

The components required for amino acid neurotransmitter signaling are present in adipose tissues

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Abstract The adipocyte does not only serve as fuel storage but produces and secretes compounds with modulating effects on food intake and energy homeostasis. Although there is firm evidence for a centrally mediated regulation of adipocyte function via the autonomous nervous system, little is known about signaling between adipocytes. Amino acid neurotransmitters are candidates for such paracrine signaling. Here, we applied immunohistochemistry to detect components required for amino acid transmitter signaling in rat fat depots. In interscapular brown adipose tissue as well as in interscapular, mesenteric, perirenal, and epididymal white adipose tissues, we demonstrate robust immunosignals for the excitatory neurotransmitter glutamate, the inhibitory neurotransmitter γ -aminobutyric acid (GABA), and the GABA-synthesizing enzyme glutamate decarboxylase (GAD) isoforms GAD65 and GAD67. Moreover, all adipose tissues stained for the vesicular glutamate transporter VGLUT1 and the vesicular GABA transporter VGAT in addition to the vesicle marker synaptophysin. Electron microscopic immunocytochemistry showed that VGLUT1 and VGAT, but not VGLUT2 or VGLUT3, are localized in vesicular organelles in adipocytes. The receptors for glutamate (subunits GluR2/3 and NR1 but not mGluR2) and for GABA (GABA_AR α 2) were present in the adipocytes. The presence of glutamate, GABA, their vesicular transporters, and their receptors indicates a paracrine signaling role for amino acids in adipose tissues.—Nicolaysen, A., R. Gammelsaeter, J. Storm-Mathisen, V. Gundersen, and P. O. Iversen. The components required for amino acid neurotransmitter signaling are present in adipose tissues. *J. Lipid Res.* 2007. 48: 2123–2132.

Supplementary key words adipocyte • γ -aminobutyric acid • glutamate • synaptic vesicle • vesicular transporter

With the increasing prevalence of overweight and obesity worldwide, a growing interest in the functions of adipocytes has been stirred. The adipose tissue is no longer considered solely as a primary energy storage organ; it has emerged also as an important endocrine organ.

Whereas the regulation of hormone synthesis and secretion from the different kinds of adipose cells is still poorly understood, many reports have shown that the release of fuel substrates from adipose tissue is at least partly governed by neuronal inputs. For example, there is extensive neuroanatomical evidence for the sympathetic innervation of white adipose tissue (WAT) (1, 2). In agreement with this, the sympathetic neuronal outflow reportedly modulates glucose and lipid metabolism in WAT of animals and humans (3). The brown adipose tissue (BAT) also receives a rich sympathetic innervation, and mediastinal BAT reportedly has parasympathetic neurons (4). In addition to its function as a heat-producing organ, alterations in BAT metabolism may affect nutritional homeostasis (5). Collectively, these data underlie the hypothesis that a dysregulated sympathetic tone might modify adipose tissues and hence contribute to obesity-related disorders (3).

The adipose depots localized in various parts of the body are seemingly different concerning gene expression profiles and whether they confer increased risk of disease (6, 7). Moreover, lipectomy in rats followed by autologous transplantation led to increased mass of fat depots not in contact with the grafted fat tissue (8, 9). Because of the lack of innervation of the surgically removed adipose tissue, these latter findings indicate important endocrine signaling between adipocytes originating from various, distant organ regions. The nature of this intercellular signaling remains unknown, but the discovery that the excitatory neurotransmitter glutamate and the inhibitory neurotransmitter γ -aminobutyric acid (GABA) can have signaling functions outside of the central nervous system (CNS),

Abbreviations: AMPA, α -amino-hydroxy-5-methyl-4-isoxazolepropionate; BAT, brown adipose tissue; CNS, central nervous system; GABA, γ -aminobutyric acid; GAD, glutamate decarboxylase; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2; NMDA, *N*-methyl-D-aspartate; SV, synaptic vesicle; WAT, white adipose tissue.

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such as in the pancreatic islets of Langerhans, opens the intriguing possibility that adipose tissue also could use these signaling substances for paracrine and/or autocrine regulation (10–13).

In the CNS, the machinery for synaptic signaling of amino acids includes *i*) the presence of a transmitter in the presynaptic terminals (e.g., glutamate and GABA); *ii*) the intraterminal synthesis of the transmitter by specific enzymes [e.g., glutamate decarboxylase (GAD) for GABA]; *iii*) the packaging of the free transmitter into synaptic vesicles (SVs) in presynaptic terminals by vesicular transporters (e.g., the vesicular glutamate transporters VGLUT1 to VGLUT3 for glutamate and the vesicular GABA transporter VGAT for GABA); *iv*) the release of transmitter to the synaptic cleft by exocytosis of SVs; and *v*) transmitter binding to the postsynaptic receptors [e.g., the *N*-methyl-D-aspartate (NMDA) receptor NMDA-R for glutamate and the GABA_A receptor GABA_AR for GABA]. Because VGAT carries GABA into SVs, the SVs could be used to mark cells that release GABA by exocytosis, like GABAergic synapses in the CNS (14). VGLUT1 to VGLUT3 are specific for glutamate; hence, their presence in a given tissue compartment suggests the release of glutamate from that particular compartment (15). Two isoforms of GAD (GAD65 and GAD67) are present in the brain (16, 17). Both enzymes catalyze the formation of GABA from glutamate. Receptors for glutamate and GABA include ionotropic and metabotropic receptors. The former type of receptor mediates fast signals via ligand-gated ion channels, whereas the latter type acts via intracellular second messenger systems. Important ionotropic glutamate receptors are the NMDA-Rs and the α -amino-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors, whereas GABA_A and GABA_C receptors constitute the ionotropic GABA receptors.

Here, we examined whether different WAT depots and interscapular BAT contain the different elements involved in the handling of transmitter amino acids. We combined the use of immunoperoxidase and immunofluorescence microscopy to localize glutamate and GABA as well as their vesicular transporters and receptors in various rat adipocyte tissues.

MATERIALS AND METHODS

Materials

Glutaraldehyde (25%) and paraformaldehyde were from Merck (Whitehouse Station, NJ). Pentobarbital (9.7 mg/ml) was from Rikshospitalets Apotek (Oslo, Norway). Dextran D-70 was from Amersham (Buckinghamshire, England). All chemicals were of analytical grade. We used two cryostats: 2800 Frigocut-E (Reichert-Jung/Leica) and HM 560 (Microm International GmbH, Walldorf, Germany).

