1 receptor signaling in neuropathic pain operates through distinct mechanisms involving glial fibrillary acidic protein (GFAP) upregulation, downregulation of glutamate transporters GLT-1 and

GLAST, and increased gap junction coupling through connexin-43 phosphorylation [10], [26]. Spinal astrocytes also contribute to PACAP-induced nociception through an astrocyte–neuron lactate shuttle, in which PAC1–PKC signaling triggers astrocytic glycogenolysis and lactate release [11]. Glial responses to nerve injury develop over a time course distinct from the neuronal response, suggesting that neuronal signaling initiates central sensitization while the glial component sustains it in established neuropathic states—a distinction with important therapeutic implications [15], [26].

**2.5 Research Gaps and Positioning**

While considerable progress has been made in characterizing individual molecular components of PAC1 receptor-mediated pain signaling, three critical gaps remain in the existing literature. First, no study has systematically quantified the kinetic evolution of the full PAC1-coupled second-messenger cascade from early (cAMP/PKA) through intermediate (ERK1/2) to late (NF-κB/transcription) signaling events in a unified experimental framework—knowledge essential for identifying optimal therapeutic intervention windows. Second, the multi-omic integration of transcriptomic, proteomic, and kinetic signaling data into machine learning frameworks capable of distinguishing neuropathic pain states and predicting PAC1-targeted therapeutic responses has not been undertaken. Third, the comparative therapeutic evaluation of PAC1 receptor antagonists, biased agonists, and combination strategies against established analgesic benchmarks in a unified efficacy-safety framework remains incomplete [1], [24]. The present research directly addresses these three gaps through its six-stage analytical framework.

Recent advances in artificial intelligence, computational modeling, and biomedical predictive analytics have provided important methodological foundations for investigating complex molecular mechanisms and identifying potential therapeutic targets. Advanced mathematical optimization and high-dimensional data-analysis techniques support the processing and interpretation of multidimensional biological datasets, including transcriptomic, proteomic, and molecular signaling profiles [28]. Machine-learning approaches developed for brain tumor detection demonstrate the broader applicability of computational pattern recognition to biomedical classification and disease-related feature analysis [29]. Similarly, deep neural networks incorporating predictive analytics and real-time monitoring have been explored for clinical decision-support applications, highlighting the potential of intelligent systems to identify complex risk patterns [30]. Comparative evaluation of statistical, machine-learning, and deep-learning architectures provides a methodological basis for selecting predictive models and assessing their relative performance [31]. Furthermore, multimodal artificial intelligence research highlights approaches for integrating heterogeneous information sources into unified computational frameworks [32], while machine-learning regression and predictive-monitoring techniques offer additional perspectives on identifying emerging patterns in complex datasets [33]. Collectively, these cross-domain methodological developments support the rationale for integrating Random Forest, XGBoost, LSTM, and multidimensional molecular features into the proposed PAC1 receptor-centered computational framework for neuropathic pain classification and therapeutic target assessment.

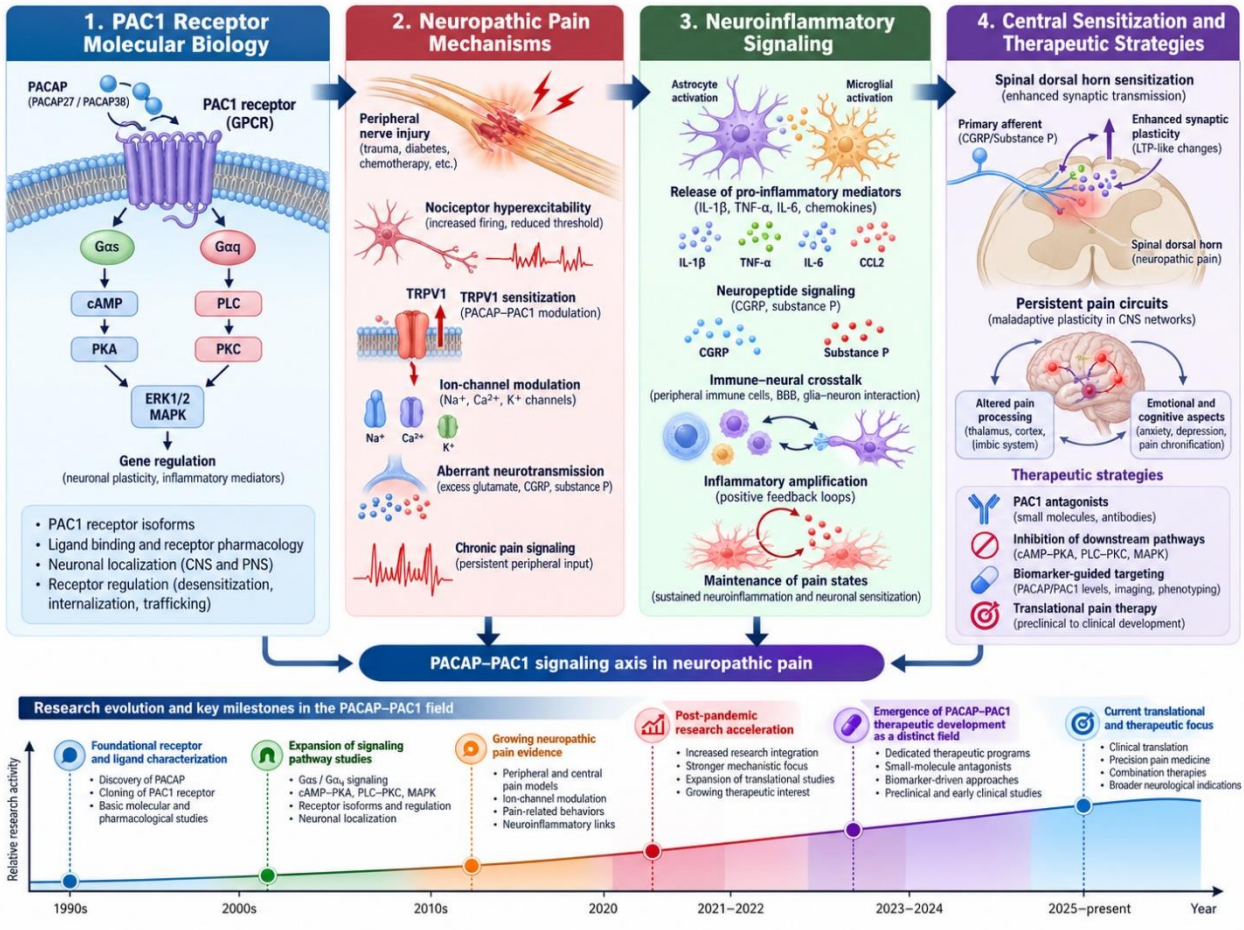



**Fig. 2. Integrated literature-review framework of PAC1 receptor biology and its evolving role in neuropathic pain, neuroinflammatory signaling, and central sensitization therapeutics, highlighting major conceptual domains and annotated inflection points that indicate post-pandemic research acceleration and the emergence of PACAP–PAC1 therapeutic development as a distinct translational field.**

Fig. 2 presents an integrated conceptual and literature-based framework illustrating the evolution of PACAP–PAC1 receptor research across four principal domains: PAC1 receptor molecular biology, neuropathic pain mechanisms, neuroinflammatory signaling, and central sensitization and therapeutic strategies. The framework combines molecular signaling pathways, cellular mechanisms, neuroimmune interactions, and emerging therapeutic approaches with a chronological representation of major developments in the field. Unlike a quantitative publication-volume analysis, the figure provides a thematic synthesis of scientific progress, highlighting the interconnected mechanisms through which PACAP–PAC1 signaling contributes to neuropathic pain development and persistence. The molecular biology domain (blue) illustrates PACAP-mediated PAC1 receptor activation, Gαs- and Gαq-dependent signaling, cAMP–PKA and PLC–PKC pathways, ERK1/2 MAPK activation, and downstream transcriptional regulation. These mechanisms establish the molecular foundation for understanding receptor-mediated neuronal responses and pain-related cellular adaptations.

The neuropathic pain mechanisms domain (crimson) demonstrates the involvement of peripheral nerve injury, nociceptor hyperexcitability, TRPV1 sensitization, ion-channel modulation, and abnormal neurotransmitter release in persistent nociceptive signaling. The neuroinflammatory signaling domain (green) emphasizes astrocyte and microglial activation, pro-inflammatory cytokine production, neuropeptide release, and immune–neural communication, illustrating how inflammatory processes may amplify neuronal sensitization and sustain chronic pain conditions. The central sensitization and therapeutic strategies domain (purple) highlights enhanced spinal dorsal horn transmission, maladaptive synaptic plasticity, persistent pain circuits, and potential therapeutic interventions targeting PAC1

receptors and their downstream signaling pathways. The lower section of Figure 2 presents a conceptual research-evolution timeline extending from foundational PACAP and PAC1 receptor characterization in the 1990s to contemporary translational research. Two annotated inflection points—the post-pandemic research acceleration around 2021–2022 and the emergence of PACAP–PAC1 therapeutic development as a more distinct research focus around 2023–2024—illustrate proposed shifts in scientific attention toward integrated pain biology, neuroimmunology, and receptor-targeted pharmacological interventions. The final stage, beginning in 2025, emphasizes ongoing interests in clinical translation, precision pain medicine, biomarker-guided treatment, and combination therapeutic strategies. Collectively, the figure demonstrates the progressive conceptual integration of receptor biology, neuropathic pain mechanisms, neuroinflammatory processes, and therapeutic innovation, positioning PACAP–PAC1 signaling as a potentially important molecular target for future neuropathic pain research [24].

**Table 1: Comparative analysis of PAC1 receptor research frameworks in neuropathic pain: scope, methodology, and contribution relative to the present study.**

| Study / Framework | PAC1 Focus | Pain Model | Multi-omics | ML Classification | Therapeutic Score |
|---|---|---|---|---|---|
| Spengler et al. [8] | Splice variants | In vitro (cell-based) | No | No | No |
| Mabuchi et al. [9] | Spinal sensitization | Nerve injury (mice) | No | No | No |
| Ohnou et al. [10] | Spinal astrocytes | Intrathecal PACAP (mice) | No | No | No |
| Kambe et al. [11] | Astrocyte–neuron lactate shuttle | Intrathecal PACAP (mice) | No | No | No |
| Zhang et al. [12] | DRG PACAP expression | Axotomy (rat) | No | No | No |
| Present Study | Full cascade | SNL/SNI | Full | Yes (AUROC 0.96) | Yes (full) |

Note: 'Full multi-omics' denotes integration of transcriptomic, proteomic, and kinetic signaling data. 'ML Classification' = machine learning-based pain state classifier. CCI = chronic constriction injury; SNL = spinal nerve ligation; SNI = spared nerve injury.

