Previous genetic analyses of neurotransmitter function have been based on screens for mutants resistant to specific neurotransmitter agonists or antagonists[11]. By contrast, we identified mutants that phenocopy laser-operated animals lacking particular GABAergic neurons. In principle our approach could identify all genes that are not redundant in function and that are required specifically for GABA function in *C. elegans*. So far we have defined five such genes.

Some of the genes we have identified seem likely to encode familiar molecules. For example, *unc-25* probably encodes the GABA biosynthetic enzyme GAD, and *unc-49* might encode a $GABA_A$ receptor subunit. Although these proteins are biochemically well characterized, the *in vivo* effects of mutations that affect such proteins have not previously been examined. For example, it has been proposed[12] that GABA has a neurotrophic role and is necessary for differentiation in early development. The rescue of AVL and DVB function with exogenous GABA in *unc-25* mutants indicates that GABA may not be necessary for the development of these neurons.

In addition, we have defined several other genes that seem likely to encode proteins that act in GABA presynaptic functions. The gene *unc-30* appears to be necessary for a number of aspects of D-type neuron synaptic development, including neurotransmitter expression and the specification of synaptic partners. The genes *unc-47* and *unc-46* are necessary presynaptically at all GABAergic synapses. These two genes appear to affect GABA release and might define components necessary for GABA vesicular loading, transport or fusion with the synaptic membrane or differentiation of the neuromuscular junction. A molecular characterization of these genes might well define novel proteins involved in GABAergic neurotransmission. □

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The GABAergic nervous system of *Caenorhabditis elegans*

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γ-AMINOBUTYRIC acid (GABA) is the most abundant inhibitory neurotransmitter in vertebrates and invertebrates[1]. GABA receptors are the target of anxiolytic, antiepileptic and antispasmodic drugs[2], as well as of commonly used insecticides[3]. How does a specific neurotransmitter such as GABA control animal behaviour? To answer this question, we identified all neurons that react with antisera raised against the neurotransmitter GABA in the nervous system of the nematode *Caenorhabditis elegans*. We determined the *in vivo* functions of 25 of the 26 GABAergic neurons by killing these cells with a laser microbeam in living animals and by characterizing a mutant defective in GABA expression. On the basis of the ultrastructurally defined connectivity of the *C. elegans* nervous system, we deduced how these GABAergic neurons act to control the body and enteric muscles necessary for different behaviours. Our findings provide evidence that GABA functions as an excitatory as well as an inhibitory neurotransmitter.

Of the 302 neurons in an adult *C. elegans* hermaphrodite[4], 26 are stained by antibodies raised against the neurotransmitter GABA (Fig. 1). These 26 GABAergic cells comprise the six DD, 13 VD, four RME, one AVL, one DVB and one RIS neurons. As described later, we use laser microsurgery to identify roles in behaviour for all of the GABAergic neurons except RIS. We did not observe any behavioural changes in animals in which RIS had been killed.

The DD and VD neurons are motor neurons that innervate the body muscles required for locomotion[4] (Fig. 2*a*). *C. elegans* locomotion involves the propagation of a sinusoidal body wave from one end of the animal to the other. For example, if an animal is touched on the head it backs by producing a body wave of alternating contractions and relaxations of the opposing ventral and dorsal body muscles (Fig. 2*b*). By contrast, if an animal in which the DD and VD neurons have been killed by laser microsurgery is touched on the head, the animal simultaneously contracts its ventral and dorsal body muscles, resulting in a shrinkage in body length rather than in backward movement (Table 1*a*; Fig. 2*c*). Thus, the DD and VD neurons coordinate the wave of body muscle contractions involved in locomotion by preventing the simultaneous contraction of opposing muscles.

Is the role of the GABAergic DD and VD neurons in locomotion mediated by GABA? Mutations in the gene *unc-25* eliminate GABA expression in all 26 GABAergic neurons of *C. elegans*, probably by eliminating the GABA biosynthetic enzyme glutamic acid decarboxylase (ref. 5; and C. Johnson, personal communication). When touched on the head, *unc-25* animals shrink just like laser-operated animals that lack the DD and VD function (Fig. 2*c*; Table 1*a*), indicating that GABA is required for DD and VD function.

