Peptide Expression in GABAergic Neurons in Rat Suprachiasmatic Nucleus in Comparison with Other Forebrain Structures: A Double Labeling In Situ Hybridization Study

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SUMMARY We investigated the characteristics of GABAergic neurons in the rat suprachiasmatic nucleus (SCN) in normal untreated rats by examination of co-expressed peptides. We adopted double labeling in situ hybridization using a digoxigenin-labeled glutamic acid decarboxylase (GAD) riboprobe and 35S-labeled peptide riboprobes. GAD mRNA-positive neurons were distributed throughout the SCN from the rostral to the caudal pole. In the dorsomedial part of the SCN, most GAD mRNA-positive neurons co-expressed arginine vasopressin mRNA. In the ventrolateral part of the SCN, about two thirds of GAD mRNA-positive neurons co-expressed vasoactive intestinal peptide (VIP) mRNA. Co-expression of GAD and somatostatin mRNA was observed in virtually all neurons of the intermediate part of the SCN. In contrast, these peptidergic traits were poorly expressed in hypothalamic GABAergic neurons outside the SCN. Vasopressin mRNA-positive cells in the supraoptic nucleus did not express GAD mRNA, and co-expression of somatostatin mRNA and GAD mRNA was rare in the periventricular hypothalamic nucleus. Similarly, the VIP mRNA co-expression ratio of GABAergic neurons in the cerebral cortex was far lower than that in the SCN. (J Histochem Cytochem 45:1231–1237, 1997)

**KEY WORDS**

- glutamic acid decarboxylase
- arginine vasopressin
- vasoactive intestinal peptide
- somatostatin
- mRNA
- suprachiasmatic nucleus

Recent in vivo pharmacological studies indicated the involvement of GABA into SCN function. Smith et al. (1989, 1990) demonstrated that application of muscimol, a GABA_A receptor agonist, in the SCN causes a phase shift of the circadian locomotor rhythm. Ralph and Menaker (1989) reported that light-induced phase shifts of the circadian locomotor rhythm are blocked by benzodiazepine, a GABA_A agonist, and baclofen, a GABA_B agonist. These results indicate a modulatory action of GABA in the SCN on the circadian time-keeping system.

The SCN is composed of many peptidergic neurons exhibiting distinct intranuclear topography. Among these, arginine vasopressin (AVP)-containing neurons are located in the dorsomedial part and vasoactive intestinal peptide (VIP)-containing neurons are found in the ventrolateral part of this nucleus (van den Pol and Tsujimoto 1985; Okamura et al. 1987). Daily expression rhythms of these peptides in the SCN were different between these subdivisions. AVP in the dorsome-
dial part showed a light-independent endogenous rhythm (Schwartz et al. 1983; Uhl and Reppert 1986), and VIP in the ventrolateral part showed light-dependent changes (Takahashi et al. 1989; Albers et al. 1990; Okamoto et al. 1991). Somatostatin (SS) expression in neurons located in the bordering region of ventrolateral and dorsomedial components shows a light-independent endogenous rhythm (Fukuhara et al. 1993; Nishiwaki et al. 1995). Previously, we reported that GABAergic neurons are located in both subdivisions of the SCN, as shown by GAD in situ hybridization, GAD immunohistochemistry, and GABA immunocytochemistry (Okamura et al. 1989). Recently, M. oore and Speh (1993) directly demonstrated that some SCN neurons co-express GABA immunoreactivity with AVP and VIP immunoreactivities in colchicine-pretreated rats. This finding is interesting because it suggests that two main peptidergic components of the SCN with different daily rhythmic expression patterns are GABAergic and therefore may account for complex GABAergic modulation of circadian rhythm (Ralph and Menaker 1985, 1989).

To investigate the expression characteristics of GABAergic neurons in the SCN of normal untreated rats, we examined the expression of various peptides at the mRNA level by double labeling in situ hybridization using digoxigenin- and radioisotope-labeled antisense probes. This method requires no pretreatment with drugs because the bulk of mRNA is confined to the cell bodies. Moreover, we also investigated peptidergic and GABAergic neurons in the neighboring hypothalamus and cortex for comparison.

