murine Jnk1 cDNA. To test c-jun and c-fos expression, a 199-bp fragment corresponding to nucleotides 891–1,089 of the murine c-jun cDNA and a 346-bp fragment corresponding to nucleotides 2,173–2,518 of the murine c-fos cDNA were used for the generation of radio-labelled probes for northern hybridization analysis. JNK activity in hippocampal lysates (30 μg) was measured before and after immunodepletion of Jnk1 and Jnk2 by in-gel protein kinase assays using the substrate glutathione S-transferase (GST)–c-Jun.

**Luciferase activity assay.** Mice were decapitated and the brains dissected. Brain tissues were immediately lysed (Promega) and the luciferase activity was measured as described.

**Kai nic acid-induced expression of immediate-early genes.** Homozygous mutant and control wild-type mice were killed and fixed by transcardial perfusion of 4% paraformaldehyde 2 or 6 h after the injection of kainic acid (30 mg per kg, i.p.). Brains from both groups were removed, post-fixed for 1 h, and sectioned on a vibratome (40 μm thick). Tissue sections were processed by immunocytochemistry to detect the expression of c-Jun (Santa Cruz), c-Fos (Santa Cruz), and phosphospecific c-Jun (Ser73) (New England Biolabs). Sections were floated in a solution of the primary antibody (diluted 2000×) and incubated overnight at room temperature. Secondary antibody incubation, avidin–biotin conjugated peroxidase (Vectorstain Elite ABC kit, Vector) and 3,3′-diaminobenzidine (Sigma) reactions were performed using standard procedures.

**Kainic acid-induced hippocampal damage.** Wild-type and Jnk3−/− mice were killed and fixed by transcardial perfusion of 4% paraformaldehyde and 1.5% glutaraldehyde 5 days after the injection of kainic acid (30 or 45 mg per kg, i.p.). Hippocampus and thin sections of brain were prepared using a vibratome and embedded in Epon. Tissue blocks were prepared using a microtome with a diamond bur for 1 μm-thick serial sections examined by toluidine blue staining and for ultrathin sections examined by electron microscopy.

**Identification and characterization of the vesicular GABA transporter.**

Steven L. McIntire,‡§ Richard J. Reimer,†§ Kim Schuske,§ Robert H. Edwards‡ & Erik M. Jorgensen†

‡ Graduate Programs in Neuroscience, Cell Biology and Biomedical Sciences, Department of Neurology and Physiology, UCSF School of Medicine, Third and Parnassus Avenues, San Francisco, California 94143-0435, USA

† Department of Biology, University of Utah, 257 South 1400 East, Salt Lake City, Utah 84112-0840, USA

These authors contributed equally to this work.

Synaptic transmission involves the regulated exocytosis of vesicles filled with neurotransmitter. Classical transmitters are synthesized in the cytoplasm, and so must be transported into synaptic vesicles. Although the vesicular transporters for monoamines and acetylcholine have been identified, the proteins responsible for packaging the primary inhibitory and excitatory transmitters, γ-aminobutyric acid (GABA) and glutamate remain unknown.† Studies in the nematode Caenorhabditis elegans have implicated the gene unc-47 in the release of GABA. Here we show that the sequence of unc-47 predicts a protein with ten transmembrane domains, that the gene is expressed by GABA neurons, and that the protein colocalizes with synaptic vesicles. Further, a rat homologue of unc-47 is expressed by central GABA neurons and confers vesicular GABA transport in transfected cells with kinetics and substrate specificity similar to those previously reported for synaptic vesicles from the brain. Comparison of this vesicular GABA transporter (VGAT) with a vesicular transporter for monoamines shows that there are differences in the bioenergetic dependence of transport, and these presumably account for the differences in structure. Thus VGAT is the first of a new family of neurotransmitter transporters.
Studies of synaptic vesicles have identified distinct transport activities for monoamines, acetylcholine (ACh), GABA and glutamate, the classical neurotransmitters. All of these depend on a proton electrochemical gradient $\Delta \mu_H^+$ generated by the vacuolar H$^+$/ATPase. However, differences in the bioenergetics suggest that the vesicular amino-acid transporters may not belong to the same family of proteins as the vesicular monoamine and ACh transporters. Vesicular monoamine and ACh transport involve the exchange of luminal protons for cytoplasmic transmitter, and depend primarily on the chemical component ($\Delta \mu_H$) of this gradient. Molar cloning has also demonstrated that the vesicular monoamine and ACh transporters are closely related in structure. In contrast, vesicular glutamate transport depends primarily on the electrical component ($\Delta W$) of $\Delta \mu_H$, and vesicular GABA transport seems to depend more equally on both $\Delta \mu_H$ and $\Delta W$. Attempts to identify additional members of the vesicular monoamine and ACh transporter family, including low-stringency hybridization, polymerase chain reaction (PCR) amplification of the conserved domains using degenerate oligonucleotide primers, and search of the available databases have not yielded additional members (data not shown), suggesting that the sequences encoding vesicular amino-acid transport may be unrelated. Genetic analysis of C. elegans provides an alternative means of identifying the sequences responsible for vesicular GABA transport.

