IMMUNOCYTOCHEMICAL LOCALIZATION OF GLUTAMIC ACID DECARBOXYLASE (GAD) AND SUBSTANCE P IN NEURAL AREAS MEDIATING MOTION-INDUCED EMESIS. EFFECTS OF VAGAL STIMULATION ON GAD IMMUNOREACTIVITY


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Abstract

Immunocytochemical methods were employed to localize the neurotransmitter amino acid γ-aminobutyric acid (GABA) by means of its biosynthetic enzyme glutamic acid decarboxylase (GAD) and the neuropeptide substance P in the area postrema (AP), area subpostrema (ASP), nucleus of the tractus solitarius (NTS) and gelatinous nucleus (GEL). In addition, electrical stimulation was applied to the right vagus nerve at the cervical level to assess the effects on GAD-immunoreactivity (GAD-IR).

GABA: GAD-IR terminals and fibers were observed in the AP, ASP, NTS and GEL. They showed pronounced density at the level of the ASP and gradual decrease towards the solitary complex. Nerve cells were not labelled in our preparations. Ultrastructural studies showed symmetric or asymmetric synaptic contacts between labelled terminals and non-immunoreactive dendrites, axons or neurons. Some of the labelled terminals contained both clear- and dense-core vesicles. Our preliminary findings, after electrical stimulation of the vagus nerve, revealed a bilateral decrease of GAD-IR that was particularly evident at the level of the ASP.

Substance P: SP-immunoreactive (SP-IR) terminals and fibers showed varying densities in the AP, ASP, NTS and GEL. In our preparations, the lateral subdivision of the NTS showed the greatest accumulation. The ASP showed medium density of immunoreactive varicosities and terminals and the AP and GEL

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displayed scattered varicose axon terminals. The electron microscopy revealed that all immunoreactive terminals contained clear-core and dense-core vesicles which made symmetric or asymmetric synaptic contact with unlabeled dendrites.

It is suggested that the GABAergic terminals might correspond to vagal afferent projections and that GAD/GABA and substance P might be co-localized in the same terminal allowing the possibility of a regulated release of the transmitters in relation to demands.

Introduction

The present report is part of a study designed to investigate the interaction between neuropeptides and conventional neurotransmitters under conditions producing motion sickness and in the process of sensory-motor adaptation.

A vast amount of literature has dealt with the cytoarchitectural organization and ultrastructural analysis of the area postrema (AP), area subpostrema (ASP), nucleus of the tractus solitarius (NTS) and gelatinous nucleus (GEL), all structures localized in the dorsal part of the medulla oblongata (e.g., Olszewski and Baxter, 1954; Taber, 1961; Gwyn and Wolstencroft, 1968; Klara and Brizzee, 1975, 1977; Chernicky et al., 1980; D'Amelio et al., 1986). Anatomical studies have provided details of their somatotopic organization in relation to visceral afferents and physiological findings have demonstrated their involvement in a variety of autonomic functions (e.g., von Euler et al., 1973; Gwyn et al., 1979; Gwyn and Leslie, 1979; Katz and Karten, 1979; Gale et al., 1980; Hamilton and Gillis, 1980; Helke et al., 1980; Kalia and Mesulam, 1980a, b; Panneton and Loewy, 1980; Cirillo et al., 1981; Kalia, 1981; Kalia and Sullivan, 1982; Helke, 1982). Neurotransmitters such as GABA, catecholamines, neuropeptides and serotonin have been identified by immunocytochemical procedures (e.g., Armstrong et al., 1981, 1982a, b; Maley and Elde, 1982; Maley et al., 1983; Kalia et al., 1984; Maley, 1985; Maley and Newton, 1985; Newton et al., 1985; D'Amelio et al., 1987; Maley et al., 1986; Newton and Maley, 1987; Nomura et al., 1987) and in some cases, synaptic interactions between neurotransmitters have been established (Pickel et al., 1979, 1984; Kubota et al., 1985). By combining autoradiography and immunocytochemistry, Sumal et al. (1983) reported synaptic interactions between vagal afferents and catecholaminergic neurons in the NTS of the rat. Gial fibrillary acidic protein and glutamine synthetase were identified in the gloiopendymal cells and astrocytes of the cat AP (D'Amelio et al., 1985, 1987). The relevance of the AP as the emetic chemoreceptor trigger zone has been corroborated (Borison and Brizzee, 1951; Carpenter et al., 1983; Borison et al., 1984) and evidence of its participation in the emetic response to motion has also been reported (Wang and Chinn, 1952, 1954; Brizzee et al., 1980; Crampion and Daunton, 1984).

