Cloning of a functional vesicular GABA and glycine transporter by screening of genome databases

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Abstract The *unc-47* locus of *Caenorhabditis elegans* has been suggested to encode a synaptic vesicle GABA transporter. Here we used hydropathy plot analysis to identify a candidate vesicular GABA transporter in genomic sequences derived from a region of the physical map comprising *unc-47*. A mouse homologue was identified and cloned from EST database information. In situ hybridization in rat brain revealed codistribution with both GABAergic and glycinergic neuronal markers. Moreover, expression in COS-7 and PC12 cells induced an intracellular, glycine-sensitive GABA uptake activity. These observations, consistent with previous data on GABA and glycine uptake by synaptic vesicles, demonstrate that the mouse clone encodes a vesicular inhibitory amino acid transporter.

Key words: Neurotransmission; Synaptic vesicle; Neurotransmitter transporter; GABA; Glycine; *Caenorhabditis elegans*

1. Introduction

Before their exocytotic release for neurotransmission, non-peptide neurotransmitters are loaded into synaptic vesicles by vesicular transporters driven by a H^+-ATPase [1,2]. Studies on isolated secretory vesicles have identified independent uptake activities for monoamines (catecholamines, serotonin, histamine), acetylcholine, glutamate, GABA and glycine, and ATP. The transporters responsible for the first two activities have been recently identified. Two isoforms of the mammalian monoamine transporter, VMAT1 and VMAT2, were characterized by cDNA expression cloning [3,4]. These isoforms, derived from distinct genes, share about 60% amino acid identity. Thereafter, a highly homologous protein was revealed by cDNA cloning and sequencing of the *Caenorhabditis elegans unc-17* gene [5]. Since this protein was shown to be localized to cholinergic synaptic vesicles, it was concluded that the *unc-17* gene encodes the vesicular acetylcholine transporter. Cloning and expression of homologous vertebrate proteins firmly established this conclusion [6,7]. These three related transporters contain ~500 amino acids, and possess 12 putative transmembrane domains (TMs) with a large, intravesicular 'loop' between TM1 and TM2 according to current topological models.

Although a majority of central nervous system synapses use amino acids as transmitters, the corresponding vesicular transporters have not yet been characterized. Here we report the cDNA cloning and expression of a vesicular transporter for GABA and glycine, based on recent genetic studies in *C. elegans*. In this animal, the reciprocal inhibition of opposing body muscles is ensured by GABAergic neurons. The selective destruction of these neurons with a laser microbeam was recently shown to induce a 'shrinker' behaviour [8] and, in a related study [9], several mutants displaying a 'shrinker' phenotype were found to be selectively impaired in GABAergic transmission. Interestingly, the absence of defects in GABA synthesis or GABA postsynaptic sensitivity in two mutants, *unc-46* and *unc-47*, led to the suggestion that the *unc-46* or *unc-47* genes might encode the vesicular GABA transporter [9]. Since the defects observed in *unc-47* animals were more severe and unambiguously presynaptic, we investigated this hypothesis for the *unc-47* gene.

2. Materials and methods

2.1. Bioinformatics

An integrated genetic and physical map of *C. elegans* (ACeDB) is accessible at the WWW site: http://probe.nalusda.gov. The positive clones of loci *stP127* and *unc-69*, cosmids RW#L127 and C46D2 respectively, were used to select the following set of sequenced cosmids: M04D8, T16G12, T16H12, ZK1128, K08E5, T20G5, R01H10, K10G9, and T07A5. These cosmids overlap, except T20G5 and R01H10 which are separated by a gap of about 40 kb. The proteins predicted from the cosmid sequences are accessible in Genbank or equivalent databases. Hydropathy profiles [10] were plotted using the DNA Strider 1.2 software, with a window size of 11. Homology searches in gene databases were performed using BLAST [11]. The significance of the highest scores was assessed further by global pairwise alignment using BESTFIT (GCG software package). Fifty alignments of randomized sequences were performed as negative controls.

2.2. Cloning of the mouse VIAAT cDNA

The mouse and human I.M.A.G.E. cDNA clones [12] corresponding to ESTs [13] W64201 (ID #385606) and H23502 (ID #52553), respectively, were obtained from GenomeSystems. The mouse clone contains a short cDNA fragment (406 bp). An EST 'bridging' strategy, using PCR on mouse brain Marathon-Ready cDNA with Advantage-HF polymerase mix (Clontech), was developed to isolate longer cDNAs. Amplifications using primers derived from the three non-overlapping mouse ESTs W64201, R75019 and R75020 indicated that they all derived from a single mRNA species. A 1277 bp partial cDNA was amplified using the primers 5′-GAATTGAACTAGGGGCGCAGAAG, derived from EST W64201, and 5′-CCA-

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Abbreviations: EST, expressed sequence tag; GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; GAT, plasma membrane GABA transporter; GLYT, plasma membrane glycine transporter; RACE, rapid amplification of cDNA ends; TM, transmembrane domain; VIAAT, vesicular inhibitory amino acid transporter; VMAT, vesicular monoamine transporter

The mouse VIAAT cDNA sequence has been submitted to the EMBL Data Bank under the accession number AJ001598.