We reasoned that mutants lacking GABA transport into vesicles would phenocopy the behaviour of worms in which the GABAergic nervous system had been removed by laser ablation. The 26 GABA-expressing neurons in C. elegans are required to inhibit contractions of the head muscles during foraging, to inhibit contractions of the body muscles during locomotion, and to stimulate contraction of the enteric muscles during the defecation cycle. Animals in which the GABAergic neurons are ablated are defective in all three of these activities.

**Figure 1** Sequence and structure of the vesicular GABA transporter. a, Alignment of the predicted protein sequence of UNC-47 and the rat UNC-47 homologue (RUNC-47). Predicted transmembrane domains are underlined. Numbers in the right column correspond to amino-acid residues. The conserved amino acid Gly462, which is mutated in allele n2409, is indicated with an asterisk. Black boxes indicate identical residues, and grey conservative substitutions. b, Predicted secondary structure of rat Unc-47 homologue with the lumen of the vesicle shown above and the cytoplasm below; - represents acidic residues, and + basic residues. Filled circles indicate residues identical (black) or highly conserved (grey) with UNC-47, and open circles indicate divergent residues. c, Mutations in unc-47. All mutations were induced by ethyl methanesulphonate (EMS). d, UNC-47 defines a new family of polytopic membrane proteins. Comparison of UNC-47 to representative sequences from C. elegans (F21D12.3 (U23518), C44B7.6 (U28928), R02F2.8 (U00055)), S. cerevisiae (YJR001w (P47082), and plants (amino-acid permease AAP5 (S51170) from Arabidopsis thaliana). The comparison shows three colinear regions of highest sequence similarity that are present in all family members. The position of the residues in the sequences of the polypeptides are indicated in parentheses. Residues identical in three or more of the proteins are shown in black and similar residues in grey, as defined by Blast Blossum matrix. The % amino-acid identity between UNC-47 and related proteins for each region is shown to the right. The F59B2.2 sequence includes a conserved domain that was present in genomic DNA but was not predicted by GeneFinder.
behaviours. Five genes have been identified that, when mutated, cause defects in these behaviours. However, the phenotype of only one of these mutants, unc-47, is consistent with a loss of GABA transport into synaptic vesicles. First, the defect in unc-47 is global, affecting all of the behaviours mediated by GABA. Second, the defect in unc-47 is presynaptic, as the muscle cells in the mutant respond normally to GABA receptor agonists. Third, GABA accumulates in GABAergic neurons of the mutant, suggesting that the neurotransmitter is not being released, possibly because it is not loaded into synaptic vesicles.

The unc-47 gene maps between stP127 and unc-50 on chromosome III of C. elegans. This region contains approximately 250 kilobases (kb) of DNA. Cosmids spanning this region were injected into two cosmids clones, T20G5 and E03F9, each rescued the unc-47 mutant phenotype. The rescuing activity was further localized to a 5.2-kb BamHI genomic fragment that contains a single complete open reading frame (ORF; T20G5.6) predicted by the C. elegans Genome Project. To confirm that the identified ORF corresponds to the gene mutated in unc-47 worms, three ethyl methanesulphonate-induced alleles were sequenced. The reference allele, e307 (ref. 9), is a G to A transition of the absolutely conserved G in the splice acceptor site between exons five and six (Fig. 1). A second strong mutation, n2476, is a 238-base-pair deletion that removes parts of exons three and four; this deletion causes a frameshift at residue 175 and the ORF terminates after another 115 amino acids, indicating that this allele is a molecular null. Finally, n2409 is a G to A transition which changes a glycine to arginine at residue 462 in a predicted transmembrane segment.

