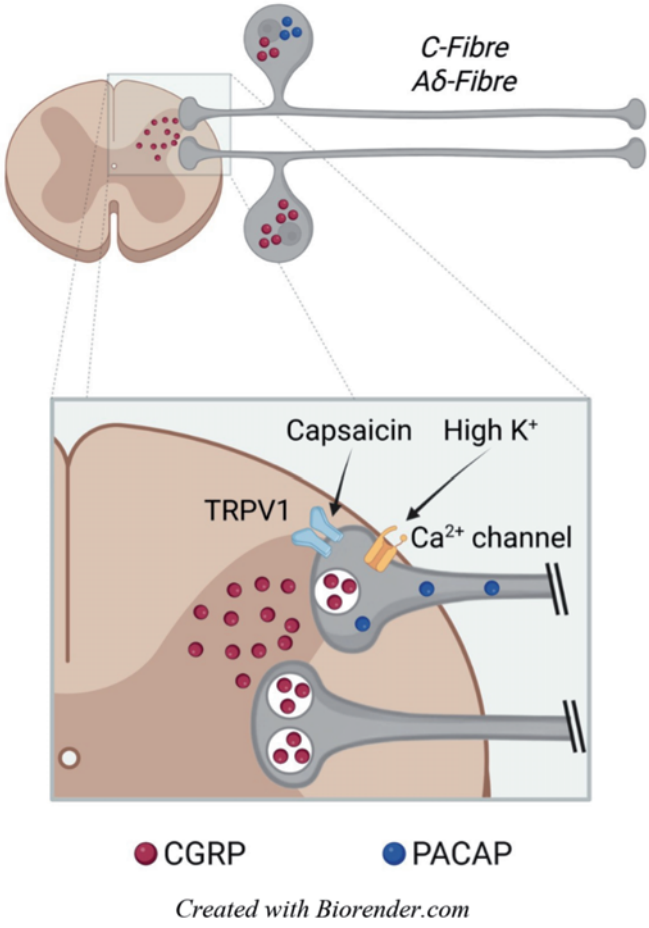
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Divergent acute release of CGRP and PACAP from mouse spinal cord tissue: implications for migraine neurobiology

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GRAPHICAL ABSTRACT



ABSTRACT

Calcitonin gene-related peptide (CGRP) and pituitary adenylate cyclase-activating polypeptide (PACAP) are both implicated in migraine, but whether they undergo similar acute release from primary sensory terminals remains uncertain. We compared stimulus-evoked CGRP-like immunoreactivity (CGRP-LI) and PACAP-like immunoreactivity (PACAP-LI) in superfused mouse spinal cord tissue. Spinal cords from 14 male C57BL/6 mice were pooled and distributed among six superfusion chambers per stimulus. After two baseline fractions, tissues were exposed to capsaicin (10 μM) or high K^+ (40 mM KCl), followed by a post-stimulation fraction. Capsaicin and high K^+ produced marked CGRP responses, with mean stimulation-minus-baseline increases of 44.89 and 47.19 pg/mg, respectively, both Holm-adjusted $p<0.001$. In contrast, PACAP responses were −0.12 and 0.009 pg/mg, with Holm-adjusted $p=0.742$ and $p=0.888$. The geometric mean stimulation-to-baseline responses were 23.23-fold and 18.57-fold for CGRP, compared with 0.86-fold and 1.00-fold for PACAP. The corresponding CGRP-to-PACAP ratios of fold responses were 26.99 and 18.51. These findings show a marked dissociation between acute CGRP and PACAP release in this pooled ex vivo spinal cord preparation. They suggest that PACAP should not be assumed to behave as a CGRP-like co-transmitter during acute stimulation of primary sensory neuron (PSN) terminals.

Key words: PACAP; CGRP; TRPV1; release; migraine.

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Introduction

Calcitonin gene-related peptide (CGRP) and pituitary adenylate cyclase-activating polypeptide (PACAP) are vasoactive neuropeptides with partly overlapping but non-identical roles in migraine and sensory signaling. CGRP has a well-established distribution in primary sensory neurons (PSNs) and a central role in migraine therapeutics. (1,2) PACAP occurs mainly as PACAP38, with PACAP27 representing a shorter bioactive form, and activates PAC1, VPAC1, and VPAC2 receptors. (3)

After a failure with a monoclonal antibody directed against the receptor PAC1, (4) interest in PACAP in migraine has been renewed by recent positive results obtained with another monoclonal antibody directed against PACAP38. (5)

These findings establish PACAP signaling as therapeutically relevant but do not identify the anatomical source of endogenous PACAP during migraine attacks. Histological studies have detected PACAP small- and medium-size dorsal root ganglion neurons and their peripheral and central fibers. (6,7) PACAP-like immunoreactivity (PACAP-LI) has also been demonstrated in human spinal cord and dorsal root ganglia (DRG). (8) Anatomical presence, however, does not necessarily imply that PACAP and CGRP undergo equivalent storage, transport, or stimulus-dependent release. Furthermore, the origin and the site of action of PACAP possibly implicated in the migraine mechanism remain to be determined.