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CATCGAAGAAGACCTGGTGC, derived from EST R75019. The PCR product was characterized by TOPO TA Cloning (Invitrogen) and automated sequencing (ABI). This product lacks both 5′ and 3′ ends of the coding sequence. To recover the 3′ end, we followed the same bridging strategy: the human clone was sequenced, providing a 733 bp 3′ untranslated sequence that was used as a query sequence for a second-round homology searching and led to the identification of a novel mouse EST, AA028280. This EST lacks coding sequence and was thus not identified in the first round (done with the T20G5.6 amino acid sequence as a query). PCR with primers 5′-CATCTTATTCACCACGAGCACACCACAC, derived from AA028280, and 5′-ACATCCTGGTCATCGCTTACTGTCTC, derived from R75019, amplified a 1018 bp product that perfectly matched the previous clone in a 652 bp overlap and extended 283 bp downstream the STOP codon. 5′-RACE [14] with the mVIAAT antisense primer 5′-GCATTTGTCACGTTCCAGCCCGCTTCC and the Marathon Adaptor Primer 1 was used to recover the 5′ end. Four consistent independent clones, extending up to 44 bp upstream the first in-phase ATG, were obtained.

Each base of the reassembled mVIAAT coding sequence was determined by sequencing both strands of 2 to 4 independent PCR clones, in order to discard possible PCR artefacts (a single point mutation was found). It has to be noted that the first 10 bases of EST W64201 mismatches the coding sequence due to a cloning artefact of the I.M.A.G.E. clone #385606. A full-length cDNA clone was constructed by ligating the longest 5′-RACE product and the 3′-RT-PCR product to the 1277 bp partial cDNA, at restriction sites *Dra*III and *Afl*II, respectively. The full-length mVIAAT cDNA was subcloned into the expression vector pcDNA3 (Invitrogen).

2.3. In situ hybridization

Single strand, [^{35}S]UTP-labelled cRNA antisense probes were synthesized with Ampliscribe kits (Epicentre Technology). The following plasmids were used as templates for the synthesis of the probes: (i) a full-length rat GAD67 cDNA in pSPT18 (a gift of J.F. Julien [15]), (ii) a RT-PCR product from mouse brain encoding the 323 C-terminal amino acids of GLYT2 [16], cloned in pCRII (Invitrogen) and (iii) for mVIAAT, the mouse I.M.A.G.E. clone #385606 in pT7T3D-PAC. The templates (50 ng) were linearized with *Pvu*II (GAD67) or *Xho*I (GLYT2 and mVIAAT), and used with SP6 (GAD67 and GLYT2) or T3 polymerase (mVIAAT). Cryostat parasagittal or coronal rat brain sections (10 μm) were mounted on slides, fixed in 4% formaldehyde and hybridized overnight at 60°C with the labelled riboprobes (2×10^6 dpm in 50 μl). Slides were treated with RNAase A, washed in 0.1×SSC buffer (30 mM NaCl, 3 mM sodium citrate) at 55°C, dried and apposed to β max Hyperfilms (Amersham) for 5 days. For observations at the cellular level, sections were dipped into emulsion (LM1, Amersham), exposed for 8 days and photographed under dark-field or bright-field light.

2.4. Expression in COS-7 and PC12 cells

COS-7 cells were grown under 5% CO_2 in glucose rich Dulbecco's Modified Eagle Medium supplemented with 7.5% foetal bovine serum (GIBCO BRL). PC12 cells were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine and 7% Supreme serum (Bio-Products). Cells (10^6 and 5×10^6 for COS-7 and PC12, respectively) were transfected by electroporation with both pcDNA3-mVIAAT and a plasmid encoding a plasma membrane transporter of GABA (rat GAT1 in pcDNA1, a gift of N. Nelson [17]) or glycine (human GLYT1b in pRcCMV [18]). Negative controls were done by replacing the VIAAT plasmid by a pcDNA3 vector bearing no insert or a cDNA for the bovine vesicular monoamine transporter VMAT2 [19]. The transfected cells were divided in 12 aliquots and cultured in 24-well (15 mm diameter) culture plates. Two or three days after transfection, GABA or glycine uptake by intact adherent cells was assayed as follows. The culture wells were washed twice with 0.5 ml uptake buffer (5 mM Tris/HEPES, pH 7.5, 140 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1 mM ascorbic acid, 5 mM D-glucose). Cells were then incubated for 10 min at room temperature in 0.2 ml uptake buffer supplemented with 30 nM [^{3}H]GABA (84 Ci/mmol; Amersham) or 300 nM [^{3}H]glycine (16 Ci/mmol; Amersham). The reaction was stopped by two brief washings with 0.5 ml of ice-cold uptake buffer. The radioactivity accumulated in the wells was recovered in 0.2 ml of 0.1 N NaOH and counted by liquid scintillation in Ready Protein$^+$ (Beckman).