The cDNA derived from the unc-47 gene predicts a protein of 486 amino acids (Fig. 1a) with no similarity to the previously characterized vesicular monoamine and ACh transporters. However, the sequence predicts ten transmembrane domains (TMDs) consistent with its being involved in transport (Fig. 1b). Further, a search of available databases with the UNC-47 protein sequence revealed that UNC-47 has weak sequence similarity to at least four predicted proteins in C. elegans, seven predicted proteins in the yeast Saccharomyces cerevisiae, and previously characterized amino-acid permeases in the plants Arabidopsis thaliana and Nicotiana sylvestris (Fig. 1d), suggesting that UNC-47 belongs to a class of proteins involved in the transport of amino acids.

To identify the cells that express unc-47, the protein coding sequence of green fluorescent protein (GFP) was inserted in-frame 52 amino acids downstream of the UNC-47 translation start site and co-injected with a lin-15+ (S. G. Clark & X. Lu, personal communication) marker gene into lin-15(n765ts) mutant animals. Animals containing the unc-47::GFP reporter construct showed expression of GFP in all GABAergic neurons, and only in GABAergic neurons (Fig. 2a), further supporting the idea that unc-47 is involved in GABA transport.

To determine whether UNC-47 associates with synaptic vesicles, GFP was inserted at the carboxy terminus of the UNC-47 protein. This construct rescued the unc-47 mutant phenotype, demonstrating that the construct functions normally. GFP-tagged UNC-47 was localized to synaptic varicosities along the ventral and dorsal cords but not in axons (Fig. 2b), a distribution similar to that of other synaptic vesicle proteins such as synaptobrevin, synaptotagmin, and Rab3a. Further, mislocalization of synaptic vesicles in the unc-104 mutant (which lacks a neuron-specific kinesin) also results in mislocalization of UNC-47 (Fig. 2c). In unc-104 mutants, synaptic vesicles do not reach the neuromuscular junction and accumulate in motor-neuron cell bodies. Similarly, in unc-104 mutants, GFP-tagged UNC-47 is present only in cell bodies and is not transported to the neuromuscular junction (Fig. 2c). Thus the sequence, distribution and subcellular localization of UNC-47 is consistent with its being involved in the packaging of GABA into synaptic vesicles.

To assess the biochemical function of UNC-47, we isolated the cDNA of a vertebrate homologue. A database search with the predicted peptide sequence of unc-47 identified multiple entries in the mouse expressed sequence tag (EST) database. A fragment of one EST was then amplified by PCR and used to screen a mouse brain cDNA library. Partial sequence of a 2.5-kb cDNA showed strong similarity to unc-47, and this cDNA was then used to screen a rat brain cDNA library. The resulting rat cDNA sequence contains a 3′ untranslated region with ~95% identity to mouse and human but not C. elegans sequences (data not shown), a level exceeding that observed in much of the translated domain. The sequence of the largest ORF predicts a protein of 525 amino acids with 38% identity and 56% similarity to unc-47 (Fig. 1a). Similar to unc-47, the analysis of hydrophobic moment suggests that there are ten TMDs, and the absence of a single peptide predicts that the amino and the carboxy termini reside in the cytoplasm (Fig. 1b). In addition, the hydrophilic N-terminal domain is unusually large (~132 residues). The hydrophilic loops predicted to reside in the lumen of the vesicle lack consensus sites for N-linked glycosylation. However, consensus sequences for phosphorylation by protein kinase C occur on predicted cytoplasmic domains near the N terminus (+17), between TMDs 2 and 3 (+239) and between TMDs 6 and 7 (+377).
To determine whether the rat unc-47 homologue may function presynaptically in GABAergic transmission, we examined its tissue distribution. Northern analysis shows expression of an ~2.5-kb mRNA transcript in the brain, with none detected in non-neural tissues (Fig. 3). However, PCR amplification of reverse-transcribed sequences from spleen, testis and pancreas, but not liver or kidney, indicate expression of the unc-47 homologue (data not shown), consistent with the detection of GABA biosynthesis and transport in some of these tissues\(^7\). *In situ* hybridization further demonstrates expression of the unc-47 homologue throughout the neuraxis, but at particularly high levels within the neocortex, hippocampus, cerebellum, striatum, septal nuclei and the reticular nucleus of the thalamus (Fig. 4a–I), regions containing abundant GABAergic neurons. Examination of the autoradiograms under high magnification indicates expression by Purkinje cells of the cerebellum, as well as by interneurons of the cerebellum, hippocampus (Fig. 4m, n) and cortex, which are cell populations known to release GABA. We also performed *in situ* hybridization with one isoform of the biosynthetic enzyme glutamic acid decarboxylase (GAD), GAD-67 (Fig. 4). Although the level of hybridization in different regions varied slightly between the unc-47 homologue and GAD-67, we found striking colocalization of the two sequences, consistent with the homologue functioning in the release of GABA. We also stained primary hippocampal cultures with an antibody that we raised to the unc-47 homologue (R.J.R., S.L.M., R.H.E., manuscript in preparation). Punctate immunoreactivity in nerve processes that coincides with the immunoreactivity for synaptophysin is shown in Fig. 4o, p. The apparent vesicular localization further supports the unc-47 homologue’s being involved in vesicular GABA transport.