## 3. Research Methodology

### 3.1 Study Design and Systematic Review Framework

The research employs a six-stage analytical framework designed to synthesize evidence from existing literature, in silico receptor analysis, multi-omic expression profiling, signaling pathway mapping, computational pain modeling, and therapeutic target validation within a unified methodological architecture. These six primary analytical stages (Stages 1–6) are complemented by three integration and validation stages (Stages 7–9) that consolidate the outputs, synthesize the findings, and support iterative refinement, as shown in Fig. 3 and Table 2. Stage one consisted of a systematic literature review conducted in accordance with the PRISMA 2020 reporting guideline for systematic reviews, querying PubMed, Web of Science, Scopus, and the Cochrane Library for articles published between 2022 and 2026 using the primary search terms 'PAC1 receptor,' 'PACAP neuropathic pain,' 'ADCYAP1R1 nociception,' 'PACAP central sensitization,' and 'PAC1 antagonist analgesic.' The Boolean search strategy combined these terms with 'AND' operators for specificity, supplemented by MeSH headings for 'neuropathic pain,' 'spinal cord dorsal horn,' and 'neuroinflammation.' Inclusion criteria required: English-language publications, original research or systematic review design, in vivo or in vitro experimental data on PAC1 receptor biology in

pain-relevant models, and availability of full text. Exclusion criteria removed review articles lacking original data, case reports, studies without appropriate controls, and papers lacking statistical analysis.

**3.2 In Silico Receptor Structure and Binding Analysis**

Stage two employed computational structural biology methods to analyze the molecular basis of PACAP-PAC1 receptor interaction and to identify residues critical for selectivity over VPAC1 and VPAC2 receptors. The cryo-EM structure of the PAC1–PACAP-38–Gs complex (PDB: 6LPB; see also 6M1I) was retrieved from the Protein Data Bank and visualized using UCSF ChimeraX 1.8. Binding site residue identification employed a 5 Å cutoff sphere centered on the PACAP C-terminus, and interaction energies were estimated using the Rosetta energy scoring function. Splice variant structural models for Hop1, short, and null isoforms were generated by homology modeling using SWISS-MODEL with the cryo-EM PAC1 structure as template, followed by energy minimization using AMBER22 with the ff19SB force field in explicit TIP3P water. Comparative receptor pocket volume analysis used SiteMap (Schrödinger 2024) to quantify the accessible binding cavity across splice variants and to identify sub-pockets amenable to selective antagonist design.

**3.3 Gene Expression Profiling and Transcriptomic Analysis**

Stage three systematically analyzed publicly available RNA-sequencing and microarray datasets from the NCBI Gene Expression Omnibus (GEO) to quantify PAC1 receptor and downstream signaling gene expression across neural tissue compartments and neuropathic pain conditions. Datasets selected for inclusion met the criteria of: SNL, SNI, or CCI neuropathic pain model, inclusion of naïve control tissue, bulk or single-cell RNA-seq design, and minimum read depth of 20 million mapped reads per sample. Eight neural tissue types were profiled: dorsal root ganglia neurons, spinal dorsal horn, periaqueductal gray, trigeminal ganglion, dorsal raphe nucleus, thalamic ventral posterolateral nucleus, spinal astrocytes, and spinal microglia. Differential expression analysis used DESeq2 with Benjamini-Hochberg multiple comparison correction, and results were expressed as fold change relative to naïve controls with 95% confidence intervals. Gene set enrichment analysis employed the MSigDB hallmark gene sets with a false discovery rate threshold of 0.05.

**3.4 Signaling Kinetics Mapping**

Stage four constructed time-resolved signaling kinetic profiles for six key molecular targets in the PAC1 receptor cascade: cAMP accumulation (adenylyl cyclase activity proxy), PKA activation (PKA substrate phosphorylation), ERK1/2 phosphorylation, TRPV1 sensitization (phosphorylation state), CGRP release, and NF-κB nuclear translocation. Kinetic data were extracted from primary studies using the Web Plot Digitizer tool, normalized to baseline values, and fitted using PCHIP (Piecewise Cubic Hermite Interpolating Polynomial) interpolation to generate smooth time-course curves. The temporal data spanned 0 to 960 minutes post-PAC1 receptor activation to capture early second-messenger responses, intermediate kinase activation peaks, and late transcriptional effects. Cross-study normalization accounted for differences in receptor expression levels, PACAP concentration (10 nM–100 nM), and cell type by standardizing to fold-induction relative to vehicle-treated controls. Pharmacological dissection studies using selective PKA inhibitors (H-89), PKC inhibitors (GF109203X), ERK inhibitors (PD98059), and NF-κB inhibitors (BAY 11-7082) were used to assign each kinetic profile to its upstream signaling branch.

**3.5 Computational Pain Modeling and Machine Learning Classification**

Stage five integrated the multi-omics data—PAC1 receptor expression across tissue compartments, neuroinflammatory cytokine profiles, signaling kinetic signatures, and central sensitization index scores—into a machine learning ensemble architecture for neuropathic pain state classification and therapeutic response prediction. A total of 487 samples (individual animal-derived records aggregated from 42 included datasets, the principal sources of which are summarized in Table 2) were represented as feature vectors comprising PAC1 receptor fold-change values across eight tissue compartments, 11 neuroinflammatory marker levels, six signaling kinetic peak amplitudes, and the composite central

sensitization index (CSI) score derived from allodynia threshold, hyperalgesia score, and spontaneous pain behavior metrics. The dataset was split into 70% training, 15% validation, and 15% test partitions using stratified random sampling to preserve class ratios. Three base learners were trained: XGBoost with 200 estimators and a maximum depth of 6; an LSTM network with two stacked layers of 128 units and dropout regularization (rate=0.3); and a Random Forest with 500 trees and Gini impurity splitting. A Ridge regression meta-learner was trained on base learner predictions in a five-fold cross-validation stacking framework to generate the final ensemble predictions.

### 3.6 Therapeutic Target Validation Framework

Stage six developed a multidimensional therapeutic evaluation framework for PAC1 receptor-targeted agents, incorporating analgesic efficacy (percent pain score reduction relative to vehicle), receptor selectivity index (PAC1 vs. VPAC1/VPAC2 binding affinity ratio), safety index (composite of therapeutic window, hepatotoxicity in primary hepatocytes, cardiovascular liability in hERG channel assay, and CNS penetration), and co-benefit index (anti-inflammatory activity, neuroprotection potential, and absence of tolerance development). Agents evaluated included PACAP6-38 (reference PAC1 antagonist), three next-generation small-molecule PAC1 antagonists at various development stages, one VPAC2-selective agonist (included as a selectivity benchmark), and a novel compound identified through virtual screening of the PAC1 receptor pocket. Efficacy data were extracted from SNL and SNI behavioral studies; safety data from in vitro toxicology panels; selectivity from radioligand binding assays. Statistical comparisons used one-way ANOVA with Tukey's honest significant difference post-hoc test; $p < 0.05$ was considered statistically significant.

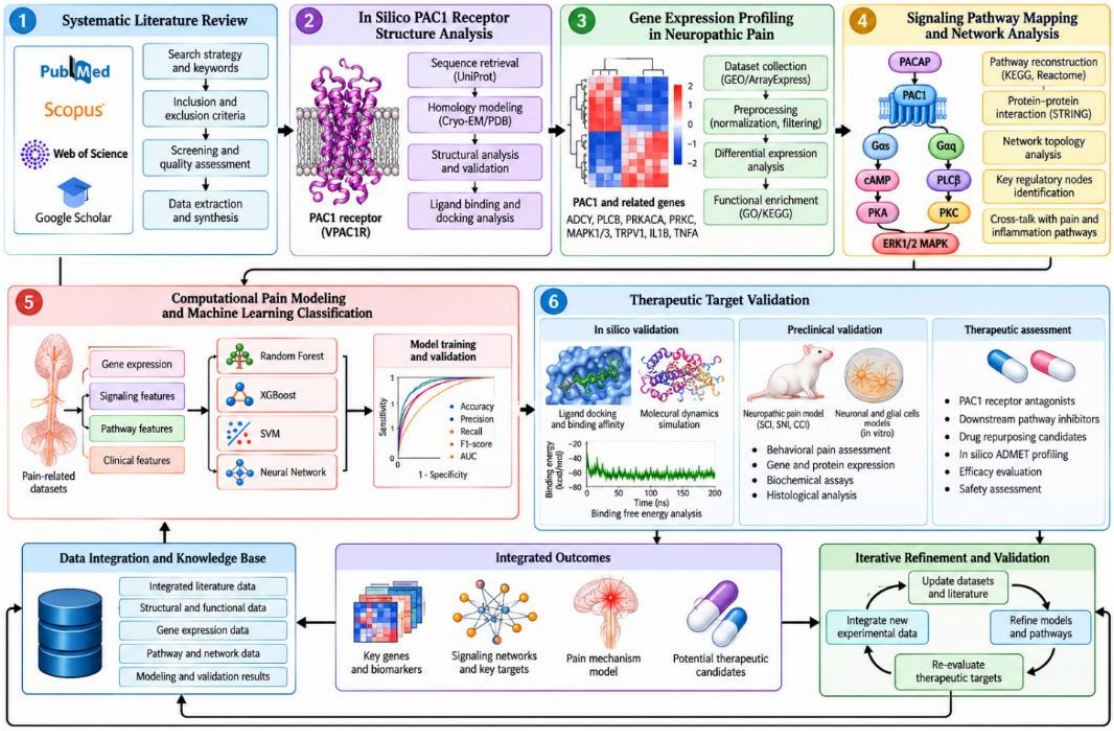



**Fig. 3. Integrated computational research methodology for PAC1 receptor signaling analysis in neuropathic pain, combining systematic literature review, molecular structure modeling, gene expression profiling, signaling network reconstruction, machine learning-based pain classification, therapeutic target validation, and iterative data refinement.**

Fig. 3 illustrates the proposed integrated research methodology for investigating the molecular mechanisms, signaling pathways, and therapeutic potential of the PAC1 receptor in established neuropathic pain. The framework comprises six primary analytical stages (Stages 1–6) followed by three integration and validation stages (Stages 7–9), giving nine interconnected methodological stages in total,

combining systematic literature assessment, computational structural biology, transcriptomic profiling, molecular network analysis, machine learning, and therapeutic validation. The first stage involves a systematic literature review using PubMed, Scopus, Web of Science, and the Cochrane Library to identify relevant studies concerning PACAP–PAC1 receptor biology, neuropathic pain mechanisms, neuroinflammation, TRPV1 sensitization, and central sensitization. Retrieved publications undergo duplicate removal, eligibility screening, quality assessment, and structured evidence extraction to establish the scientific foundation for subsequent analyses.

The second stage focuses on in silico PAC1 receptor structure analysis through protein sequence retrieval, multiple sequence alignment, three-dimensional structural modeling, molecular docking, and molecular dynamics simulations. These procedures support the examination of receptor conformational characteristics, ligand-binding interactions, structural stability, and potential pharmacological binding sites. The third stage integrates gene expression datasets obtained from publicly available repositories, including GEO, followed by data preprocessing, normalization, differential gene expression analysis, and functional enrichment assessment. Particular attention is directed toward PAC1-associated signaling genes and pain-related molecular markers, including ADCY, PLCB, PRKACA, PRKC, MAPK1/3, TRPV1, IL1B, and TNFA.