In this report we will describe the light microscopic distribution and ultrastructural appearance of GAD- and SP-immunoreactivity, the preliminary observations on the effects of electrical stimulation of the vagus nerve on GAD-IR and discuss some of our views with respect to the relationship between neurotransmitter action and distribution pattern and degree of density of the immunoreactive structures.
Immunocytochemical Localization of GAD and Substance P

Material and Methods

Animals

Adult cats were employed for this study. They were housed in air-conditioned rooms and given regular dry pellet cat food and water ad libitum.

Antisera

The well characterized GAD-antiserum (code #P3) was kindly provided by Dr. Jang-Yen Wu (Pennsylvania State University, Hershey Medical Center, Hershey, PA) (for review, see Wu et al., 1982).

The monoclonal antiserum for substance P was obtained from Pel-Freeze Biologicals, code MAS 035b.

Immunocytochemical Procedures

The peroxidase-antiperoxidase method developed by Sternberger (1979) was employed to visualize the immunoreactivity of both GAD and SP. Dilution of antisera was 1:1000. The details of the general procedures concerning fixation of the brain and treatment of the sections have been previously published (D'Amelio et al., 1987).

Electrical Stimulation of the Vagus Nerve

The animals were tranquilized with an intramuscular injection of 0.5 ml ketamine HCl, after which they were anesthetized with an intravenous injection of sodium pentobarbital (30-35 mg/kg). The right cervical vagus nerve was exposed between the branching points of the superior pharyngeal and the recurrent nerves. Bipolar electrodes were positioned on the nerve and biphasic square-wave pulses of 0.6 sec duration were applied at 1-10 ma and 60 Hz. The current was steadily increased from 1 ma to 10 ma to determine a threshold response from the nerve. The threshold response was hyperventilation as observed visually or recorded on a polygraph via a respirometer. The nerve was stimulated continuously for a total duration of one hour. During the last 5 minutes of stimulation the thoracic cavity was opened and the perfusion procedure via the heart was started (see D'Amelio et al., 1987).

Results

GAD Immunocytochemistry

The details of GAD-immunoreactivity in the AP, ASP, NTS and GEL have been published elsewhere (D'Amelio et al., 1987). Briefly, the light microscopic examination revealed variable degree of density of the GAD-IR terminals and fibers along the rostrocaudal axis. Their distribution is exemplified in Figures 1A, B and C.

It is obvious that the ASP is distinguished from the AP and NTS by its high concentration of GAD-IR pre-terminal fibers and boutons which are seen at all levels examined. No labelled neurons were observed in our preparations.
Fig. 1. A: Rostral segment at the level of the area postrema (AP). The area subpostrema (ASP) is distinguished by the high density of GAD-IR terminals. The decrease in density is evident towards the gelatinous nucleus (GEL). B: GAD-IR is present in the lateral sub-division of the nucleus of the tractus solitarius (NTS). The medial region of the nucleus (M) shows lighter immunoreactivity. C: Medial segment of the AP. Patches of GAD-IR terminals are visible throughout the AP. The high density of the ASP is also apparent at this level. D: Medial segment of the AP. In both the AP and ASP there is extreme depletion of immunoreactivity after electrical stimulation. Only scattered GAD-IR immunoreactive boutons are visible in the ASP. IV, fourth ventricle; T, solitary tract. Magnifications: A, x150; B, C and D, x250.

The ultrastructural study demonstrated that the GAD-IR boutons corresponded to immunoreactive terminals with occasional staining of the pre-terminal segment. The immunoprecipitate outlined clear synaptic vesicles, mitochondria and the inner surface of the plasma membrane. Many of the profiles contained dense-core vesicles in variable number. Synaptic contacts, either symmetric or asymmetric were observed between labelled terminals and unlabelled dendrites, axons or neurons (Fig. 2).