3. Results

We used a positional cloning strategy to identify a vesicular GABA transporter candidate from nematode genomic sequences [20]. The nearest neighbouring cloned loci of *unc-47* on the current genetic map III of *C. elegans*, namely *stP127* and *unc-69*, were used to define a region of the physical map comprising *unc-47*. Nearly 90% of this region was sequenced at the time of our search. The known sequence, encompassing 280 kb, was distributed on two sets of overlapping cosmids separated by a 40 kb gap. In order to select transporter candidates, we analyzed the hydropathy plots of the 60 proteins predicted from this sequence. Nine hypothetical proteins had at least 6 maxima reaching a hydropathic index of 2 or more. Considering the size range of most known ion-coupled transporters, 5 proteins of less than 400 or more than 900 amino acids were not analyzed further. Homology searching showed that three of the four remaining candidates shared about 40% overall amino acid identity with functionally characterized mammalian transporters: the Na^+/dicarboxylate cotransporter [21], for hypothetical protein K08E5.2, and the brain-specific Na^+/phosphate cotransporter [22], for both K10G9.1 and T07A5.3. This value of 40% was considered as suggestive of functional similarity based on pairwise alignments of functionally characterized orthologous proteins, such as the rat and nematode vesicular acetylcholine transporter [6,23]. The last candidate, T20G5.6, showed no homology with functionally known proteins and was thus considered as the leading candidate for the *unc-47* gene product.

We then performed a homology search for T20G5.6 in the EST division of Genbank [24]. This led to the identification of four ESTs from mouse or human brain, sharing 40–50% identity with T20G5.6 in 60–90 amino acid stretches: W64201, R75019, R75020 and H23502. The clones corresponding to ESTs W64201 (from mouse) and H23502 (from human) were available from the I.M.A.G.E. Consortium [12]. Since both of them are partial clones, a combination of RT-PCR and 5′-RACE [14] was used to recover a full-length coding sequence from mouse brain (see Section 2.2). As shown in Fig. 1a, the amino acid sequence deduced from the mouse cDNA is 38% identical to the nematode sequence for T20G5.6. Since in situ hybridization and expression studies indicate that the mouse protein is a vesicular transporter of GABA and glycine (see below), the protein will be designated as mVIAAT, for 'mouse vesicular inhibitory amino acid transporter', to refer to the usual role of GABA and glycine in neurotransmission.

Hydropathy plots [10] of mVIAAT and T20G5.6, shown in

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Fig. 1. Structure of mVIAAT. a: Optimal alignment of the mouse VIAAT and nematode T20G5.6 sequences. The alignment was determined using BESTFIT. The hydrophobic core of putative transmembrane domains are lined above the mouse sequence and numbered. Since the second in-frame ATG codon of the mouse sequence has a better context for the initiation of translation (GCC AGG *ATG* G) than the first one (AGC GGC *ATG* T), the mouse polypeptide may possibly begin at residue M4. b: Hydropathy plot of the mouse sequence. c: Topological model of mVIAAT.

a

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mVIAAT:   MFARMGFQAA TDEEAVGFAH CDDLDFEHRQ GLQMDILKSE GEPCGDEGAE 50
                                 :  .:::. ::.: .:..  ....::..:.
T20G5.6:                         MCTSQKR SFAVRTWPPL QSRGSNPPPH 27