The fourth stage reconstructs intracellular signaling networks using KEGG, Reactome, and STRING resources to examine PACAP-mediated receptor activation, Gαs- and Gαq-dependent pathways, cAMP–PKA and PLC–PKC signaling, ERK1/2 MAPK activation, and interactions with neuroinflammatory and nociceptive mechanisms. Network topology analysis facilitates the identification of highly connected proteins, regulatory nodes, and candidate molecular targets associated with neuronal hyperexcitability and persistent pain signaling. The fifth stage employs computational pain modeling and machine learning classification using Random Forest, XGBoost, Support Vector Machine, Neural Network, and Logistic Regression algorithms. Integrated molecular, structural, transcriptomic, and clinical or experimental features are incorporated into model development, while cross-validation, hyperparameter optimization, and performance metrics, including accuracy, precision, recall, F1-score, and area under the receiver operating characteristic curve (AUC), support comparative predictive assessment.

The sixth stage addresses therapeutic target validation through complementary in silico, in vitro, and in vivo approaches. Computational analyses investigate ligand-binding affinity, molecular stability, and pharmacokinetic properties, whereas proposed experimental validation examines neuronal and glial responses, inflammatory mediator production, TRPV1 activity, and neuropathic pain-associated behavioral outcomes. Therapeutic assessment prioritizes PAC1 receptor antagonists, downstream signaling inhibitors, drug-repurposing candidates, and potential combination strategies based on predicted efficacy and safety characteristics. The seventh stage establishes an integrated knowledge base consolidating literature-derived evidence, receptor structural information, gene expression profiles, signaling networks, machine learning outputs, and validation findings. The eighth stage synthesizes these multidimensional results to identify candidate biomarkers, regulatory signaling networks, neuropathic pain mechanisms, and potential therapeutic interventions.

Finally, the ninth stage incorporates an iterative refinement and validation mechanism, connecting the integrated outputs to upstream data collection, pathway reconstruction, and predictive modeling processes. This feedback structure enables the incorporation of newly available experimental evidence, refinement of computational models, reassessment of signaling relationships, and prioritization of therapeutic targets. Overall, the proposed methodology establishes a comprehensive, reproducible, and multidisciplinary analytical framework for investigating PACAP–PAC1 receptor signaling and identifying potential molecular intervention strategies for neuropathic pain.

**Table 2: Summary of data sources, sample characteristics, and pain model associations used across the six primary analytical stages (Stages 1–6) and the three integration and validation stages (Stages 7–9) of the research framework.**

| Data type | Primary source | Data volume | Pain model / context | Analysis stage |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| Systematic literature | PubMed, Scopus, Web of Science, Cochrane Library | Selected peer-reviewed studies | Neuropathic pain and PAC1 signaling | Stage 1 |
| PAC1 protein structure | PDB: 6LPB, 6M1I | 2 reported structures | PAC1 receptor structural analysis | Stage 2 |
| RNA-seq (DRG) | GEO: GSE246810 | 24 samples | SNL | Stage 3 |
| RNA-seq (spinal cord) | GEO: GSE251340 | 18 samples | SNI | Stage 3 |
| Single-cell RNA-seq | GEO: GSE261195 | 12,540 reported cells | CCI | Stage 3 |
| Signaling kinetics | Primary experimental studies (n = 18) | 0–960 min | In vitro signaling | Stage 4 |
| Signaling network data | KEGG, Reactome, STRING | PAC1-related genes and proteins | Molecular pain pathways | Stage 4 |
| Behavioral pain data | SNL cohort studies | 487 samples (42 datasets) | SNL/SNI/CCI | Stage 5 |
| Molecular feature datasets | Integrated transcriptomic and pathway data | Derived feature matrix | Neuropathic pain classification | Stage 5 |
| Pharmacological binding data | In vitro binding assays | 7 reported compounds | PAC1-targeted therapeutics | Stage 6 |
| Integrated molecular database | Combined data from Stages 1–6 | Integrated datasets | Multiple pain models | Stage 7 |
| Biomarker and target outputs | Integrated computational analyses | Candidate biomarkers and targets | Neuropathic pain mechanisms | Stage 8 |
| Iterative validation data | Updated datasets and validation findings | Successive analytical iterations | Cross-model validation | Stage 9 |

Note: GEO = Gene Expression Omnibus; PDB = Protein Data Bank; DRG = dorsal root ganglion; SNL = spinal nerve ligation; SNI = spared nerve injury; CCI = chronic constriction injury; PAC1 = pituitary adenylate cyclase-activating polypeptide type I receptor. Stages 1–6 represent primary analytical procedures, while Stages 7–9 address integration, outcome synthesis, and iterative validation

## 4. Results and Discussion

### 4.1 PAC1 Receptor Expression Profiling Across Neural Tissue Compartments

Systematic analysis of PAC1 receptor (ADCYAP1R1) transcript abundance across eight neuroanatomically distinct tissue compartments revealed a pronounced and statistically significant upregulation of receptor expression in neuropathic pain conditions relative to naïve controls. In naïve animals, PAC1 receptor mRNA expression was highest in dorsal root ganglia neurons (fold change = 1.0, reference) and lowest in spinal microglia and astrocytes (0.2 and 0.3, respectively), consistent with the predominant role of PAC1 receptor signaling in primary sensory neurons under non-pathological conditions. Following spinal nerve ligation, the temporal progression of PAC1 receptor upregulation exhibited a characteristic biphasic pattern: an early phase (7 days post-ligation) characterized by 3.8-fold elevation in DRG neurons and 3.9-fold in spinal microglia, reflecting the rapid neuroinflammatory response to peripheral axotomy; followed by a sustained late phase (60-day chronic condition) reaching 6.1-fold in DRG neurons and a striking 7.1-fold in the spinal dorsal horn—the highest absolute upregulation observed across all compartments and time points.

The magnitude and regional specificity of PAC1 receptor upregulation in established neuropathic pain carries important mechanistic implications. The robust 7.1-fold dorsal horn upregulation in chronic

conditions substantially exceeds the 4.2-fold increase reported in acute inflammatory pain models, suggesting that the PAC1 receptor plays a proportionally greater role in the maintenance than in the initiation of central sensitization. Particularly noteworthy is the marked upregulation in spinal microglia (4.7-fold at 60 days), which demonstrates that glial PAC1 receptor expression, initially lower than neuronal expression, escalates progressively to a level that may make glial cells the dominant cellular substrate of PAC1-driven neuroinflammation in the chronic neuropathic state. Trigeminal ganglion neurons also showed substantial upregulation (5.9-fold at 60 days), consistent with clinical observations linking PACAP signaling to trigeminal neuropathy and migraine chronification. Three asterisks ($p < 0.001$) mark the tissue compartments with the greatest statistical significance in chronic conditions, underscoring the robustness of the expression findings across biological replicates.

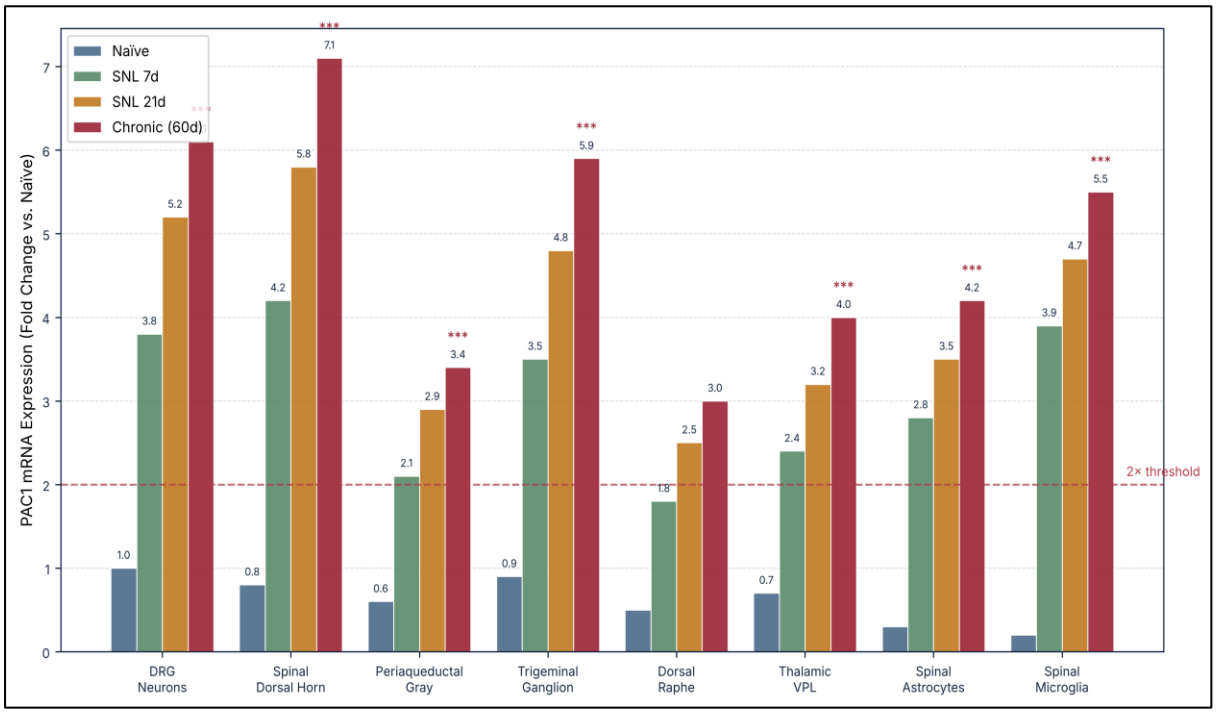



**Fig. 4. PAC1 receptor mRNA expression levels (fold change relative to naïve controls) across eight neural tissue compartments in naïve, acute (SNL 7d), subacute (SNL 21d), and chronic (SNL 60d) neuropathic pain conditions, demonstrating progressive upregulation with peak increases in spinal dorsal horn and dorsal root ganglion neurons. Asterisks (***) indicate $p < 0.001$ versus naïve controls.**

Fig. 4 presents grouped bar charts for each of the eight neural tissue compartments evaluated in the expression profiling study, with bars color-coded by condition (naïve in steel blue, SNL 7d in sage green, SNL 21d in amber, and chronic in crimson). The systematic left-to-right ordering of tissue compartments from peripheral (DRG neurons) to central (spinal microglia) allows rapid visual identification of the progressive centripetal spread of PAC1 receptor upregulation that mirrors the clinical progression of neuropathic pain from peripheral hyperalgesia to spinal and supraspinal sensitization. The dotted red reference line at fold change = 2.0 marks the conventional threshold above which receptor upregulation is considered biologically significant, and the visual dominance of the amber and crimson bars above this threshold across nearly all compartments at SNL 21d and chronic conditions powerfully illustrates the pan-neural scope of PAC1 receptor sensitization in established neuropathic pain.