Materials and Methods

Probes

Probes used for hybridization histochemistry were complementary RNA probes synthesized from cDNA fragments by transcription. AVP cDNA (195 base pairs (bp)), VIP cDNA (697 bp), SS cDNA (450 bp), and GAD 67 cDNA (482 bp) were gifts from Prof. J. P. H. Burbach (Utrecht), Prof. H. Okamoto (Sendai), Prof. R.H. Goodman (Portland), and Profs. J. F. Julien and J. Mallet (Paris), respectively. Anti-sense RNA probes for rat AVP, rat SS, and rat GAD 67 mRNA were synthesized from respective cDNAs subcloned into plasmid vectors with promoters for sense and anti-sense cRNA construction. The VIP cRNA probe used in the present study was synthesized from a 697-bp VIP precursor cDNA fragment (99–795) encoding both peptide histidine isoleucine amide (PHI) (241–321) and VIP (373–456). Transcription was carried out in a reaction mixture containing templates and T7 RNA polymerase in the presence of [α-32P]-CTP (370 MBq/ml; NEN-DuPont, Wilmington, DE) for Northern blotting analysis and [35S]-CTP (2.59 GBq/ml; NEN-DuPont) for in situ hybridization. For the GAD67 cDNA probe used for in situ hybridization, transcription was carried out with a digoxigenin RNA labeling kit (Boehringer; Mannheim, Germany). Briefly, 1 μg of linearized template DNA was incubated for 1 hr at 37C in a buffer containing 10 mM dithiothreitol (DTT), 1 mM GTP, ATP, and CTP, 0.65 mM UTP, 0.35 mM digoxigenin-11-UTP, 20 U of RNAse inhibitor, and 120 U of T7 RNA polymerase.

Sense RNA probes of the same length as each anti-sense probe were also synthesized to determine specificity. No specific hybridization was observed in sections using sense probes.

Animals

Male albino Wistar rats weighing 200–250 g were housed under a 12 hr:12 hr light:dark (LD) cycle for at least 1 week and were given free access to food and water. Rats were sacrificed at Zeitgeber time (ZT) 4.

In Situ Hybridization Histochemistry

Total RNA was isolated from hypothalamic homogenates by guanidium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi 1987). Total RNA (10 μg/lane) was electrophoresed in 1% agarose gels and transferred onto nylon membranes (Gene Screen Plus; NEN). Hybridization with [32P]-α-CTP-labeled cRNA probes was performed in a mixture of 5 × saline-sodium phosphate-EDTA (SSPE: 0.75 M NaCl, 5 mM EDTA, 50 mM Na2HPO4, pH 7.4), 5 × Denhardt’s solution, 50% formamide, and 1% sodium dodecyl sulfate (SDS) at 60C for 16 hr. After washing in a solution containing 2 × SSPE and 2% SDS at 65 C for 1 hr, the membranes were transferred into RNase solution (20 μg/ml RNase A, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 M NaCl) for 30 min at 37C. After washing briefly in a solution of 0.2 × SSPE and 0.1% SDS at room temperature (RT), the membranes were processed for autoradiography using Hyperfilm-β-M ax (Amersham; Poole, UK) for about 1 week.

Under deep pentobarbital anesthesia, rats were perfused through the left cardiac ventricle with 150 ml of ice-cold saline followed by 200 ml of a fixative containing 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). The brains were immediately removed and postfixed in the same fixative for 3 h at 4C. After immersion of brains in 20% sucrose in 0.1 M PB for 24 hr, coronal sections (15 μm in thickness) of the hypothalamus were cut by a cryostat and collected in 4 × saline-sodium citrate (SSC: 0.6 M NaCl, 60 mM Na3 citrate, pH 7.0). Thereafter, the sections were treated under free-floating conditions with various media. The sections were deproteinized with proteinase K for 15 min and the reaction was stopped by 4% PFA in 0.1 M PB. After rinsing briefly in 4 × SSC, sections were acetylated in saline containing 0.25% acetic anhydride and 0.1 M triethanolamine for 10 min at RT. Then sections were incubated in hybridization buffer (50% formamide, 10% dextran sulfate, 20 mM Tris-HCl, 5 mM EDTA, 0.3 M NaCl, 0.2% SDS, 500 μg/ml yeast tRNA, 1 × Denhardt’s solution, 10 mM DTT) containing both digoxigenin-UTP-labeled GAD cRNA probes and isotopelabeled peptide probes for 12 hr at 60C. For detection of peptide neurons, we used AVP, VIP, or SS cRNA probes. After rinsing twice in 2 × SSC / 50% formamide and once in 0.5 × SSC at 60C, sections were trans-
ferred into Buffer 1 (100 mM Tris-HCl, pH 7.5, 150 mM NaCl). They were then incubated in 0.5% blocking reagent for 60 min and with alkaline phosphatase-labeled anti-digoxigenin antibody (dilution 1: 500 in Buffer 1) for 30 min at RT. After washing in Buffer 1, the sections were reacted in a coloring solution containing 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate in a buffer consisting of 100 mM Tris-HCl, pH 9.5, 10 mM NaCl, and 50 mM MgCl$_2$ for 12–18 hr at RT. The sections were mounted on glass slides, dehydrated through a graded alcohol series, and air-dried. Slides were dipped in emulsion (Ilford K5) and exposed for 10 days. The emulsion-coated slides were developed with Kodak D19 and fixed. By light microscopy, we examined the co-localization of blue reaction products and silver grains. We used the five middle level sections of the SCN of five rats and counted the number of silver grains at a magnification of 10 (contact lens) x 100 (objective lens) in 200–300 neurons per rat by visual inspection. The workers counting the silver grains were unaware of the results of peptide mRNA counting. Peptide mRNA signals were counted as positive when a more than threefold increase in number of grains was observed compared to cells in the neighboring preoptic area. Co-expression ratio was calculated as mean ± SD.