PACAP, first isolated from the sheep hypothalamus and found to stimulate adenylate cyclase activity in cultured rat anterior pituitary cells, (3,9) belongs to the secretin family of regulatory peptides, which includes vasoactive intestinal polypeptide (VIP). The main signaling pathway activated by PACAP receptors increases adenylyl cyclase activity to produce cyclic adenosine monophosphate, leading to the subsequent engagement of protein kinase A and mitogen-activated protein kinase. PACAP exhibits the highest affinity and selectivity for the PAC1 receptor, whereas VPAC1 and VPAC2 are also activated by VIP, with significant differences in agonist affinity determined by splice variants. (3) PACAP38 accounts for about 90% of total PACAP, indicating this isoform as the most physiologically relevant. (3,9)

The highest levels of PACAP have been found in the hypothalamus, although it is present in additional areas of the central and peripheral nervous systems. (3,9) Importantly for the present study, PACAP has been visualized in DRGs (10) and the trigeminal ganglion (TG). (11) Notably, the release literature is discordant across preparations and findings.

Early evidence (12) showed a remarkable release of CGRP and a considerable smaller increase in PACAP outflow by capsaicin, which selectively targets the transient receptor potential vanilloid 1 (TRPV1) in PSNs, in rat cerebrospinal fluid. In contrast to this previous evidence, a more recent study failed to detect any PACAP release from an *ex vivo* preparation of rat dura mater (hemi-skull preparation) superfused with capsaicin or the non-selective depolarizing stimulus, high K^+, (11) which, nevertheless, released substantial amounts of CGRP. (13,14) Interestingly, PACAP release was detected in the TG but not from peripheral nerve terminals in the dura. (11) The latter observation suggests that PACAP release may depend on cellular compartment and tissue context.

PSNs project their central terminals to the dorsal spinal cord, resulting in an extraordinary density of terminals and a very high concentration of their neuropeptide content in this anatomical area. In agreement with this concept, remarkable amounts of CGRP have been detected from rodent spinal cord tissue upon exposure to capsaicin. (15,16) Although PACAP release from spinal sensory pathways was first reported nearly three decades ago, its reproducibility and relationship to CGRP release remain insufficiently investigated. Therefore, we sought to re-examine stimulus-evoked PACAP release by directly comparing CGRP and PACAP responses to capsaicin and high K^+ in superfused mouse spinal cord slices, measuring both neuropeptides in corresponding superfusate fractions. Both stimuli induced robust CGRP release, whereas neither produced a consistent increase in PACAP-LI under the conditions tested.

Results

CGRP assay. A marked increase in CGRP-like immunoreactivity (CGRP-LI) over baseline values was observed in the six chambers containing the pooled spinal cord preparations and superfused with capsaicin (10 μM) (**Figure 1A**, **Tables 1 and 2**). Mean

Table 1. CGRP-LI and PACAP-LI across sequential superfusate fractions.

Peptide	Stimulus	B1	B2	S1	S2	Post
CGRP-LI	Capsaicin	2.11±0.31	1.95±0.23	46.59±4.47	47.27±4.41	17.51±3.06
CGRP-LI	High K^+	2.46±0.41	2.94±0.22	46.50±4.56	53.28±3.91	18.93±2.81
PACAP-LI	Capsaicin	1.04±0.13	0.74±0.12	0.63±0.05	0.89±0.12	0.63±0.16
PACAP-LI	High K^+	1.03±0.11	0.72±0.08	0.95±0.11	0.83±0.08	0.75±0.11

CGRP-LI, calcitonin gene-related peptide-like immunoreactivity; PACAP-LI, pituitary adenylate cyclase-activating polypeptide-like immunoreactivity. Values are mean ± standard error of the mean in pg/mg across six superfusion chambers derived from one pooled biological preparation. B1 and B2 denote the two baseline fractions; S1 and S2 denote the two fractions collected during stimulation; Post denotes the post-stimulation fraction. Sequential fractions obtained from the same chamber are repeated measurements.

Table 2. Changes in CGRP-LI and PACAP-LI from baseline to stimulation.

Peptide	Stimulus	Baseline mean ± SEM (pg/mg)	Stimulation mean ± SEM (pg/mg)	Mean paired change (pg/mg)	95% CI	Ratio of group means	Holm-adjusted p
CGRP-LI	Capsaicin	2.04±0.23	46.93±4.30	44.90	33.95-55.85	23.05	<0.001
CGRP-LI	High K^+	2.71±0.20	49.89±2.99	47.19	39.41-54.97	18.44	<0.001
PACAP-LI	Capsaicin	0.89±0.08	0.76±0.07	−0.12	−0.46-0.20	0.86	0.742
PACAP-LI	High K^+	0.88±0.05	0.89±0.07	0.009	−0.15-0.17	1.01	0.888

SEM, standard error of the mean; CI, confidence interval; CGRP-LI, calcitonin gene-related peptide-like immunoreactivity; PACAP-LI, pituitary adenylate cyclase-activating polypeptide-like immunoreactivity. The 95% CIs were not adjusted for multiplicity.

