ULTRASTRUCTURAL LOCALIZATION OF GABA IN THE SUPRAOPTIC NUCLEUS AND NEURAL LOBE

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Abstract—Antibodies directed against the neurotransmitter γ-aminobutyric acid (GABA) enabled the ultrastructural localization of GABA in conventional glutaraldehyde fixed and osmium postfixed material of the rat supraoptic nucleus and neural lobe. GABA was visualized using immunogold postembedding staining in axonal profiles that terminate on dendrites, axons or cell bodies throughout the supraoptic nucleus, also in the neural lobe. Here GABA axons were found to terminate synaptically on pituicytes and axonal profiles containing large dense core vesicles.

These results emphasize, from an anatomical point of view, the potency of GABA to influence, as a transmitter, the release of vasopressin and oxytocin, both at the level of the cell body and of the neural lobe.

Several neuronal systems control the release of arginine vasopressin (AVP) and oxytocin at the level of the supraoptic (SON) and paraventricular nuclei (PVN) and/or at the level of the neural lobe (see Refs 20 and 21 for reviews). Morphological and physiological evidence indicates that, e.g. dopamine and noradrenaline are involved in the control of the release of these peptides, mainly by innervation of the AVP and oxytocin containing cell bodies in the PVN and SON.3,18,22 This aminergic input is considered to be stimulatory to AVP and oxytocin release.5,6,11,12,15 The GABAergic innervation of the SON, PVN and neural lobe is regarded as an important inhibitory input. Electrophysiological evidence indicates that GABA depresses the electrical activity of AVP and oxytocin containing neurons.1,11,12,15 GABA also depresses the electrical activity of AVP and oxytocin neurons at the level of the neural lobe.9,34 The anatomical basis of these findings is the light-microscopic demonstration of a GABAergic innervation, which has been shown to be present around cell bodies in the SON and also in the neural lobe.3,22,29,32 Light and electronmicroscopic observations on glutamate decarboxylase (GAD), the enzyme in the synthetic pathway of GABA, did not permit any conclusions to be drawn concerning the exact mode of termination in the SON or neural lobe.25

Recently an antibody against GABA became available.19 It permits an optimum fixation with a high concentration of glutaraldehyde. Since GABA is a very small stable molecule it seemed possible to use OsO₄ as a postfixative and to embed the material in epon to demonstrate GABA by means of post-embedding staining and the immunogold technique. This method indeed appeared to be compatible with an ultrastructural localization of GABA.23,29 It had the advantage that all ultrastructural details were preserved. In the present paper it led to the description of the ultrastructural localization of GABA containing terminals in the SON and neural lobe.

EXPERIMENTAL PROCEDURES

Twelve male Wistar rats (220–260 g) were perfused intracardially after pentobarbital anaesthesia (0.1 mg/100 g body wt), subsequently with 50 ml 0.9% saline, 500 ml 5% glutaraldehyde in 0.1 M Na-cacodylate, pH 7.3. The brain was removed from the skull, the neural lobe and a brain slice containing the whole hypothalamus were postfixed for 1 h in the same fixative. After postfixation the supraoptic nucleus was dissected and cut transversely into slices of approximately 500 μm. The tissue was then postfixed for 1.5 h in 0.1 M cacodylate-buffered 1% osmium, pH 7.4 or in 0.1 M phosphate buffer containing 1% OsO₄ with 1.5% potassium ferriyane. After postfixation the tissue was dehydrated in graded ethanol propylene oxide series, embedded in epon and polymerized at 60°C. Ultrathin silver-coloured sections were made on a Reichert ultramicrotome and the quality of the ultrastructure was examined after lead and uranyl contrasting. For the ultrastructural localization of GABA only tissue material was used that yielded an optimum ultrastructure. For the GABA staining as a rule silver-gold coloured sections were used. Initially the effect of various etching media on the positivity of the presumed GABA localization and on background staining was evaluated. The background staining was estimated by counting the gold particles in the labeled profiles and those in the
same magnocellular area opposite to the labelled profile. The labelling in the profile was considered specific when the number of particles counted was at least 10 times more than in the same area of the postsynaptic structure. For this purpose a saturated solution of sodium metaperiodate was used with various incubation times, ranging from 10 to 120 min, 1 or 5% peridate solutions, or a combination of sodium metaperiodate 1% and peridate 1% was used. In addition, also various concentrations of H₂O₂, ranging from 2 to 16% and various incubation times ranging from 2 to 40 min were used to study the effect of these procedures on GABA labelling.