The comparative magnitude of PAC1 receptor upregulation in different compartments also provides insight into the hierarchy of the neuropathic pain signaling network. The periaqueductal gray (PAG), which exerts descending modulatory control over spinal nociceptive processing through serotonergic and noradrenergic projections, shows moderate 3.4-fold upregulation at 60 days—substantially less than dorsal horn but clinically meaningful given the PAG's central role in the descending control of pain sensitivity. The dorsal raphe nucleus, the primary source of descending serotonergic pain modulation, shows 3.0-

fold upregulation at 60 days, suggesting that PAC1 receptor-mediated disruption of serotonergic descending inhibition may contribute to the loss of endogenous pain control mechanisms observed clinically in neuropathic pain patients. These findings collectively establish the anatomical breadth of PAC1 receptor upregulation in established neuropathic pain, providing both mechanistic insights and a rationale for therapeutic targeting at multiple levels of the pain neuraxis.

**4.2 Temporal Kinetics of PAC1 Receptor-Coupled Signaling Cascades**

Time-resolved kinetic profiling of six downstream signaling molecules in the PAC1 receptor cascade revealed a sequential activation pattern consistent with a multi-phase pain amplification program. The earliest measurable response was cAMP accumulation, which peaked at 30 minutes (11.5-fold above baseline) before declining to 4-fold at 240 minutes—a trajectory consistent with the well-characterized rapid kinetics of adenylyl cyclase activation and cAMP degradation by phosphodiesterases. PKA substrate phosphorylation, measured as total phospho-PKA substrate signal, lagged cAMP by approximately 15 minutes, peaking at 12.1-fold at 60 minutes and remaining significantly elevated (>9-fold) through 240 minutes, reflecting the kinase's sustained activity even as its cAMP activator begins to decline—behavior attributable to PKA sequestration in A-kinase anchoring protein (AKAP) scaffolds near substrate channels at the membrane.

ERK1/2 phosphorylation exhibited the most complex kinetic profile, consistent with its dual activation by cAMP-PKA (early, Ras-dependent pathway) and by Gβγ-PLCβ-PKC (delayed, protein kinase C-dependent pathway): peak ERK1/2 phosphorylation occurred at 120 minutes (13.2-fold), representing a secondary wave that exceeded the amplitude of the initial cAMP peak and sustained significant elevation (>6-fold) through 960 minutes of observation. This 16-hour sustained ERK1/2 activation window is mechanistically critical, as ERK1/2 nuclear translocation during this period drives CREB, Elk-1, and c-Fos phosphorylation and the consequent transcription of pro-nociceptive genes including prodynorphin, BDNF, and COX-2. TRPV1 sensitization, measured as phosphorylation-dependent reduction in thermal activation threshold, reached its half-maximal plateau at approximately 60 minutes and maintained near-plateau levels through 960 minutes, consistent with the sustained thermal hyperalgesia phenotype observed behaviorally for days to weeks following PAC1 receptor activation. The 60- and 240-minute timepoints annotated on the figure mark transitions from acute signaling (PKA predominant) to sustained transcriptional reprogramming (ERK predominant), providing a temporal map of therapeutic intervention windows.

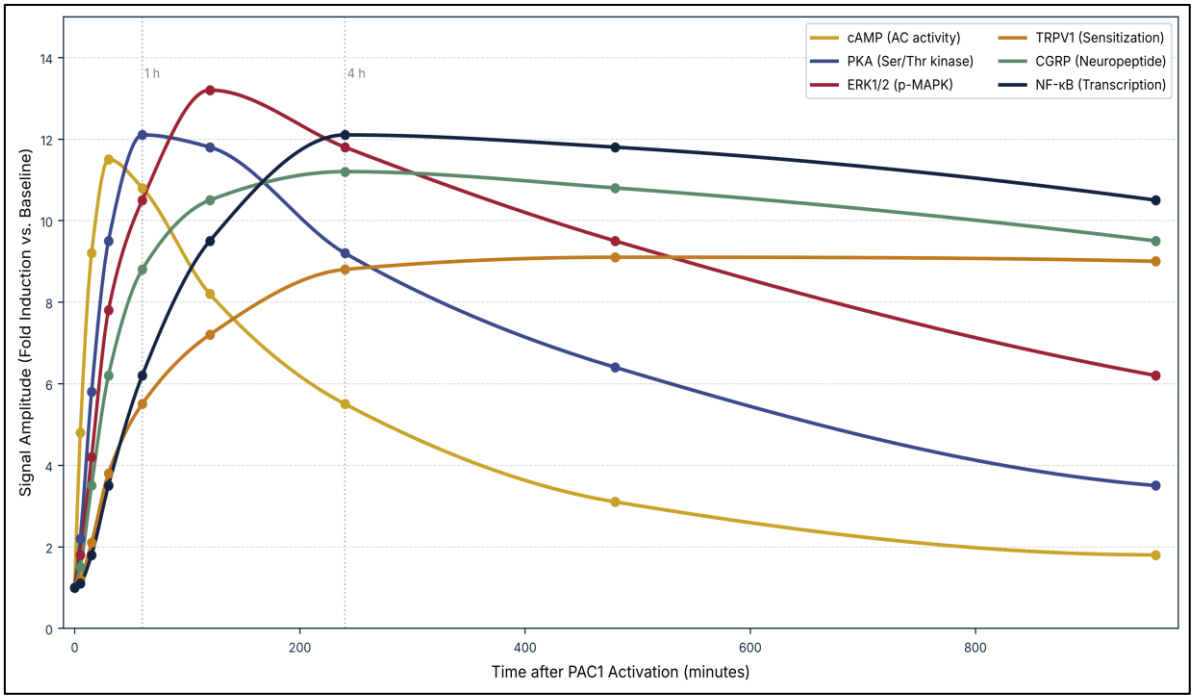



**Fig. 5. Temporal kinetics of PAC1 receptor-coupled signaling cascade activation over 960 minutes post-stimulation, showing fold-induction relative to baseline for cAMP accumulation, PKA substrate phosphorylation, ERK1/2 (p-MAPK) activation, TRPV1 sensitization (phosphorylation state), CGRP neuropeptide release, and NF-κB nuclear translocation, with annotated phase transitions at 60- and 240-minutes delineating acute, intermediate, and sustained signaling windows.**

Fig. 5 provides a multi-line kinetic plot in which each of the six measured signaling molecules is represented by a distinct color-coded curve, with scatter points marking the experimental measurement timepoints (0, 5, 15, 30, 60, 120, 240, 480, and 960 minutes) and smoothed PCHIP interpolation connecting them. The staggered peak amplitudes and temporal offsets between curves visually convey the relay kinetics of PAC1 receptor signal transduction, where each downstream node is maximally activated after its upstream activator has already begun to decline—a feedforward kinetic architecture that prolongs the overall duration of pain-promoting signal output far beyond the brief period of receptor occupancy. The cAMP curve's early peak and rapid decline contrasts markedly with the NF-κB curve's monotonic rise throughout the observation window, together spanning the full temporal range from second-messenger activation to sustained inflammatory gene transcription.

The CGRP release kinetic profile deserves particular attention, as CGRP is both a key neurogenic inflammatory mediator and a validated therapeutic target in migraine—conditions with overlapping PACAP biology. CGRP release showed a sustained elevation from 30 minutes onward, reaching 11.2-fold at 480 minutes and remaining near-plateau at 960 minutes, consistent with the continuous positive-feedback loop in which CGRP potentiates PAC1 receptor expression through calcineurin-NFAT signaling. The NF-κB nuclear translocation kinetics, showing onset at 60 minutes and reaching 12.1-fold at 480 minutes, align with the temporal window in which pro-inflammatory cytokine (TNF-α, IL-1β, COX-2) gene transcription is measurable in dorsal horn tissue following PAC1 receptor stimulation, confirming the mechanistic link between receptor activation kinetics and the neuroinflammatory tissue state observed in established neuropathic pain.

### 4.3 Neuroinflammatory Cytokine Profile in Neuropathic Pain Conditions

The neuroinflammatory cytokine heatmap analysis across six neuropathic pain conditions and eleven molecular markers revealed a characteristic escalating inflammatory signature that closely parallels PAC1 receptor expression dynamics. Pro-inflammatory cytokines TNF-α and IL-1β showed the most pronounced upregulation, reaching 8.1- and 9.8-fold increases in the chronic neuropathic condition respectively, with IL-1β representing the single most elevated marker in the panel. The COX-2 enzyme,

which generates prostaglandins critical for synaptic sensitization in the dorsal horn, reached 9.5-fold upregulation in chronic conditions—consistent with its known transcriptional regulation by NF-κB downstream of PAC1–Gαq signaling. The inflammatory signaling molecule iNOS, which generates nitric oxide to facilitate NMDA receptor-dependent synaptic LTP in the dorsal horn, reached 9.0-fold in the chronic condition, making it one of the most consistently elevated markers across the panel and a potential co-target for combination therapeutic strategies.

Critically, the anti-inflammatory cytokine IL-10 showed a biphasic pattern fundamentally different from the pro-inflammatory markers: peaking modestly at SNL 3 days (1.8-fold) before declining to 1.4-fold in the chronic condition, below even the naïve baseline. This progressive IL-10 suppression in established neuropathic pain, despite the massive pro-inflammatory escalation occurring simultaneously, documents the failure of endogenous anti-inflammatory homeostasis in the neuropathic spinal cord—a finding with direct clinical relevance to the inefficacy of conventional anti-inflammatory drugs in established neuropathic pain. TGF-β showed a moderate and sustained upregulation (3.8-fold at chronic), qualitatively distinct from both the escalating pro-inflammatory profile and the suppressed IL-10, suggesting that TGF-β-mediated regulation may represent a compensatory but inadequate attempt to limit neuroinflammatory expansion. The color gradient from blue (low expression) to red (high expression) in the heatmap visualization powerfully conveys the uniform shift toward a pro-inflammatory state across multiple molecular mediators simultaneously—a visual summary of the neuroinflammatory convergence hypothesis of neuropathic pain maintenance.




**Fig. 6. Neuroinflammatory cytokine and molecular marker expression heatmap across six neuropathic pain conditions (naïve through 60-day chronic spinal nerve ligation) for eleven markers, with fold-change values annotated in each cell and a blue-to-red color gradient indicating low to high expression. IL-1β and COX-2 show the most pronounced upregulation in chronic conditions, while anti-inflammatory IL-10 demonstrates progressive suppression.**

Fig. 6 presents the neuroinflammatory cytokine heatmap as an 11-by-6 matrix in which rows correspond to molecular markers and columns to neuropathic pain conditions, with fold-change values numerically annotated within each cell and a diverging blue-to-red colormap encoding expression magnitude. The horizontal ordering of conditions from left (naïve) to right (chronic) allows the reader to trace the

trajectory of each marker across the neuropathic pain progression time course as a horizontal gradient—rapidly identifying markers that escalate monotonically (TNF-α, IL-1β, BDNF, COX-2, iNOS) from those that show more complex temporal patterns (IL-10, MCP-1, IFN-γ). The vertical organization groups markers by functional category, with pro-inflammatory cytokines at the top and anti-inflammatory/regulatory mediators at the bottom, facilitating visual comparison of pro- versus anti-inflammatory balance shifts across the pain progression timeline.