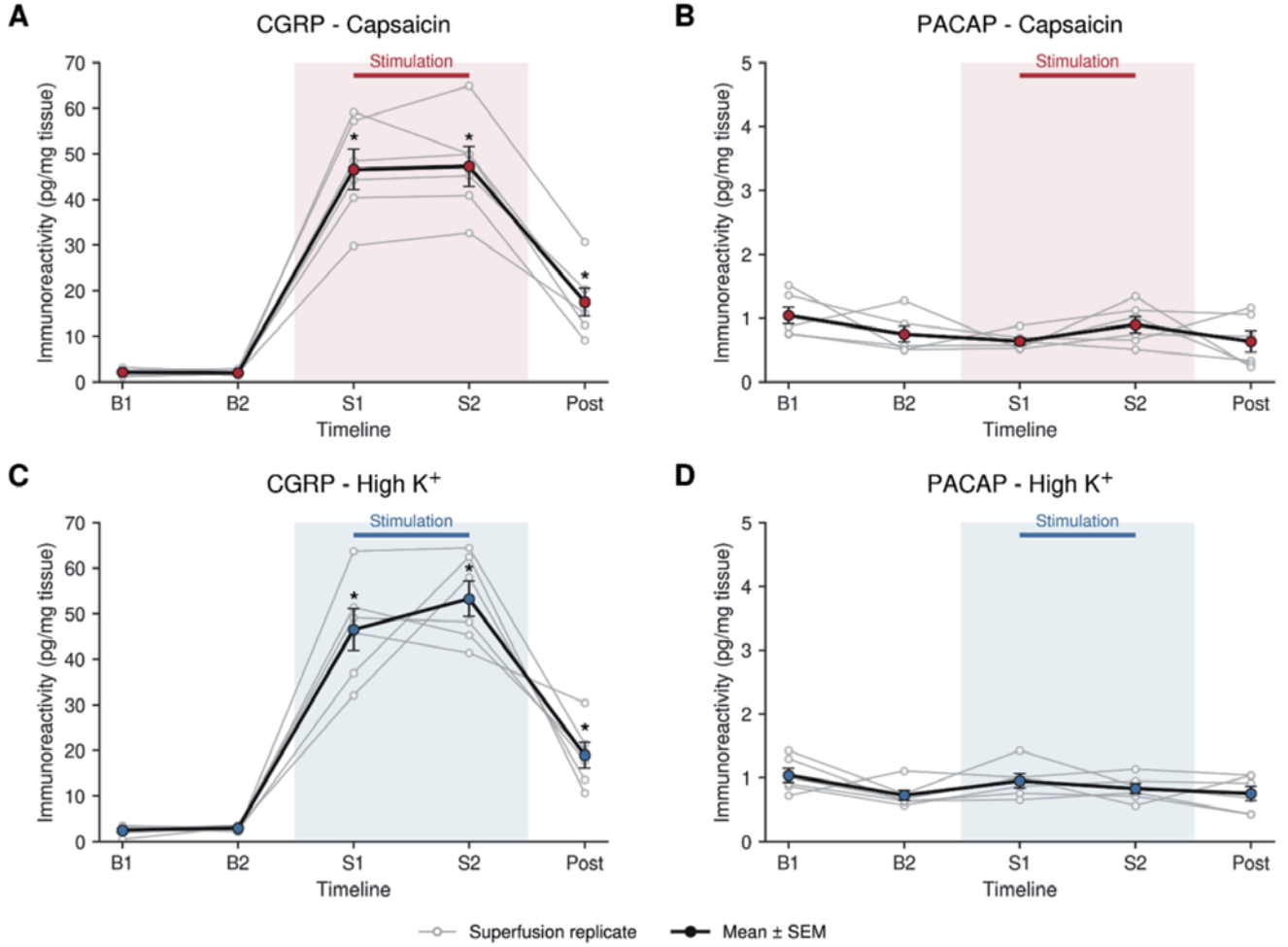
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level (B1 and B2 mean) was 2.04±0.23 pg/mg during baseline and 46.93±4.30 pg/mg during stimulation (S1 and S2 mean). The mean stimulation-minus mean baseline was 44.90 pg/mg (95% confidence interval [CI]: 33.95-55.85, Holm-adjusted p<0.001), corresponding to a 23.1-fold ratio of the stimulation and baseline group means, as well as S1 and S2 being 22.9-fold and 23.2-fold higher than mean baseline, respectively. Post-stimulation (Post) release declined but remained 8.6-fold above baseline; all three fraction-specific comparisons remained significant after correction (**Supplementary Table 1**). The overall fraction effect was also detected by repeated-measures analysis of variance (ANOVA) and the Friedman test (both p<0.001; **Supplementary Table 2**).