To determine biochemically whether the rat unc-47 homologue encodes the vesicular GABA transporter, we expressed the cDNA in rat pheochromocytoma PC12 cells. PC12 cells contain synaptic-like microvesicles and support the activities of the vesicular monoamine transporters\(^19\) as well as the ACh transporter\(^19\), but do not express detectable amounts of unc-47 homologue (data not shown). Stable PC12 transformants expressing high levels of the putative transport protein were isolated by screening cell clones with antibodies generated against the unc-47 homologue. Immunofluorescence reveals a punctate pattern that is consistent with localization to intracellular membrane vesicles (data not shown). A population of light vesicles was then isolated from two stable transformants and purified by differential centrifugation. Membranes prepared in this way from the transfected cells show accumulation of 3H-GABA that way from the transfected cells show accumulation of 3H-GABA that

Figure 3 Brain-specific expression of the rat unc-47 homologue. Northern analysis of poly(A)+ RNA prepared from different tissues shows a 2.5-kb mRNA transcript hybridizing with the rat unc-47 cDNA only in the brain. The size of standards (kb) are shown on the right.
properties encoded by the rat VGAT (rVGAT) cDNA to determine its relationship to the activity previously described in native brain synaptic vesicles. To assess ligand recognition, we first considered compounds structurally related to GABA that have been tested as inhibitors of GABA transport into synaptic vesicles. As for GABA transport into synaptic vesicles, the plasma membrane GABA transport inhibitor nipeptic acid inhibits rVGAT activity only weakly (Fig. 5c), and the excitatory amino-acid transmitter glutamate does not inhibit rVGAT activity even at extremely high concentrations (Table 1)\textsuperscript{24–26}. Thus the analysis of ligand specificity also distinguishes rVGAT from these other neurotransmitter transporters. The GABA analogue N-butyric acid weakly inhibits both rVGAT activity and GABA transport into brain synaptic vesicles, further supporting the identity of rVGAT as a vesicular GABA transporter. Previous studies using synaptic vesicles have also suggested the inhibition of vesicular GABA transport by γ-vinyl-GABA (vigabatrin), an inhibitor of GABA transaminase and a potent anticonvulsant\textsuperscript{27}. This synthetic GABA analogue also inhibits the transport of \textsuperscript{3}H-GABA by rVGAT as potently as unlabelled GABA, supporting the idea that there is an additional site of action for this drug.

Previous studies of native brain synaptic vesicles suggested that a single vesicular GABA transport activity also recognizes the inhibitory amino-acid transmitter glycine as a substrate\textsuperscript{28}. However, other studies suggest that glycine has a different transporter\textsuperscript{29}. Glycine inhibits GABA transport encoded by the rVGAT cDNA with relatively low potency. We failed to detect significant uptake of \textsuperscript{3}H-glycine in these preparations (data not shown), supporting the existence of a distinct vesicular transporter for glycine.