Can the known synaptic connectivity of the DD and VD neurons account for their function in locomotion? Based on the connectivities of these neurons, White *et al.*[4] suggested that the DDs and VDs act as contralateral inhibitory neurons[4]. Electrophysiological studies by Stretton and co-workers[6,9] of the larger parasitic nematode *Ascaris lumbricoides* have provided functional evidence for such a role for the *Ascaris* GABAergic motor neurons that correspond to the *C. elegans* VD neurons. Our studies of the behaviours of operated and mutant animals provide functional evidence for such a role for the *C. elegans* DD and VD neurons and strongly support the model that GABA is essential for contralateral inhibition. Specifically, it seems that the DD and VD motor neurons provide positive feedback to

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bending of the body by releasing GABA onto muscles that antagonize those that cause contraction. For example, when the VA and VB motor neurons cause the ventral muscles to contract, these neurons also excite the DD motor neurons, which in response release GABA and inhibit contraction of the dorsal muscles (Fig. 2*a*). The elimination of DD and VD neuron functions, either by selective killing with a laser or by mutation in *unc-25* animals, prevents this reciprocal inhibition and results in the simultaneous contraction of ventral and dorsal muscles. Our results indicate that the reciprocal inhibition provided by this circuit ensures that contraction of muscles on one side of the animal is linked to relaxation of muscles on the other side during locomotion.

The GABAergic RME neurons innervate muscles in the head necessary for foraging (Fig. 3*a*). During normal foraging the tip of the nose moves rapidly from side to side within a narrow arc of movement, as shown in Fig. 3*b* (J.K. and H.R.H., unpublished observations). Killing the four RME motor neurons by laser microsurgery resulted in worms with an abnormal foraging behaviour described as 'loopy' (Table 1*b*; Fig. 3*c*). Specifically, flexures of the nose became grossly exaggerated. Thus, the RMEs appear to limit the extent of head deflection during foraging. *unc-25* animals display the same defect as that produced by killing the RME neurons, suggesting that GABA is the relevant neurotransmitter (Table 1*b*; Fig. 3*c*).

Does the connectivity of the RMEs explain the ability of these neurons to attenuate head deflections? The RMEs receive input from putative sensory neurons of the head and nose[4] and, like the DDs and VDs, have output to contralateral muscles. For example, as shown in Fig. 3*a*, the RMED neuron receives input from the SMBD neuron directly and from the SAAD neuron indirectly. Both of these neurons have long undifferentiated processes that run anteriorly and posteriorly along the dorsal side of the head, and White *et al.* proposed that these neurons provide proprioceptive information about head position[4]. Our results suggest that these putative sensory neurons might act through the RMEs: bending the head towards the ventral side might activate SAAD and SMBD as dorsal stretch receptors, which then activate RMED, which in turn relaxes the muscles on the ventral side of the head and restores a straightened head posture. Other sensory neurons might act through the RMEs in an analogous fashion.

The GABAergic motor neurons AVL and DVB each send a process to the enteric muscles (which consist of the intestinal, sphincter and anal depressor muscles)[4], all of which contract during the defaecation cycle (Fig. 4*a*) (ref. 10). The defaecation cycle is initiated by a contraction of the posterior body muscles and is followed by a contraction of the anterior body muscles and then a contraction of the enteric muscles[10]. Killing AVL alone severely decreased the frequency of contractions of the anterior body muscles (Table 1*c*). Killing either AVL or DVB alone moderately decreased the frequency of enteric muscle contractions (Table 1*d*). Killing AVL and DVB together severely decreased the frequency of contractions of the enteric muscles, resulting in distention of the lumen of the gut (Fig. 4*b*, *c*). These results indicate that AVL and DVB are partially redundant in function and that these neurons are necessary for muscle contraction, indicating that they are stimulatory rather than inhibitory. *unc-25* mutants lack enteric muscle contractions, suggesting that GABA mediates this stimulatory function of AVL

