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Localization of Vesicular Inhibitory Amino Acid Transporter (VIAAT) in the Submandibular Salivary Gland Throughout the Postnatal Development of Mice

Localización del Transportador Vesicular de Aminoácidos Inhibidores (VIAAT) en la Glándula Salival Submandibular Durante el Desarrollo Postnatal de Ratones

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SUMMARY: According to recent studies, it is highly possible that the occurrence of vesicular inhibitory amino acid transporter (VIAAT) is a good marker of GABA-signaling not only in the brain, but also in extra-brain tissue cells containing GABA and GAD. In view of this, the present study was attempted to localize VIAAT-immunoreactivity in the submandibular gland of mice. In the present study, the submandibular glands of male mice at various postnatal developmental stages were examined for detailed localization of VIAAT-immunoreactivity in immunohistochemistry at light microscopic level. The immunoreactivity for VIAAT was localized in epithelial cells of proximal and distal excretory ducts with the striated portion more intensely immunopositive at young postnatal stages. No significant immunoreactivity was seen in the acinar cells throughout the postnatal development. In addition, the immunoreactivity for VIAAT was detected in the salivary parasympathetic ganglionic neurons, but not in any nerve fibers surrounding the glandular cells. Furthermore, VIAAT-immunoreactivity was found in smooth muscle cells forming the outermost layer of intralobular arterioles. From the present findings, it is possible that GABA plays roles as paracrine and autocrine regulators in the saliva secretion as well as the gland development.

KEY WORDS: VIATT-immunoreactivity; GABA; Salivary glands; Arteriolar smooth muscle.

INTRODUCTION

A vesicular transporter of inhibitory amino acids such as GABA (γ-aminobutylic acid) and glycine, which is termed VIAAT (vesicular inhibitory amino acid transporter), has been identified in rodent brain and it localizes the amino acids to synaptic vesicles of central inhibitory neurons (Bormann, 2000; Chaudhry *et al*., 1998; Chebib & Johnston, 1999; Hsu *et al*., 1999; McIntire *et al*., 1997; Sagné *et al*., 1997; Watanabe *et al*., 2002). There have recently been studies suggesting that the occurrence of VIAAT is a good marker of GABAergic signaling in extra-brain tissue cells containing GABA and its synthesizing enzyme termed GAD (glutamate decarboxylase), such as the pancreatic islets (Chessler *et al*., 2002; Gammelsaeter *et al*., 2004; Suckow *et al*., 2006). With regard to the extra-brain GABAergic signaling-implicated tissues, it should be noted that a substantial concentration of GABA and the activity of GAD, GAD67 as the major isoform, have been detected in major salivary glands of rodents, and that the GABA levels in the glands have been significantly decreased by administration of a GAD inhibitor, and further that GABA inhibits cellular functions in the salivary glands (Kawaguchi & Yamagishi, 1996; Kosuge *et al*., 2009; Sawaki *et al*., 1995; Shida *et al*., 1995). These findings suggest that the possibility of VIAAT as a marker for the GABA-signaling is the case also for the salivary glands.

Considering this suggestion, together with the fact that the development and differentiation of the duct-acinus system of the salivary glands progress at early postnatal stages of mice (Denny *et al*., 1990; Gresik, 1980; Patel & Hoffman, 2014; Zajicek *et al*., 1985), the present study was undertaken to localize VIAAT in the salivary submandibular gland of mice during the postnatal development by immunohistochemistry at light microscopic level.