mVIAAT:   APVEGDIHYQ RGGAPLPPSG SKDQAVGAGG EFGGHDKPKI TAWEAGWNVT 100
          ..:|.  .    ::.| ...  .|::.. .:| .  |.....| .|::|:||||
T20G5.6:  DRLEPIQESV VSEQPQKDDI NKQEEAKDDG H..GEASEPI SALQAAWNVT 75
                              1_____________________
mVIAAT:   NAIQGMFVLG LPYAILHGGY LGLFLIIFAA VVCCYTGKIL IACLYEENED 150
          |||||||::| ||.|:  ||: :::  :: .|  ||::|| :| |.||||:.
T20G5.6:  NAIQGMFIVG LPIAVKVGGW WSIGAMVGVA YVCYWTGVLL IECLYENG.. 123
                                          2_____________________
mVIAAT:   GEVVRVRDSY VAIANACCAP RFPTLGGRVV NVAQIIELVM TCILYVVVSG 200
             |: |..|  .||: :      |.:| :|:  .||:.||:  |||:|:|:.:
T20G5.6:  ...VKKRKTY REIADFYK.. ..PGFGKWVL .AAQLTELLS TCIIYLVLAA 165
                        3_____________________     4_________
mVIAAT:   NLMYNSFPGL PVSQKSWSII ATAVLLPCAF LKNLKAVSKF SLLCTLAHFV 250
          :|: .:||:   |.. :| :| ..| ||.|.| |.:|. ||:: |:: .:.|::
T20G5.6:  DLLQSCFPS. .VDKAGWMMI TSASLLTCSF LDDLQIVSRL SFFNAISHLI 213
          ________                          5_____________________
mVIAAT:   INILVIAYCL SRARDWAWEK VKFYIDVKKF PISIGIIVFS YTSQIFLPSL 300
          :|:::: ||| | ..:|.:.. :.| :::..: |. :|::||: |||:||||.|
T20G5.6:  VNLIMVLYCL SFVSQWSFST ITFSLNINTL PTIVGMVVFG YTSHIFLPNL 263
                              6_____
mVIAAT:   EGNMQQPSEF HCMMNWTHIA ACVLKGLFAL VAYLTWADET KEVITDNLPG 350
          ||||..|.:| :.|:.|.||| |.|:|.:|:: :::||::: | .| |.:.||.
T20G5.6:  EGNMKNPAQF NVMLKWSHIA AAVFKVVFGM LGFLTFGELT QEEISNSLPN 313
                    7_____________
mVIAAT:   .SIRAVVNLF LVAKALLSYP LPFFAAVEVL EKSLFQEGSR AFFPACYGGD 399
           |:: :|||: ||.|||||| |||:|||::| ...|| : .. . |..||:.|
T20G5.6:  QSFKILVNLI LVVKALLSYP LPFYAAVQLL KNNLFLGYPQ TPFTSCYSPD 363
                    8_____________________     9_____________
mVIAAT:   GRLKSWGLTL RCALVVFTLL MAIYVPHFAL LMGLTGSLTG AGLCFLLPSL 449
           .|:.|::|| |. ||:|||: :|: ||.::.  ||||.|.:|| . |:|::|.|
T20G5.6:  KSLREWAVTL RIILVLFTLF VALSVPYLVE LMGLVGNITG TMLSFIWPAL 413
          _                         10____________________
mVIAAT:   FHLRLLWRKL LWHQVFFDVA IFVIGGICSV SGFVHSLEGK FAGLET     495
          ||| :  :.|    :  || : |:::|. .:: ||.  |  :  : :::.
T20G5.6:  FHLYIKEKTL NNFEKRFDQG IIIMGCSVCI SGVYFSSMEL LRAINSADS  462
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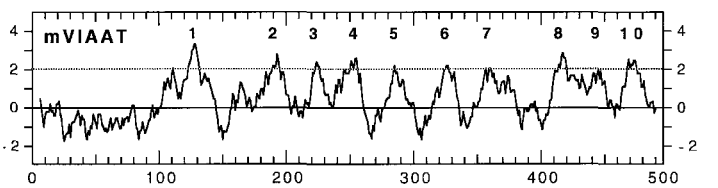
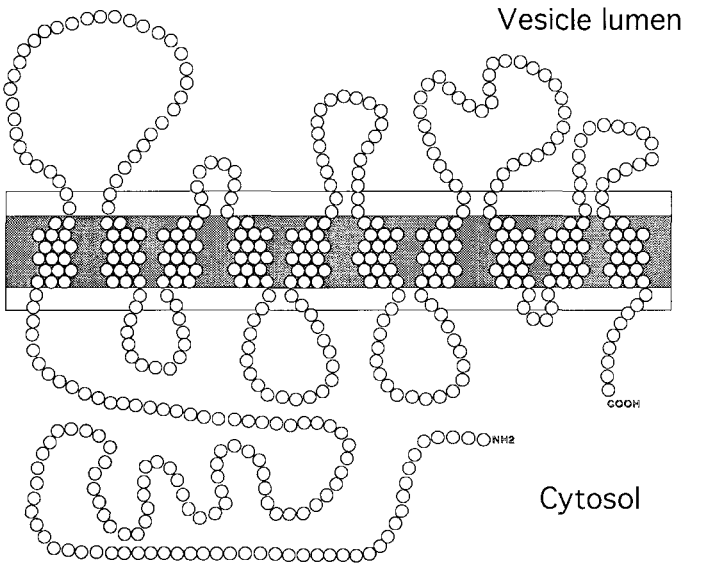
b