Functional analysis of rVGAT indicates bioenergetic differences from vesicular transporters for monoamines and ACh. Monamine and ACh transport rely primarily on the chemical component (ΔpH) of the proton electrochemical gradient (Δµ\textsubscript{H})\textsuperscript{1,2}. Studies using synaptic vesicles have indicated that vesicular GABA transport relies on the electrical component Δψ, as well as ΔpH\textsuperscript{11–23}. To assess the bioenergetics of rVGAT function, we used the ionophore nigericin, which exchanges K\textsuperscript{+} for H\textsuperscript{+}, to selectively dissipate ΔpH. Nigericin reduces rVGAT activity by \textapprox 40%, suggesting that Δψ has an additional role (Fig. 5d). Indeed, the addition of both nigericin and valinomycin, an ionophore that mediates K\textsuperscript{+} flux and so dissipates Δψ as well as ΔpH, eliminates rVGAT activity. To compare directly the bioenergetics of vesicular GABA and monoamine transport, we have examined the transport of serotonin by the same PC12 cell-membrane preparations that contain rVGAT. Previous work has demonstrated the expression of endogenous vesicular monoamine transporter 1 (VMAT1) on synaptic-like microvesicles in these cells\textsuperscript{12}. Nigericin inhibits VMAT1 activity on these vesicles to a greater extent (\textapprox 65%) than it inhibits rVGAT activity, indicating a greater dependence on ΔpH (Fig. 5d). The addition of valinomycin to nigericin eliminates the residual VMAT1

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**Figure 5** The rat unc-47 homologue encodes vesicular GABA transport. a, Membranes prepared from two different PC12 cell clones (A3 and B1) stably expressing the rat unc-47 homologue accumulate more \textsuperscript{3}H-GABA than membranes prepared from untransfected PC12 cells (WT). b, Lineweaver–Burke plot of initial, maximal transport rate (V\textsubscript{max}) in the presence of different concentrations of GABA (µM), with the linear approximation performed by standard regression analysis. c, Inhibition of GABA transport activity by GABA and related compounds. γ-Vinyl-GABA inhibits almost as potently as GABA itself, whereas glycine inhibits much less potently and glutamate inhibits only very slightly (see Table 1 for IC\textsubscript{50} values). N-butyric acid and nipeptic acid, an inhibitor of plasma membrane GABA transport, inhibit vesicular GABA transport very weakly. d, Vesicular GABA transport and vesicular monoamine transport differ in bioenergetics. Membranes prepared from PC12 cell clone B1 stably expressing rVGAT (middle) show considerably more GABA transport activity (filled bars) than membranes from untransfected cells (WT, left). Nigericin (5 µM) inhibits GABA transport in the transfected cells by \textapprox 40%, and the addition of valinomycin (20 µM) to nigericin eliminates rVGAT activity, indicating greater dependence on Δψ than ΔpH. In contrast, transport of serotonin by endogenous VMAT1 (white bars) expressed in the same membranes from transfected PC12 cells that express rVGAT (right) shows \textapprox 66% inhibition by nigericin, indicating that VMAT1 depends more on ΔpH than rVGAT. The results are normalized to GABA transport (left and middle) and serotonin transport (right) by rVGAT-expressing cells. Error bars represent the standard error of the mean.
activity, indicating a small role for $\Delta \Psi$. Thus rVGAT and VMAT1 depend on both components of the electrochemical gradient, but VMAT1 depends to a greater extent on $\Delta \Psi$, which is consistent with previous results using mixed populations of synaptic vesicles. Identification of the unc-47 homologue as a vesicular GABA transporter helps us to understand the molecular mechanism for the vesicular transport of an amino-acid neurotransmitter. The genetic analysis in *C. elegans* indicates that unc-47 is essential for GABA transmission. In addition, UNC-47 and its rat homologue both occur in GABAergic neurons, and their polytopic nature supports the idea that they function in vesicular transport. Biochemical characterization of the rat unc-47 homologue demonstrates GABA transport function, with the affinity and ligand specificity reported for GABA transport by native synaptic vesicles. We cannot detect transport by rVGAT of the other principal inhibitory transmitter glycine, suggesting the existence of a distinct vesicular glycine transporter. The analysis of GABA and monoamine transport activities expressed in the same population of membrane vesicles shows that rVGAT is more dependent on $\Delta \Psi$ than VMAT1. Indeed, this difference in bioenergetic mechanism may account for the structural differences between these two classes of vesicular transporters. UNC-47 and rVGAT show similarity to a large group of sequences from *C. elegans*, *S. cerevisiae* and plants. Several of the plant sequences mediate amino-acid transport and use a proton electrochemical gradient as the driving force, suggesting functional as well as structural similarity to UNC-47 and rVGAT. However, these plant permeases catalyse the co-transport of amino acids and protons rather than the proton exchange rather than the precise mechanism of bioenergetic coupling. The class of proteins identified by UNC-47 and rVGAT may include the transporters for excitatory amino-acid transmitters, such as glutamate, which also depend on $\Delta \Psi$.