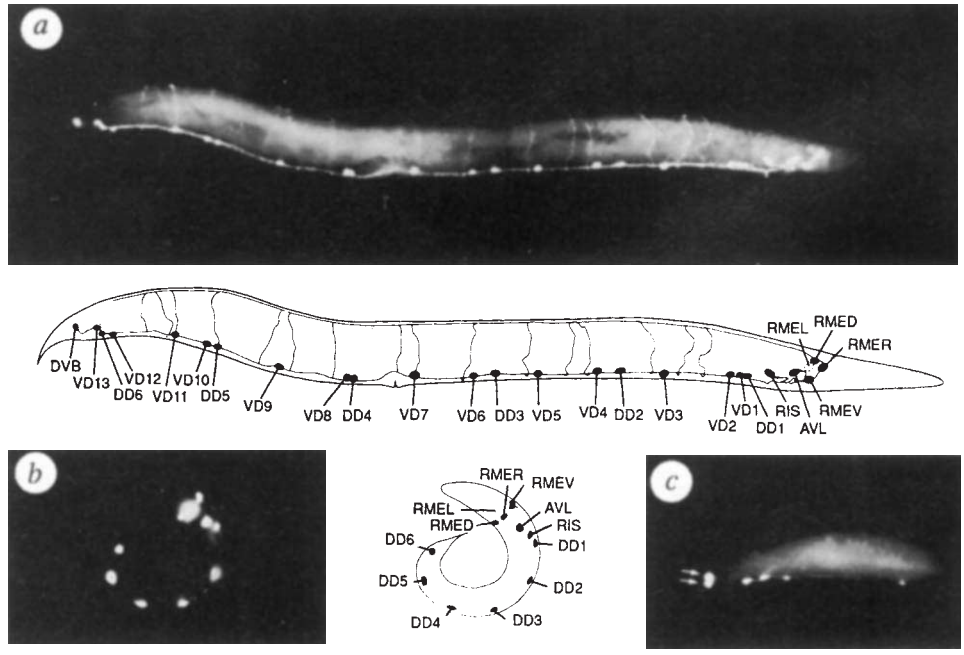

FIG. 1 GABA immunoreactivity in *C. elegans*. *a*, Fluorescent photomicrograph and a tracing of the right side of a wild-type adult hermaphrodite stained with an antiserum raised against GABA. The names of the 26 cells stained with the anti-GABA antisera are indicated. Anterior is to the right, and dorsal is up. The pharynx is indicated by the dotted line. *b*, Fluorescent photomicrograph and tracing of a young L1 larva stained with an anti-GABA antiserum. Only the six DD neurons are stained in the ventral nerve cord and DVB is absent from the tail since this neuron is generated after the L1 stage[17]. *c*, Fluorescent photomicrograph of a *lin-4(e912)* animal stained with an anti-GABA antiserum. In *lin-4* mutants the mother of DVB undergoes an extra division and generates two DVB-like cells (arrowed)[24].

METHODS. Animals were stained with anti-GABA antisera[20] using a whole-mount procedure[21]. The GABAergic neurons were identified by the following criteria: (1) All cells in *C. elegans* have relatively invariant positions relative to other cells[4,17]. The GABA immunoreactive cells occupy positions characteristic of particular classes of cells. The positions of immunoreactive cells were determined by comparison with the positions of other cells, as visualized with the nuclear stain 4-6-diamidino-2-phenylindole dihydrochloride (DAPI)[22] (data not shown). (2) The process morphologies of the DD, VD and RME neurons are unique[4] and matched those of the stained cells. (3) The number of the homologous members of each neuron class[4,17] matched that of the stained cells. (4) The known time of generation of the neurons as identified[17] was consistent with the stage at which these neurons could first be stained with anti-GABA antisera. (5) The AVL cell body was identified based upon its known position relative to other cell nuclei (using Nomarski optics) and then killed with a laser microbeam; the operated animal was shown to lack the corresponding GABA-immunoreactive cell (data not shown). (6) *egl-5* and *lin-4* mutants, which are known to generate abnormal numbers of presumptive DVB neurons as a result of cell lineage defects[23,24], were found to have abnormal numbers of stained DVB like neurons (data not shown). In the larger parasitic nematode *Ascaris lumbricoides*, there are cells with GABA immunoreactivity at positions and with morphologies similar to the DD, VD, RME and DVB neurons[25]. It is unclear whether there are GABAergic homologues of AVL and RIS.