After etching, which was performed on petri dishes in droplets containing the etching medium on paraffin, the grids were washed in a beaker containing aquadest (3 times) and 0.05 M Tris 0.9% NaCl pH 7.6 (2 times). For washing, the grids were held between forceps, kept in the beaker in which the buffer was stirred constantly. After the grids had been washed, they were stored in a droplet of Tris-NaCl. Subsequently they were transferred into a droplet of GABA antibody diluted 1:1000 in 0.05 M Tris-0.9% NaCl, 0.5% Triton X-100 pH 7.6 for a 20 min incubation. After 30 min the grids were again washed in Tris-NaCl (3 times) and transferred to a droplet of goat anti-rabbit IgG labelled with gold particles (diameter 15-20 nm; Janssen Pharmaceuticals), diluted 1:40 in Tris-NaCl Triton for 20 min. After this final incubation the grids were washed in Tris-NaCl (2 times), aquadest (2 times), and contrasted with uranyl and lead. The sections were examined with a Philips 400 electron microscope.

Specificity of staining was only studied for that procedure that yielded an optimum ratio of GABA staining/background staining. As a control for specificity sections were incubated with a GABA antibody that was absorbed with GABA conjugated with glutaraldehyde to a protein carrier (usually albumin). In addition, the GABA antibody was absorbed either with β-alanine or glutamate or aspartate, or with glycine conjugated with glutaraldehyde to a protein carrier.10

RESULTS

The light microscopical localization of GABA with the antibody employed revealed a dense innervation in all neurons in the SON. In the neural lobe a far less dense innervation was seen scattered across the entire neural lobe. The innervation disappeared after adsorption of the antibody with GABA conjugated with glutaraldehyde to a protein carrier while the innervation remained unchanged after adsorption of the antibody with β-alanine, glutamate, aspartate or glycine conjugated with glutaraldehyde to a protein carrier.

The influence of various etching media and the concentration of etching substances on the maintenance of the immunorecognition of GABA and ultrastructural preservation of morphology were determined by using the SON as a control. Gold labelling in neurons containing large AVP or oxytocin containing dense core vesicles was regarded as background staining while labelling in structures containing small clear or dense core vesicles was considered to be positive. It turned out that several factors influenced both recognition and/or ultrastructure preservation. In general, after using the various etching media, the best ultrastructural preservation was obtained with the osmium postfixed material to which KFe(CN)₆ was added. Without the addition of KFe(CN)₆ a more rapid loss of contrast was obtained and the sections proved to be "bleached" more easily. Increasing concentrations of H₂O₂ and longer incubation times also resulted in increased bleaching. For the immunocytochemical labelling no etching resulted in no specific labelling and a relative high background, increasing the concentration of the etching medium or increasing the duration of etching resulted in an increase in specific labelling and a decrease in background to a certain optimum where the density of gold particles was very high in axonal profiles and low in the surrounding tissue. Usually non-specific labelling was recognized, apart from being present in magnocellular SON neurons, to be present also on epon material containing no tissue at all. At the moment when gold labelling on the epon disappeared, it also disappeared from the SON neurons. Using higher concentrations of etching medium or very long etching periods caused a decrease in specific labelling, whereas the background labelling increased. The optimum and most reproducible labelling was found with H₂O₂, between 8 and 10%, with an incubation time between 10 and 20 min depending on the fixed brain. Etching with other oxidants such as Na₃IO₃ or H₂JIO₃ or mixtures of these usually resulted in a less well-preserved ultrastructure, a higher background and lower specific labelling. Absorption tests with GABA,
Figs 1–6. Positive reacting neurons in the neural lobe 1–5 and supraoptic nucleus stained with immunogold with an antibody against GABA. Bars = 250 nm.
Figs 7–9. Immunogold-labelled terminals stained with an antibody against GABA in the neural lobe (Fig. 7) and SON (Figs 8 and 9). Bars = 250 nm.

Fig. 7. Several positively reacting terminals stained for GABA are present close to a pituicyte and axons containing large dense core vesicles. pvs, perivascular space.

Figs 8 and 9. GABA positive terminal forms synapse with a magnocellular neuron in the SON (arrows). Note the presence of large dense core vesicles (arrows) and of a high labelling intensity over the mitochondria in the positively reacting terminals.
Fig. 10. GABA positive terminal forms a synapse (double arrow) with a magnocellular neuron in the dorsal part of the SON. Note the presence of large dense core vesicles (single arrows). Bar = 250 nm.

