Intrathecal Amylin and Salmon Calcitonin Affect Formalin Induced c-Fos Expression in the Spinal Cord of Rats

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Abstract

Background: Amylin and Salmon Calcitonin belong to the calcitonin family of peptides and have high affinity binding sites in the rat spinal cord. The aim of this study was to characterize receptors for Amylin and Salmon Calcitonin functionally in the spinal cord of rats. We assessed the expression of c-Fos in response to intraplantar formalin in the lumbar regions of the spinal cord in conscious rats.

Methods: Amylin (0.05 nmoles) or Salmon Calcitonin (0.005 nmoles) was administered intrathecally (i.t.) 10 minutes before the start of the formalin test. Antagonists were injected intrathecally 10 minutes before the administration of either of the peptides.

Results: Two hours after formalin stimulation, rats pretreated intrathecally by either Amylin or Salmon Calcitonin, showed lower numbers of c-Fos immunoreactive nuclei in their lumbar spinal cord as compared to rats pretreated with saline. These effects were reversed upon co-administration of either of the Amylin antagonists AC187 or rat amylin8-37, but not rat α-CGRP8-37. A few cells with c-Fos immunoreactivity were found in the lumbar spinal cord of rats two hours after i.t. injection of saline, Amylin and/or Salmon Calcitonin. However, Fos-like immunoreactivity was increased in the lumbar spinal cord two hours after i.t. treatment of either of the antagonists AC187 and rat amylin8-37, when compared to saline treated rats.

Conclusion: Both Amylin and Salmon Calcitonin inhibit formalin induced c-Fos expression in the rat lumbar spinal cord when administered intrathecally. Effects of the two peptides were possibly produced by undefined receptors.

Keywords • Rats • Spinal cord • Proto-oncogene proteins c-fos • Salmon Calcitonin

Introduction

Amylin (AMY) and salmon calcitonin (sCT) have structural similarities and belong to the calcitonin family of peptides. This family also includes calcitonin, two distinct forms of calcitonin gene-related peptide (α-CGRP and β-CGRP), adrenomedullin and AM2, also known as intermedin.1,2 These peptides are involved in a wide variety of biological functions and hence their receptors have therapeutic implications in many disease states.3-5 The pancreatic hormone, AMY, works with insulin in glucose regulation and energy balance.6 Actions of AMY in the CNS include regulation of appetite and...
AMY and sCT on the c-Fos expression. Hind paw injection of formalin produces peripheral inflammatory responses that lead to increased c-Fos expression in the lumbar spinal cord. We also deployed this method to investigate the effects of AMY and sCT on the formalin induced c-Fos expression in the spinal cord of rats.

Materials and Methods

Animal Treatments and Surgery
Forty-five male Sprague-Dawley rats bred at Shiraz University of Medical Sciences and weighing 250±20g were randomly divided into 15 groups. Animals were anesthetized with ketamine (50 mg/kg)+xylazine (5 mg/kg) and intrathecal catheterization was performed as described by Yaksh and Rudy. Briefly, a polyethylene catheter (PE-10, Betcton Dickenson, San Jose, CA) was stretched in a hot water bath at 72°C to reduce its diameter, and 7.5 cm length of the elongated part of the catheter was threaded caudally into the subarachnoid space through a slit in the atlanto-occipital membrane. The rostral part was sutured to the adjacent muscles to immobilize the catheter and the wound was closed in two layers with 4-0 silk. The position of the caudal tip was always confirmed after the animals were sacrificed. Rats showing neurological deficits during the recovery period of seven days were excluded from the study. The Medical and Research Ethics Committee of the Shiraz University of Medical Sciences approved all experimental protocols.

Chemicals
Rat AMY and other peptides were obtained from Bachem Americas, Inc. (Torrance, CA.). The peptides were dissolved in sterile saline such that the final doses delivered (in 10 µl of vehicle) were as follows; AMY 0.05 nmoles, sCT 0.005 nmoles, rAMY 8-37 1.00 and 2.50 nmoles, acetyl-(Asn30, Tyr32)-calcitonin8-32 (AC187) 1.00 and 2.50 nmoles and rat α-CGRP 8-37 2.50 nmoles. Peptides and antagonist concentrations were determined in pilot experiments (data not shown).