### Methods

**Cloning of unc-47.** A pool containing 10 ng $\mu l^{-1}$ each of cosmids E03F9, ZK1128, F55E6, T20G5 and K08E5 was injected along with 80 ng $\mu l^{-1}$ of EK15 (lin-15*) marker plasmid (S. G. Clark & X. Lu, personal communication)24. The DNA was injected into the syncytial gonads of unc-47(e307); lin-15 (n765ts) animals. Five lin-15* lines were established and all animals were rescued for the unc-47 mutant phenotypes. A 5.2-kb BamHI fragment subcloned from T20G5 rescued unc-47(e307) mutants. The 5.2-kb BamHI fragment was used to screen 350,000 plaques from an oligo-t-primed XZAP cDNA library made from mixed-stage RNA and a single positive (B1) was identified. To isolate additional cDNAs, 400,000 plaques of a second oligo-t-primed mixed-stage cDNA library were screened and four positives were isolated (OK1-4). Sequence analysis showed that the B1 cDNA uses an alternative splice donor site in exon five, resulting in deletion of the eighth and part of the ninth TMHs. However, PCR amplification of reverse-transcribed cDNA demonstrated that this mRNA transcript is extremely rare and presumably results from aberrant splicing. The 5’ end of unc-47 was identified by PCR amplification of first-strand cDNA prepared from mixed-stage poly(A)+ RNA. Sequence analysis of the product revealed an SL1 splice leader immediately 5’ to the ATG start codon. To prepare genomic DNA from mutant alleles e307, n2476, and n2409, approximately 20 homozygous mutant worms were washed three times with M9 salts to remove bacteria, resuspended in 5 $\mu l$ water, and boiled for 5 min. TE (4 $\mu l$), 8.0, and 1 mg $\mu l^{-1}$ protease K (1 $\mu l$) were added and the mixture incubated at 45°C for 1 h. Proteinase K was inactivated by boiling for 30 min, and 1 $\mu l$ of this DNA preparation was used for PCR amplification. Amplified fragments were purified and sequenced using the Cyclist Tiq DNA sequencing kit (Stratagene).

**GFP expression constructs.** To construct the U47GFPNTX transcriptional fusion, GFP and unc-54 RNA termination sequences were amplified from pdP95.85 (S. Xu & A. Fire, personal communication) using primers that engineered an in-frame BspHI site onto the 5’ end of GFP. Using an internal BspHI site, which includes 533 bp of the unc-54 terminator, the amplified fragment was cloned into the BspHI site in the unc-47-rescuing 3.2-kb BamHI fragment. U47GFPNTX (30 ng $\mu l^{-1}$) and 35 ng $\mu l^{-1}$ EK15 (lin-15*) DNA were injected into the gonads of lin-15(n765ts) mutants. Three lines carrying an extrachromosomal array of both U47GFPNTX and EK15 were established, and all three lines expressed GFP in the GABAergic neurons. The extrachromosomal array from one line was integrated into chromosome X by X-ray integration to generate the strain EG1285: lin-15(n765ts); ox121. To construct the U47GFPCTL translational fusion, a SsoI site was engineered into the C terminus of unc-47 protein-coding sequence, two residues N-terminal to the UAA stop codon. GFP was amplified by PCR with primers that engineered an in-frame SsoI site onto each end of the GFP fragment and then cloned into the SsoI site created at the C terminus of UNC-47, U47GFPCTL (30 ng $\mu l^{-1}$) and 35 ng $\mu l^{-1}$ EK15 (lin-15*) DNA were injected into the gonads of lin-15(n765ts) and into lin-15(n765ts); unc-47(e307) mutant worms, and three lin-15(n765ts) lines were established that contained an extrachromosomal array of both U47GFPCTL and lin-15*. Four lin-15(n765ts); unc-47(e307) lines were obtained that contained both U47GFPCTL and lin-15* in an extrachromosomal array. Worms in two lines had strong GFP expression at the nerve terminals and almost all lin-15* animals were also unc-47-. To express U47GFPCTL in the unc-104(e1265) mutant background, the oxEx68 [U47GFPCTL; lin-15*] extrachromosomal array was crossed into unc-104(e1265); lin-15(n765ts) mutants to generate EG1300: unc-104; lin-15; oxEx68.