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MATERIAL AND METHOD

Male and female ICR mice at the stage of 6 week-age were purchased from National Laboratory Animal Center, Mahidol University, Thailand and they were grown until 8 week-age under standard laboratory conditions with a 12-h light/12-h dark cycle and free access to standard food and water until use for experiments. All subsequent procedures were conducted in accordance with Guidelines for the Care and Use of Laboratory Animals at Khon Kaen University. The study was reviewed and approved by the ethical board with the ethics number AEKKU 4/2014. Female mice were used only for mating to generate five male mice on each stage of postnatal 1day (P1D), 1 week (P1W), P3W, P4W, P5W, P8W. The male mice were perfused with 10 ml PBS, followed by 10 ml 4% paraformaldehyde/0.1 M phosphate buffer. The submandibular gland was extirpated, then postfixed with the same fixative for 2 h. Specimens were dipped into 30% sucrose/0.1 M phosphate buffer for cryoprotection. Cryosections of 20 μm thickness were made on a cryostat, mounted on glass slides. They were permeabilized with 0.1% TritonX-100/ PBS for 30 min at room temperature, incubated with 0.3% H_2O_2/methanol for 10 min, and then 5% normal goat serum/PBS for 30 min. The sections were incubated with anti-mouse VIAAT rabbit IgG (1 μg/ml) overnight at room temperature. The specificity of the antibodies was confirmed previously (McIntire *et al*.). The sections were subsequently incubated for 1 h at room temperature with biotinylated anti-rabbit IgG secondary antibody (Vector Laboratories, Burlingame, CA USA) diluted at 1:200 for DAB reaction by VECTASTAIN Elite ABC kit (Vector Laboratories, Burlingame, CA USA).

In control experiments, omission of the primary antibody alone was applied to the sections.

RESULTS

On postnatal 1 day (P1D), weak to moderate immunoreactivity for VIAAT was detected in a substantial number of acinar cells. The immunopositive acinar cells occurred in random distribution and the immunopositive materials were homogeneously throughout the cells. The immunoreactivity was also seen in the plasmalemma of the proximal and distal ductal cells with the luminal and basal contours of the ducts in cross sections in a distinct appearance (Fig.1).

On postnatal 1 week (P1W), no immunoreactivity was seen in almost all the acinar cells. In contrast, the immunoreactivity was evident in the plasmalemma of ductal cells from the distal to proximal ducts (Fig. 2).

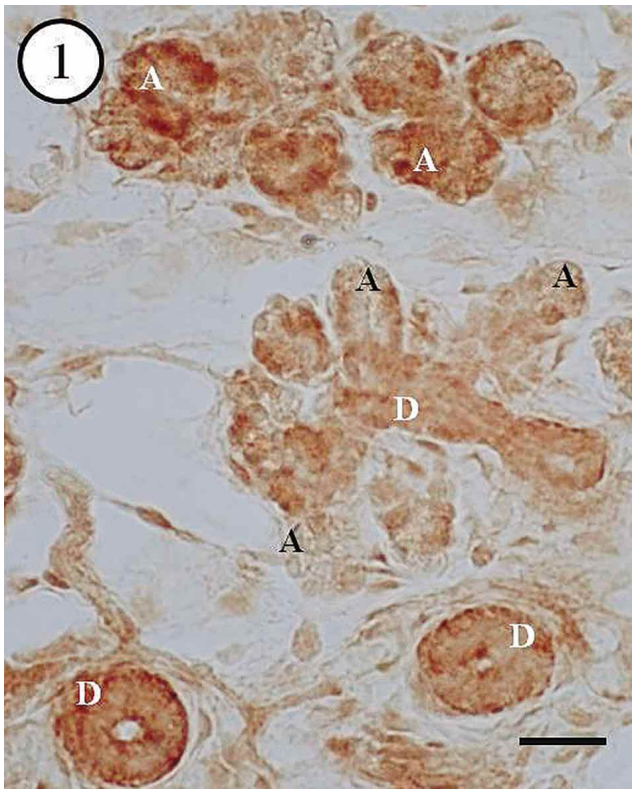

Fig. 1 Immuno-positive (white A) and immuno-negative (black A) acinar cells and immuno-positive ductal cells (white D) of distal excretory duct at P1D. Bar represents 20 μm.

On P3W, the immunoreactivity in almost all the ductal cells, except for the intercalated portions, was condensed in the plasmalemma, resulting in appearance of tight spreading of immunostained polygonal flagstones, especially when the ductal striated portions and proximal excretory ducts were cut obliquely (Fig. 3). The acinar cells remained immunonegative.

On P4W, the immunoreactivity remained positive in the plasmalemma of the ductal cells of the striated portions, while it was weaker in the plasmalemma of ductal cells in the granular convoluted portions which were well-developed at this stage (Fig. 4).

On P5W and P8W, the immunoreactivity in the ductal cells decreased progressively, and no significant immunoreactivity was seen in the gland (Fig. 5).