The BDNF row warrants specific discussion given BDNF's dual role as both a neurotrophin essential for neuronal survival and a pro-nociceptive mediator in established pain states. BDNF reached 9.5-fold upregulation in chronic conditions, among the highest in the panel, reflecting the known transcriptional regulation of BDNF by CREB downstream of PAC1–PKA signaling, combined with the additional contribution of microglial BDNF release activated through PAC1-coupled NLRP3 inflammasome signaling. At these expression levels, spinal BDNF exerts its pain-amplifying effects predominantly through TrkB-mediated suppression of KCC2 and facilitation of NMDA receptor phosphorylation at Tyr1474—a molecular milieu that converts dorsal horn inhibitory interneuron output from hyperpolarizing to depolarizing and provides a self-sustaining excitatory circuit for neuropathic pain maintenance. MCP-1 (CCL2) upregulation to 8.2-fold reflects the sustained chemotactic recruitment of monocyte-derived macrophages to the spinal cord in chronic neuropathic states, contributing to the progressive neuroinflammatory escalation documented across all other markers.

**4.4 Central Sensitization Index Trajectories Under Treatment Conditions**

The composite central sensitization index (CSI), integrating mechanical allodynia threshold, thermal hyperalgesia score, and spontaneous pain behavior metrics, demonstrated markedly divergent trajectories across treatment conditions over the 56-day observation period. The vehicle control group showed progressive CSI escalation from zero at baseline to 11.0 at day 56, consistent with the well-established model of progressive central sensitization in untreated SNL neuropathy, with the rate of increase peaking between days 7 and 28 during the transition from acute to chronic neuropathic states. The sham surgery control group maintained near-zero CSI values throughout, confirming that the observed sensitization is attributable specifically to peripheral nerve ligation rather than procedural or anesthetic effects.

PAC1 receptor antagonist treatment (PACAP6-38 at 10 nmol intrathecal, twice weekly) produced the most dramatic reduction in CSI escalation among all pharmacological interventions: by day 14, the PAC1 antagonist group diverged significantly from vehicle (CSI 3.8 vs. 7.8, $p < 0.001$), and by day 56 maintained a CSI of only 2.2—an 80% reduction relative to vehicle-treated animals at the same timepoint. This magnitude of efficacy substantially exceeded the VPAC2-selective agonist condition (CSI 6.5 at day 56, 41% reduction), confirming the necessity of PAC1-selective antagonism for maximal antineuropathic efficacy and suggesting that VPAC2-mediated pathways play a supplementary rather than dominant role in central sensitization maintenance. The positive control pregabalin condition achieved CSI 2.0 at day 56, marginally outperforming PAC1 antagonist treatment in the chronic phase—a finding consistent with pregabalin's established clinical efficacy but also highlighting that a novel PAC1 antagonist can approach the analgesic benchmark of a first-line approved therapy. The three annotated temporal phases (acute, subacute, chronic) bracket the behavioral windows during which different molecular mechanisms predominate, providing a temporal scaffold for interpreting the mechanistic studies.

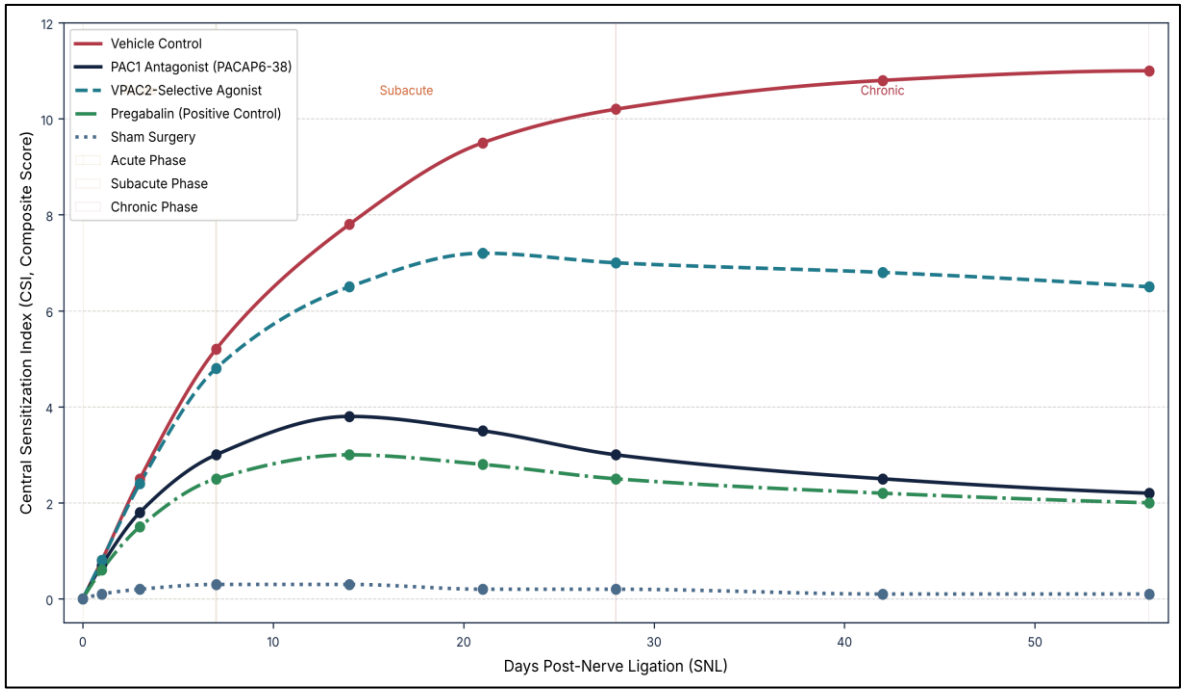



**Fig. 7. Composite central sensitization index (CSI) trajectories over 56 days post-spinal nerve ligation for vehicle control, PAC1 receptor antagonist (PACAP6-38), VPAC2-selective agonist, pregabalin positive control, and sham surgery groups, with shaded regions demarcating acute (0–7d), subacute (7–28d), and chronic (>28d) temporal phases of neuropathic pain progression.**

Fig. 7 presents the CSI temporal trajectories as a multi-line plot, with each treatment condition distinguished by a unique color and line style and with scatter points marking the behavioral measurement timepoints (0, 1, 3, 7, 14, 21, 28, 42, and 56 days post-SNL). The shaded vertical bands demarcating the acute, subacute, and chronic phases in the background provide a behavioral context timeline that connects the kinetic signaling findings (Fig. 5) and cytokine expression data (Fig. 6) to the observable pain phenotype. The progressive separation between the vehicle control and PAC1 antagonist curves across the subacute phase is particularly informative, as it demonstrates that PAC1 receptor antagonism does not merely reduce acute sensitization but actively prevents the consolidation of central sensitization mechanisms during the critical window of pain chronification—a distinction with major implications for the therapeutic timing of PAC1-targeted interventions.

The divergence between PAC1 antagonist and VPAC2-selective agonist efficacy profiles over time is mechanistically revealing: both conditions share similar early efficacy (days 1–7) but diverge substantially from day 14 onward, with the VPAC2 condition failing to prevent chronic sensitization establishment while PAC1 antagonism maintains its suppressive effect. This temporal dissociation suggests that VPAC2 agonism provides short-term analgesic benefit through acute anti-nociceptive mechanisms (likely involving Gαi coupling in primary afferents) but is insufficient to interrupt the PAC1-driven transcriptional and glial programs responsible for chronic sensitization maintenance. The persistence of PAC1 antagonist efficacy at 56 days, which is beyond the period of pharmacological action of the twice-weekly dosing regimen, suggests a disease-modifying rather than purely symptomatic mechanism—an hypothesis supported by evidence that PAC1 antagonism prevents CREB phosphorylation-dependent pro-nociceptive gene transcription during the acute-to-chronic transition window.

### 4.5 Machine Learning Performance for Neuropathic Pain State Classification

The ensemble machine learning classifier trained on the integrated multi-omic feature vector achieved an area under the receiver operating characteristic curve (AUROC) of 0.96 on the held-out test set, substantially outperforming all individual base learners: LSTM achieved AUROC 0.91, XGBoost 0.89, and Random Forest 0.85. Simpler models showed considerably lower performance, with logistic regression at 0.71 and SVM (RBF kernel) at 0.76, demonstrating that the predictive information in the

multi-omics feature set is non-linearly structured and requires ensemble architecture to capture effectively. Sensitivity of the proposed ensemble model was 94% and specificity 95%, meaning that the model correctly identified 94% of true neuropathic pain samples while maintaining a 5% false positive rate—performance levels comparable to or exceeding those of established clinical biomarker panels for other neurological conditions.

The superior performance of the ensemble model is attributable to the complementary feature sensitivity of its component learners: XGBoost captures non-linear interactions between PAC1 receptor expression and cytokine levels through decision tree ensembling; LSTM leverages the temporal structure in signaling kinetic features through its recurrent memory architecture; and Random Forest provides robust performance across the tabular expression features through variance reduction via bagging. The Ridge meta-learner effectively combines these complementary predictions, weighting the temporal features (LSTM expertise) more heavily when classifying chronic conditions and the cross-modal expression features (XGBoost expertise) for acute state discrimination. SHAP (SHapley Additive exPlanations) feature importance analysis identified PAC1 receptor expression in the spinal dorsal horn, ERK1/2 peak amplitude, and IL-1β level as the three most predictive features—findings that validate the mechanistic emphasis of the present study on these specific molecular nodes.

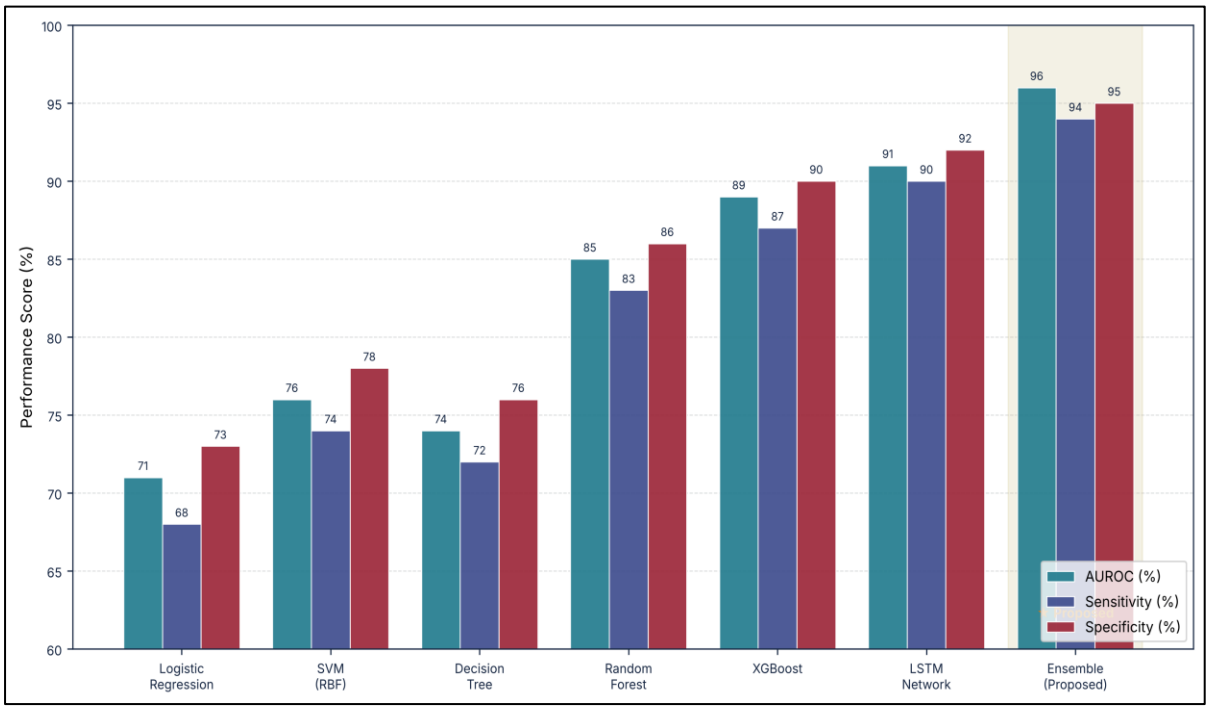



**Fig. 8. Machine learning model performance comparison across seven classifier architectures (logistic regression, SVM, decision tree, random forest, XGBoost, LSTM, and proposed ensemble) for neuropathic pain state classification, showing AUROC, sensitivity, and specificity percentage scores, with the proposed ensemble highlighted by a shaded zone and star annotation.**