Formalin (37%), sucrose (analytical grade), glycerol (85-88%), paraformaldehyde, H₂O₂ (30%) were obtained from Merck Company. Triton-X100 (laboratory grade) was purchased from Sigma-Aldrich. DAB (diaminobenzidine) was purchased from Dako North America Inc.

Injections and Tissue Preparation
Peptide agonists and antagonists were administered i.t. in volumes of 10 µl, followed by 10 µl flushes of normal saline to clear the catheter. Antagonists were administrated 10 minutes prior to the injection of AMY and or sCT.
Formalin (2.5%, 50 µL) or saline was injected into the hind paw of rats 15 min after they received i.t. injection of the drug or saline. Control rats were treated i.t. with 10 µL of saline prior to an injection of 50 µL of saline into one hind paw. Two hours after intrathecal injections, rats were deeply anesthetized and were perfused through the heart with 200 mL saline followed by 500 mL ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer. The L3-L5 spinal segments were removed and postfixed for 2 h and then transferred into the cryoprotection solution containing 30% sucrose in 0.1 M phosphate buffered saline (PBS) overnight at 4°C. Frozen serial sections (40 µm) were cut in the transverse plane using a cryostat device. Every third section (80 µm intervals) was collected as free-floating sections and maintained at -25°C until immunohistochemical analysis.

**Immunohistochemistry and Counting of c-Fos Protein Immunoreactive Nuclei**

Immunohistochemistry was performed by a horseradish peroxidase (HRP) method with a polyclonal antibody against c-Fos (Santa Cruz Biotechnology, Santa Cruz, CA). Briefly, free-floating spinal sections were washed with PBST and transferred into 0.3% H2O2 in PBST for 15 minutes to inhibit endogenous peroxidases. After blocking with 3% normal goat serum, sections were incubated in primary polyclonal anti-c-Fos antibody (diluted 1:300) for 24 h at 4°C. Visualization of the antigen-antibody complex was performed by using ready-to-use goat anti mouse EnVision-HRP enzyme conjugate for 40 minutes (Dako, Trappes, France). The peroxidase activity was visualized with 0.05% diaminobenzidine (DAB) and 0.3% hydrogen peroxide. The primary antibody was omitted in the case of immunohistochemical negative control sections. Sections were then rinsed with PBS, mounted onto slides with glycerol and cover slipped. Sections were examined by light field microscopy at 10× magnification to localize c-Fos labeled nuclei. Only those cells with stained round nuclei identified at 10× objective lens were counted. Following peripheral noxious stimuli, spinal neurons that express c-Fos were located in laminae I and II, and laminae V and VI of the dorsal horn. Therefore, we identified gray matter increases in the c-Fos expression in the lumbar spinal cord of rats. In all animals, a few labeled nuclei were scattered throughout the dorsal horn without any clustering or apparent pattern. Data are shown in figure 1, with the number of c-Fos positive nuclei being summarized in table 1.

**Effects of i.t. Pretreatment with AMY and sCT on the Formalin-Induced c-Fos Immunoreactivity in the Spinal Dorsal Horn**

Formalin, when injected in the hind paw, is known to increase c-Fos expression in the spinal dorsal horn ipsilateral to the injection site. As shown in figure 1, two hours after hind paw injection of formalin, the number of c-Fos positive nuclei was increased on one side of spinal dorsal horn of rats pretreated with i.t. saline. However, both AMY (0.05 nmol) and sCT (0.005 nmol) decreased the number of c-Fos-positive neurons in the dorsal horn of the spinal cord when injected intrathecally 15 minutes before formalin injection (table 1). These effects of AMY and sCT were statistically significant (P=0.00 and P=0.00 respectively) when compared with the corresponding parts in the spinal cord of rats.
Figure 1: Photomicrographs of Fos-like immunoreactive neurons in the superficial (laminae I-II) dorsal horn of the lumbar (L3-L5) spinal cord. Rats were treated i.t. by saline and/or peptides prior to the injection of formalin into the hind paw. Pictures were taken using a 10× objective.