Antibodies

The polyclonal antibodies directed against glutamate and GABA were raised in rabbits essentially as originally described by immunizing with the amino acids coupled to BSA by a

glutaraldehyde-formaldehyde mixture (18). The anti-glutamate (No. 607) and anti-GABA (No. 990) sera have been extensively characterized previously (19–21). The antibodies do not bind to amino acid residues in proteins, as they selectively recognize the free amino acids bound to tissue proteins via the aldehyde fixative. We used an overnight (4°C) preabsorption with glutaraldehyde-formaldehyde complexes (weight proportion, 2.5:1) to block cross-reactivities as follows. The glutamate 607 antiserum was preabsorbed with complexes of glutamine (200 μ M) and aspartate (200 μ M), whereas the GABA 990 antiserum was preabsorbed with complexes of β -alanine (200 μ M) and glutamine (200 μ M). As a negative control, we performed blocking experiments by preadsorbing the antisera with aldehyde complexes (500 μ M) of the amino acids against which the respective antisera were raised. This abolished the glutamate and GABA staining of the tissue sections.

Guinea pig anti-VGLUT1, anti-VGLUT2, and anti-VGLUT3 antibodies and rabbit anti-NMDA receptor subunit NR1 antibody as well as mouse anti-synaptophysin, anti-microtubule-associated protein 2 (MAP2), anti-GAD65, and anti-GAD67 antibodies were purchased from Chemicon International (Temecula, CA). The mouse anti-glial fibrillary acidic protein (GFAP) antibody was purchased from Sigma-Aldrich (St. Louis, MA), and the anti-VGAT antibody was raised in rabbit and purchased from Synaptic Systems (Goettingen, Germany). The rabbit anti-GABA_A receptor α 2 antibody was purchased from Alomone Laboratories, Ltd. (Jerusalem, Israel), whereas the rabbit AMPA receptor subunit antibody (anti-GluR2/3) was a gift from R. Wenthold (Bethesda, MD). The mouse anti-metabotropic glutamate receptor mGluR2 subunit antibody (mG2Na-s) was purchased from Abcam, Ltd. (Cambridge, UK). The anti-RECA1 antibody, which binds to the RECA antigen expressed on vascular endothelium, was raised in mouse and purchased from Serotec Immunological Excellence (Oxford, UK).

Fluorescence-coupled anti-rabbit, anti-mouse, and anti-guinea pig secondary antibodies with excitation wavelengths of 488 nm (Alexa A488), 555 nm (Alexa A555), and 594 nm (Alexa A594) and ProLong-Gold Antifade Reagent were purchased from Molecular Probes (Eugene, OR). Biotin-conjugated donkey anti-rabbit secondary antibody for immunoperoxidase and streptavidin-coupled horseradish peroxidase complex were purchased from Amersham. Gold particle-conjugated secondary antibodies [goat anti-guinea pig (10 nm) and goat (Fab fragment) anti-rabbit (15 nm)] were from BBI (Cardiff, UK).

Perfusion fixation

Four Wistar rats (body weight, 150–350 g) were obtained from M&B (Ry, Denmark). The protocol was in conformity with the Public Health Service Policy on the Use of Laboratory Animals and was approved by local ethical committees. The animals had free access to food (standard low-protein pellet diet) and tap water. After an overnight fast, they were deeply anesthetized with intraperitoneal injections of pentobarbital (50–75 mg/kg). Before cessation of spontaneous respiration, the rats were perfused for 15 min through the left ventricle with different fixatives (room temperature) in 0.1 M sodium phosphate buffer, pH 7.4 (50 ml/min), preceded by a brief flush (10–15 s) of 4% (w/v) dextran (molecular weight, 70,000) in the same buffer without fixative. The animals were fixed with 2.5% glutaraldehyde and 1% formaldehyde (amino acid immunoperoxidase histochemistry) or with 4% formaldehyde with or without 0.1% glutaraldehyde (immunofluorescence histochemistry). After fixation, we carefully removed WAT specimens from interscapular, mesenteric, perirenal, and epididymal depots as well as interscapular BAT. In addition, the brains were removed, and together with the

WAT and BAT specimens they were postfixed (either for 4 h at room temperature or for 3 days at 4°C) in the same fixative used for the perfusion. Samples of pancreas were obtained from separate animals that were perfusion-fixed in the same manner. Formaldehyde was freshly made from paraformaldehyde, and the glutaraldehyde was added immediately before fixation. The tissues prepared for immunoperoxidase histochemistry were stored in the fixative at 4°C, whereas tissues prepared for immunofluorescence were stored in the fixative diluted 1:10 in sodium phosphate buffer.

Immunohistochemistry

For immunofluorescence, the fixed tissues were treated for 2 days at 4°C with 30% sucrose for cryoprotection, quick-frozen in a mixture of isopentane and dry ice (−75°C), and stored at −30°C. The WAT and BAT samples and the brain were sectioned (20 μm) on a cryostat and collected on coated glass slides. These slides were stored at −20°C until they were processed with the antibodies in single- and double-labeling experiments according to an indirect immunofluorescence method (22). The sections were first incubated with a single primary antibody or a mixture of primary antibodies from two different species, followed by one or a mixture of species-specific secondary IgG antibodies that were coupled to different fluorochromes.

For immunoperoxidase microscopy, the sections were incubated with the antibodies visualized using a streptavidin-biotin-peroxidase method (23).

The antibodies were used at the following dilutions: anti-glutamate, 1:1,000 to 1:3,000; anti-GABA, 1:1,000 to 1:3,000; anti-VGLUT1, 1:300 to 1:3,000; anti-VGLUT2, 1:2,500; anti-VGLUT3, 1:1,500; anti-VGAT, 1:300 to 1:1,000; anti-synaptophysin, 1:1,000; anti-GAD65, 1:300 to 1:3,000; anti-GAD67, 1:1,000; anti-RECA1, 1:1,000; anti-GABA_A receptor α2, 1:500; anti-NR1, 1:200; anti-GluR2/3, 1:400; anti-mGluR2, 1:500 to 1:1,000; anti-GFAP, 1:500; and anti-MAP2, 1:500. The fluorescence-conjugated secondary antibodies (Alexa A488, A555, and A594) were used at dilutions of 1:1,000. The biotinylated secondary antibody (anti-rabbit Ig) and the streptavidin-biotinylated horseradish peroxidase complex were used at dilutions of 1:100.

In control immunofluorescence experiments, in which the primary antibodies were omitted or substituted with preimmune sera, there was no staining of the tissue sections. To support the specificity of the adipose tissue labelings, brain sections were incubated with the antibodies as positive controls in all experiments along with the adipose tissues.

The localization of immunoreactivities was analyzed with a Zeiss LSM5 laser-scanning confocal microscope. We report replicate data from two to four rats.