Furthermore, distinct immunoreactivity was discerned in cells forming the outermost cell layer of arterioles in association with the interlobular excretory ducts

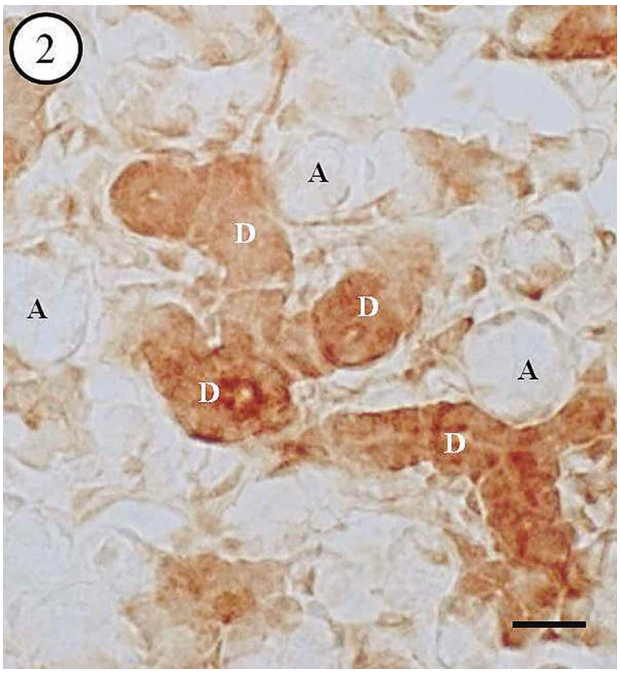

Fig. 2 Immuno-positive ductal cells (white D) of distal excretory ducts in contrast to immuno-negative acinar cells (black A) at P1W. Bar represents 20 μm.

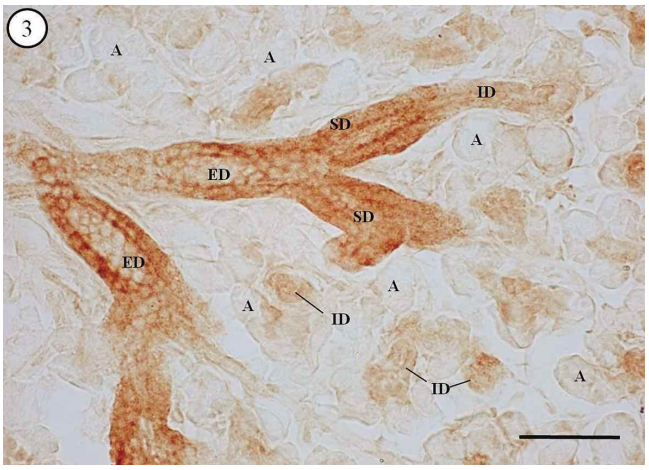

Fig. 3 Immuno-positive ductal cells of striated (SD) and intercalated (ID) ducts and initial portions of proximal excretory ducts (ED) at P3W. Note weaker immunoreactivity in ID and also note the immunoreactive deposits distinctly in the peripheral cytoplasm, resulting in clear contours of individual cells. A: immuno-negative acinar cells. Bar represents 50 μm.