and DVB (Table 1*d*; Fig. 4*c*). Interestingly, *unc-25* mutants are not defective in anterior body contractions (Table 1*c*). One possibility for this difference between *unc-25* mutants and AVL-defective animals is that this function of AVL is mediated by a second neurotransmitter in this cell.

Do AVL and DVB directly excite the enteric muscles, or do they stimulate the muscle contractions indirectly, for example by inhibiting an inhibitory neuron? All evidence suggests that DVB and AVL are primary excitors. First, the connectivity is consistent with this hypothesis: DVB synapses directly to the enteric muscles, AVL forms varicosities (which often indicate sites of transmitter release) at the enteric muscles, and no other neurons are known to innervate the enteric muscles[4,11]. Second, killing

TABLE 1 The roles of GABAergic neurons

a

Genotype	Cells killed	No. animals	No. shrinking/ no. animals
Wild type	None	21	0.00
Wild type	DD2-DD5, VD3-VD11	6	1.00
unc-25 (GABA$^-$)	None	23	1.00

b

Genotype	Cells killed	No. animals	No. loopy foraging/ no. animals
Wild type	None	12	0.00
Wild type	RMEV, RMEL, RMER, RMED	15	0.93
unc-25 (GABA$^-$)	None	10	1.00

c

Genotype	Cells killed	No. animals	No. def. cycles	Anterior body contractions/ def. cycle
Wild type	None	6	151	0.99
Wild type	AVL DVB	10	183	0.05
Wild type	AVL	14	153	0.03
Wild type	DVB	13	50	0.98
unc-25 (GABA$^-$)	None	7	75	0.96

d

Genotype	Cells killed	No. animals	No. def. cycles	Enteric muscle contractions/ def. cycle
Wild type	None	6	206	0.95
Wild type	AVL DVB	10	199	0.01 + 0.04*
Wild type	AVL	14	154	0.53
Wild type	DVB	13	143	0.71
unc-25 (GABA$^-$)	None	7	76	0.12

Cells were killed using a laser focused through a Zeiss Axioplan microscope as previously described[15,16]. All behaviours were assayed by studying 4-day-old wild-type (two days after surgery) or *unc-25(e156)* animals. *a*, The DD and VD neurons were killed in second stage (L2) larvae and were identified on the basis of their positions in the ventral nerve cord relative to other neurons[17]. Because we could not reliably identify the DD and VD neurons in the retrovesicular and the preanal ganglia, the cells DD1, DD6, VD1, VD2 and VD13 were not killed. Our analysis of animals double-stained with anti-GABA antisera and DAPI (data not shown), and reconstructions from electron micrographs[18] indicate that although the ventral nerve cord is largely invariant in its order of neuron types, the positions of the VDs occasionally vary; therefore VDs might have remained in the mid-section of the ventral nerve cord in some operated animals. Based upon the degree of the observed variability, we estimate that fewer than one-third of our operated animals should have had more than one of the nine VDs in the ventral cord (VD3–VD11) remaining after surgery. *b*, Animals displaying exaggerated flexures of the head when moving forward were scored as 'loopy foraging'. The RMEs were identified in first-stage (L1) animals on the basis of their positions relative to other neurons[19]. Operated animals were scored for loopy foraging in a double-blind assay. *c* and *d*, The defaecation cycle (def. cycle) begins with a posterior body contraction (PBOC), which is followed about 4 s later by an anterior body contraction and an enteric muscle contraction[10]. Contractions were assayed by direct observation; in all operated animals the frequency of the defaecation cycle was normal (~45 s)[10]. The difference in the number of enteric muscle contractions between *unc-25* mutants and the AVL$^-$ DVB$^-$ animals might be a consequence of undetectable levels of GABA remaining in *unc-25* mutants or of a second neurotransmitter in these cells. Asterisk indicates that this value represents enteric muscle contractions that occurred between defaecation cycles, that is, more than 10 s following one PBOC and preceding the next. AVL and DVB were identified by position[17] relative to other neurons in early L2 animals. Wild-type control animals, in which no cells were killed, were anaesthetized with the experimental animals but were not subjected to laser microsurgery. The following neurons were killed in various combinations without significantly reducing enteric muscle contractions: all neurons in the preanal ganglion, the lumbar ganglion, the retrovesicular ganglion, the ventral ganglion (other than AVL), the dorsal ganglion, the dorsal rectal ganglion (other than DVB) and those neurons in the lateral ganglia that send processes to the preanal ganglion, that is, AVA, AVB, AVD, AVH and AVJ.