A remarkable increase in CGRP-LI over baseline values was also found in the six chambers superfused with a high (40 mM) K^+ (**Figure 1C**, **Tables 1 and 2**). Mean release was 2.71±0.20 pg/mg during baseline and 49.90±2.99 pg/mg during stimulation. The mean stimulation-minus mean baseline was 47.19 pg/mg (95% CI: 39.41-54.97; Holm-adjusted p<0.001), an 18.4-fold ratio of group means. S1, S2, and Post were 17.2-fold, 19.7-fold, and 7.0-fold above mean baseline, respectively; all three comparisons remained significant after correction (**Supplementary Table 1**). The overall fraction effect was detected by both omnibus methods (both p<0.001; **Supplementary Table 2**).

PACAP assay. Capsaicin (10 µM) superfusion did not produce any significant increase in PACAP-LI (**Figure 1B**, **Tables 1 and 2**). Mean baseline level was 0.89±0.08 pg/mg and mean stimulation level was 0.76±0.07 pg/mg. The mean paired difference was −0.12 pg/mg (95% CI: −0.46-0.20; Holm-adjusted p=0.742). Relative to mean baseline, S1, S2, and Post were 0.71-fold, 1.00-fold, and 0.71-fold, respectively. Although unadjusted comparisons suggested lower values in S1 and Post, neither remained significant after Holm correction (**Supplementary Table 1**). Neither omnibus analysis identified a fraction effect (**Supplementary Table 2**).

Likewise, high K^+ superfusion produced no consistent increase in PACAP-LI (**Figure 1D**, **Tables 1 and 2**). Mean baseline level was 0.88±0.05 pg/mg and mean stimulation level was 0.89±0.07 pg/mg. The mean stimulation-minus mean baseline was 0.009 pg/mg (95% CI: −0.15-0.17; Holm-adjusted p=0.888). S1 was 1.08-fold, S2 was 0.94-fold, and Post was 0.86-fold relative to mean baseline; none differed significantly from baseline



CGRP, calcitonin gene-related peptide; PACAP, pituitary adenylate cyclase-activating polypeptide; *p<0.001.

Figure 1. Sequential CGRP-LI (A, C) and PACAP-LI (B, D) levels in the superfusate of slices of mouse spinal cord during capsaicin (10 mM) or high (40 mM) K^+ stimulation. B1 and B2 indicate baseline fractions, S1 and S2 indicate stimulation fractions, and Post indicates the post-stimulation fraction. Open grey points and connecting lines represent individual superfusion replicates. Filled points and error bars represent mean ± standard error of the mean (SEM).

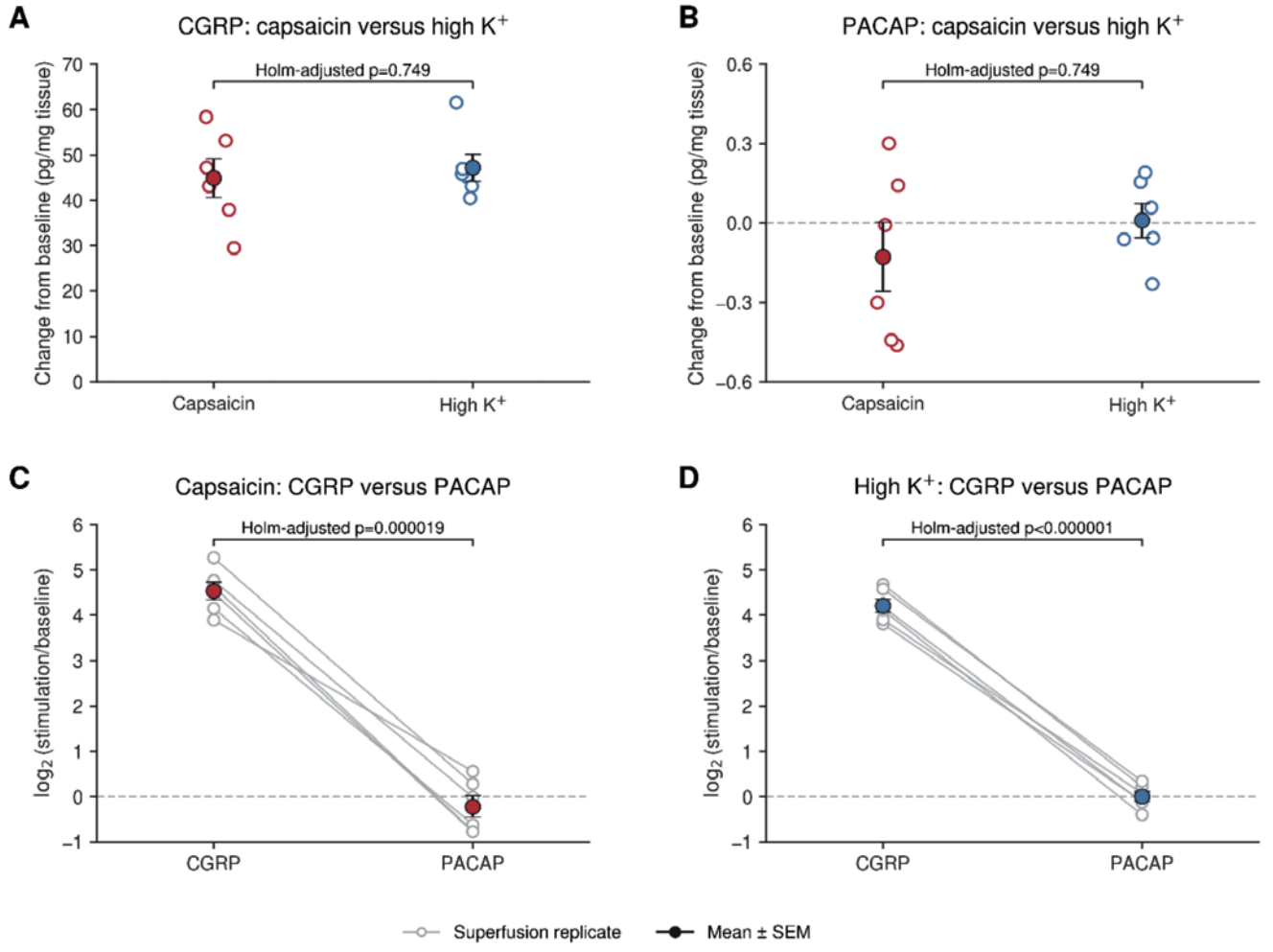
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after correction (**Supplementary Table 1**). Neither omnibus analysis identified a fraction effect (**Supplementary Table 2**). Because of these negative results, and to exclude underestimation of PACAP in the superfusate, exogenous PACAP was added to four wells containing non-stimulated freeze-dried superfusates reconstituted with the assay buffer (400 pg were added to two wells and 800 pg to two additional wells). The percentage recovery of PACAP-LI in these samples was approximately 80%, warranting the ability of the assay to detect neuropeptide increases in the superfusate.