Fixed BAT and perirenal WAT tissues for electron microscopy were embedded in Lowicryl HM20 according to a freeze-substitution protocol, and immunogold labeling was performed as described (13). Ultrathin sections were processed with the primary antibodies at the following dilutions: anti-VGAT from rabbit (1:100), anti-VGLUT1 from guinea pig (1:500), and anti-VGLUT2 from guinea pig (1:1,000). In double-labeling experiments of BAT and perirenal WAT, rabbit anti-VGAT was visualized with goat anti-rabbit (10 nm gold particles; BBI) and guinea pig anti-VGLUT1 (15 nm gold particles; BBI) or VGLUT2 with goat anti-guinea pig (15 nm gold particles; BBI) secondary antibodies (1:20). In each experiment, ultrathin brain sections were processed along with the BAT and WAT sections, ascertaining the specificity of the labeling produced, and negative controls (primary antibody omitted) were included.

The sections were viewed with a Philips CM10 electron microscope.

RESULTS

To investigate whether glutamate and GABA, which are the most abundant excitatory and inhibitory transmitters in the brain, may have signaling functions in adipose tissue, we first examined the immunohistochemical localization of these free amino acids in adipocytes from various anatomical regions. **Figure 1** shows that adipocytes of interscapular BAT and WAT were stained with antibodies recognizing glutamate and GABA. We could also identify these two amino acids using immunofluorescence, and there was a similar glutamate and GABA staining pattern of the other WAT depots included in the study (data not shown). In both WAT and BAT, the amino acid staining was clearly confined to the cytoplasmic part of the adipocyte and absent from the lipid droplets (Fig. 1).

We further assessed whether the presence of GABA in the adipose tissues could be attributable to intracellular

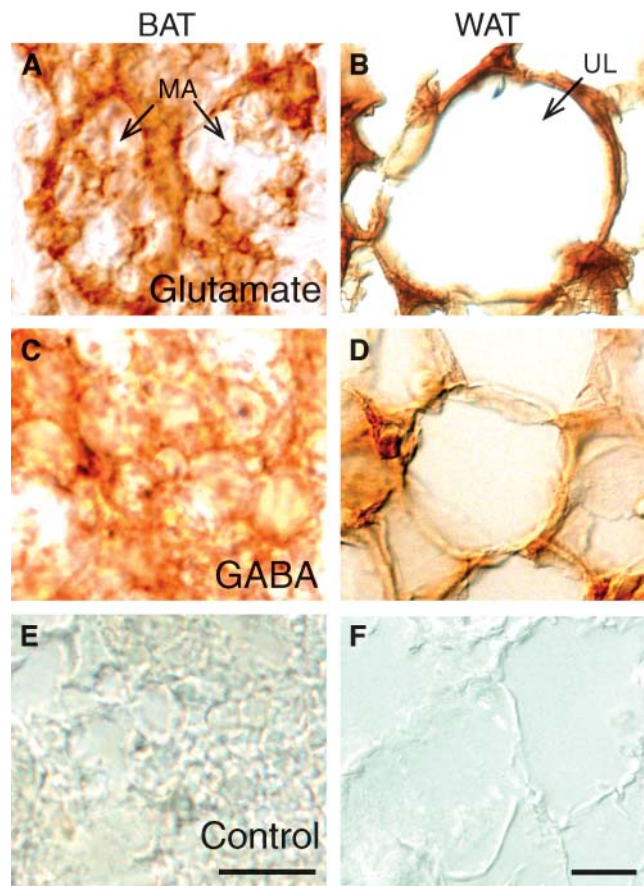


Fig. 1. Light micrographs showing sections of adipose tissue labeled with antibodies against glutamate and γ -aminobutyric acid (GABA) according to an immunoperoxidase method. Staining for glutamate (A, B) and GABA (C, D) is present in the cytoplasm of interscapular brown adipose tissue (BAT) cells (A, C) as well as in interscapular white adipose tissue (WAT) cells (B, D). When the primary antibodies were omitted, there was no staining of either BAT (E) or WAT (F) (negative controls). The multilocular (MA) features of BAT are denoted with arrows (A). The characteristic unilocular (UL) appearance of individual WAT cells is marked with an arrow (B). Bars = 10 μm.

synthesis using immunofluorescence labeling with antibodies raised against the GABA-synthesizing enzyme glutamate decarboxylase (GAD) 65. We found that GAD65 was present in BAT and mesenteric WAT (Fig. 2) as well as in the other studied WAT depots; similar data were obtained with antibodies recognizing the GAD67 isoform (data not shown).

Glutamate plays an important role in the energy metabolism of most cells; hence, its presence does not necessarily

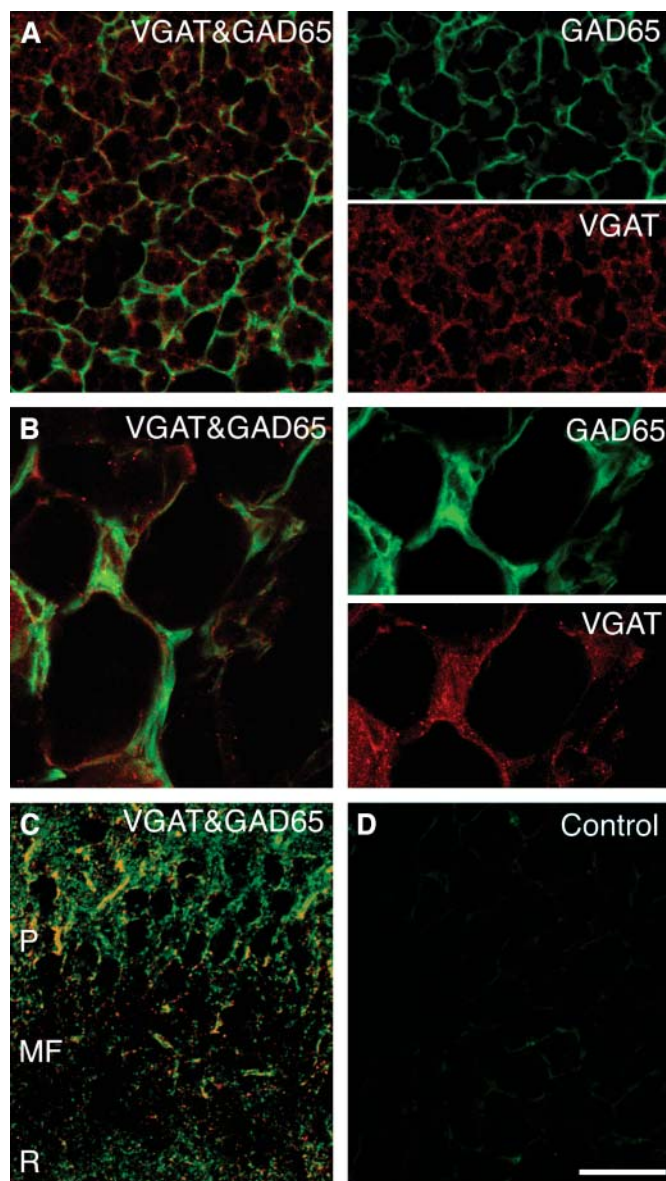


Fig. 2. Confocal images showing the immunofluorescence labeling of the GABA-synthesizing enzyme glutamate decarboxylase (GAD) 65 and the vesicular GABA transporter VGAT in adipose tissue. Both GAD65 (green) and VGAT (red) were present in interscapular BAT (A) and mesenteric WAT (B) cells. The left panels show overlays of the single-labeling images in the right panels. We also included a positive control from the brain (C; CA3 of the hippocampus). P, MF, and R indicate the pyramidal layer, mossy fiber, and radiatum layer, respectively; note the abundance of colocalized VGAT and GAD65 in GABAergic terminals in the pyramidal layer (29). In the negative control, the primary antibodies were omitted (D; BAT). Bar = 20 μ m.