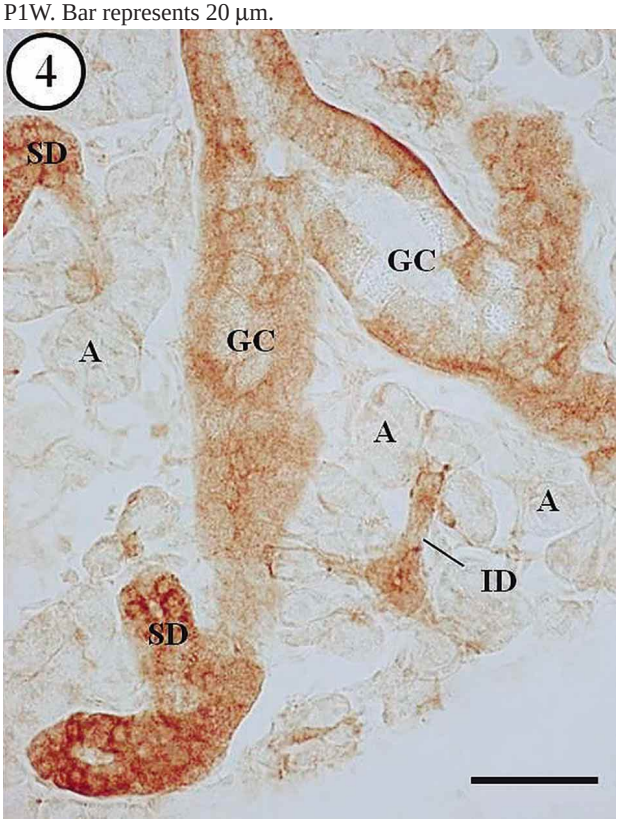

Fig. 4 Distinct immunoreactivity in the peripheral cytoplasm in striated ductal cells (SD) in contrast to immunonegative acinar cells (A) at P4W. Weak immunoreactivity was seen in basal cytoplasm of ductal cells of granular convoluted (GC) portions. ID: immunopositive intercalated portions. Bar represents 50 μm.

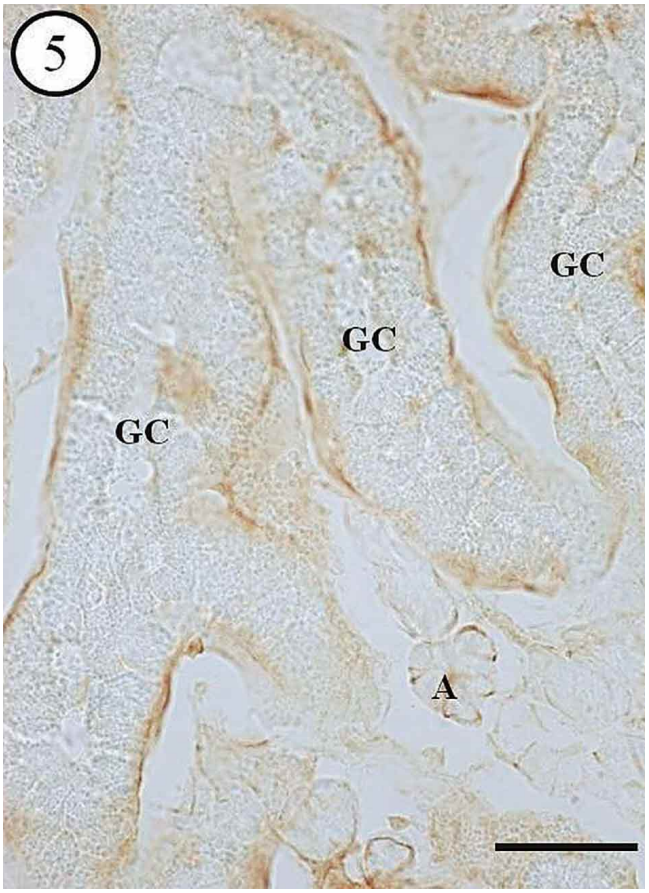

Fig. 5 Distinct immunoreactivity was seen in basal cytoplasm but not in the cytoplasmic granules of ductal cells of the granular convoluted (GC) portions at P5W. No immunostained nerve fibers surrounding the acinar cells (A). Bar represents 50 μm.

throughout the postnatal development till adult stages. The immunoreactive cells were oblong in shape and their long axis was arranged in a direction oblique or spiral to that of the blood-flow (Fig 6).

In control experiments, omission of the primary antibodies alone resulted in the abolition of all immunostaining (not shown).

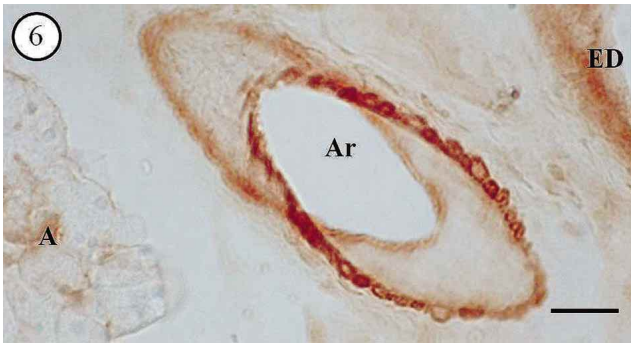

Fig 6 Distinct immunoreactivity in cells (arrowheads) forming the outermost cell layer of arteriole (Ar) in association with the interlobular excretory ducts (ED) at P4W. A: immunonegative acinar cells. Bar represents 50 μm.