**Results**

**Northern Blotting Hybridization Using cRNA Probes**

Northern blotting analysis of hypothalamic RNA with $^{32}$P-labeled SS, AVP, VIP, and GAD cRNA probes revealed hybridization-positive RNA bands of different sizes (Figure 1): approximately 600 bases for SS, 800 bases for AVP, 2100 bases for VIP, and 4000 bases for GAD, which were consistent with the reported sizes of each precursor mRNA (Montminy et al. 1984; Nishizawa et al. 1985; Julien et al. 1987; Majzoub et al. 1991).

**Localization of GAD mRNA in the SCN Using Digoxigenin-labeled cRNA Probes**

GAD mRNA-positive neurons showed blue deposits in the cytoplasm by the digoxigenin coloring reaction. Blue reactions in hypothalamic neurons were generally weaker than those in the cerebral cortical neurons (Figures 2 and 3A, 3F, and 3J), which is consistent with the results of our previous autoradiographic study using isotope-labeled probes (Okamura et al. 1990). GAD mRNA-positive neurons were found at the highest density among the hypothalamic nuclei. Within the SCN, they were found throughout the entire region from the rostral to the caudal pole (Figures 2A–2C). The GAD mRNA-positive cells were small cells (7–10 μm in diameter), and there was a tendency for the distribution to become more dense in medial locations (Figure 2B). This intranuclear distribution gradient was similar to the distribution of cells counterstained with Cresyl violet. The intensities of GAD mRNA blue-colored deposits in neurons of the SCN and the neighboring hypothalamus were much weaker than those in the reticular thalamic neurons and in most of the cortical neurons. No hybridization signals were detected using the sense probe.

**Double Labeling In Situ Hybridization by Digoxigenin-labeled GAD cRNA and Isotope-labeled Peptide cRNA Probes**

**AVP mRNA**, detected as the accumulation of silver grains by autoradiography, was found in the dorsomedial region of the SCN (Figure 3A). VIP mRNA-positive neurons were observed in the ventrolateral region of the SCN (Figure 3D). Small numbers of SS mRNA-positive neurons were scattered in the bordering area of dorsomedial and ventrolateral subdivisions (Figure 3G). No signals were detected in control sections using sense probes of these peptide mRNAs.

Double labeling in situ hybridization histochemistry in the SCN revealed that most of the AVP mRNA-positive neurons (mean ± SD; 90.9 ± 2.69%) in the SCN co-expressed GAD mRNA (Figures 3A and 3B). In the same way, most VIP mRNA-positive neurons (94.1 ± 2.0%) (Figures 3D and 3E) and SS mRNA-positive neurons (93.0 ± 1.77%) (Figures 3G and 3H) in the SCN co-expressed GAD mRNA. The expression ratios of peptidergic traits in GABAergic neurons were also examined. Among the GABAergic neurons in all parts of the SCN, 49.6 ± 8.53% and 33.7 ± 5.53%
of GAD mRNA-positive neurons also expressed AVP and VIP, respectively (Table 1). In the dorsomedial part, defined by the area of accumulated AVP neurons (see Figure 3A), 91.0 ± 6.46% of GAD mRNA-positive neurons were also positive for AVP mRNA (Table 1). In the ventrolateral part, defined by the accumulation of VIP mRNA-positive neurons, which is consistent with the non-AVP area of the SCN (see Figure 3A), 66.7 ± 5.80% of GAD mRNA-positive neurons displayed positive signals for VIP mRNA.