reflect a transmitter function. The amino acid vesicular transporters VGLUT and VGAT can be used to identify cells that release glutamate and/or GABA. To investigate whether the various subpopulations of adipocytes contained one or both of these transporter molecules, we took advantage of immunofluorescence microscopy and labeled the tissue sections with antibodies against VGAT and VGLUT1 (which is the most abundant VGLUT type in the brain). We found that both VGAT and VGLUT1 (Fig. 3) were present in BAT as well as in perirenal WAT depots.

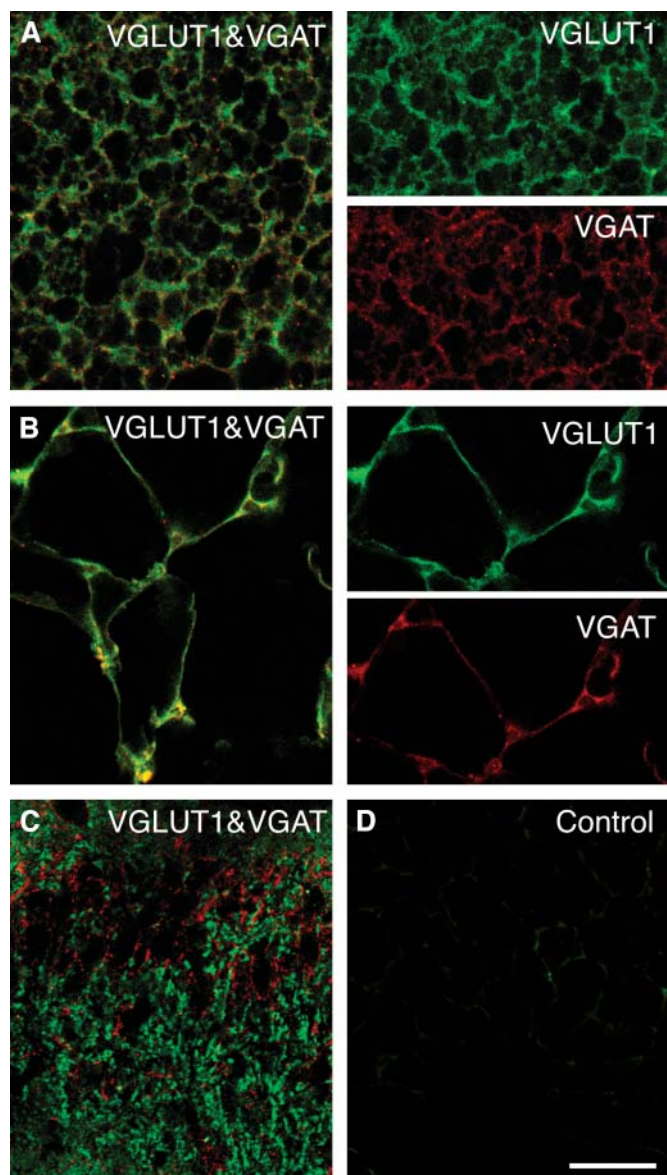


Fig. 3. Confocal images of the vesicular transporters VGLUT1 and VGAT in adipose tissue. VGLUT1 (green) and VGAT (red) were partly colocalized in interscapular BAT (A) and in perirenal WAT (B) cells. The left panels show overlays of the single-labeling images in the right panels. We also included a positive control of the brain showing that VGAT and VGLUT1 are located in separate structures (i.e., separate sets of nerve endings) (C; CA1 of the hippocampus) (29, 30). In the negative control, the primary antibody was omitted (D; BAT). Bar = 20 μ m.