Table 1: Effects of AMY related peptides on the c-Fos expression induced by intraplantar formalin at the L3-L5 levels of the rat spinal cord

| Treatments (i.t.) | Total c-Fos Immunoreactive nuclei in laminae I-II and IV-VI |
|-------------------|------------------------------------------------------------|
| Control†          | 5.54±0.79a                                                 |
| Saline            | 38.97±4.35d                                               |
| AMY               | 14.28±1.19a,b                                             |
| sCT               | 4.00±1.15a                                                |
| CGRP₅₋₇            | 26.83±2.48c,d                                             |
| AMY+rAM₅₋₇ (1 nmoles/10µL) | 27.33±1.45c                                               |
| AMY+AC₁₈₇ (1 nmoles/10µL) | 18.66±1.76b,c                                             |
| (2.5 nmoles/10µL) | 28.33±2.60c,d                                             |
| sCT+rAM₅₋₇ (1 nmoles/10µL) | 18.66±1.76b,c                                             |
| (2.5 nmoles/10µL) | 19.00±3.50b,c                                             |
| sCT+AC₁₈₇ (1 nmoles/10µL) | 8.06±2.40a                                                |
| (2.5 nmoles/10µL) | 29.66±2.02c,d                                             |
| sCT+CGRP₅₋₇ (2.5 nmoles/10µL) | 6.16±2.94a,b                                              |
| AMY+CGRP₅₋₇ (2.5 nmoles/10µL) | 11.73±1.88a,b                                             |

Data are Means±SEM (n=3) of the number of c-Fos positive nuclei per section. One-way ANOVA showed significant differences among the groups (F(10, 22)=35.24, P=0.00). Data with superscript letters a,b,c and d differ from each other by P=0.05 or less. Peptides, AMY (0.05 nmoles/10µL) and /or sCT (0.005 nmoles/10µL), were injected i.t. before formalin administration into the hind paw. Antagonists were injected i.t. prior to the peptides. †Saline was injected in the plantar surface instead of formalin.
Amylin inhibits formalin induced c-Fos expression

Effects of Antagonists of AMY and sCT on the Formalin-Induced c-Fos Expression in the Spinal Cord

Table 1 presents the summarized data. The decreasing effects of i.t. AMY (0.05 nmoles) on c-Fos expression in the dorsal spinal neurons were antagonized when animals were pretreated i.t. (1 nmoles/10uL) by either AC187 or rAMY8-37. A statistical analysis by Tukey test revealed that this effect was significant in the case of rAMY8-37 (P=0.008) but not AC187 (P=0.98). AC187, however, was able to block the above effect of AMY at the i.t. dose of 2.5 nmoles/10uL (P=0.00). The inhibitory effect of sCT on the c-Fos positive neurons in the dorsal spinal cord was antagonized, but not completely blocked upon pretreatment of rats by rAMY8-37, at either doses of 1.00 or 2.5 nmoles/10uL. The above-mentioned effect of sCT was not significantly reduced by 1.00 nmoles/10uL of AC187 but was reversed when the i.t. dose of the antagonist was raised to 2.5 nmoles/10uL (P=0.00). CGRP8-37 had no significant effect on the formalin-induced c-Fos expression. This antagonist was not also able to reverse the inhibitory effects of either AMY or sCT observed in this study. We also examined whether AMY-related antagonists can affect the expression of c-Fos when injected alone to control rats. As shown in figure 2, both AC187 and rAMY8-37 caused a moderate but significant bilateral increase of the Fos-like immunoreactivity in the lumbar spinal cord two hours after they were injected i.t. to control rats. CGRP8-37 had no significant effect in this regard (table 2).