The most ostensible finding of our preliminary observations after electrical stimulation of the vagus nerve was a noticeable bilateral decrease in density of the GAD-IR terminals of the ASP. In non-stimulated animals the density of these terminals was clearly higher in ASP than in the underlying structures. The decrease with stimulation seemed to involve all levels
Fig. 2. A: Symmetric synaptic contact (arrow) between a GAD-IR axon profile and an unlabelled axon containing clear-core vesicles and scattered dense-core vesicles (arrowhead). B: GAD-IR profile forms a long symmetric contact with a dendrite (D). C: A GAD-IR axon terminal which contains clear-core vesicles and a few dense-core vesicles forms symmetric contact with a dendrite (arrowhead). In close apposition to the immunoreactive terminal is seen a non-immunoreactive axon profile forming an asymmetric contact with the same dendrite (arrow). D: Symmetric contact (arrow) between a GAD-IR axon profile containing both clear- and dense-core vesicles and a dendrite. E: Asymmetric (arrow) and symmetric (arrowhead) contacts between two GAD-IR terminals with the same dendrite (D). F: GAD-IR pre-terminal segment and bouton. Several dense-core vesicles are seen in addition to the clear-core ones. Magnifications: A, x16,000; B, x25,000; C and D, x20,500; E and F, x25,000.
of the ASP (Fig. 1D). The level of GAD-IR in the AP, NTS and GEL also showed a decrease in stimulated animals, with some inter-animal variation in the different regions. This finding has to be evaluated with further quantitative assessment.

Substance P Immunocytochemistry

The pattern of immunoreactivity of this neuropeptide within the AP, ASP and NTS is largely consistent, with some variations, with that found by other investigators (Maley and Elde, 1982; Newton et al., 1984). In the ventromedial part of the ASP, SP-IR punctate structures and varicose axons appeared to be more abundant than in the dorsolateral region. In the AP, NTS and GEL, varying densities of immunoreactivity were found along the rostrocaudal axis, arranged into aggregates of ill-defined boundaries. The AP proper contained mainly varicose terminals, randomly distributed. In our preparations the NTS exhibited labelled terminals and varicosities in all topographical subdivisions with distinct pronounced density in the lateral subdivision. We did not find labelled neurons in any of the regions under study (Fig. 3).

We concentrated our electron microscopic studies mainly on the AP and ASP. The most significant finding was that all the immunoreactive terminals contained dense-core vesicles in variable number (2-10) together with clear-core vesicles bound by immunoprecipitate. The majority of the dense-core vesicles showed immunoreactivity. The synaptic contacts were mainly between labelled axons and dendrites, either symmetric or asymmetric (Fig. 4).
Fig. 4. A: SP-IR immunoreactive bouton in which the dense-core vesicles are markedly immunoreactive, except for one (arrowhead). B, C and D: Symmetric synaptic contacts between SP-IR terminals and unlabelled dendrites (arrowheads). E: Two SP-IR terminals, one weakly labelled (arrowhead) and the other strongly immunoreactive (arrow), forming symmetric contacts with the same dendritic profile. F: Two axon terminals, one SP-IR and one unlabelled (Ax), in close apposition. Magnifications: A, x25,000; B and C, x20,500; D, x16,000; E and F, x20,500.
Discussion

It is our contention that extreme caution should be applied in assessing the distribution and ‘mapping’ of neurotransmitters in the central nervous system by means of immunocytochemical techniques. It is frequently neglected that the difference in immunocytochemical images in various areas of the brain obtained by different investigators is, in many instances, not the product of methodological procedures, source or sensitivity of antiserum, etc., but of the dynamic character of intercellular signaling among neurons, which also frequently accounts for inter-animal variation. This signaling is the reflection of the actual ‘motion’ of neurotransmitters within a functional system in response to external (environmental) or internal (homeostatic changes) conditions which in turn might affect the rate of biosynthesis of the transmitter or its precursor and hence its release. In consequence, we prefer to consider the distribution of a particular neurotransmitter as ‘provisional’ and rely upon procedures such as tract-tracing methods, autoradiography, and physiological methods, combined with ultrastructural and light microscopic immunocytochemistry, to try to define communication lines among neural regions.