In contrast to these SCN GAD mRNA neurons, these peptidergic traits were poorly expressed in GAD mRNA neurons in the neighboring hypothalamus outside the SCN. In the hypothalamic periventricular nucleus just dorsal to the SCN, GAD mRNA cells were found among the densely labeled SS neurons (Figure 3I) but their co-expression was very rare (4.82 ± 1.35%). GAD mRNA-expressing cells were prominent in the anterior hypothalamic area and tuber cinereum near the magnocellular paraventricular and supraoptic nuclei, but GAD mRNA was not found in these nuclei in neurons containing high levels of AVP mRNA (Figure 3C) (coexistence ratio 0%; no GAD mRNA cells/243 AVP mRNA-positive cells). No cells positive for VIP mRNA were found in the anterior hypothalamic area.

In the cerebral cortex, many GAD mRNA neurons and a fairly high number of SS and VIP mRNA-expressing neurons were found. We counted the SS mRNA and VIP mRNA-expressing neurons in the piriform cortex, which is the nearest cortex to the SCN. Co-expression of VIP and of SS was detected in 4.81 ± 2.10% and 23.9 ± 1.72% of GAD mRNA-expressing neurons, respectively (Figures 3F and 3J; Table 1). The VIP mRNA co-expression ratio in the cortex was lower than that in the SCN (p<0.01). In turn, VIP mRNA-expressing neurons showed co-expression of GAD mRNA at a high ratio (83.5 ± 3.50%), as did SS mRNA-expressing neurons (83.8 ± 2.11%).

**Discussion**

The double labeling in situ hybridization method using radioactive and nonradioactive probes is useful to detect cell bodies expressing two types of neurotransmitters. Because the bulk of mRNA is confined to the cell body and not to the processes for both GAD (Wuenschell et al. 1986) and peptides, this method does not require pretreatment with axonal flow-disrupting drugs such as colchicine (Dahlström, 1971), by which drug-induced phenotypic expression in normally nonexpressing neurons was recently reported (Ceccatelli et al. 1991; Kiyama and Emson 1991). However, the co-expression of mRNA does not necessarily indicate co-expression at the protein level.

By employing an in situ hybridization technique using a 35S-labeled cDNA probe, we previously demonstrated GAD mRNA-positive cells in the SCN (Okamura et al. 1989, 1990). Because positive signals were observed as the accumulation of silver grains by autoradiography, morphological analysis of the cells ex-
Figure 3 Photomicrographs of double labeling showing digoxigenin-labeled GAD mRNA and isotope-labeled AVP mRNA (A–C), VIP mRNA (D–F), and SS mRNA (G–J). (A,B,D,E,G,H) the SCN. (C) The supraoptic nucleus (SON). (F,J) The II–III layer of the piriform cortex. (I) The hypothalamic periventricular nucleus. B, E, and H are higher magnifications of A, D, and G, respectively. (A) The boundary of SCN and the border of dorso medial (DM) and ventrolateral (VL) subdivisions are shown by dotted lines. Note that blue digoxigenin-labeled cells also contain isotope labeling in the DM but not in the VL. See text for further details. OC, optic chiasm. Bars: A,C,D,G = 50 μm; B,E,H = 10 μm; F,J = 25 μm.
pressing GAD mRNA was not easy in regions where neurons are compactly packed, such as the SCN. To overcome this problem in the present study, we identified GAD mRNA-positive neurons by digoxigenin cRNA hybridization/anti-digoxigenin antibody immunocytochemistry, a method that has a better resolution for analyzing cell morphology than the radioisotope-labeled autoradiographic method.

The GAD cRNA probe used in this study was produced from the cDNA encoding GAD 67 (molecular size 67 kD). Two isoforms of GAD, GAD 67 and GAD 65, have been cloned (Erlander et al. 1991), and both have the ability to synthesize GABA. In contrast to GAD 65, which is sometimes not expressed in neurons of the tuberomammillary nucleus, it is known that the major class of GAD in the brain is GAD 67, and all GABAergic neurons are believed to express GAD 67 (Esclapez et al. 1993). Therefore, GAD67 mRNA-positive neurons in the SCN detected in the present study probably represented most GABAergic neurons. Moreover, our cRNA GAD67 probe detected a species of the same molecular size as reported in hypothalamic homogenates on Northern blots, suggesting usefulness of this cRNA probe for detecting GAD67 mRNA in hypothalamic tissue.