c



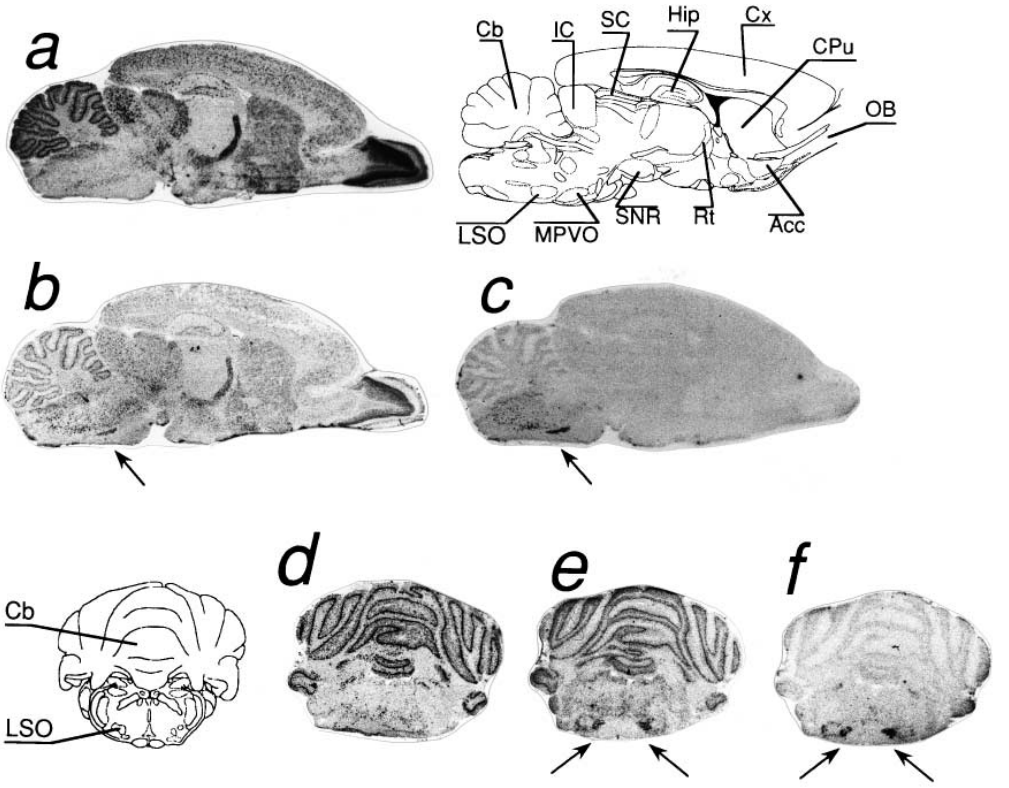


Fig. 2. Regional distribution of the VIAAT mRNA in rat brain. The distribution of the VIAAT mRNA (b and e) is compared with those of GAD67 (a and d) and GLYT2 (c and f) in parasagittal (a, b and c) and coronal (d, e and f) sections. Arrows in b, c, e and f point at glycinergic nuclei. A diagram depicts the main structures for each section. Abbreviations: Acc, nucleus accumbens; Cb, cerebellum; CPu, caudate putamen; Cx, cortex; Hip, hippocampus; IC, inferior colliculus; LSO, lateral superior olivary nucleus; MPVO, medioventral periolivary nucleus; OB, olfactory bulb; Rt, reticular thalamic nucleus; SC, superior colliculus; SNR, substantia nigra pars reticulata.

Fig. 1b for the mouse sequence, predict the existence of 10 transmembrane domains (TMs), with a large, hydrophilic N-terminal domain (Fig. 1c). The latter domain lacks a signal peptide and, due to its size, may fold before the first TM is synthesized and inserted in the reticulum membrane. The N-terminus is thus assumed to be cytosolic. A consequence of this topological model is that mVIAAT may not be N-glycosylated, since the first putative glycosylation site (N98) is cytosolic and the second one (N315), albeit luminal, is too close to TM6 to reach the active site of oligosaccharide transferase [25]. Eight protein kinase C or casein kinase II consensus sites are present in the mouse sequence, among which three, present in the N-terminal domain (T11, S39 and T91), might have a biological significance. The other sites are luminal or close to the TMs, and thus should not be accessible to protein kinases. An interesting feature shown by the alignment (Fig. 1a) is the presence of three conserved, charged residues in TM2 (E187), TM6 (K325) and TM7 (K363), which might prove critical for the activity of the proteins.