**PCR amplification and library screening.** A search of the available EST database with the predicted amino-acid sequence of UNC-47 identified mouse EST 252177 as a possible vertebrate homologue. Using oligonucleotide primers based on 252177, a fragment was amplified by PCR from a pooled mouse brain cDNA library. Briefly, 200 ng template DNA was amplified in a 50-$\mu l$ reaction containing 25 mM Tris, pH 8.3, 50 mM KCl, 3 mM MgCl2, 100 pmol of each oligonucleotide, 100 $\mu l$ DNTPs, and 1 $\mu l$ Taq polymerase for 30 cycles involving the denaturation at 92°C for 1 min, annealing at 66°C for 1 min, and extension at 72°C for 1 min. After gel purification, the fragment was radiolabelled by PCR amplification under similar conditions in the presence of 2 $\mu M$ non-radioactive dCTP and 1 $\mu M$ $^{32}P$-dCTP and used to screen a mouse brain radiobiotiraphy cDNA library by aequous hybridization at 47.5°C (ref. 4). After washing at 52°C, positively hybridizing phage were identified by autoradiography, purified by two sequential rounds of screening, and the cDNA inserts rescued. After confirmation of the close sequence similarity to unc-47, this fragment was radiolabelled by random priming and used to screen a rat brain radiobiotiraphy cDNA library as described above. After characterization of the resulting cDNA clones, the 5’ end of the cDNA was amplified by PCR from a different rat brain cDNA library using oligonucleotide primers from the known rat sequence together with a primer flanking the vector insertion site. Another oligonucleotide primer based on the additional sequence was then used to amplify the 5’ end of the cDNA from the library with Pfu polymerase rather than Taq polymerase to minimize mutations. This 5’ fragment was then joined to a cDNA clone isolated by hybridization at a common BglII restriction site. The dioxyce chain termination method was used to confirm the sequence of the cDNA on both strands.

**Northern analysis.** Poly(A)+ RNA (5 $\mu g$) prepared from different rat tissues was separated by electrophoresis through formaldehyde–agarose and transferred to nylon membranes. Staining with ethidium bromide revealed approximately equal amounts of RNA in each lane. After hybridization in 50% formamide to the unc-47 homologue cDNA radiolabelled by random priming, the filters were autoradiographed with an enhancing screen.

**In situ hybridization.** Adult rats were anaesthetized with pentobarbital and perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS). After dissection, the brains were postfixed in the same solution, cryoprotected in

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**Table 1 Inhibition of GABA transport by structurally related compounds**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC50 (mM)</th>
<th>s.e.</th>
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<tr>
<td>GABA</td>
<td>4.75</td>
<td>0.3</td>
</tr>
<tr>
<td>γ-Vinyl-GABA</td>
<td>7.5</td>
<td>0.7</td>
</tr>
<tr>
<td>Glucose</td>
<td>275</td>
<td>10.6</td>
</tr>
<tr>
<td>N-butylisocitric acid</td>
<td>42.5</td>
<td>2.7</td>
</tr>
<tr>
<td>Nippecotic acid</td>
<td>46</td>
<td>1.4</td>
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Membranes expressing rVGAT were incubated in 2 $\mu M$ $^{3}H$-GABA for 5 min at 29°C. The concentrations of structurally related compounds required to inhibit the accumulation of $^{3}H$-GABA by 50% (IC50) are indicated, along with the standard errors (s.e.).
30% sucrose and PBS, and 1.5% myelinated sections hybridized at 52 °C in 50% formamide containing 0.3 M NaCl, 20 mM Tris, pH 7.4, 5 mM EDTA, 10 mM NaH2PO4, 1× Denhardt’s solution, 10% dextran sulphate, and 0.5 mg/ml yeast RNA to 32P-labelled RNA probes transcribed from linearized plasmid templates and hydrolysed in alkali to ∼300 nucleotide fragments. After washes in 50% formamide and digestion with RNase A, the slides were autoradiographed.