Fig. 11. Several GABA positive terminals forming a synapse (arrows) with magnocellular elements in the lateral part of the SON. Bar = 250 nm.
Fig. 12. Two GABA immunoreactive terminals forming a synapse (double arrow) with a process of a magnocellular neuron in the SON. Single arrow points to a large dense core vesicle. Bar = 250 nm.
β-alanine, glutamate aspartate and glycine conjugated with glutaraldehyde to a protein carrier, resulted in either disappearance or maintenance of the positive staining just like the light microscopical results.

The immunocytochemical ultrastructural localization of gold particles indicating the presence of GABA in the SON was usually obtained in axonal profiles containing many clear vesicles (+50 nm) and an occasional larger vesicle with a light dense core (+80 nm) (Figs 8–12). In addition, a strong positive gold labelling was observed on mitochondria in several axonal profiles (Figs 1 and 9–11). GABA immunoreactive axonal profiles were frequently seen to form symmetrical synaptic contacts with cell bodies or their processes (Figs 8–12) and dendrites (Fig. 6) containing large dense core vesicles, which indicate the presence of vasopressin or oxytocin. Synaptic contacts were also seen between GABA positive terminals and axons containing large dense core vesicles.

In the neural lobe gold labelling was seen on axonal profiles with similar features as compared to the GABA axons in the SON. These axons were also filled with clear vesicles. In addition, irregularly shaped vesicles could be seen ranging in size from +30 to 100 nm (Fig. 1), but this can be a result of fixation problems, as in this brain region the blood brain barrier is lacking. In the neural lobe the GABA positive axons seemed to contain a few more dense core vesicles of +80 nm. GABA positive terminals were usually found in clusters scattered across the neural lobe, but most frequently in the region where the axons enter the neural lobe. The axonal profiles were found to be in neuropil synapse with pituitary cell bodies and their processes (Figs 1, 4 and 5). Moreover, GABA terminals were found to be in neuropil contact with axons containing large dense core vesicles. In longitudinally sectioned axons the immunogold labelling seemed to be associated with microtubules (Fig. 2).

DISCUSSION

The GABA staining

The present light microscopical results with the localization of GABA confirmed the results with the localization of GAD on the SON13,25 and the results with a GABA antibody.29 Up till recently no ultrastructural data were available on the localization of GABA containing profiles in the SON. Antibodies to GAD21 and GABA20 provided evidence for the presence of synaptic terminals of GABA containing axons on magnocellular neurons in the SON. In the present paper the advantage of the ultrastructural localization of GABA, apart from demonstrating the transmitter directly, was the maintenance of an optimum ultrastructural preservation of the tissue. Since ultrastructural details, especially in the neural lobe, are quickly lost when the preembedding staining procedure is used, an immediate attempt was made to localize GABA in ultrathin sections of conventional glutaraldehyde fixed osmium tetroxide postfixed material. For the selection of the optimum conditions for ultrastructural GABA localization it was necessary to select a structure in which no GABA containing cell bodies were present and thus, background staining could easily be recognized. Therefore the SON was selected since it is known to contain only magnocellular cell bodies containing no GABA. GABA containing cell bodies were shown to be present only in the immediate surroundings of the SON.25 Soon it appeared that etching of ultrathin sections is necessary to obtain a specific reaction.

Although the specificity of the antiserum had previously been demonstrated for light microscopical localization of GABA,19 it was necessary to demonstrate this specificity again since a different procedure of fixation and embedding was used. This kind of procedure might interfere with the GABA antigenicity or with other molecules in such a way that false-positive localization could be obtained.14 The specificity of the reaction was determined by applying the following criteria: the antiserum was adsorbed with the homologous antigen (GABA) or the heterologous ones (Asp.Glu.Bala.Glyc.). The disappearance of all gold labelling after adsorption with the homologous antigen and the maintenance of labelling after adsorption with the heterologous one indicated the specificity of the method employed. Additional support for the specificity of the localization was the observation that no concentrations of gold particles are seen on axonal profiles or cell bodies containing large dense core vesicles. The high density of positive labelling on mitochondria indicates the presence of a high concentration of GABA in mitochondria of nerve terminals containing GABA. This observation is in line with the finding that GABA synthesis from labelled glucose largely occurs at a very high speed4 and is therefore likely to be associated with mitochondria. Further support for this specificity is lent by the fact that, in serial sections, positively or negatively reacting axons remained positive or negative in all sections.