Neither the mouse VIAAT nor the nematode protein T20G5.6 have homology with functionally characterized proteins. In particular, no significant homology could be detected with the vesicular monoamine or acetylcholine transporters (Table 1). However, homology searching allowed the detection of a weak similarity of mVIAAT with several 'orphan' proteins predicted from genomic sequences. The results of

Table 1
Homologies of mVIAAT

Species	Protein	Status	% Identity	Quality score	Average quality score after 50 randomizations (±S.D.)
Caenorhabditis elegans	T20G5.6	hypothetical	37.6	352.6	146.1±5.5
Arabidopsis thaliana	ATU90439	hypothetical	27.0	218.6	143.8±5.1
Saccharomyces cerevisiae	YJR001w	hypothetical	21.5	213.1	165.0±4.9
Saccharomyces cerevisiae	YNL101w	hypothetical	20.2	221.4	168.3±5.4
Saccharomyces cerevisiae	YEL064c	hypothetical	20.1	197.0	148.5±4.9
Rattus norvegicus	rVMAT2	translated (cDNA)	13.5	161.9	159.1±4.6
Rattus norvegicus	rVAChT	translated (cDNA)	19.0	163.6	162.6±5.5

Results of BESTFIT alignments with mVIAAT are shown. Statistically significant alignments have a quality score above the average score obtained with randomized sequences. Proteins predicted from genomic sequences are mentioned as 'hypothetical' and designated by their individual codes, except for the *A. thaliana* protein, which corresponds to an unannotated open reading frame (bases 30749–31990) in the BAC clone T06D20 from chromosome II (accession #U90439). Data for the rat vesicular monoamine transporter (rVMAT2; acc. #M97381) and acetylcholine transporter (rVAChT; acc. #U09211) are shown for comparison.

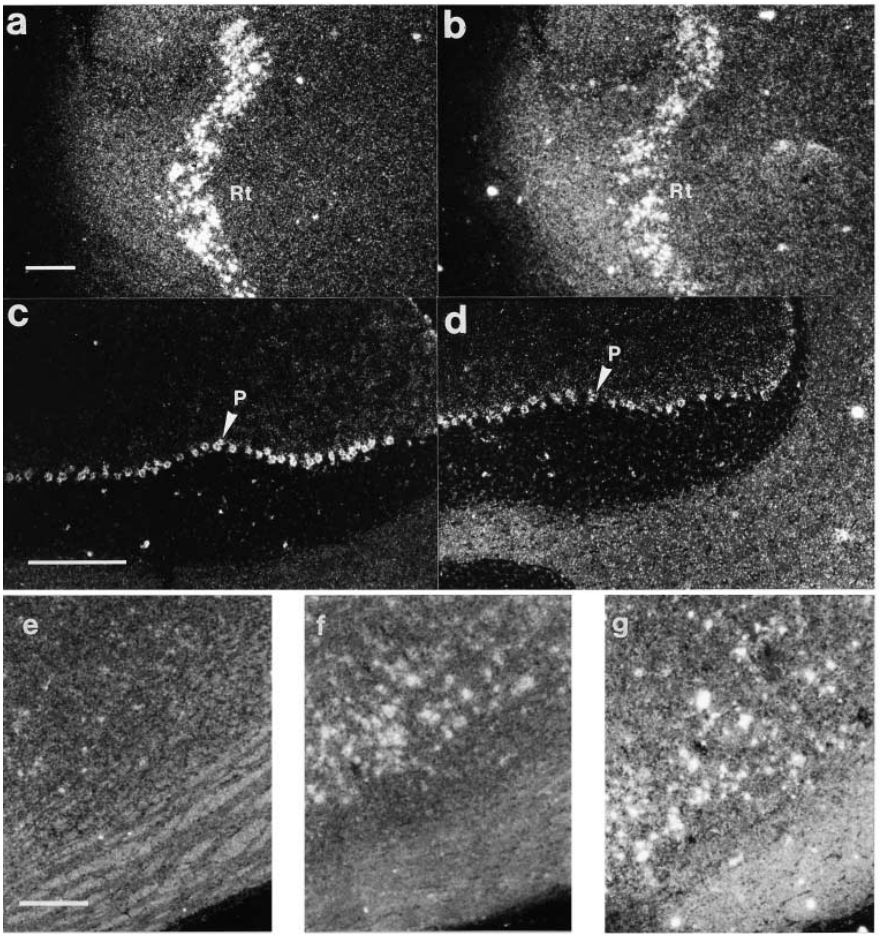


Fig. 3. Cellular distribution of the VIAAT mRNA in rat brain. Dark-field microphotographs of emulsion-coated sections are shown for the reticular thalamic nucleus (a and b), the cerebellum (c and d) and the lateral superior olive in the brainstem (e, f and g). The slides were labelled with probes for the GAD67 (a, c and e), VIAAT (b, d and f) or GLYT2 (g) transcripts. Abbreviations: Rt, reticular thalamic nucleus; p, Purkinje cell layer. Scale bar, 300 μm.