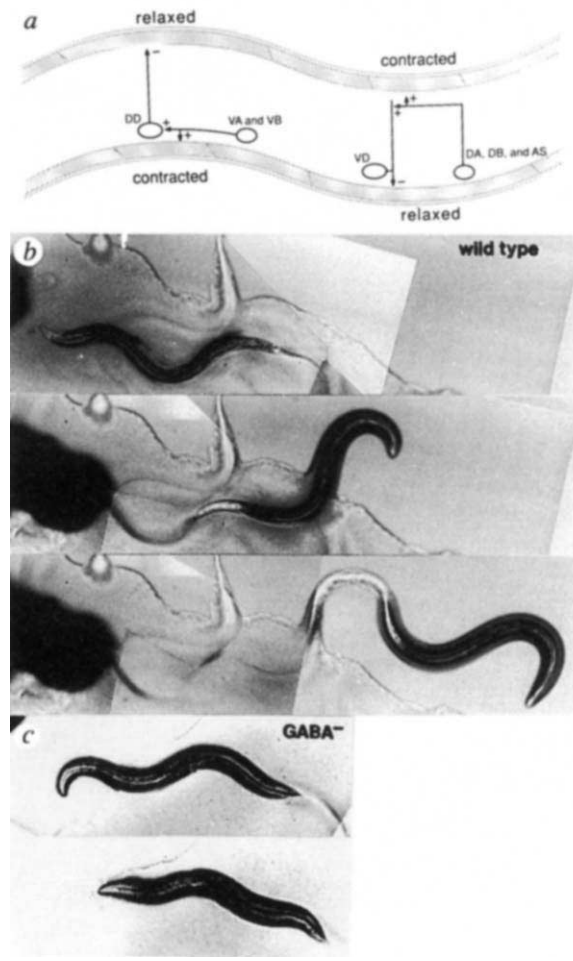

FIG. 2 The role of the DD and VD neurons in locomotion. *a*, Model for DD and VD neuronal function. Excitatory cholinergic motor neurons[6] cause body muscle contractions: the VA and VB neurons cause contraction of ventral muscles, and the DA, DB and AS neurons cause contraction of dorsal muscles. These excitatory motor neurons also activate the GABAergic DD or VD neurons, which cause relaxation of the dorsal and ventral muscles, respectively. Presumptive excitatory synapses are designated by a plus sign, and inhibitory synapses are designated by a minus sign. Muscles are shaded. *b* and *c*, Locomotory behaviour in *b*, a wild-type animal, and *c*, an *unc-25(e156)* mutant, which lacks GABA expression[5]. The head of each animal was touched with a platinum wire (shadow to the left of the wild type) and photographed at intervals of ~1 s. Touch to the head of the wild-type animal resulted in backward movement characterized by deep sinusoidal waves. Touch to the head of the *unc-25* mutant caused the ventral and dorsal muscles to contract simultaneously, resulting in no substantial backward movement, reduced flexures of the body and a shrinkage of body length. *unc-25* animals are still capable of some forward movement (not shown), although the amplitude of the body flexures is greatly reduced.