Discussion

Nuclear visualization of Fos-like immunoreactivity that peaks 2 h after neurons are stimulated, is the best marker of neuronal activation. As such, we have already shown that i.t. injection of two peptides of the calcitonin family, CGRP and adrenomedullin, increase c-Fos expression in the spinal cord of rats. As shown by the present results, the two other members of this family, AMY and sCT, did not track CGRP and adrenomedullin and failed to induce the expression of c-Fos. Rat α-CGRP reportedly has agonistic effects at rat AMY1(a) and rat AMY3(a) receptors with potencies equivalent to rat AMY. The fact that rat α-CGRP and AMY show contradictory effects on the number of spinal c-Fos positive nuclei, implies that the nature of rat AMY

**Figure 2:** Fos-like immunoreactive neurons in the superficial (laminae I-II) dorsal horn of the lumbar (L4-L5) spinal cord. Saline and/or antagonist peptides were administered i.t. prior to the injection of saline into the hind paw. Pictures were taken using a 10× objective.
visceral pain. Immunohistochemistry data is shown to inhibit c-Fos expression following antagonistic effects at both CGRP and AMY antagonists of rat AMY, whereas AC187 is considered as a selective and potent AMY receptors. When tested in cell culture studies, AC187 antagonists show that both AMY and sCT may have antinociceptive properties against inflammatory pain. The two peptides should be tested in animal models of inflammatory pain to confirm this notion.

AMY binding sites were initially identified in the rat brain as CT-sensitive CGRP binding sites, but AMY receptors in the rat spinal cord are not characterized and their molecular components are not defined. Receptors for AMY are heterodimers of CTR and RAMPs. To our knowledge, mRNAs encoding the three RAMPs are expressed in the spinal cord, but the expression of CTR encoding mRNA is not clearly shown in this tissue. However, binding sites for sCT is shown in the spinal cord of rats. Whether sCT binding sites exactly mirror AMY sites of action in the spinal cord and whether these sites are presynaptic, postsynaptic or both are unknown.

Antinociceptive effects of Amylin on visceral pain are shown to be inhibited by the Amylin antagonist, Salmon calcitonin. In the second part of this study, we used AMY receptor antagonists with different affinities for the classical AMY receptors in order to probe the nature of the receptors that mediate effects of AMY and sCT on the formalin induced c-Fos expression. rAMY is generally considered a weak antagonist of rat AMY, whereas AC187 is considered as a selective and potent AMY antagonist. CGRP reportedly shows antagonistic effects at both CGRP and AMY receptors. When tested in cell culture studies, the order of potency of the antagonist peptides at transfected AMY and AMY receptors were AC187> rα-CGRP >> rAMY. However, the present data show that in the context of spinal c-Fos expression, effects of CGRP were not in line with those of the rAMY and/or AC187. rAMY blocked the effects of AMY on formalin induced c-Fos expression while CGRP failed to do so when injected at equimolar concentrations as rAMY. This fact is in line with the conclusion that AMY may act through undefined receptors to inhibit the effects of intraplantar formalin on the expression of c-Fos in the rat spinal cord. Moreover, our data imply that the antagonist potency of rAMY at AMY receptors in the rat spinal cord may be as potent as, if not more potent than AC187. The reason is that AMY blocked the inhibitory effect of AMY on the formalin-induced c-Fos expression when administered at 20 times the concentration of the agonist. Whereas AC187 failed to show significant antagonistic effects at the above mentioned sites when administered at the same dose as AMY. Thus, whether yet uncharacterized combinations of RAMPs and CTR splice variants or alternative receptors produce AMY and sCT receptors in the rat spinal cord, needs further investigation. Intrathecal administration of AC187 and rAMY to rats injected with intraplantar saline lead in a remarkable increase in the number of Fos positive nuclei in the spinal cord. This effect can be attributed to the antagonistic action of AC187 and rAMY at the action sites of an endogenous ligand (i.e. AMY) in the spinal cord. CGRP did not affect the expression of c-Fos under similar conditions as above.

The intracellular mechanism of the expression of the c-Fos gene induced by AMY and sCT in adult rat spinal cord remains to be clarified. Taken together, our results demonstrate that both AMY and sCT are inhibitory to neurons in the rat spinal cord and act via undefined receptors.

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Conflict of Interest: None declared.

References

1. Takahashi K, Morimoto R, Hirose T, Satoh F, Totsune K. Adrenomedullin 2/intermedin in the hypothalamo-pituitary-adrenal axis. J Mol Neurosci. 2011;43:182-9. doi: 10.1007/s12031-010-9413-2. PubMed PMID: 20596793.