pairwise global alignments are shown in Table 1 for the five highest scores of the BLAST search [11], excluding ESTs. Low homologies (20–27% identity) were observed with four plant or yeast proteins. A randomization test showed that these homologies, which are scattered along the overall alignments (not shown), are statistically significant (Table 1). The biological significance of these alignments is further supported by the fact that these orphan proteins are also homologous to one another; for example, the *Arabidopsis thaliana* protein is 27% identical to the *Saccharomyces cerevisiae* protein YNL101w (not shown). These data suggest that mVIAAT, its nematode homologue and the distantly related yeast and plant proteins may have evolved from a common ancestor.

As a first step to determining the function of VIAAT, we characterized the distribution of its cognate mRNA in rat

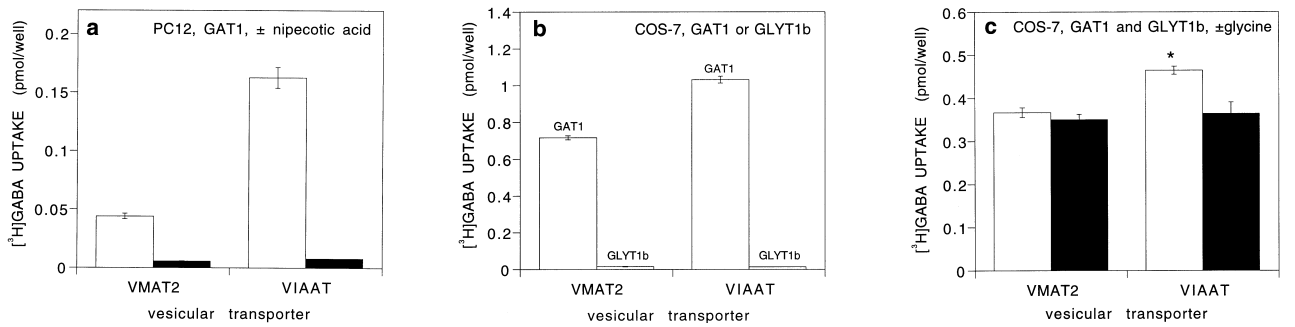


Fig. 4. Expression of the mouse VIAAT in PC12 and COS-7 cells. Cells were transfected with the VIAAT or VMAT2 cDNA, as well as with the GAT1 and/or GLYT1b cDNAs. [^{3}H]GABA uptake was assayed as described in Section 2.4. Black bars correspond to the addition of 0.2 mM nipecotic acid (a) or 10 mM glycine (c) to the uptake medium. Data are means ± S.E.M. of 3 to 6 determinations. The asterisk (*) in (c) denotes a statistically significant difference with the other bars ($P < 0.005$; $n = 4$).

brain by in situ hybridization, and compared it with that of markers of GABAergic and glycinergic neurons. We used the GAD67 isoform of the GABA biosynthetic enzyme glutamic acid decarboxylase as a marker of GABAergic neurons [15,26] and the plasma membrane glycine transporter GLYT2 as a marker of glycinergic neurons [16,27–29]. The GLYT2 protein has been shown recently by electron microscopic immunocytochemistry to be almost exclusively localized in axons and terminals of neurons thought to be glycinergic [29]. As shown in Fig. 2a, b, d and e, the regional distribution of the VIAAT mRNA paralleled that of GAD67 in the olfactory bulb, the thalamic reticular nucleus and the cerebellum, as well as in more diffusely labelled areas such as the superior and inferior colliculi, the caudate-putamen, the hippocampus and the substantia nigra. However, in the brainstem, the VIAAT mRNA appeared concentrated in nuclei of the superior olivary complex, which were not detected with the GAD67 probe but were intensely labelled by the GLYT2 probe (Fig. 2c and f, arrows). These nuclei are documented as glycinergic [29]. The overall intensity observed with the three markers indicated that the level of VIAAT transcript is in the same range as that of GLYT2, but much lower than that of GAD67. The correlation of the mVIAAT mRNA distribution with those of GAD67 and GLYT2 was also investigated at the cellular level. Similar patterns were observed for the VIAAT and GLYT2 mRNAs in the lateral superior olive (Fig. 3f and g). In known GABAergic structures [26], such as the reticular nucleus (Fig. 3a and b), the inferior colliculus, the hilus of the hippocampal dentate gyrus and the olfactory bulb (not shown), similar patterns were observed for the VIAAT and GAD67 mRNAs. In the cerebellar cortex, the GABAergic Purkinje neurons were unambiguously labelled by both the VIAAT and GAD67 mRNA probes (Fig. 3c and d, arrowheads). Taken together, these observations suggest that VIAAT is selectively expressed in both GABAergic and glycinergic neurons of the rat brain.

