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Research Report

# An immunohistochemical study on a unique colocalization relationship between substance P and GABA in the central nucleus of amygdala

*Naoki Shigematsu*[a,b,1], *Kenji Yamamoto*[b], *Shun Higuchi*[a], *Takaichi Fukuda*[c,*]

[a]*Clinical Pharmacokinetics, Division of Clinical Pharmacy, Department of Medico-Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582, Japan*
[b]*Department of Pharmacology, Graduate School of Dental Science, Faculty of Dental Science, Kyushu University, Fukuoka 812-8582, Japan*
[c]*Department of Anatomy and Neurobiology, Graduate School of Medical Sciences, Kyushu University, Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*

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ABSTRACT

Substance P (SP) is a neuropeptide contained in axon terminals. Various classical neurotransmitters coexist with SP in mammalian brains, but there has been no information on the colocalizing substances in the central nucleus of amygdala (CeA), where both SP and its specific receptor are highly concentrated. The present study aimed at determining the colocalizing neurotransmitter in SP terminals in CeA by multi-label immunohistochemistry combined with digitized quantitative analysis. Unexpectedly, most of SP-containing boutons did not show immunoreactivities for any of the transmitters or their marker proteins examined (GABA, glycine, glutamate, acetylcholine, serotonin, or dopamine). Electron microscopy demonstrated small clear vesicles in addition to dense core vesicles within SP-positive terminals that formed symmetrical synapses, indicating the presence of some classical neurotransmitter, most likely GABA. Therefore tissues were fixed by zinc–aldehyde to enhance immunoreactivity for a low level of glutamic acid decarboxylase (GAD), the GABA synthetic enzyme. This led to weak but consistent labeling for GAD in the majority of SP-positive boutons in CeA. By contrast, definite GAD-immunoreactivity was confirmed in SP-containing boutons in the substantia nigra pars reticulata even in specimens treated with a conventional fixative, indicating that negligible GAD labeling in CeA is not ascribed to methodological problems such as interference by the presence of SP but actually reflects low GAD content. These data suggest a unique mode of synaptic transmission at amygdalar SP-containing terminals where slowly-acting SP is concentrated but both GABA and its synthetic enzyme are maintained at low levels, possibly underlying long-lasting responses in emotions.



* *Corresponding author*. Fax: +81 92 642 6059.
E-mail address: fukuda@a3rd.med.kyushu-u.ac.jp (T. Fukuda).

*Abbreviations:* AchE, acetylcholine esterase; BLA, basolateral nucleus; BSA, bovine serum albumin; CeA, central nucleus of amygdala; CeC, capsular division of CeA; CeL, lateral division of CeA; CeM, medial division of CeA; CLSM, confocal laser scanning microscopy; DAB, 3,3′-diaminobenzidine tetrahydrochloride; GABA, gamma amino butyric acid; GAD, glutamic acid decarboxylase; MeA, medial nucleus of amygdala; NK-1, neurokinin-1; PB, phosphate buffer; PBS, phosphate buffered saline; SERT, serotonin transporter; SP, substance P; SN, substantia nigra; TH, tyrosine hydroxylase; vAChT, vesicular acetylcholine transporter; vGluT, vesicular glutamate transporter

[1] N. Shigematsu's present address: Division of Cerebral Circuitry, National Institute for Physiological Sciences, Myodaiji, Okazaki 444-8787, Japan.

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## 1. Introduction

Substance P (SP) is an undeca-neuropeptide processed from its precursor protein prerprotachykinin-A (Nawa et al., 1983; Kawaguchi et al., 1986). Cleavage of prerprotachykinin-A produces three other peptides, neurokinin A, neuropeptide K, and neuropeptide γ, as well as SP. All these peptides, termed tachykinins, are thought to act as neuromodulators involved in slow and long-lasting modification of synaptic transmission. Effects of each tachykinin on neural systems have been investigated usually through analysis of the response to that tachykinin or its agonist administered as single reagents at different levels from cells in vitro to behaving animals (Elliott, 1988; Reid et al., 1990; Krase et al., 1994; Aguiar and Brandão, 1996; Kramer et al., 1998; Maubach et al., 2001; Stacey et al., 2002; Ogier and Raggenbass, 2003). However, it is uncertain whether tachykinin alone is released and engaged in synaptic response within the intact brain as if it were in an experimental setting. Therefore, for better understanding of a functional role of SP in a particular neuronal circuit, it is essential to know what kind of classical neurotransmitter (amino acids, monoamines, and acetylcholine) colocalizes in SP terminals. Surprisingly, this fundamental issue has been addressed in limited regions: glutamate was detected in SP-containing terminals in the spinal cord (De Biasi and Rustioni, 1988; Merighi et al., 1991), and GABA was demonstrated in SP terminals in the substantia nigra (Bolam and Smith, 1990) and neocortex (Jakab et al., 1997). In other areas colocalization is known at the somatic level, e.g. SP is contained in serotonin neurons in the lower medulla oblongata (Hökfelt et al., 1978), in cholinergic neurons in the pons (Vincent et al., 1983), and in norepinephrine neurons in the ventrolateral medulla (Lorenz et al., 1985). It is possible to deduce colocalizing transmitters at terminals when areas targeted by these neurons are considered, but this provides only indirect information.