DISCUSSION

Although the occurrence of immunoreactivity for GABA and GAD in the ductal cells of rat submandibular gland was briefly described without showing any micrographic evidences in a previous immunohistochemical study by others (Kosuge *et al*.), the present study is the first to show the possible occurrence of GABA in the glandular cells, mainly ductal cells, of the exocrine glands of mice at the early postnatal stages, but not at the adult stage by means of VIAAT-immunoreactivity. The immunoreaction in the ductal cells was noted to occur in the plasmalemma representing the plasma membranes and submembranous cell cortex rich in vesicles and vacuoles. In the endocrine cells of the previous study (Gammelsaeter *et al*.), VIAAT-immunoreactivity was shown at ultrastructural levels to be localized in the membranes of synaptic-like microvesicles (SLMVs), equivalent to the neuronal synaptic vesicles in the central nervous system, as well as in the membranes of endocrine secretory granules. It is therefore necessary to examine the ultrastructural localization of VIAAT- immunoreactivity in the plasmalemmal domains of ductal cells of the salivary gland, which is now under way.

The present study did not detect any VIAAT-immunopositive nerve fibers and terminals innervating the acini and ducts, although GABA-ergic nerve terminals have been clearly demonstrated in the central nervous system of mice by immunohistochemistry using the same antibody against VIAAT as this employed in the present study (Miyazaki *et al*., 2003). On the other hand, there has been a study reporting the occurrence of autonomic nerve fibers immunoreactive for GAD around the acini, but not within any glandular cells, of the submandibular gland of adult rats (Kosuge *et al*.). Although the species difference between mice and rats should be considered in the interpretation of any experimental results, it should also be noted that the immunostained fibrous structures surrounding the glandular acini in the previous study (Kosuge *et al*.) did not show varicose features which are characteristic of autonomic nerve terminals (Gabella, 1976). It should be further noted that the previous study did not describe the presence or absence of GAD-immunoreactivity in the intra-glandular ganglionic neurons which are considered to be parasympathetic autonomic neurons innvervating the glandular cells (Gabella). Therefore, further studies are necessary to confirm their GAD-immunostained fibrous structures to be truly autonomic nerve terminals themselves by immuno-electron microscopy. It is thus likely from the present findings that the amount of GABA and GAD already detected biochemically in the salivary gland in the previous studies (Kawaguchi & Yamagishi; Kosuge *et al*.; Sawaki *et al*.; Shida *et al*.) is attributed mainly to the VIAAT-immunoreactivity in the glandular ductal cells.

With regard to the functional significance of the present findings, there has been a study reporting the localization of immunoreactivity for GABA receptor in the salivary gland of rats (Shida *et al*.). According to their finding, the immunoreactivity for GABA receptor was localized intensely in the ductal cells and less intensely in the acinar cells of the submandibular gland in young adult rats. Assuming that the localization of the receptor- immunoreactivity in the mouse gland is similar to that in the rat, it is possible to consider that GABA produced/secreted by the presently detected VIAAT-immunopositive cells binds with GABA-receptors in these young glandular cells by way of paracrine or autocrine routes, and that it plays inhibitory roles in the saliva secretion based on the available electrophysiological data so far published (Mayer *et al*., 1983).

On the other hand, it should be noted that VIAAT-immunoreactivity decreased markedly in the acinar cells at P1W, and progressively increased at P1W-P3W in the ductal cells and decreased at P4W and thereafter in the present analysis of postnatal development. These findings are in contrast to the fact that most physiological studies on the effect of GABA in the saliva secretion have been done in adult rodents (Okubo & Kawaguchi, 2013; Ouchi *et al*., 2011). From this chronological feature of the present immunoreactivity, it is more plausible to consider the regulatory role of GABA in the development of the glandular cells rather than the saliva