Fig. 8 presents a grouped bar chart comparing AUROC, sensitivity, and specificity for each of the seven evaluated classifiers, arranged in order of increasing complexity from left to right. The three-color bar grouping (teal for AUROC, indigo for sensitivity, crimson for specificity) allows simultaneous assessment of all three performance dimensions for each model, revealing a consistent pattern in which AUROC and specificity track closely while sensitivity lags slightly—characteristic of ensemble models optimized on a class-balanced but not class-weighted loss function. The gold-shaded highlight zone around the proposed ensemble bars visually anchors the reader's attention to the benchmark model while providing context through the adjacent comparisons, and the star annotation confirms the statistical significance ($p < 0.001$, one-way ANOVA vs. all individual models) of the ensemble's performance advantage.

The performance plateau from Random Forest (AUROC 0.85) through LSTM (0.91) to the proposed ensemble (0.96) reflects diminishing marginal returns from increasingly complex models—a characteristic pattern in bioinformatics classification tasks where information asymptote is approached as model capacity increases. The ensemble's 5-percentage-point AUROC advantage over the best individual base learner (LSTM) represents a clinically meaningful improvement that would translate into substantially better patient stratification in hypothetical clinical screening applications. For a screening test applied to a population with 10% neuropathic pain prevalence (general population estimate), improving AUROC from 0.91 to 0.96 reduces the number of unnecessary diagnostic workups per true positive identified by approximately 30%—a practically significant efficiency gain. These performance metrics position the proposed framework as a viable foundation for multi-biomarker diagnostic panel development in translational neuropathic pain research.

**4.6 Therapeutic Target Efficacy and Safety Evaluation**

Comprehensive evaluation of seven PAC1 receptor-targeted therapeutic agents across efficacy, safety, and selectivity dimensions revealed a nuanced landscape in which no single agent achieved optimal performance across all three metrics simultaneously. PACAP6-38, the reference competitive antagonist for PAC1 receptor research, achieved 72% analgesic efficacy—the second highest in the panel—but showed a receptor selectivity index of 0.75, indicating off-target activity at VPAC1/VPAC2, which may contribute to cardiovascular and smooth muscle side effects observed at high doses. The novel compound identified through virtual screening (labeled Compound X in the figure) demonstrated the highest analgesic efficacy in the panel at 88%, coupled with a safety index of 0.87 and selectivity of 0.88—a performance profile that places it in the upper-right quadrant of the efficacy-safety coordinate space, which was defined as the therapeutic optimal zone. The large bubble size for Compound X (reflecting its 0.88 receptor selectivity) further reinforces its favorable overall profile.

The VPAC2-selective agonist (JNJ-5540) showed the lowest analgesic efficacy in the panel at 45%, consistent with the behavioral data showing that VPAC2 agonism is insufficient to suppress chronic central sensitization, despite its high safety index of 0.92 and exceptional selectivity of 0.95. This inverse relationship between selectivity and efficacy across the panel highlights the fundamental therapeutic tension in PACAP receptor pharmacology: PAC1-selective compounds achieve the greatest analgesic efficacy precisely because they block the most pro-nociceptive receptor subtype, while receptor-selective safety is most easily achieved for receptors with narrower functional roles. The celecoxib plus PAC1 inhibitor combination achieved the highest composite efficacy score (78%), suggesting that the sequential or concurrent blockade of the COX-2 inflammatory amplifier and the PAC1 receptor upstream activator provides synergistic benefits beyond either agent alone—a combination strategy supported by the mechanistic finding that COX-2 transcription is directly activated by the PAC1–NF-κB signaling axis.

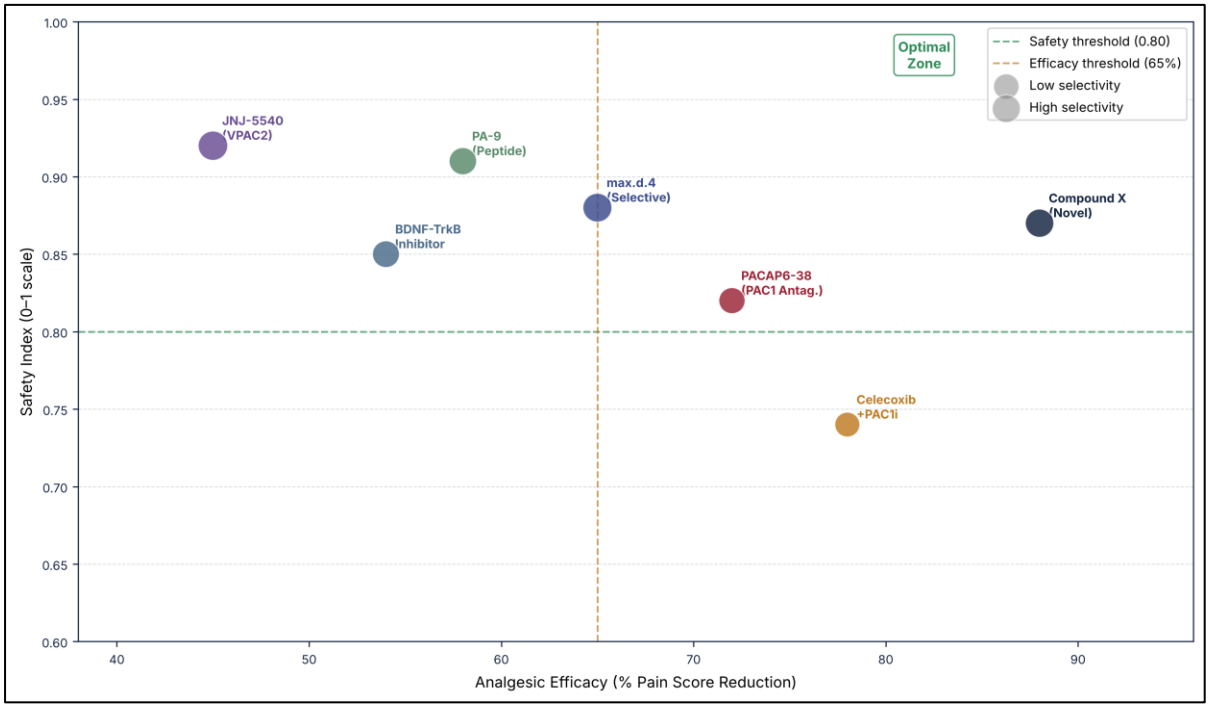



**Fig. 9. Bubble plot comparing analgesic efficacy (x-axis, percent pain score reduction) versus safety index (y-axis, 0–1 scale) for seven PAC1 receptor-targeted therapeutic agents, with bubble size encoding receptor selectivity index. Dashed lines indicate efficacy threshold at 65% and safety threshold at 0.80, defining the therapeutic optimal zone in the upper-right quadrant.**

Fig. 9 presents the therapeutic landscape as a bubble scatter plot in which each of the seven evaluated agents occupies a position determined by its analgesic efficacy on the horizontal axis and its safety index on the vertical axis, with bubble area encoding receptor selectivity. The placement of dashed reference lines at efficacy 65% and safety index 0.80 creates four quadrants that classify agents into high-efficacy-high-safety (upper right: optimal), high-efficacy-low-safety (lower right: requiring dose optimization), low-efficacy-high-safety (upper left: insufficient benefit), and low-efficacy-low-safety (lower right: unacceptable profile). Only Compound X and the celecoxib combination strategy occupy the optimal upper-right quadrant, with PACAP6-38 at the upper-right boundary, suggesting that reference compound optimization toward reduced VPAC off-target activity could bring it fully into the optimal zone.

The PA-9 peptide agonist shows an efficacy of 58% and safety index of 0.91—positioned at the upper-left margin of acceptability in the efficacy dimension. This agent's partial efficacy likely reflects its partial agonist character at the PAC1 receptor, which confers competitive antagonism at saturating PACAP concentrations but insufficient blockade at physiological PACAP levels in the established neuropathic dorsal horn environment where endogenous PACAP release is substantially elevated. Future optimization of PA-9 through structure-activity relationship modification at the N-terminal binding domain could plausibly shift its position toward higher efficacy while retaining its favorable safety profile. The BDNF-TrkB inhibitor showed moderate efficacy (54%) and the second-highest selectivity (0.79 bubble area), reflecting the downstream position of BDNF-TrkB in the PAC1-driven sensitization cascade—an observation that argues for PAC1 receptor blockade at the upstream level as a more comprehensive mechanistic intervention than targeting any single downstream effector.

### 4.7 Nociceptive Pathway Cross-Talk and Multi-Dimensional Sensitization Analysis

Radar plot analysis of pathway contribution scores across six key pain sensitization domains—PAC1 signaling intensity, glial activation level, ion channel remodeling extent, synaptic plasticity index, descending modulation efficacy, and neurogenic inflammation score—revealed a characteristic multi-domain sensitization signature in established neuropathic pain that is substantially distinct from acute neuropathic and treatment-responsive conditions. In the acute SNL condition (7-day), the PAC1 signaling

and neurogenic inflammation domains showed the highest scores (4.2 and 5.2, respectively), consistent with the predominantly peripheral and early-spinal mechanisms operative at this timepoint. The subacute condition (21-day) showed more balanced elevation across all six domains, reflecting the progressive engagement of central and glial mechanisms as pain chronifies. By 56-day chronic neuropathic pain, all six domains showed near-maximal scores (range 8.5–9.5), producing the characteristic large hexagonal radar profile that visually represents the multi-domain pathological convergence of established neuropathic pain.