Comparison between capsaicin and potassium stimuli. The mean stimulation-minus mean baseline CGRP-LI response was 44.90 pg/mg after capsaicin and 47.19 pg/mg after high K^+. The direct difference was 2.29 pg/mg (95% CI: −9.52-14.11; Holm-adjusted p=0.749) (**Table 3**, **Figure 2A**). For PACAP-LI, the corresponding responses were −0.12 and 0.009 pg/mg, giving a between-stimulus difference of 0.13 pg/mg (95% CI: −0.20-0.47; Holm-adjusted p=0.749) (**Table 3**, **Figure 2B**). These technical comparisons did not identify a difference between stimuli within either peptide.

Comparison between PACAP and CGRP. Under capsaicin, the geometric mean stimulation-to-baseline response was 23.23-fold for CGRP and 0.86-fold for PACAP. The paired CGRP-LI to PACAP-LI ratio of fold responses was 26.99 (95% CI: 15.76-46.23; Holm-adjusted paired *t*-test p<0.001) (**Table 4**, **Figure 2C**). Under high K^+, the corresponding geometric mean responses were 18.57-fold and 1.00-fold, yielding a ratio of 18.51 (95% CI: 15.09-22.69; Holm-adjusted paired *t*-test p<0.001) (**Table 4**, **Figure 2D**). The proportional CGRP-LI response exceeded the PACAP-LI response under each stimulus.



CGRP, calcitonin gene-related peptide; PACAP, pituitary adenylate cyclase-activating polypeptide.

Figure 2. Direct comparisons between stimulus-associated and peptide-specific release responses. A, B) Chamber-level change from mean baseline to mean stimulation for CGRP and PACAP, respectively. Capsaicin (10 mM) or high (40 mM) K^+ responses were compared using Welch independent-samples t-tests, with Holm adjustment across the two peptide comparisons. C, D) Comparison between CGRP and PACAP stimulation-to-baseline responses following capsaicin and elevated K^+, respectively. Fold responses are displayed on a log scale (statistical comparisons were performed on natural-log-transformed ratios). Connected points represent corresponding CGRP and PACAP measurements from superfusion replicates. Peptide responses were compared using paired t-tests of natural-log-transformed fold changes, with Holm adjustment across the two stimulus conditions. Filled points and error bars represent mean ± standard error of the mean.

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Discussion

Recent positive clinical results obtained by targeting PACAP38 in individuals with migraine who did not respond to previous preventive therapies, including anti-CGRP treatments, (5) highlight the role of PACAP and raise the question of the source, acute release mechanism and site of action of this neuropeptide. Current literature generally supports the hypothesis that PACAP, like CGRP, is a sensory neuropeptide expressed and released from PSNs from which it contributes to neurogenic inflammation and migraine attacks, mimicking CGRP acute release and effects. (3,6) However, this view is supported by limited and partly inconsistent neurochemical evidence. In the only direct *in vivo* study of spinal release from the rat spinal cord, intrathecal capsaicin increased PACAP27 by 177% and PACAP38 by 93% in rat cerebrospinal fluid, compared with a 692% increase in CGRP. (12) Thus, PACAP release was detectable but substantially smaller than CGRP.