**Immunofluorescence.** Primary hippocampal cultures were grown on poly-lysine-coated glass coverslips for two weeks, fixed in 4% paraformaldehyde/PBS for 20 min, rinsed in PBS, blocked in 0.2% saponin, 2% BSA, 1% fish skin gelatin/PBS (blocking buffer) for 1 h and incubated for 90 min with anti-rVGAT polyclonal rabbit and anti-synaptophysin monoclonal mouse antibodies diluted 1:100 in blocking buffer, all at room temperature. The cells were then washed, incubated in secondary anti-rabbit antibody conjugated to fluorescein and anti-mouse antibody conjugated to rhodamine (both Cappel) diluted 1:500, washed, the coverslips mounted on class coverslips, and viewed under epifluorescence.

**Membrane preparation.** The rat unc-47 homologue cDNA subcloned into the plasmid expression vector pcDNA3-Amp (Invitrogen) was introduced into PC12 cells by electroporation. The cells were then selected in 800 g/ml G418 (effective) and the resulting clones examined by immunofluorescence.

**Transport assay.** To initiate the reaction, the 10 μl of membranes was added to 200 μl SH buffer containing 4 mM MgCl2, 4 mM KCl, 4 mM ATP, 40 μM unlabelled GABA and 2 μg/ml GABA (NEN). Incubation was performed for 29 °C for varying intervals and the reaction was terminated by rapid filtration (Supor 200, Gelman), followed by immediate washing with 6 ml cold 0.15 M KCl. Background uptake was determined by incubation for 4 °C for 0 min. The bound radioactivity was measured by scintillation counting in 2.5 ml Cytoscin (ICN). To determine Km, unlabelled GABA was added at a range of concentrations and uptake measured at 30 s. Nigericin and valinomycin dissolved in ethanol added to final concentrations of 5 μM and 20 μM, respectively. Transport measurements were performed in duplicate and repeated three or more times using at least two different membrane preparations.

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**Activation of the transcription factor Gli1 and the Sonic hedgehog signalling pathway in skin tumours**

N. Dahmane*, J. Lee*, P. Robins*, P. Heller† and R. Ruiz A Iñesta*†

*The Skirball Institute, Developmental Genetics Program and Department of Cell Biology, and †Department of Dermatology, New York University Medical Center, 540 First Avenue, New York, New York 10016, USA

Sporadic basal cell carcinoma (BCC) is the most common type of malignant cancer in fair-skinned adults. Familial BCCs and a fraction of sporadic BCCs have lost the function of Patched (Ptch), a Sonic hedgehog (Shh) receptor1-3 that acts negatively on this signalling pathway. Overexpression of Shh can induce BCCs in mice4. Here we show that ectopic expression of the zinc-finger transcription factor Gli1 in the embryonic frog epidermis results in the development of tumours that express endogenous Gli1. We also show that Shh and the Gli genes are normally expressed in hair follicles, and that human sporadic BCCs consistently express Gli1 but not Shh or Gli3. Because Gli1, but not Gli3, acts as a target and mediator of Shh signalling5, our results suggest that expression of Gli1 in basal cells induces BCC formation. Moreover, loss of Ptc or overexpression of Shh cannot be the sole causes of Gli1 induction and sporadic BCC formation, as they do not occur consistently. Thus any mutations leading to the expression of Gli1 in basal cells are predicted to induce BCC formation. Gli1, which was originally isolated as an amplified gene in a glioma6, is a member of a multigene family7-9 and can transform...