The optimum etching procedure showed a gold labelling representing GABA, only on axonal profiles in the SON and not in cell bodies. These profiles were usually found to terminate synaptically on cell bodies containing much RER and large ±120 nm dense core vesicles indicative for AVP and oxytocin neurons.30 It has been demonstrated that AVP neurons in the rat brain are localized preferentially in the ventral and central SON and oxytocin neurons in the dorsal and rostral and caudal SON.24,28 Thus the observation that GABAergic terminals are present in the SON from rostral to caudal and from ventral to dorsal indicates that both AVP and oxytocin neurons are innervated by GABA neurons.

GABA terminals on magnocellular elements

It was observed that in a single ultrathin section
often more than one GABAergic terminal impinged on a single neuron and that GABA terminals were found to synapse on dendrites and axonal structures. These observations suggest that GABA in the SON has a potentially very powerful action on the release of AVP and oxytocin. Electrophysiological data suggest an inhibition of GABA on the spontaneous firing of phasic and continuously firing neurons in the SON.1-3 In in vitro experiments the inhibitory action of GABA on AVP and oxytocin axons was extensively demonstrated by means of electrophysiological studies.9,17,34 Application of GABA to a preparation containing the rat median eminence, pituitary stalk and neural lobe reduced the amplitude of the antidromic compound action potential recorded from hypothalamo-neurohypophyseal axons in response to electrical stimulation of the posterior pituitary. It was concluded that the peptidergic neurons of the hypothalamus neurohypophyseal system are endowed with receptors for GABA.35 These observations are in agreement with the present results which demonstrate the overall presence of GABAergic terminals on the magnocellular neurons of the SON. Moreover, the present results show that GABA containing synapses can be visualized on magnocellular axonal profiles in the neural lobe. The present results corroborate the results of Tappaz et al.25 and Van den Pol,29 who demonstrated positive terminals around SON neurons, using GAD or GABA immunocytochemistry, respectively. In addition, Tappaz25 showed that GAD axons were in close contact with magnocellular axons and pituicytes in the neural lobe. Thanks to the optimum preservation of ultrastructure, the present results allow the conclusion that GABA containing fibers indeed terminate synaptically on magnocellular axons in the neural lobe.

GABA terminals on pituicytes

GABA axons can also be visualized, terminating synaptically on pituicytes in the neural lobe. Such synaptic structures can frequently be observed in classically fixed material of the neural lobe35 but up till now the poor ultrastructural preservation after GAD immunocytochemistry did not allow the conclusion that GABA is involved in the direct modulation of pituicytes. Various studies suggest a role for the pituicytes in the control of AVP and OXT release. It has been proposed that pituicytes might be able to modulate the ionic environment of the neurons of the neural lobe,3 or that they envelope these neurons when no high release levels are needed.7 Under conditions in which AVP or OXT release is enhanced during thirsting or milk ejection, it has been found that the pituicytes are likely to retract in order to facilitate AVP or oxytocin release.3,26,27 As yet it has been unclear what mechanisms cause the pituicytes to retract, but it is feasible that the GABAergic input now demonstrated to be present on pituicytes may play a role in this process. The autoradiographic demonstration of a peripheral type of benzodiazepine receptors present in the rat neural lobe25 and the view that this type of receptors is associated with glia16 corroborates the present findings of a GABAergic innervation of pituicytes. GABA might then, just like enkephalin,30,31 play a role in the regulation of the release of AVP and oxytocin by influencing the level or envelopment of AVP and oxytocin terminals in the neural lobe by the pituicytes.

In the present study no indication was found that the gold labelling of pituicytes was higher than that of the rest of the neural lobe. Therefore no evidence for the suggested presence of GABA in the pituicytes10 could be obtained.

The origin of the GABAergic innervation

At present it is unclear where the neurons of origin that have their GABA projection on the neural lobe and SON are located. If the population of GABA neurons that innervates the neural lobe, is different from the one that innervates the SON, there is a possibility that the GABAergic inhibition of AVP or oxytocin release is regulated differentially. Consequently, the present observations indicate that GABA is able to influence the release of AVP and oxytocin at all levels of the hypothalamo-neurohypophyseal tract: the dendrite, the cell body, the axonal process and the axonal terminal.

It remains to be established, however, where the neurons of this GABAergic innervation are located. Regions just above the SON or within the diagonal band of Broca are a possible source for GABAergic innervation of the SON. Further studies will be necessary to help clarify this problem.

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REFERENCES


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