Additional indirect evidence was obtained in an organ-cultured transected rat vagus nerve preparation, in which PACAP expression, axonal transport, and release increased after nerve injury. (17) However, this model does not closely reproduce acute stimulus-coupled secretion from PSN terminals. Importantly, both capsaicin and high K^+ elicited substantial CGRP release but failed to induce detectable PACAP release from PSN terminals of the rat dura mater, although a small increase in PACAP release was detected from the TG exposed to these two stimuli. (11)

The present study identified a marked dissociation between the acute release profiles of CGRP and PACAP in superfused mouse spinal cord tissue. Capsaicin and elevated extracellular K^+ produced large increases in CGRP-like immunoreactivity, whereas neither stimulus produced a significant increase in PACAP-LI outflow. Direct comparisons confirmed a substantially greater proportional response for CGRP than for PACAP after both capsaicin (approximately 27-fold) and elevated K^+ (approximately 19-fold; both adjusted $p<0.001$). These findings provide direct evidence that CGRP and PACAP do not share the same acute stimulus-coupled release behavior in this preparation.

The consistency of this dissociation across two mechanistically different stimuli strengthens the biological interpretation. Capsaicin selectively engages the TRPV1 channel, (18,19) expressed by a subpopulation of PSNs, whereas elevation of extracellular K^+ induces a generalized membrane depolarization in all excitable cells. Failure to detect an increase in PACAP outflow after capsaicin may reflect a limited PACAP expression within TRPV1-positive neurons. However, the absence of a PACAP response after high K^+ depolarization argues against the hypothesis that PACAP is released from spinal cord cells or fibers. Several hypotheses may be advanced to explain the failure to detect PACAP release by capsaicin or high K^+, including that PACAP is stored in smaller and negligible quantities in releasable pools; may be stored or trafficked differently from CGRP; may require different temporal or physiological patterns of stimulation; or finally, may be more susceptible to metabolizing factors. The present findings should not be interpreted as conclusive evidence that PACAP is completely absent from PSNs. Other that the anatomical evidence discussed above with related limitations, (6,7) PACAP has also been identified in subsets of CGRP- and substance P-containing PSNs, and its expression changes after inflammation, axonal injury, and peripheral nerve compression. (10,12,20)

The distinction between peptide expression and stimulus-evoked terminal secretion is therefore essential when interpreting our findings. The present findings refine rather than contradict the prior neurophysiologic literature indicating that PACAP does not behave as a conventional CGRP-like sensory co-transmitter during acute stimulation within the dorsal spinal cord.

The trigeminal literature further supports anatomical compartment as a potential determinant of PACAP release. In rats, capsaicin and high K^+ elicited CGRP release from both the TG and dura, whereas PACAP was released only from the TG, despite PACAP38 immunoreactivity in neurons is sometimes being co-expressed with CGRP. (11) However, PACAP expression in and release from TG or DRG neurons do not automatically imply its neurosecretion from their central (present findings) or peripheral terminals. (11)

The spinal and trigeminal systems are nevertheless not fully equivalent. The present preparation contains central terminals of DRG neurons projecting to the spinal dorsal horn, whereas migraine-relevant meningeal afferents originate predominantly from the TG and project centrally to the trigeminocervical complex. Although these neurons share an identical pseudo-unipolar anatomical and neurophysiological organization, they can differ molecularly and functionally. (21,22) The present results therefore cannot be directly extrapolated to trigeminovascular PACAP secretion but demonstrate distinct PACAP and CGRP release behavior within central terminals of DRG PSNs.

Although PSNs may not be the source of extracellular PACAP in cranial tissues, other sources of PACAP might be implicated in migraine mechanism: high PACAP expression has been reported

Table 3. Direct comparison of responses to capsaicin and high K^+.

Peptide	Capsaicin response mean ± SEM (pg/mg)	High K^+ response mean ± SEM (pg/mg)	Between-stimulus difference (pg/mg)	95% CI	Raw p	Holm-adjusted p
CGRP-LI	44.90±4.26	47.19±3.02	2.29	−9.52-14.11	0.671	0.749
PACAP-LI	−0.12±0.13	0.009±0.06	0.13	−0.20-0.47	0.374	0.749

SEM, standard error of the mean; CI, confidence interval; CGRP-LI, calcitonin gene-related peptide-like immunoreactivity; PACAP-LI, pituitary adenylate cyclase-activating polypeptide-like immunoreactivity. The 95% CIs were not adjusted for multiplicity.

Table 4. Direct comparison of proportional CGRP and PACAP responses.