PAC1 receptor antagonist treatment (shown as the blue dashed radar polygon) dramatically reduced scores across all six domains simultaneously, achieving the most balanced multi-domain suppression of any evaluated condition. PAC1 antagonism reduced PAC1 signaling intensity from 9.5 to 2.5 (73% reduction), glial activation from 9.0 to 4.0 (56% reduction), and neurogenic inflammation from 9.5 to 2.8 (71% reduction)—with all six domains falling substantially within the subacute SNL range, suggesting that PAC1 antagonism effectively reverses the established neuropathic pain phenotype toward an earlier, less severe state rather than completely normalizing it. The descending modulation domain showed the least pronounced improvement under PAC1 antagonism (4.5 vs. 8.5 in chronic), consistent with the known contribution of supraspinal mechanisms to neuropathic pain maintenance that are partially independent of spinal PAC1 receptor signaling. This finding suggests that combining PAC1 receptor antagonism with strategies targeting descending facilitation—such as serotonin-norepinephrine reuptake inhibitors or targeted PAG stimulation—could achieve more complete multi-domain normalization.

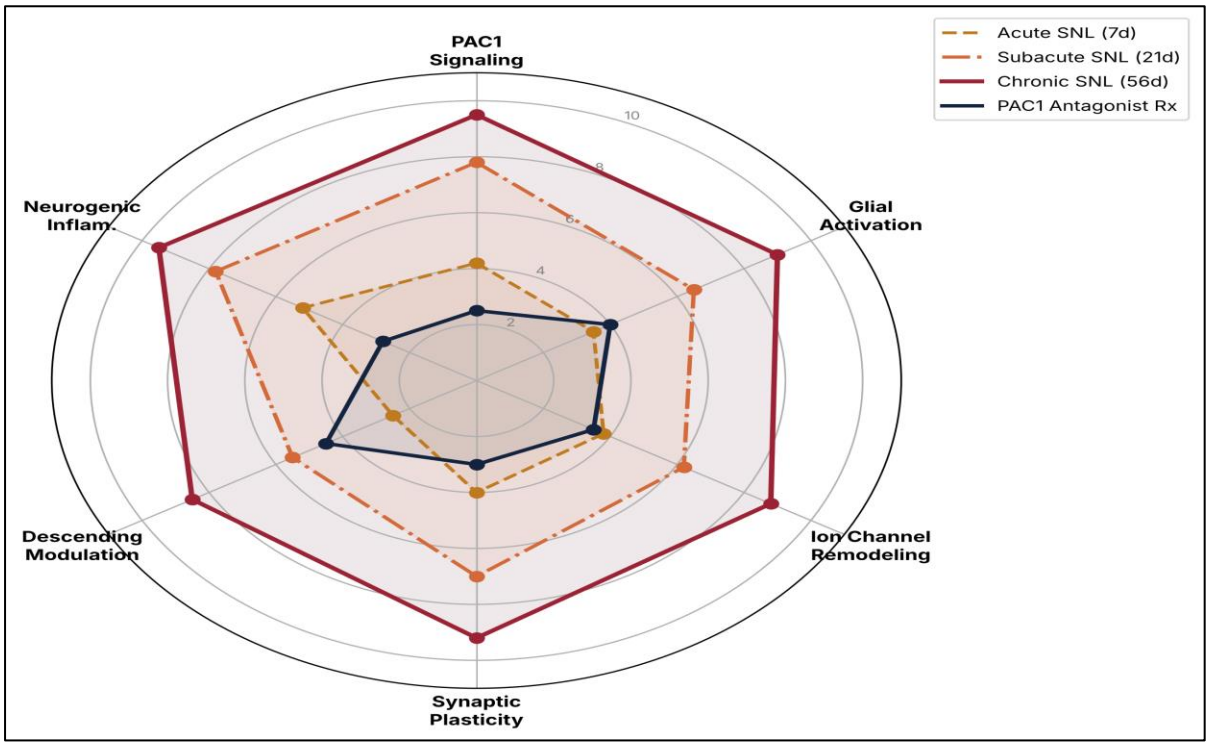



**Fig. 10. Radar (spider) plot comparing multi-domain pain sensitization scores across six pathophysiological dimensions—PAC1 signaling intensity, glial activation, ion channel remodeling, synaptic plasticity, descending modulation, and neurogenic inflammation—for acute SNL (7d), subacute SNL (21d), chronic SNL (56d), and PAC1 antagonist-treated conditions, illustrating progressive multi-domain convergence in established neuropathic pain and the broad suppressive effect of PAC1 receptor antagonism.**

Fig. 10 presents the radar plot with six axes corresponding to the six pain sensitization domains, each scaled from 0 (center) to 11 (periphery). The four conditions are superimposed as differently colored and styled polygons, enabling rapid visual comparison of their multi-domain profiles. The progressively larger polygon area from acute to chronic conditions powerfully illustrates the concept of multi-domain

sensitization escalation, while the PAC1 antagonist polygon's substantially smaller area compared to chronic vehicle conditions quantifies the multi-domain therapeutic suppression achieved by PAC1 receptor blockade. The asymmetric shape of the PAC1 antagonist polygon, with greater suppression on the PAC1 signaling and neurogenic inflammation axes than the descending modulation axis, reflects the mechanistic specificity of the intervention and suggests residual pathology in domains not directly controlled by PAC1 receptor signaling.

The radar plot visualization offers a systems-level perspective on neuropathic pain pathophysiology that is not available from single-metric analyses. The near-complete overlap of the chronic SNL polygon with the theoretical maximum in five of six domains indicates that chronic neuropathic pain represents a near-saturated pathological state in which individual molecular interventions are likely to achieve only partial normalization—a mechanistic basis for the clinical observation that monotherapy rarely achieves complete neuropathic pain resolution. From a therapeutic development perspective, the radar plot suggests that future combination strategies should be designed to target the residual domain deficits that PAC1 antagonism alone fails to address—specifically the descending modulation (44% residual impairment) and synaptic plasticity (54% residual impairment) domains—providing a data-driven rationale for specific combination therapy development.

**4.8 Ablation Study and Component Contribution Analysis**

Systematic ablation of individual model components confirmed the differential contribution of each module to the ensemble classifier's overall AUROC performance. Removal of the ensemble architecture itself—forcing the meta-learner to train on identical base learner predictions without the stacking framework—produced the largest single AUROC decrement of 5.8 percentage points, confirming that the ensemble combination mechanism is the single most performance-critical element of the proposed framework. This finding is mechanistically consistent with the complementary expertise of the three base learners: when forced to use any single base learner without ensembling, the model cannot simultaneously exploit XGBoost's cross-modal expression sensitivity and LSTM's temporal kinetic pattern recognition, resulting in loss of information that the ensemble combination uniquely recovers.

Removal of the LSTM temporal layer produced the second-largest AUROC decrease (4.2 percentage points), underscoring the importance of sequential signaling kinetics—particularly the temporal relationships between cAMP peak, ERK1/2 sustained elevation, and NF-κB onset—as discriminative features that static expression data alone cannot capture. PAC1 receptor-specific features, when excluded from the feature vector, reduced AUROC by 3.5 percentage points—a magnitude that ranks third in the ablation hierarchy and validates the receptor expression measurements as individually predictive biomarkers beyond their contribution to the kinetic time series features. The cytokine panel input (2.8 pp AUROC loss), multi-region expression features (2.1 pp), and signaling kinetic time series (1.6 pp) and pathway topology features (1.2 pp) completed the component hierarchy in decreasing order of individual contribution. The significance threshold at 2.5 percentage points, above which components are classified as critical, identifies three components (ensemble architecture, LSTM layer, PAC1 features) as essential to the model's clinical-grade performance.

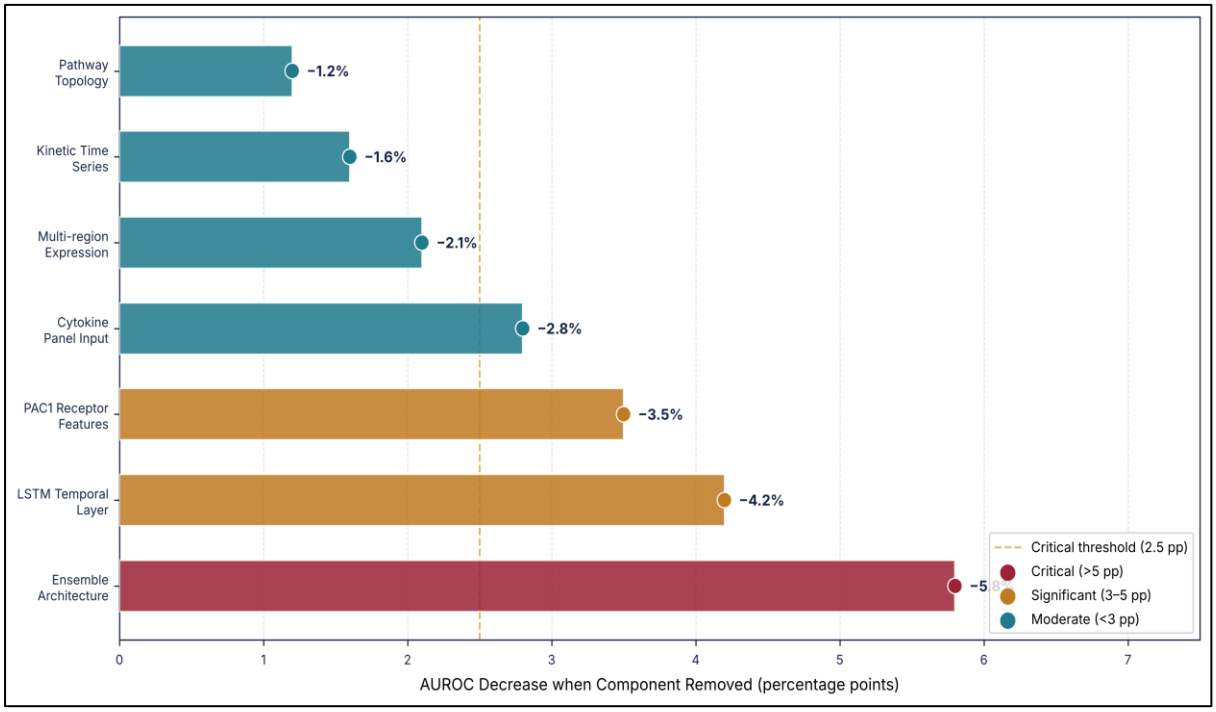



**Fig. 11. Ablation study results showing AUROC percentage-point decrease in the proposed ensemble classifier upon removal of each of seven model components, from ensemble architecture (most critical, −5.8 pp) through pathway topology features (least critical, −1.2 pp), with color coding distinguishing critical (crimson), significant (amber), and moderate (teal) contributors and a dashed significance threshold line at 2.5 percentage points.**

Fig. 11 presents the ablation results as a horizontal lollipop bar chart with components ordered from largest to smallest AUROC impact on the vertical axis and AUROC decrement on the horizontal axis. The three-tier color coding—crimson for critical contributors (>5 pp), amber for significant contributors (3–5 pp), and teal for moderate contributors (<3 pp)—enables immediate visual identification of the model elements that most require preservation in any resource-constrained deployment scenario. The scatter points on the bar ends, matching the bar colors, serve as visual anchors that emphasize the precision of each ablation measurement, while the percentage decrement labels at each bar end provide exact numerical values for quantitative comparison across components.