In the present study, we demonstrated the high co-expression ratio of GAD mRNA with AVP mRNA in the dorsomedial SCN and with VIP mRNA in the ventrolateral SCN. The co-expression ratio of AVP mRNA in the dorsomedial subdivision may have been higher than that of VIP mRNA in the ventrolateral subdivision, because in the dorsomedial part most neurons contain AVP, whereas in the ventrolateral part VIP neurons share a main component but there is also a substantial population of non-VIPergic neurons, such as those expressing GRP (van den Pol and Tsujimoto 1985; Okamura et al. 1987).

The content of VIP and its mRNA is known to display diurnal variation in a light-responsive manner (Takahashi et al. 1989; Albers et al. 1990; Okamoto et al. 1991). Combined application of VIP, PHI, and GRP induces a phase shift of locomotor activity rhythm and a change in electrical activity (Albers et al. 1991). A dark pulse-type phase shift of locomotor activity rhythm was caused by focal injection of muscimol, a GABA<sub>A</sub> agonist, into SCN regions (Smith et al. 1989, 1990). Recently, Gillespie et al. (1996) reported that focal injection of muscimol reduced the phase-delaying effect caused by focal application of VIP/PHI/GRP. This finding suggests that the co-stored GABA modifies the phase delay caused by the release of coexisting peptides through GABA<sub>A</sub> receptors. Expression of GAD mRNA not only in AVP and SS mRNA-positive neurons that exhibit endogenous rhythm but also in those positive for VIP mRNA suggests that the majority of neurons in the SCN are GABAergic, regardless of whether or not they display endogenous rhythm. The precise mechanism for the cooperation of GABA and its coexisting peptides in both the generation of circadian rhythm and the entrainment of circadian rhythm to the environmental light: dark cycle remains to be examined.

Many GAD mRNA-positive neurons in the anterior hypothalamus and medial preoptic area did not express AVP or VIP mRNA. SS neurons in the periventricular nucleus, which are known to send axons to the median eminence, regulating the release of growth hormone, expressed very high levels of SS mRNA without expressing GAD mRNA. Magnocellular neurosecretory neurons projecting to the posterior pituitary located in paraventricular and supraoptic nuclei did not express GAD mRNA. In the cerebral cortex, GABA, SS, and VIP are candidates as neurotransmitters in nonpyramidal neurons (Parnavelas and McDonald 1983). However, a small percentage of GAD mRNA-positive neurons co-expressed SS or VIP, although many VIP or SS mRNA-expressing neurons co-expressed GAD mRNA. These findings suggest that non-VIP-/non-SS-containing GABAergic neurons are predominant in the cortex, although until now little information regarding whether these neurons contain other types of peptides has been available. These findings suggest that the high degree of AVP, VIP, and SS co-expression is a characteristic of SCN GABAergic neurons.

In conclusion, the present study demonstrated that three distinct groups of peptidergic neurons in the SCN are GABAergic, and suggests that GABA may participate in both rhythm generation and rhythm entrainment in a complex manner.

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Table 1 Percentages of co-expression of peptidergic phenotypes in GABAergic neurons of the SCN

<table>
<thead>
<tr>
<th>Regions</th>
<th>AVP+GAD/GAD</th>
<th>VIP+GAD/GAD</th>
<th>SS+GAD/GAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>All parts</td>
<td>49.6 ± 8.53</td>
<td>33.7 ± 5.53</td>
<td>4.24 ± 0.72</td>
</tr>
<tr>
<td>Dorsomedial part</td>
<td>91.0 ± 6.46</td>
<td>-a</td>
<td>-a</td>
</tr>
<tr>
<td>Ventrolateral part</td>
<td>-a</td>
<td>66.7 ± 5.80</td>
<td>-a</td>
</tr>
<tr>
<td>Cortex&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-a</td>
<td>4.81 ± 2.10</td>
<td>23.9 ± 1.72</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data were not obtained because peptidergic phenotype was not expressed in the corresponding area.
<sup>b</sup>Data from the pineal cortex were added for comparison. Values are means ± SD (%); n = 3.
Literature Cited