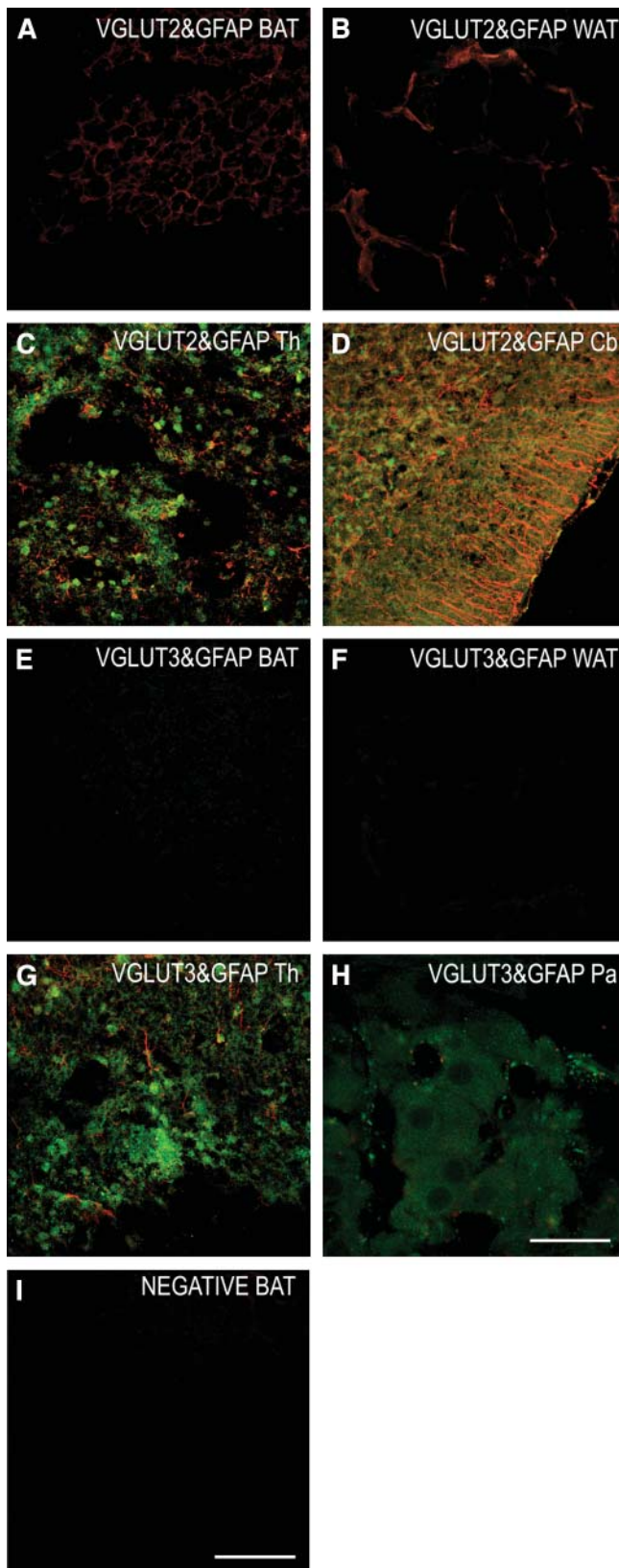


Fig. 4. Confocal images of the vesicular transporters VGLUT2 and VGLUT3 costained with glial fibrillary acidic protein (GFAP). Neither VGLUT2 (green) nor GFAP (red) was detected in adipose tissue. A, B: Staining in BAT (A) and perirenal WAT (B). C, D: VGLUT2 and GFAP labeling in brain sections was as expected in thalamus (Th; C) and cerebellum (Cb; D) (30). E–G: Similarly, VGLUT3 and GFAP could not be detected in BAT (E) or perirenal WAT (F), whereas positive staining in brain was as expected (G) (31). H: VGLUT3, but not GFAP, stained endocrine pancreas (Pa) as expected. I: In the negative control, the primary antibody was omitted (BAT). Bar = 50 μ m for all panels except H, where the bar = 20 μ m.

WAT. The intracellular staining pattern produced by antibodies against VGAT was similar to that obtained with the VGLUT1 antibodies (i.e., a fine granular appearance), compatible with the association with organelles, whereas the GAD65 labeling appeared more uniform, compatible with the localization of the enzyme primarily in cytosol (Fig. 2A, B). Similar results were obtained for VGAT

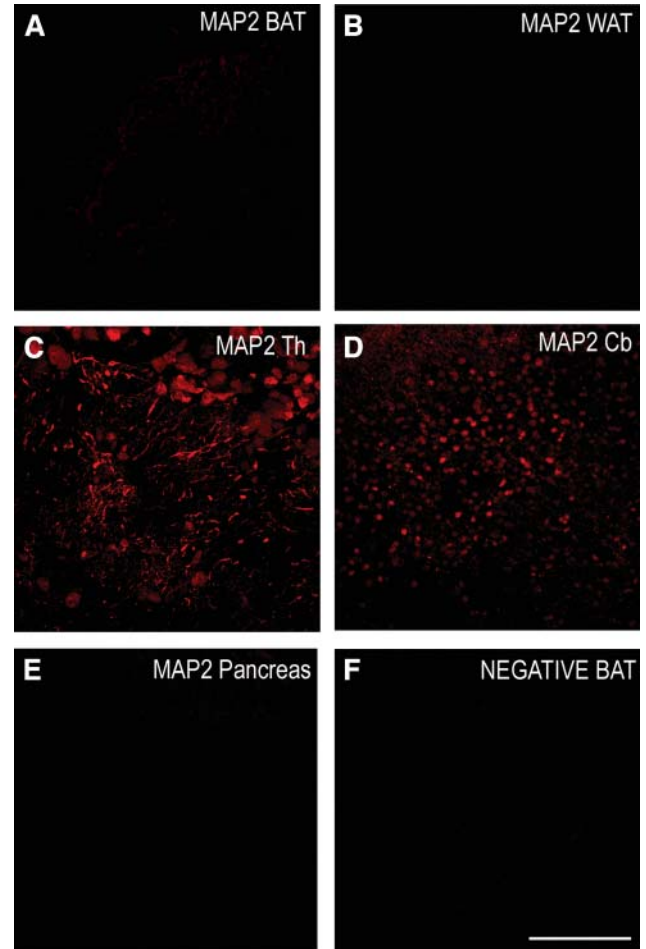


Fig. 5. Confocal images of the brain-specific protein microtubule-associated protein 2 (MAP2). MAP2 (red) staining was absent in BAT (A) and perirenal WAT (B). In contrast, MAP2 labeled brain sections as expected in thalamus (Th; C) and cerebellum (Cb; D). MAP2 did not stain pancreas (E). In the negative control, the primary antibody was omitted (F; BAT). Bar = 50 μ m.

In addition, we observed that GAD65 and VGAT were partly colocalized in these BAT and WAT depots (Fig. 2). Furthermore, as shown in Fig. 3, VGAT and VGLUT1 were present in the same adipocytes in BAT and in renal

and VGLUT1 in the other studied WAT depots (data not shown).

To further substantiate the presence of certain neurotransmitter components in adipose tissue, we performed a number of additional experiments. **Figures 4 and 5** show that although VGLUT2, VGLUT3, GFAP, and MAP2 were not present in either BAT or perirenal WAT, these antigens could be identified in CNS. VGLUT3, but not GFAP or MAP2, was present in endocrine pancreatic tissue, as expected.

Next, we studied whether VGAT, VGLUT1, and VGLUT2 were present in adipose tissue using the electron microscopic immunogold method. **Figure 6** shows that although VGAT and VGLUT-1 labeled vesicular organelles in both BAT and perirenal WAT, the level of VGLUT2 staining was not above background signal, corroborating our immunofluorescence findings.

To explore whether the vesicular transporter molecules were restricted to adipocytes or localized in other cell types, such as endothelial cells of blood vessels, we double-stained the adipose tissues with antibodies against the endothelial cell surface antigen RECA1 and VGAT or VGLUT1. There was no overlap between VGAT/VGLUT1

and RECA1 labeling; VGAT and VGLUT1 were both located exclusively in the adipocytes, whereas the RECA1 signal could be detected only in endothelial cells delineating blood vessels (**Fig. 7**) in BAT and in epididymal WAT. Corresponding distributions of VGAT/VGLUT1 and RECA1 were also found in all other WAT depots included in the study (data not shown).

VGAT and VGLUT1 are an integral part of the membrane of SVs. To explore whether such vesicles, termed synaptic-like microvesicles in peripheral organs, are present in adipocytes, we labeled the tissues with antibodies against the SV marker synaptophysin. Immunofluorescence labeling showed that synaptophysin was present in adipocytes of both BAT and perirenal WAT (**Fig. 8**) as well as in adipocytes of the other WAT depots studied (data not shown). In the adipocytes, synaptophysin was partly colocalized with both VGAT and VGLUT1.

Like the amino acid labelings (**Fig. 1**), the GAD65 (**Fig. 2**), VGAT, VGLUT1, and synaptophysin labelings (**Fig. 8**) were all localized throughout the cytoplasmic part of BAT and WAT adipocytes, leaving the lipid droplets unstained.