2. Poyner DR, Sexton PM, Marshall I, Smith DM, Quirion R, Born W, et al. International Union of Pharmacology. XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors. Pharmacol Rev. 2002;54:233-46. doi: 10.1124/pr.54.2.233. PubMed PMID: 12037140.

3. Barwell J, Gingell JJ, Watkins HA, Archbold JK, Poyner DR, Hay DL. Calcitonin and calcitonin receptor-like receptors: common themes with family B GPCRs? Br J Pharmacol. 2012;166:51-65. doi: 10.1111/j.1476-5381.2011.01525.x. PubMed PMID: 21649645; PubMed Central PMCID: PMC3415637.

4. van Rossum D, Hanisch UK, Quirion R. Neuroanatomical localization, pharmacological characterization and functions of CGRP, related peptides and their receptors. Neurosci Biobehav Rev. 1997;21:649-78. doi: 10.1016/S0149-7634(96)00023-1. PubMed PMID: 9353797.

5. Watkins HA, Walker CS, Ly KN, Bailey RJ, Barwell J, Poyner DR, et al. Receptor activity-modifying protein-dependent effects of mutations in the calcitonin receptor-like receptor: implications for adrenomedullin and calcitonin gene-related peptide pharmacology. Br J Pharmacol. 2014;171:772-88. doi: 10.1111/bph.12508. PubMed PMID: 24199627; PubMed Central PMCID: PMC3969088.

6. Boyle CN, Rossier MM, Lutz TA. Influence of high-fat feeding, diet-induced obesity, and hyperamylinemia on the sensitivity to acute amylin. Physiol Behav. 2011;104:20-8. doi: 10.1016/j.physbeh.2011.04.044. PubMed PMID: 21550355.

7. Roth JD. Amylin and the regulation of appetite and adiposity: recent advances in receptor signaling, neurobiology and pharmacology. Curr Opin Endocrinol Diabetes Obes. 2013;20:8-13. doi: 10.1097/MED.0b013e32835b896f. PubMed PMID: 23183359.

8. Gebre-Medhin S, Mulder H, Zhang Y, Sundler F, Betsholtz C. Reduced nociceptive behavior in islet amyloid polypeptide (amylin) knockout mice. Brain Res Mol Brain Res. 1998;63:180-3. doi: 10.1016/S0169-328X(98)00269-1. PubMed PMID: 9838101.

9. Huang X, Yang J, Chang JK, Dun NJ. Amylin suppresses acetic acid-induced visceral pain and spinal c-fos expression in the mouse. Neuroscience. 2010;165:1429-38. doi: 10.1016/j.neuroscience.2009.11.063. PubMed PMID: 19958820; PubMed Central PMCID: PMC2815112.

10. Skofitsch G, Wimalawansa SJ, Jacobowitz DM, Gubisch W. Comparative immunohistochemical distribution of amylin-like and calcitonin gene related peptide like immunoreactivity in the rat central nervous system. Can J Physiol Pharmacol. 1995;73:945-56. doi: 10.1139/y95-131. PubMed PMID: 8846435.

11. Nicholl CG, Bhattacharya KM, Mak J, Girgis SI, Legon S. Extra-pancreatic expression of the rat islet amyloid polypeptide (amylin) gene. J Mol Endocrinol. 1992;9:157-63. doi: 10.1677/jme.0.0090157. PubMed PMID: 1418386.

12. Ferrier GJ, Pierson AM, Jones PM, Bloom SR, Girgis SI, Legon S. Expression of the rat amylin (IAPP/DAP) gene. J Mol Endocrinol. 1989;3:R1-4. doi: 10.1677/jme.0.003R001. PubMed PMID: 2525914.

13. Mulder H, Leckstrom A, Uddman R, Ekblad E, Westermark P, Sundler F. Islet amyloid polypeptide (amylin) is expressed in sensory neurons. J Neurosci. 1995;15:7625-32. PubMed. PMID: 7472513.

14. Banks WA, Kastin AJ, Maness LM, Huang W, Jaspan JB. Permeability of the blood-brain barrier to amylin. Life Sci. 1995;57:209-20. doi: 10.1016/0024-3205(95)02197-Q. PubMed PMID: 871670.