Following this line of argument for the region under study, we think that the distribution pattern of GABAergic terminals in the AP, NTS, ASP, and gelatinous nucleus, closely resembles that of vagal afferent projections found by means of horseradish peroxidase (HRP) injections of the proximal cut ends of the vagus nerves (Ciriello et al., 1981), following HRP or [3H]leucine injections of the nodose ganglion (Gwyn et al., 1979) and with the use of degeneration methods after the removal of the nodose ganglion (Gwyn and Leslie, 1979). Furthermore, our preliminary findings of the depletion of GAD-IR in those areas after electrical stimulation of the vagus nerve seem to confirm that at least part of the GABAergic projections correspond to vagal afferents. The bilaterality of the GAD-immunoreactivity depletion seems also to coincide with the tract-tracing studies of Kalia and Mesulam (1980), who found bilateral sensory labelling of the AP and NTS after HRP injections of the right nodose ganglion. There also appears to be evidence that the depletion in GAD-IR is not due to a widespread effect of the vagal stimulation, since some areas of the histological sections, e.g., the fetal nucleus of the inferior olive, show prominent GAD-IR in both non-stimulated and stimulated cats. As for the causes of GAD-IR depletion, it is an early stage in this research to attempt an explanation, since the analysis of sub-cellular and molecular mechanisms has not been initiated.

With respect to SP-IR in the AP, NTS and ASP, it is interesting to notice that although the density gradients differ from those of GAD-IR, they follow a similar pattern of localization, with the lateral sub-division of the NTS showing the greatest accumulation of SP-IR terminals and fibers. At this point, and in keeping with the opening remarks of our discussion, it is important to emphasize that it is not density, considered in a rigid context, of immunoreactive fibers and terminals that is expected to provide meaningful data to assess the functional significance of neurochemical phenomena in a given area of the nervous system. For example, in previous studies dealing with the immunoreactivity of substance P in the NTS of the cat (Maley and Elde, 1982) and Rhesus monkey (Maley et al., 1987), it was reported that the respiratory subdivisions displayed a low level of immunoreactivity. These findings led the researchers to speculate that substance P does not play a major role in the mediation of respiratory functions. However, measurements of substance P by microdialysis
in the cat NTS (Lindefors et al., 1986) and microinjections of substance P in the NTS of the
rat (Carter and Lightman, 1985) have supplied significant evidence for the relevance of
substance P in respiratory functions. In our opinion, the significance of the presence of a
neurotransmitter within a particular structure will not be properly understood until its source
and synaptic relations with other functional systems are clearly defined.

One feature that deserves to be stressed is the presence in all SP-IR terminals of dense-core vesicles, a characteristic already shown in the NTS and other regions of the nervous system (Maley, 1985; Pickel et al., 1979). The possibility of peptide storage by those vesicles has been suggested by Pickel et al. (1979). Interestingly, many GAD-IR terminals also contain dense core vesicles in addition to the clear-core ones. This observation suggests that both messengers, GABA and substance P, may coexist within the same terminal, as has been previously reported for other areas of the nervous system. For example, 95-98% of SP-IR cortical neurons have been found to be also immunoreactive for GABA and GAD (Jones and Hendry, 1986). Additional observations account for the possibility of such co-existence. Immunocytochemical studies of substance P in other species have shown its presence within the neurons of the nodose ganglion, which is known to send sensory projections to the solitary complex and AP (Katz and Karten, 1980). Since, according to our observations and those of others, both GAD and substance P are consistently present in those regions, it is reasonable to assume that the sensory cells of the nodose ganglion might regulate the genetic expression of GABA, substance P and their biosynthetic enzymes. Our own preliminary studies after infra-nodose electrical stimulation of the vagus nerve provide further support to this hypothesis. Naturally, the possibility of the existence of separate neuronal populations in the nodose ganglion expressing either GAD/GABA or substance P, cannot be excluded.

The co-existence of both neuronal messengers in fibers and terminals of the region under
study would once again demonstrate the extensive scope of the transmission process and the
adaptive capacities of brain circuitry, with variable and regulated responses for the release
of a neurotransmitter (or neuromodulator) according to the imposition of a given stimulus.

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