Stimulus	CGRP-LI geometric mean fold response (95% CI)	PACAP-LI geometric mean fold response (95% CI)	CGRP-LI to PACAP-LI ratio of fold responses (95% CI)
Capsaicin	23.23 (16.38-32.96)	0.86 (0.57-1.31)	26.99 (15.76-46.23)
High K^+	18.57 (14.37-23.99)	1.00 (0.83-1.22)	18.51 (15.09-22.69)

CGRP-LI, calcitonin gene-related peptide-like immunoreactivity; PACAP-LI, pituitary adenylate cyclase-activating polypeptide-like immunoreactivity; CI, confidence interval. Holm correction was applied across the two stimulus-specific peptide comparisons. Holm-adjusted $p<0.001$ for both paired t-tests.

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in the sphenopalatine and otic ganglia, and PACAP-containing neurons, fibers, and receptors have been shown in rat and human parasympathetic ganglia. (7,23) Activation of the trigeminal-autonomic reflex engages sphenopalatine projections to cranial vessels, the dura, and the lacrimal gland. (24) PACAP released from these terminals could therefore reach the meningeal environment independently of trigeminal sensory endings. Collectively, these observations support a model in which CGRP is predominantly released from terminals of PSNs, whereas PACAP may originate from different compartments, including sensory ganglia and cranial parasympathetic projections. However, it must be underlined that direct neurochemical proof that PACAP is released from any type of peripheral neuron remains to be demonstrated.

The main strengths of this study are the simultaneous assessment of CGRP and PACAP in corresponding sequential superfusate fractions, the use of two mechanistically distinct stimuli, and the inclusion of repeated baseline and post-stimulation measurements. The robust CGRP responses provided an internal functional control, while direct within-chamber comparisons allowed the relative release profiles of the two peptides to be evaluated without relying on separate significance tests.

However, some limitations need to be considered. The first limitation is that immunoreactivity of either neuropeptide was measured, without any confirmation of the actual identity of the two molecules. However, the CGRP data, confirming previous literature reporting its abundant release from central endings of PSNs in rodents, (15,16) support the validity of the present results. Moreover, the fact that the PACAP assay does not distinguish between PACAP38 and PACAP27 reduces the risk of failing to detect one of the two PACAP isoforms. However, we cannot exclude that, for unidentified reasons, the present experimental conditions do not enable the detection of a small amount of released PACAP. Another limitation is that the results have been obtained in mice (only male) and not in humans, where PACAP might be expressed and released from PSNs. Notably, *in vivo* data from healthy volunteers show that the cutaneous vasodilatation induced by the application of capsaicin cream to the human forearm was completely attenuated by a CGRP receptor antagonist. (25) These findings, while not supporting a major role, cannot rule out a minor contribution of PACAP released from TRPV1-expressing PSNs in this cutaneous response to capsaicin. One alternative hypothesis, that PACAP is released in humans from TRPV1-negative peripheral nerve terminals, remains to be explored. Other limitations include the absence of a time-matched unstimulated control, the use of only male mice, and the use of whole spinal cord slices rather than isolated dorsal horn tissue. Consequently, the absence of a detectable PACAP increase cannot exclude low-level release, different release kinetics, extracellular degradation, or limited analytical recovery.

Conclusions

Capsaicin and elevated extracellular K^+ elicited robust CGRP release from a pooled *ex vivo* mouse spinal cord preparation, whereas neither stimulus produced a consistent increase in PACAP. This marked dissociation indicates that CGRP and PACAP have different acute stimulus-coupled release profiles and should not be assumed to behave as functionally equivalent co-transmitters in PSNs. The findings do not exclude completely PACAP release from other anatomical compartments, in response to different stimulation patterns, or over longer time scales. Together with previous evidence of PACAP release from the TG but not from dural sensory terminals, the results support the hypothesis that PACAP neurosecretion is more compartment- and stimulus-dependent than CGRP neurosecretion. Independent replication and peptide-specific analytical validation are required to confirm this interpretation.

Materials and Methods

Animals, tissue preparation, and setting. The research complies with all relevant ethical regulations according to the European Union (EU) guidelines for animal care procedures and Italian legislation (DLgs 26/2014) and the application of the EU Directive 2010/63/EU. All animal studies were approved by the Animal Ethics Committee of the University of Modena and Reggio Emilia and the Italian Ministry of Health (permit no. 381/2020-PR; PRIN2022 Prot. 202277LAA7). We used a total of 14 C57BL/6 (20-30 g, 6-8 weeks of age) male mice (Charles River Laboratories, Italy), which were anesthetized with isoflurane (4-5%) and sacrificed by decapitation. The spinal cord was pushed gently out of the vertebral canal (26) and maintained at 4°C for the entire preparation period in ice-cold oxygenated (96% O_2 and 4% CO_2) normal Krebs solution of the following composition (mM): NaCl 119; $NaHCO_3$ 25; KH_2PO_4 1.2; $MgSO_4$ 1.5; CaCl2 2.5; KCl 4.7 and glucose 11. Thick transverse slices (0.5 mm) of the whole spinal cord were prepared using a MacIlwain tissue chopper (Campden Instruments) and 80-110 mg of tissue were transferred to each 2 mL superfusion chamber.