To further substantiate the putative roles of the detected amino acids as signaling substances in adipose tis-

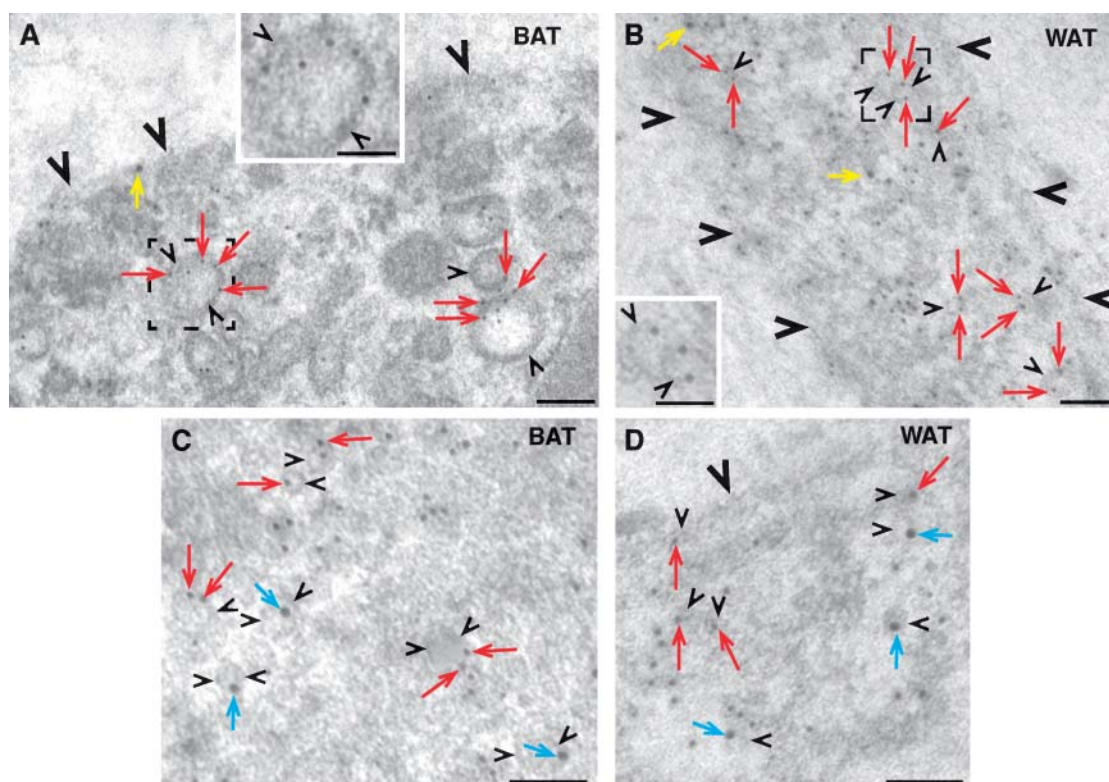


Fig. 6. VGAT and VGLUT1 in vesicular organelles in adipose tissue. Electron micrographs of double-labeled sections showing VGAT (small gold particles)/VGLUT2 (large gold particles) in BAT (A) and perirenal WAT (B) as well as VGAT (small gold particles)/VGLUT1 (large gold particles) in BAT (C) and perirenal WAT (D). As the lateral resolution of the immunogold method is ~ 30 nm, most VGAT gold particles (red arrows) and VGLUT1 gold particles (blue arrows) are located in such a way that they signal transporters situated in the membranes of vesicular organelles (small arrowheads). The density of VGLUT2 (yellow arrows) was very low and not different from the background level. Large arrowheads indicate the surface of the adipocytes. Insets show larger magnifications of the areas indicated by broken rectangles. Bars = 100 nm (50 nm in insets).

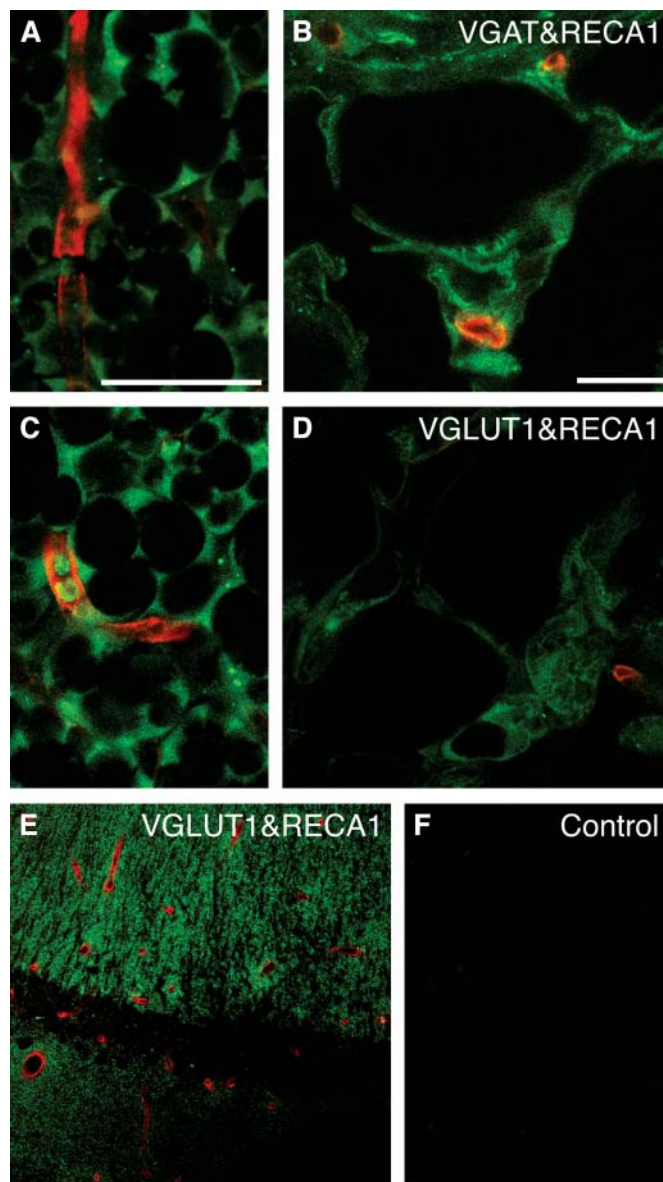


Fig. 7. VGAT/VGLUT1 and the endothelium marker RECA1 have different distributions in adipose tissue. Images of VGAT and RECA1 are from BAT (A; VGAT is green and RECA1 is red) and epididymal WAT (B), whereas images of VGLUT1 and RECA1 are from BAT (C) and epididymal WAT (D). The positive control is from the brain (E; CA1 of the hippocampus). In the negative control, the primary antibodies against VGAT and RECA1 were omitted and only the secondary antibodies were used (F; BAT). A similar negative control was obtained with omission of the primary antibodies against VGLUT1 and RECA1, using only the secondary antibodies (data not shown). Scale bars are the same for A and C and for B, D, E, and F. Bars = 20 μ m for all panels.

sues, we studied the localization of glutamate and GABA receptors in BAT and WATs. By the use of immunofluorescence microscopy, we found that the AMPA-type glutamate receptor subunit GluR2/3 and the NMDA-type glutamate receptor subunit NR1 were present in BAT and in all WAT depots studied (Fig. 9A, B). Furthermore, GABA_A receptor subunit α 2 labeling could also be iden-

tified in all adipose tissues examined (Fig. 9C). In contrast to these ionotropic receptors, the metabotropic glutamate receptor mGluR2 was not detected in any of the adipose tissues studied (Fig. 9D).