secretion itself. In this regard, a previous finding by Amano & Iseki (1998) should be noted, in which the localization of cAMP response element-binding protein (CREB) was examined in the postnatal development of the submandibular gland of rats. According to their study, CREB-immunoreactivity was detected mainly in ductal cells from birth till young adult stages. The chronological localization pattern of CREB-immunoreactivity in rats is similar to that of VIAAT in the present mouse gland. Since there have been studies suggesting that CREB signalling is a central pathway in adult hippocampal neurogenesis, regulating the development and survival of new hippocampal neurons downstream of GABA-mediated excitation (Fukuchi *et al*., 2014; Jagasia *et al*., 2009), the similarity in the chronological localization between CREB and VIAAT/GABA may lead us to consider the possibility that the signalling cascade of the two molecules plays roles in the postnatal development of the salivary glandular cells. In support of this possibility in the peripheral organ, there has recently been accumulating evidence on the role of tonic GABAergic currents during early brain development (Kilb *et al*., 2013).

The present study also disclosed VIAAT-immunoreactivity in cells of inra-glandular small arterioles. Judging from the oblong shape of the immunoreactive cells and their arrangement in a direction oblique or spiral to that of the blood-flow, the immunoreactive cells are regarded as the outermost smooth muscle cells of the thin vascular wall. The same immunostaining pattern of intraglandular arterioles was obtained with an antibody against a-actin (data not shown), confirming the cellular identification as the outermost smooth muscle cells of arterioler walls. The previous immunohistochemical study cited above (Kosuge *et al*.) has not reported the occurrence of GABA- or GABA-receptor-immunoreactivity within interlobular arterioles of the gland. Considering the importance of GABA-ergic regulation of microvascular tones in some organs (Hinds *et al*., 2013; Roberts & Krause, 1982), it is thus necessary to examine first the authenticity of VIAAT in the smooth muscle cells by in situ hybridization histochemistry. In addition, regardless of the nature of the vascular muscle-specific immunoreactivity, it is necessary to examine whether the occurrence of VIAAT-immunoreactivity in the arterioles is specific to the salivary gland arterioles or it is also found in some other organs.

Since VIAAT is also known to work as a transporter for glycine, which is, like GABA, an inhibitory neurotransmitter in the brain (Chaudhry *et al*.; Sagné *et al*.), the present finding leads us to examine whether glycine plays some inhibitory roles in the salivary gland via VIAAT. It has been shown that glycine has inhibitory effects on the growth factor signalling and cell-cycle of progenitor cells derived from mouse salivary glands (Nakamura *et al*., 2009). The inhibitory effect may be exerted via the VIAAT-immunoreactivity detected in the acini on P1D.

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TOOMSAN, Y.; CHOMPHOO, S.; PAKKARATO, S.; WATANABE, M.; KONDO, H. & HIPKAEO, W. Localización del transportador vesicular de aminoácidos inhibidores (VIAAT) en la glándula salival submandibular durante el desarrollo postnatal de ratones. ***Int. J. Morphol., 33(1)***:113-118, 2015.

RESUMEN: Según estudios recientes, es altamente posible que la aparición del transportador vesicular de aminoácidos inhibidores (VIAAT) sea un buen marcador de señalización de GABA no sólo en el cerebro, sino también en células de tejido extra-cerebrales que contienen GABA y GAD. En el presente estudio se intentó localizar inmunoreactividad a VIAAT en la glándula submandibular de ratones. En el presente estudio, se examinaron las glándulas submandibulares de ratones machos en las distintas etapas del desarrollo postnatal para la localización detallada de inmunoreactividad a VIAAT inmunohistoquímicamente a nivel de microscopía óptica. La inmunorreactividad para VIAAT se localizó en las células epiteliales de los conductos excretores proximal y distal, con mayor intensidad en la porción estriada en las etapas tempranas. No se observó inmunoreactividad significativa en las células acinares durante el desarrollo postnatal. Además, se detectó la inmunoreactividad para VIAAT en las neuronas ganglionares parasimpáticas salivales, pero no en las fibras nerviosas que rodean las células glandulares. Además, la inmunoreactividad a VIAAT se encuentra en las células del músculo liso que forman la capa más externa de las arterias interlobulillares. En base a estos hallazgos, es posible que GABA tenga una función como regulador autocrino y paraparacrino en la secreción de saliva, así como en el desarrollo de la glándula.

PALABRAS CLAVE: Transportador vesicular de aminoácidos inhibidores (VIAAT); Inmunoreactividad a VIAAT; GABA; Glándulas salivales; Músculo liso arteriolar.

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