In the present study we investigated colocalization of SP and various neurotransmitters in the central nucleus of amygdala (CeA). This subnucleus works as an output device of amygdala, sends information processed in the amygdala to hypothalamus and brain stem nuclei, thereby mediates expression of emotion (Davis, 1998; LeDoux, 2000). CeA has been also related to pathological conditions such as depression. Selective antagonists to SP-specific receptor (NK-1) have antidepressant effects (Kramer et al., 1998). Though both SP (Nilsson et al., 1974; Emson et al., 1978) and NK-1 (Mantyh et al., 1989; Nakaya et al., 1994) are highly concentrated in CeA, to date there has been no information as to which classical neurotransmitter colocalizes in SP-containing terminals there. Therefore, determining the co-expressing transmitters will provide clues not only to mechanisms through which SP affects signal transmission but also to physiological as well as pathological roles of SP in behaviors.

As transmitters colocalizing in SP terminals differ considerably among regions, it is necessary to examine a wide

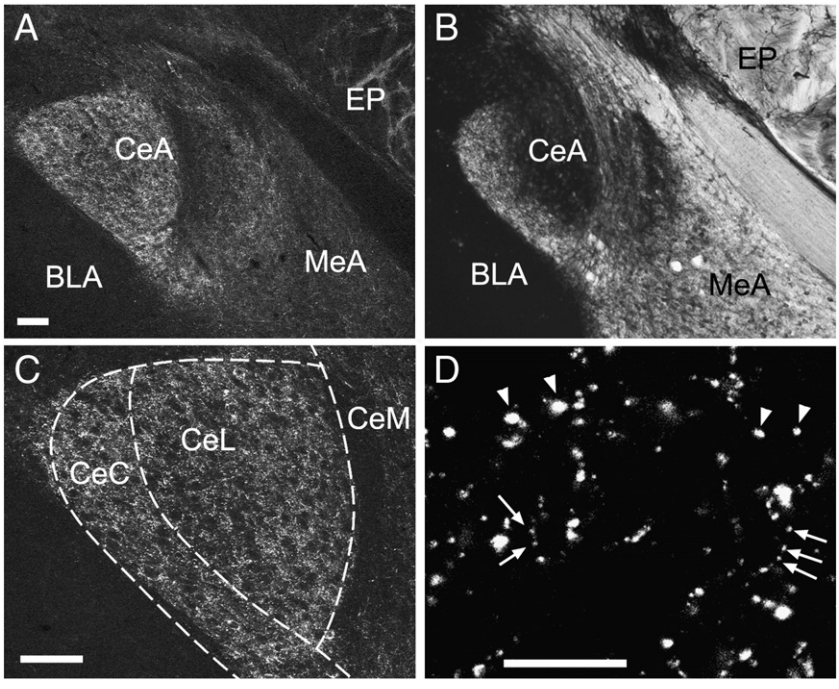


**Fig. 1 – SP-immunoreactivity in the amygdala. (A) The immunoreactivity is most conspicuous in CeA and moderate in MeA, but hardly observable in BLA, with a sharp boundary between CeA and BLA. (B) Acetylcholine esterase staining in a section next to (A) delineates CeA and surrounding areas. (C) Comparison of SP-immunoreactivity among three subdivisions (CeC, CeL, CeM) of CeA. Dotted lines indicate the border of subnuclei of CeA according to acetylcholine esterase staining. (D) High magnification image in CeL. SP-immunoreactivity is seen in either boutons of medium to large size (arrowheads) or an array of much smaller puncta (arrows). Scale bars = 100 μm (A–C) and 10 μm (D).**