To examine more directly the function of VIAAT, COS-7 and PC12 cells were transiently transfected with both the full-length mVIAAT clone and a clone for a plasma membrane GABA or glycine transporter (GAT1 [17] and GLYT1b [18], respectively). [^{3}H]GABA or [^{3}H]glycine uptake by intact cells was assayed 2 or 3 days after transfection. As shown in Fig. 4a and b, GAT1- and VIAAT-transfected cells exhibited an increased amount of accumulated [^{3}H]GABA over negative controls in which VIAAT was replaced by the vesicular monoamine transporter VMAT2 [19]. It should be noted that when GAT1 was inhibited by nipecotic acid or replaced by GLYT1b, no GABA uptake was observed in VIAAT-transfected cells. This dependence of VIAAT activity on the plasma membrane transport of GABA suggests that VIAAT uptake occurs in an intracellular compartment. To test the ability of VIAAT to recognize glycine, COS-7 cells were transfected with the three plasmids GAT1, GLYT1b and VIAAT, and unlabelled glycine was added to the uptake medium (Fig. 4c). Glycine at 10 mM fully inhibited the VIAAT-mediated [^{3}H]GABA uptake without affecting the GAT1-mediated one. Moreover, we reproducibly observed a small but significant increase in [^{3}H]glycine uptake (9.5% ± 1% S.D.) when PC12 cells transfected by both GLYT1b and VIAAT were compared with controls transfected by GLYT1b alone (6 transfections). We thus conclude that VIAAT cDNA encodes a GABA and glycine transporter.

4. Discussion

In this study, we describe the cDNA cloning and expression of a mouse GABA and glycine transporter, designated VIAAT, based on the identification of candidate mouse and nematode vesicular GABA transporters by database screening. The characteristics displayed by VIAAT are consistent with biochemical data on GABA and glycine transport into synaptic vesicles. GABA and glycine are taken up by isolated rat brain vesicles in an ATP-dependent manner [30–32]. Competition experiments [33,34], as well as the fact that the ratio of GABA to glycine uptake was similar in vesicle preparations of predominantly GABAergic or glycinergic origin [35], have led to the conclusion that these two transmitters are taken up by similar, if not identical, transporters (but see [32]). Morphological studies have even suggested that some GABAergic terminals might co-store and co-release glycine [36,37]. In contrast, GABA and glycine are highly discriminated by their plasma membrane transporters (for example, see [16]). Therefore, the GABA- and glycine-specific profile of both in situ hybridization and amino acid uptake data strongly supports the proposal that VIAAT is a vesicular transporter. This conclusion is strengthened by the likely intracellular localization of VIAAT in COS-7 and PC12 cells.

The identification of VIAAT as a vesicular GABA and glycine transporter means that the putative protein T20G5.6 is likely to represent the nematode homologue. We may also predict with some confidence that, as initially suggested by our database screening, T20G5.6 corresponds to the *unc-47* gene product. Although genetic experiments are needed to confirm this last conclusion, the successful identification of VIAAT already demonstrates the considerable time- and cost-saving benefits of genome databases. Database screening allows one to go rapidly from an hypothesis on what a gene product might do to what the protein sequence might be, thereby greatly facilitating experimental testing. A simple hydropathy profile criterion was used here to extract 4 candidate transporters from a ~300 kb genomic sequence. Other structural features, such as ligand-binding or leucine zipper motifs, etc., based on knowledge of the presumed protein family, might prove useful in identifying other genes. In addition, the easy access to sequences from other organisms by homology searching facilitates the design of the most efficient experimental test.

The molecular characterization of VIAAT should facilitate anatomical and physiological studies on GABAergic and glycinergic transmission. In particular, the availability of probes for both the vesicular and plasma membrane transporters will help to determine the relative contributions of these transporters in the modulation of GABA and glycine signalling. On the other hand, the presence of VIAAT at both GABAergic and glycinergic terminals, as suggested by our in situ hybridization data, may provide a means to generally survey inhibitory neuron plasticity or degeneration. In this context, the search for VIAAT inhibitors using heterologous expression systems might find potential diagnostic applications in the development of in vivo imaging radioligands.

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