DISCUSSION

Amino acid transmitters have been mostly studied in the brain. However, previous results suggest that both glutamate and GABA have a signal function in the endocrine pancreas (10–13). We now extend the evidence for a peripheral signal function of amino acids, presenting novel data strongly indicating that both BAT and various WAT depots contain the necessary compounds involved in transmitter amino acid signaling. Thus, we could demonstrate that the excitatory amino acid transmitter glutamate and the inhibitory amino acid transmitter GABA were present within adipocytes of BAT and WAT depots. In addition, the GABA-synthesizing enzyme isoforms GAD65 and GAD67 and the vesicular transporters VGLUT1 and VGLUT1, as well as the SV marker synaptophysin, could be detected in the same adipose tissue specimen. Together with the localization of the glutamate receptor subunits NR1 and GluR2/3 as well as the GABA_A receptor subunit α 2 on these BAT and WAT cells, these findings indicate that glutamate and GABA are released from intracellular synaptic-like microvesicles to evoke receptor effects on neighboring adipocytes through paracrine mechanisms.

The consistent inclusion of negative and positive controls derived from adipose tissue, brain samples, and extra-CNS tissue supported our findings. Specifically, immunoreactivities for VGLUT2 and VGLUT3, MAP2, GFAP, and mGluR were not detected in either BAT or WAT, whereas they could be demonstrated in relevant positive controls (i.e., in the brain).

Our study has some limitations. The mere identification of the various components of such signaling systems does not necessarily imply a function of amino acids in signal transmission within adipose tissues. Definite proof would require electrophysiological recordings of their activity at baseline and during perturbations of adipose metabolism. Although considered outside the scope of this study, further support for a role of amino acid transmitters in adipose signaling might be gained by the quantification of amino acids and their vesicular transporters and receptors using immunogold labeling and electron microscopy of adipose tissue in different states of metabolism. Moreover, there are several glutamate and GABA receptors in the CNS, and possibly there could be more amino acid receptors expressed on adipocytes than were examined in this study.

It is possible that intracellular concentrations of glutamate and GABA also reflect the synthesis of glutamate and GABA from mitochondrial citric acid cycle activity in addition to and/or instead of a possible paracrine signaling role. If so, these amino acids could be useful markers of metabolic activity in adipose tissues, monitor-

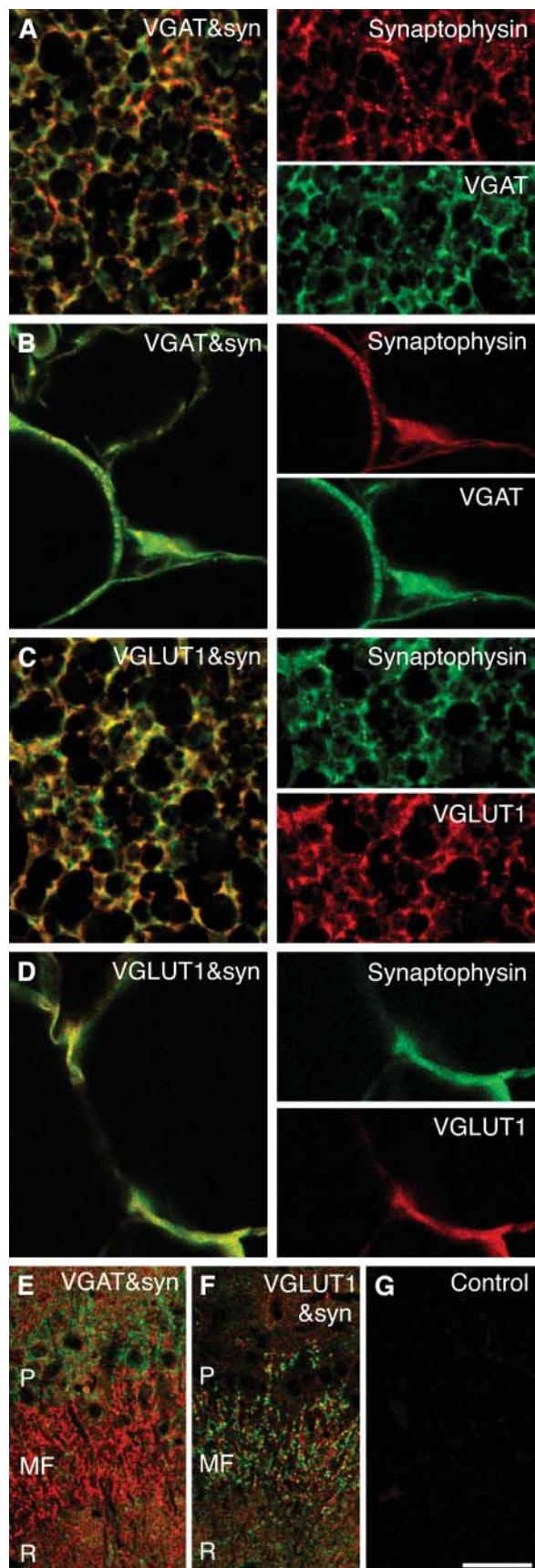


Fig. 8. Confocal images showing that the vesicular transporters VGAT and VGLUT1 were colocalized with synaptophysin (syn) in adipose tissues. A, B: Colocalization of VGAT (green) and synaptophysin (red) in interscapular BAT (A) and perirenal WAT (B) cells. C, D: Colocalization of VGLUT1 (red) and synaptophysin (green) in interscapular BAT (C) and perirenal WAT (D) cells. The left panels show overlays of the single-labeling images in the right panels. E, F: In the positive controls of brain (CA3 of the hippocampus; P, MF, and R indicate the pyramidal layer, mossy fiber, and radiatum layer, respectively), VGAT (green) and synaptophysin (red) (E) and VGLUT1 (red) and synaptophysin (green) (F) were colocalized, but in different sets of nerve endings, together accounting for most nerve endings (as identified by labeling for synaptophysin) (29, 30). G: In the negative control, the primary antibodies against VGAT and synaptophysin were omitted and only the secondary antibodies were used (BAT). A similar negative control was obtained with omission of the primary antibodies against VGLUT1 and synaptophysin, using only the secondary antibodies (data not shown). Bar = 20 μ m.

ing cell metabolism and signaling this to neighboring cells, possibly coordinating the release or uptake of free fatty acids and other metabolic substrates to and from the blood.