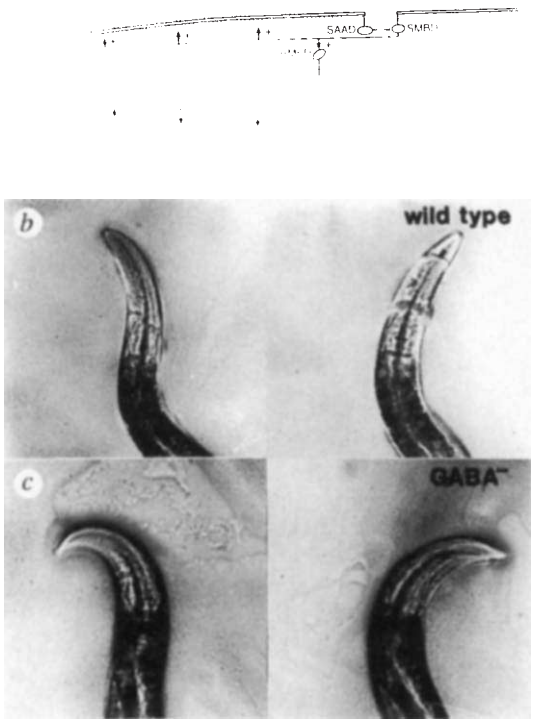

FIG. 3 The role of the RME neurons in foraging. *a*, Model for RME neuronal function. There are four RME neurons: RMED, RMEV, RMEL and RMER. Based upon the anatomically-defined connectivity of the *C. elegans* nervous system[4], each RME neuron receives inputs from a variety of sensory neurons. For example, the dorsal RME (RMED) receives inputs from the putative stretch receptor neurons SAAD and SMBD as well as from the putative mechanosensory neurons OLQ, OLL, IL1 and IL2. For simplicity, this figure illustrates only the inputs from SAAD and SMBD; inputs from the other sensory neurons could function analogously. The circuit of RMEV is similar to that of RMED. In addition, RMED and RMEV have long undifferentiated processes in the dorsal and ventral nerve cords; these processes might act as sensory endings on their own (not shown). RMEL and RMER are components of analogous circuits on the left and right sides, but these neurons do not receive input from the SMBs. ⊢, Gap junction; arrows, chemical synapses; unfilled bars, sensory processes. *b* and *c*, Foraging in *b*, a wild-type and *c*, an *unc-25(e156)* mutant. Lateral deflections of the nose and head are very restricted in wild-type animals but are very sweeping in *unc-25* animals, which lack GABA.