The slices were superfused with the oxygenated (96% O_2 and 4% CO_2) normal Krebs solution containing 0.1% bovine serum albumin and the peptidase inhibitor thiorphan (10 µM) and maintained at 37°C, at a constant flow of 0.4 mL/min to collect 2 mL fractions every 5 min. After a 30-min equilibration period, two basal fractions were collected. Subsequently, to stimulate release, the chambers were superperfused for 10 min with 10 µM capsaicin or 40 mM KCl, and two fractions were collected. To obtain the necessary high K^+ 40 mM solution, NaCl in the normal Krebs solution was replaced isotonically with 40 mM KCl.

Neuropeptide assays. Fractions were collected in tubes containing acetic acid (2 N final concentration) and freeze-dried. At the end of the experiments, the tissues were blotted 2-3 times on filter paper and weighed. CGRP-LI and PACAP-LI were measured using two commercially available enzyme-linked immunosorbent assay (ELISA) kits: for CGRP-LI and PACAP-LI, we used the rat CGRP ELISA kit (Bertin, France, Cat. N. A05482; range: 4-500 pg/mL) and the mouse PACAP ELISA kit (Antibodies.com, UK, Cat. N. A75687; range: 15.6-1000 pg/mL), respectively, according to the protocols provided by the vendors. The PACAP ELISA kit does not differentiate between PACAP38 and PACAP27. Concentrations were normalized to wet tissue weight and are expressed as pg/mg of spinal cord tissue.

Statistical analysis. Tissues from 14 mice were pooled and distributed among superfusion chambers. Three chambers per stimulus in each of two separate experiments (for a total of six chambers) provided a five-fraction profile. CGRP-LI and PACAP-LI were measured in the superfusate from corresponding chamber samples. Thus, the chamber was the unit of stimulus application within one pooled biological preparation. The five sequential fractions were measured within each chamber: two baseline fractions (B1 and B2), two stimulation fractions (S1 and S2), and one post-stimulation fraction (Post). Values are presented as mean ± standard error of the mean across chambers. In the first experiment, spinal cords from 6 mice were collected. Pooled spinal cord slices were distributed into 6 chambers and superfused. A second experiment was conducted with spinal cords collected from 6 additional mice, following the same procedure of the first experiment. Finally, spinal cords were collected from 2 mice and slices were pooled and distributed into 2 chambers and superfused with the superfusion medium. PACAP (400 or 800 pg)

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was added to these freeze-dried samples reconstituted with 1 mL of the assay buffer.

For the CGRP and PACAP release analyses, each chamber's baseline was defined as the mean of B1 and B2, and stimulation as the mean of S1 and S2. The primary response was stimulation minus baseline within the chamber. Paired *t*-tests assessed this response separately for the four peptide-stimulus conditions. Holm correction was applied across these four primary comparisons. Mean paired differences are reported with unadjusted 95% CIs. Ratios of group mean stimulation to group mean baseline were calculated descriptively.

Overall variation across the five fractions was assessed separately for each condition using repeated-measures ANOVA with Greenhouse-Geisser correction. Friedman tests provided distribution-free sensitivity analyses of these profiles (omnibus test). In secondary analyses, S1, S2, and Post were each compared with the chamber-specific mean baseline using paired *t*-tests. Holm correction was applied to the three secondary comparisons within each peptide-stimulus condition.

For the comparison between stimuli (capsaicin *vs.* high K^+), the chamber-specific stimulation-minus-baseline responses under capsaicin and high K^+ were compared separately for CGRP and PACAP using two-sided Welch *t*-tests. Differences are reported as high K^+ minus capsaicin, with unadjusted 95% CIs. Holm correction was applied across the two between-stimulus tests.

Finally, for the comparison between peptides, CGRP and PACAP measurements from corresponding samples were paired. The stimulation-to-baseline ratio was calculated and natural-log transformed. The paired difference between CGRP and PACAP log responses was assessed separately under capsaicin and high K^+ using two-sided paired *t*-tests with Holm correction. Exponentiating the mean log response produced the geometric mean fold response for each peptide; exponentiating the mean paired log difference produced the CGRP-to-PACAP ratio of fold responses. Unadjusted 95% CIs were calculated on the log scale and back-transformed. Two-tailed p-values <0.05 were considered statistically significant, regardless of whether they were adjusted for multiple comparisons. Statistical analyses were performed in SPSS version 30.0. The original graphs were prepared in GraphPad Prism version 10.0 and the figures presented here were modified (*i.e.*, style and format) in Python.

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Online supplementary material:

Supplementary Table 1. *Comparisons of individual stimulation and post-stimulation fractions with mean baseline.*

Supplementary Table 2. *Overall changes across the five sequential superfusate fractions.*

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