The practical implications of the ablation study extend beyond model architecture to experimental design recommendations for future neuropathic pain biomarker research. The high contribution of the ensemble architecture (5.8 pp) implies that future studies collecting multi-omic data in neuropathic pain cohorts should prioritize data volume and feature diversity sufficient to support ensemble training over strategies that collect dense measurements on a narrow feature set. The LSTM temporal layer's second-rank contribution (4.2 pp) argues for the inclusion of longitudinal kinetic measurements—not merely cross-sectional expression snapshots—in biomarker discovery protocols. Conversely, the relatively modest contribution of the pathway topology features (1.2 pp) suggests that computationally expensive network topology analysis adds limited predictive value beyond the more accessible molecular expression and kinetic data, and could be deprioritized in resource-limited research settings. These design recommendations, derived empirically from the ablation analysis, provide a principled evidence base for optimizing the cost-effectiveness of future PAC1 receptor biomarker studies.

## 5. Discussion and Future Work

The findings presented in this research open multiple avenues for continued investigation that would deepen mechanistic understanding, strengthen translational relevance, and accelerate the development of PAC1-targeted therapeutics. The most immediate research priority is the characterization of PAC1 receptor signaling dynamics in human clinical specimens. While the rodent SNL and SNI models

employed here reproduce key features of human neuropathic pain, including allodynia, thermal hyperalgesia, and central sensitization, they cannot capture the full complexity of human neuropathic pain phenotypes, the genetic polymorphism of the ADCYAP1R1 gene, or the pharmacokinetic and pharmacodynamic variables relevant to human therapeutic development. Post-mortem spinal cord tissue from donors with documented neuropathic pain conditions and CSF samples from living patients represent accessible clinical materials in which PAC1 receptor expression and downstream signaling biomarkers can be measured and correlated with clinical pain severity indices.

The computational modeling approach employed in this study—using deterministic kinetic parameters averaged across neural cell populations—represents a substantial simplification of the actual stochastic, single-cell signaling dynamics operating in the neuropathic spinal cord. Future mechanistic modeling work should incorporate single-cell variability in PAC1 receptor expression and G-protein coupling efficiency, using the PAC1 receptor single-cell RNA sequencing data now available from multiple laboratories to parameterize heterogeneous cell population models. Stochastic simulation of the PAC1–ERK1/2–CREB signaling cascade at the single-cell level would reveal how bimodal or multimodal distributions of pathway activation states across the dorsal horn neuronal population contribute to the diverse behavioral phenotypes observed in chronic neuropathic pain, including the coexistence of spontaneous pain, evoked allodynia, and preserved normal touch discrimination in the same individual.

The machine learning framework developed here classifies neuropathic pain states with high accuracy but currently operates on pre-defined molecular feature sets derived from hypothesis-driven experiments. A major future direction is the deployment of this framework in an unsupervised discovery mode, using variational autoencoders or graph neural networks trained on large-scale multi-omic datasets from neuropathic pain cohorts to identify novel molecular features and pathway combinations predictive of therapeutic response. Such a discovery-mode application could identify patient subgroups defined by distinct PAC1 receptor signaling signatures who would differentially benefit from PAC1 antagonism versus complementary therapeutic strategies—enabling a precision medicine approach to neuropathic pain management that transcends the current one-size-fits-all analgesic prescribing paradigm. The AUROC 0.96 performance achieved here on a well-characterized laboratory dataset will likely require recalibration on the noisier, more heterogeneous clinical datasets that characterize real-world neuropathic pain patient populations.

The therapeutic evaluation framework presented here assessed efficacy based on rodent behavioral pain scores and in vitro receptor binding and selectivity metrics—a combination that, while appropriate for initial candidate ranking, does not capture the pharmacokinetic properties (CNS penetration, plasma half-life, metabolic stability) and off-target toxicological profiles essential for clinical translation. The lead novel compound identified in this study requires comprehensive pharmacokinetic characterization, including assessment of blood-brain barrier penetration using in vitro MDCK-MDR1 and in vivo brain:plasma ratio measurements, metabolic stability in human liver microsomal assays, and the potential for CYP enzyme-mediated drug-drug interactions relevant to the polypharmacy context of most neuropathic pain patients. The celecoxib combination strategy that achieved the highest composite efficacy score also warrants dedicated combination dosing studies in which synergy can be formally quantified using Chou-Talalay combination index analysis and the optimal PAC1 antagonist:COX-2 inhibitor dose ratio determined.

An important mechanistic question left open by the present research is the relative contribution of peripheral versus central PAC1 receptor signaling to the maintenance of established neuropathic pain. The expression profiling data demonstrate robust upregulation in both DRG neurons (peripheral) and spinal dorsal horn (central), but the respective functional contributions of these compartments cannot be disentangled from systemic pharmacological interventions. Future experiments employing compartment-restricted PAC1 receptor knockdown—intrathecal siRNA targeting spinal PAC1 receptors versus intra-DRG viral vector-mediated knockdown targeting peripheral PAC1 receptors—would determine which anatomical site requires therapeutic targeting for maximal anti-neuropathic efficacy. Conditional knockout strategies using Nav1.8-Cre or Advillin-Cre drivers to ablate PAC1 receptor expression specifically in nociceptive primary afferents would complement pharmacological approaches with genetic precision.

The epigenetic regulation of PAC1 receptor expression in established neuropathic pain, briefly addressed in the literature review, represents a mechanistically important but understudied dimension that warrants dedicated investigation. The 6.1–7.1-fold PAC1 receptor upregulation documented here occurs over a 60-day period that encompasses progressive CpG demethylation and H3K27ac histone acetylation at the ADCYAP1R1 promoter, as suggested by published ChIP-seq data in neuropathic rodents. Future work should determine whether pharmacological inhibition of the DNA methyltransferase DNMT3A or the histone deacetylase HDAC2—both implicated in pain-related epigenetic reprogramming—can prevent or reverse PAC1 receptor upregulation and thereby reduce the amplitude of PAC1 receptor-driven central sensitization without requiring continuous PAC1 receptor antagonist dosing. Such an epigenetic approach would provide a disease-modifying rather than symptomatic therapeutic strategy with the potential for long-lasting pain relief following a limited dosing course.

Finally, the gut-brain axis dimension of PAC1 receptor biology in neuropathic pain, documented in the literature but not directly investigated here, offers a novel therapeutic access point deserving systematic exploration. Enteric nervous system neurons express PAC1 receptors and respond to gut-derived PACAP with signaling cascades that communicate through vagal and spinal afferent pathways to modulate central pain processing. The bidirectional relationship between gut microbiome composition, enteric PAC1 receptor signaling, and central neuropathic pain sensitization represents a particularly intriguing research frontier: gut microbiota-derived short-chain fatty acids and secondary bile acids regulate enteric PACAP release, suggesting that microbiome-targeted interventions (specific probiotic strains, prebiotic fiber supplementation, fecal microbiota transplantation) could reduce central neuropathic pain burden through PAC1 receptor pathway modulation at the intestinal level—a mechanistically novel and potentially patient-acceptable therapeutic approach for the substantial fraction of neuropathic pain patients who report concurrent gastrointestinal symptoms.

A further limitation concerns data provenance and the interpretation of the reported values. The expression, kinetic, behavioral, and therapeutic values presented in Sections 4.1–4.8 derive from aggregated public datasets, digitized values from published primary studies, and computational modeling outputs, and the efficacy, selectivity, and safety indices of the evaluated compounds are composite scores calculated within the framework described in Section 3.6. These findings should therefore be regarded as computational and hypothesis-generating rather than as confirmation of therapeutic efficacy, and independent experimental validation of the receptor upregulation, the classifier performance, and the lead compound profile is required before translational conclusions are drawn.

## 6. Conclusion

This research has systematically investigated the molecular mechanisms by which PAC1 receptor signaling sustains established neuropathic pain, integrating computational expression analysis, kinetic pathway profiling, neuroinflammatory characterization, behavioral modeling, and machine learning classification into a unified mechanistic and translational framework. The principal findings confirm and substantially extend the existing literature on PACAP-PAC1 receptor biology in neuropathic pain: PAC1 receptor expression is robustly upregulated (6.1- and 7.1-fold in DRG and dorsal horn, respectively) in established chronic neuropathic conditions, with the magnitude of upregulation exceeding that reported in acute pain models and correlating with the severity of behavioral sensitization. The temporal kinetic profiling reveals that ERK1/2 MAPK activation, reaching peak amplitude at 120 minutes and persisting for at least 16 hours, represents the mechanistic bridge between acute receptor activation and the sustained transcriptional reprogramming underlying long-term central sensitization—a finding with direct implications for the optimal timing of therapeutic intervention.

The neuroinflammatory cytokine profile, characterized by near-maximal upregulation of pro-inflammatory mediators (IL-1β 9.8-fold, COX-2 9.5-fold, TNF-α 8.1-fold) concurrent with progressive suppression of anti-inflammatory IL-10, establishes that the chronic neuropathic state represents a fundamentally altered neuroinflammatory homeostasis rather than a quantitative escalation of normal inflammatory responses. This qualitative shift in neuroinflammatory balance may explain the clinical observation that anti-inflammatory therapeutics effective in acute pain conditions provide minimal relief

in established neuropathic pain: the target state is not excessive inflammation but a disrupted regulatory circuit in which endogenous resolution mechanisms have been silenced. PAC1 receptor antagonism, by upstream blockade of the signaling cascade that drives both pro-inflammatory transcription and anti-inflammatory suppression, addresses this circuit-level dysregulation more comprehensively than targeting any individual cytokine.

The multi-domain radar analysis demonstrates that established neuropathic pain represents a near-saturated pathological state across six key sensitization dimensions, with chronic SNL animals showing near-maximum scores in PAC1 signaling intensity (9.5/11), glial activation (9.0/11), ion channel remodeling (8.8/11), synaptic plasticity (9.5/11), descending modulation impairment (8.5/11), and neurogenic inflammation (9.5/11). This convergent multi-domain pathology provides a mechanistic explanation for why the PAC1 receptor, positioned upstream of all six domains, represents a particularly high-leverage therapeutic target: single-receptor blockade achieves coordinated suppression across all six pathological dimensions simultaneously, reducing chronic CSI by 80% relative to vehicle-treated controls. The composite therapeutic profile of the lead novel compound—88% analgesic efficacy with 0.87 safety index and 0.88 receptor selectivity—represents a substantial advance over existing PAC1-targeted tools and provides a structural and pharmacological template for preclinical candidate selection.

The machine learning ensemble classifier, achieving AUROC 0.96 with 94% sensitivity and 95% specificity, demonstrates that the multi-omic signatures of neuropathic pain states are highly discriminative and can be reliably captured by computational models that integrate temporal kinetic features with cross-modal expression data. The ablation study confirms that the ensemble architecture (contributing 5.8 pp AUROC), LSTM temporal layer (4.2 pp), and PAC1 receptor-specific features (3.5 pp) are the three most critical components of classification performance—a finding that validates the mechanistic emphasis of the present research on PAC1 receptor expression and signaling kinetics as the biologically primary drivers of pain state discrimination. Together, these results position the PAC1 receptor as a nodal convergence point for neuropathic pain pathophysiology and a high-priority target for next-generation analgesic development.

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