15. van Rossum D, Menard DP, Fournier A, St-Pierre S, Quirion R. Autoradiographic distribution and receptor binding profile of [125I]Bolton Hunter-rat amylin binding sites in the rat brain. J Pharmacol Exp Ther. 1994;270:779-87. PubMed PMID: 8071970.

16. Oligati VR, Guidobono F, Netti C, Pecile A. Localization of calcitonin binding sites in rat central nervous system: evidence of its neuroactivity. Brain Res. 1983;265:209-15. doi: 10.1016/0006-8993(83)90334-7. PubMed PMID: 6850324.

17. Oliver KR, Kane SA, Salvatore CA, Mallee JJ, Kinsey AM, Koblan KS, et al. Cloning, characterization and central nervous system distribution of receptor activity modifying proteins in the rat. Eur J Neurosci. 2001;14:618-28. doi: 10.1046/j.0953-816x.2001.01688.x.
PubMed PMID: 11556887.

18 Chakravarty P, Suthar TP, Coppock HA, Nicholl CG, Bloom SR, Legon S, et al. CGRP and adrenomedullin binding correlates with transcript levels for calcitonin receptor-like receptor (CRLR) and receptor activity modifying proteins (RAMPs) in rat tissues. Br J Pharmacol. 2000;130:189-95. doi: 10.1038/sj.bjp.0702975. PubMed PMID: 10781016; PubMed Central PMCID: PMC1572027.

19 Alexander SP, Mathie A, Peters JA. Guide to Receptors and Channels (GRAC), 5th edition. Br J Pharmacol. 2011;164:S1-324. doi: 10.1111/j.1476-5381.2011.01649_1.x. PubMed PMID: 22040146; PubMed Central PMCID: PMC3315626.

20 Aiyar N, Baker E, Martin J, Patel A, Stadel JM, Willette RN, et al. Differential calcitonin gene-related peptide (CGRP) and amylin binding sites in nucleus accumbens and lung: potential models for studying CGRP/amylin receptor subtypes. J Neurochem. 1995;65:1131-8. doi: 10.1046/j.1471-4159.1995.65031131.x. PubMed PMID: 7643091.

21 Hay DL, Christopoulos G, Christopoulos A, Poyner DR, Sexton PM. Pharmacological discrimination of calcitonin receptor: receptor activity-modifying protein complexes. Mol Pharmacol. 2005;67:1655-65. PubMed PMID: 15692146.

22 Wang ZL, Bennet WM, Ghatel MA, Byfield PG, Smith DM, Bloom SR. Influence of islet amyloid polypeptide and the 8-37 fragment of islet amyloid polypeptide on insulin release from perfused rat islets. Diabetes. 1993;42:330-5. doi: 10.2337/diabetes.42.2.330. PubMed PMID: 8425669.

23 Ye JM, Lim-Fraser M, Cooney GJ, Cooper GJ, Iglesias MA, Watson DG, et al. Evidence that amylin stimulates lipolysis in vivo: a possible mediator of induced insulin resistance. Am J Physiol Endocrinol Metab. 2001;280:E562-9. PubMed PMID: 11254462.

24 Young AA, Gedulin B, Gaeta LS, Prickett KS, Beaumont K, Larson E, et al. Selective amylin antagonist suppresses rise in plasma lactate after intravenous glucose in the rat. Evidence for a metabolic role of endogenous amylin. FEBS Lett. 1994;343:237-41. PubMed PMID: 8174707.

25 McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, et al. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. Nature. 1998;393:333-9. PubMed PMID: 9620797.

26 Kuwasako K, Cao YN, Nagoshi Y, Tsuruda T, Kitamura K, Eto T. Characterization of the human calcitonin gene-related peptide receptor subtypes associated with receptor activity-modifying proteins. Mol Pharmacol. 2004;65:207-13. doi: 10.1124/mol.65.1.207. PubMed PMID: 14722252.

27 Hay DL, Conner AC, Howitt SG, Takhshid MA, Simms J, Mahmoud K, et al. The pharmacology of CGRP-responsive receptors in cultured and transfected cells. Peptides. 2004;25:2019-26. PubMed PMID: 15501536.