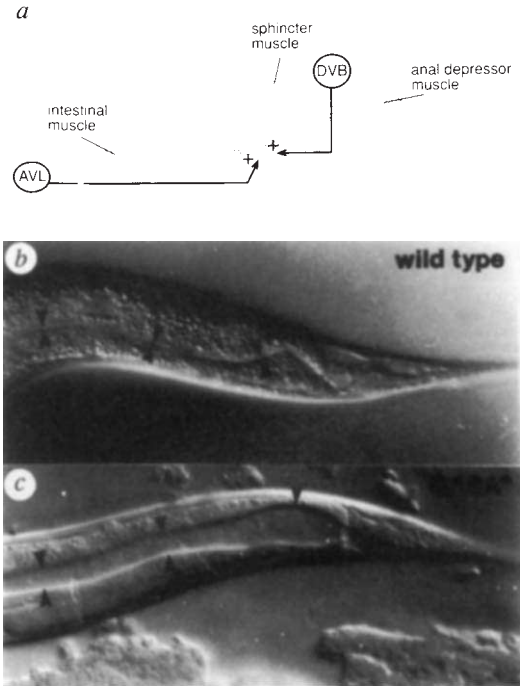

FIG. 4 The role of AVL and DVB in enteric muscle contractions. *a*, Model for AVL and DVB neuronal function. The four enteric muscles involved in expulsion comprise the anal depressor, the sphincter, and the two bilaterally symmetric intestinal muscles[4]. These muscles are connected by gap junctions and send arms to the preanal ganglion, where DVB synapses to the intestinal muscles. The sphincter appears to be relaxed before expulsion, allowing filling of the rectum; it then contracts with the anal depressor and intestinal muscles to prevent reflux of the rectal contents into the gut (our unpublished observations). Although AVL has not been observed to form synapses with enteric muscles, its process runs closely apposed to the enteric muscles and forms gap junctions with DVB. The AVL cell body is located far anteriorly in the ventral ganglion of the head. *b* and *c*, The anal region *b*, of a wild-type animal, and *c*, of an *unc-25(e156)* GABA-deficient mutant. Note that the lumen of the gut (arrowheads) is greatly distended in the *unc-25* worm as a consequence of abnormal enteric muscle function.

all other neurons with processes in the vicinity of the enteric muscles did not cause defects in defaecation (Table 1 legend). Third, we have genetic and pharmacological evidence that the inhibitory and stimulatory activities of GABA are mediated by distinct receptors (ref. 5; and E.J., unpublished results). Although GABA is generally thought to have an inhibitory effect on postsynaptic membranes[12], our results suggest that GABA has both inhibitory and excitatory effects in *C. elegans*.

How might GABA direct excitation? $GABA_A$ receptor activation in vertebrates directs inhibition by maintaining a negative membrane potential as a result of an inward Cl conductance. It is possible that excitatory effects of GABA are mediated by an outward Cl conductance through a $GABA_A$ receptor, leading to membrane depolarization; such an effect of GABA has been proposed to occur in the developing mammalian brain[13]. Alternatively, GABA might be acting directly on a novel cationic channel or indirectly on such a channel through a G-protein-linked receptor such as the $GABA_B$ receptor[14], and so cause depolarization as a consequence of cation influx.

To understand the neuronal and neurochemical bases of behaviour, it is necessary to integrate behavioural studies with anatomical, biochemical and physiological analyses of the nervous system. Here we have identified all GABA-immunoreactive neurons present within an entire nervous system. Each of these neurons is known in terms of its developmental ancestry and its synaptic connectivities. Although GABA is usually considered

to be an inhibitory neurotransmitter, our data provide evidence that GABA can have excitatory as well as inhibitory effects on postsynaptic membranes. Our observations reveal how a specific neurotransmitter can act in known neural circuits to control the behaviour of an intact, living animal. □

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Mechanosensitive channels transduce osmosensitivity in supraoptic neurons

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VASOPRESSIN is a peptide hormone synthesized by neurons of the supraoptic and paraventricular nuclei, which project axon terminals to the neurohypophysis. Consistent with its antidiuretic properties, vasopressin release rises as a function of plasma osmolality[1–3], a response that results from accelerated action potential discharge[4–6]. Previous studies have shown that increases in fluid osmolality depolarize supraoptic neurons in the absence of synaptic transmission[7–9], suggesting that these cells behave as intrinsic osmoreceptors. The mechanism by which changes in osmolality are transduced into an electrical signal is unknown, however. Here we report that changes in cell volume accompany physiological variations in fluid osmolality and that these modulate the activity of mechanosensitive cation channels in a way that is consistent with the macroscopic regulation of membrane voltage and action potential discharge. These findings define a function for stretch-inactivated channels in mammalian central neurons.