Although the autonomous innervation of BAT and WAT is well established, a dysfunctional role of particularly the sympathetic system in the pathogenesis of obesity and the metabolic syndrome has only recently gained attention. Accumulating evidence indicates that altered input from the CNS to adipose tissues might modify the normal regulation of body weight and energy homeostasis as well as insulin sensitivity (3). Whereas the CNS possibly can affect various fat depots in a coordinated manner, direct cell-to-cell signaling might also modify the metabolic activity among distantly located adipocytes. This may be of importance, because data from humans have shown important differences among WAT depots, including distinct gene expression profiles, variabilities in the activities of enzymes involved in lipid metabolism, and changed expression of adipokines (6, 24–26). Most importantly, certain differences in WAT depots might impose alterations in the risk of disease. For example, visceral WAT compared with subcutaneous WAT is consistently associated with increased morbidity (e.g., from cardiovascular disease and obesity) (26–28). In this study, the various components of the amino acid transmitter signaling system could be identified in both interscapular BAT and all WAT depots studied, suggesting a possible ubiquitous role of this signaling system. However, the present qualitative data do not allow inference regarding the relative importance of amino acid signaling in different categories of fat depots.

In conclusion, these findings could form the basis for the development of further research strategies to understand how individual adipose cells coordinate the storage and release of fat from their intracellular fat droplets and/or how they might coordinate growth and cell division. **||**

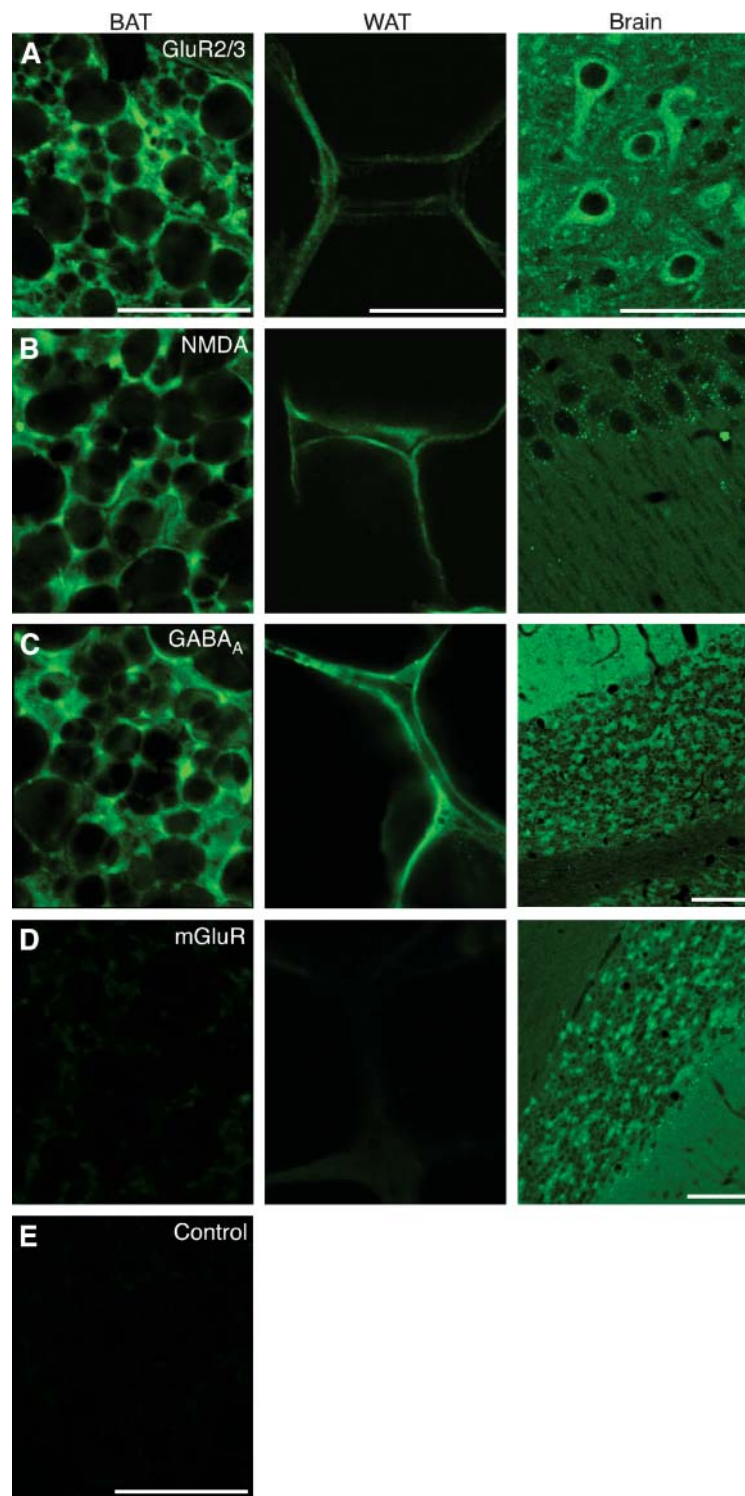


Fig. 9. Glutamate and GABA receptors in BAT and WAT cells. A: α -Amino-hydroxy-5-methyl-4-isoxazolepropionate receptor subunits GluR2/3 were found on interscapular BAT (left panel) and on interscapular WAT (middle panel) cells as well as in pyramidal cells of the brain cortex (positive control; right panel) (32). B: *N*-Methyl-D-aspartate (NMDA) receptor subunit NR1 was located on BAT (left panel) and on intestinal WAT (middle panel) cells as well as on pyramidal cells of CA1 hippocampus (positive control; right panel) (33). C: The GABA_A receptor subunit α 2 was also found on BAT (left panel) and on renal WAT (middle panel) cells as well as in the cerebellum (positive control; right panel) (34). D: The metabotropic glutamate receptor mGluR2 was present neither on BAT (left panel) nor on interscapular WAT (middle panel) cells but showed the expected localization in the cerebellum (positive control; right panel) (35). E: In the negative control, the primary antibodies (GluR2/3, NR1, and GABA_A) were omitted and only the secondary antibody was used (BAT). A similar negative control was obtained with omission of the primary antibody detecting mGluR2, using only the secondary antibodies (data not shown). The scale bar is the same for all panels except the right panels of C and D. Bars = 20 μ m for all panels.

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