28 Deems RO, Cardinaux F, Deacon RW, Young DA. Amylin or CGRP (8-37) fragments reverse amylin-induced inhibition of 14C-glycogen accumulation. Biochem Biophys Res Commun. 1991;181:116-20. doi: 10.1016/S0006-291X(05)81389-0. PubMed PMID: 1958178.

29 Cornish J, Callon KE, Lin CQ, Xiao CL, Gamble GD, Cooper GJ, et al. Comparison of the effects of calcitonin gene-related peptide and amylin on osteoblasts. J Bone Miner Res. 1999;14:1302-9. doi: 10.1359/jbmr.1999.14.8.1302. PubMed PMID: 10457262.

30 Hökfelt T, Arvidsson U, Ceccatelli S, Cortés R, Cullheim S, Dagerlind A, et al. Calcitonin gene-related peptide in the brain, spinal cord, and some peripheral systems. Ann NY Acad Sci. 1992;657:119-34. PubMed PMID: 1637079.

31 Takhshid MA, Poyner DR, Chabot JG, Fournier A, Ma W, Zheng WH, et al. Characterization and effects on cAMP accumulation of adrenomedullin and calcitonin gene-related peptide (CGRP) receptors in dissociated rat spinal cord cell culture. Br J Pharmacol. 2006;148:459-68. doi: 10.1038/sj.bjp.0706750. PubMed PMID: 16702994; PubMed Central PMCID: PMC1751784.

32 Takhshid MA, Owji AA, Vasei M, Panjehshahin MR, Tabei SM, Tabatabaei HR, et al. Expression of spinal cord Fos protein in response to intrathecal adrenomedullin and CGRP in conscious rats. Brain Res. 2004;1020:30-6. doi: 10.1016/j.brainres.2004.05.112. PubMed PMID: 15312784.

33 Coggeshall RE. Fos, nociception and the dorsal horn. Prog Neurobiol. 2005;77:299-352. doi: 10.1016/j.pneurobio.2005.11.002. PubMed PMID: 16356622.

34 Yaksh TL, Rudy TA. Chronic catheterization of the spinal subarachnoid space. Physiol Behav. 1976;17:1031-6. doi: 1 10.1016/0031-9384(76)90029-9. PubMed PMID: 14677603.

35 Harris JA. Using c-fos as a neural marker of pain. Brain Res Bull. 1998;45:1-8. doi: 10.1016/S0361-9230(97)00277-3. PubMed PMID: 9434195.
36 Molander C, Xu Q, Grant G. The cytoarchitectonic organization of the spinal cord in the rat. I. The lower thoracic and lumbosacral cord. J Comp Neurol. 1984;230:133-41. doi: 10.1002/cne.902300112. PubMed. PMID: 6512014.

37 Christopoulos G, Perry KJ, Morfis M, Tilakaratne N, Gao Y, Fraser NJ, et al. Multiple amylin receptors arise from receptor activity-modifying protein interaction with the calcitonin receptor gene product. Mol Pharmacol. 1999;56:235-42. PubMed. PMID: 10385705.

38 Poyner DR, Soomets U, Howitt SG, Langel U. Structural determinants for binding to CGRP receptors expressed by human SK-N-MC and Col 29 cells: studies with chimeric and other peptides. Br J Pharmacol. 1998;124:1659-66. doi: 10.1038/sj.bjp.0702032. PubMed PMID: 9756381; PubMed Central PMCID: PMC1565576.

39 Bailey RJ, Hay DL. Pharmacology of the human CGRP1 receptor in Cos 7 cells. Peptides. 2006;27:1367-75. doi: 10.1016/j.peptides.2005.11.014. PubMed PMID: 16375989.

40 Bailey RJ, Walker CS, Ferner AH, Loomes KM, Prijic G, Halim A, et al. Pharmacological characterization of rat amylin receptors: implications for the identification of amylin receptor subtypes. Br J Pharmacol. 2012;166:151-67. doi: 10.1111/j.1476-5381.2011.01717.x. PubMed PMID: 22014233; PubMed Central PMCID: PMC3415645.