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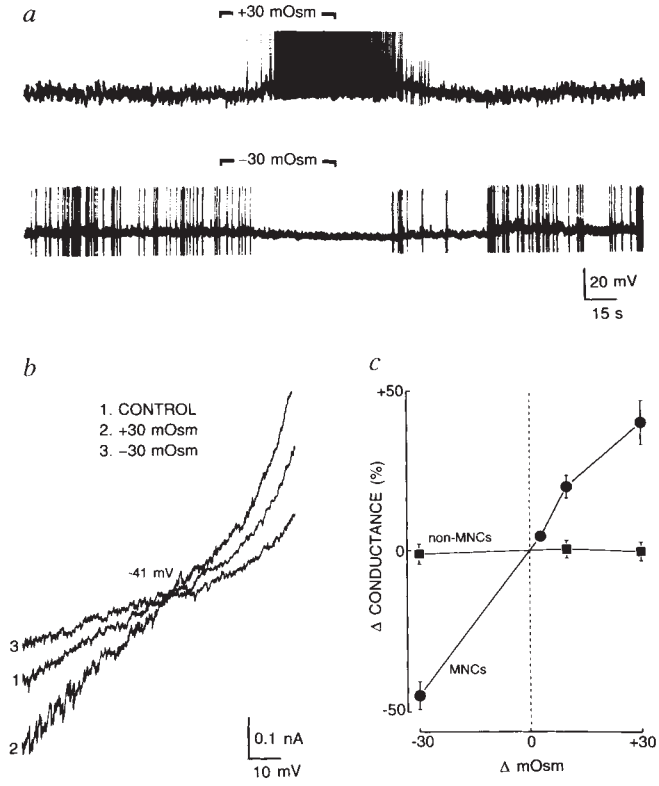

FIG. 1 *a*, Whole-cell voltage recordings from isolated supraoptic MNCs. Superfusion of a hypertonic solution (+30 mOsm) provoked a reversible depolarization and increased spike discharge (full amplitude not shown). In contrast, application of a hypotonic solution (−30 mOsm) induced membrane hyperpolarization and inhibited spike discharge. The traces in *b* show current responses to voltage ramps (16 mV s^{-1}) recorded from a supraoptic MNC. Application of hypertonic (2) and hypotonic solutions (3), respectively, increased and decreased slope conductance compared to control (1) and wash (not shown). Note that all three current ramps intersect near −41 mV. The graphs in *c* plot mean (±s.e.m.) changes in conductance (as per cent of control) evoked in MNCs ($n = 19$; filled circles) and non-MNCs ($n = 13$; filled squares) upon application of various osmotic stimuli.
METHODS. Petri dishes containing cells prepared as described[9] were perfused with a solution (295 ± 2 mOsm; pH 7.4) comprising (mM): NaCl (140), KCl (3), $CaCl_2$ (1), $MgCl_2$ (1), HEPES (10) and mannitol (30). Osmolality was adjusted by adding or removing mannitol as required. All voltage-clamp measurements were made in the presence of tetrodotoxin (TTX; 1 μM). Recordings (at 22–25 °C) were obtained either from MNCs (diameter ≫15 μm)[9] or from non-MNCs using the whole-cell configuration of the patch-clamp technique[17]. Non-MNCs included neighbouring non-neuroendocrine cells (diameter < 10 μm)[9] isolated from the same blocks of tissue used to obtain MNCs, as well as larger neurons similarly isolated from temporal cortex, cerebellum, or from the hypothalamic ventromedial and arcuate nuclei. Both oxytocin[18] and vasopressin[2,3] are released in response to hypertonic stimulation in the rat, which is in agreement with the apparent osmosensitivity of both types of MNCs[4]. Therefore, no attempt was made to characterize the cells beyond their identification as MNCs. Recording electrodes (2–5 MΩ) were filled with a solution (pH 7.15) comprising (mM): KCl (150), $MgCl_2$ (1), HEPES (10), EGTA (1.6), Na_2–ATP (1.5) and cAMP (0.2). Membrane currents (DC; 200 Hz) were digitized by a labmaster interface and analysed using pCLAMP software.

Patch-clamp recordings were obtained from magnocellular neurosecretory cells (MNCs) acutely isolated from the supraoptic nuclei of adult rats[9]. Bath-application (0.5–10 min) of hypertonic solutions evoked a sustained and reversible membrane depolarization accompanied by increased firing in each of 24 MNCs tested (Fig. 1*a*). Conversely, reducing the osmolality