remipedes^{9,11}. Our findings indicate that such convergent changes may have been achieved by similar developmental changes (involving similar posterior shifts in the expression boundary of *Ubx-abdA*) on several independent occasions. This suggests that, given a particular developmental system, there may be limited ways for achieving a particular morphological result.

It is often thought that the study of development mechanisms can provide a rigorous way to assess the homologous or convergent origin of morphological characteristics (see refs 7, 16, 24 for example). Our observations introduce one complication: this expectation will be true only in the extent to which convergent events are driven by distinguishable genetic changes. In this respect, it will be interesting to establish whether the observed convergent shifts in Hox expression can be attributed to distinguishable changes at a fine molecular-genetic level. □

Methods

The monoclonal antibody against Ubx and AbdA (FP6.87) is described in ref. 13; the monoclonal antibodies against Engrailed (4D9 and 4F11) are described in ref. 25. Immunohistochemical staining is described in refs 25, 26. Cuticularized embryos and larvae were sonicated briefly to allow efficient penetration of reagents²⁶. Further details on collection, rearing, fixation, sonication and staining are available upon request.

Received 25 March; accepted 10 June 1997.

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Acknowledgements. We thank M. Akam and F. Ferrari for discussion; K. Rützel and the Smithsonian Institution for hosting our collecting expeditions at Carrie Bow Cay in Belize; R. White for the FP6.87 antibody; G. Wyngaard for *Mesocyclops*; E. Chang for *Honnarus* embryos; M. Sepanski for help with electron microscopy; A. Crittenden and L. Brown for technical assistance; and M. Palopoli, R. Chasan and S. Cohen for comments on the manuscript. This work was supported by the Wellcome Trust (M.A.), the Carnegie Institution of Washington, and the Howard Hughes Medical Institute (N.H.P.).

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Recruitment of functional GABA_A receptors to postsynaptic domains by insulin

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Modification of synaptic strength in the mammalian central nervous system (CNS) occurs at both pre- and postsynaptic sites^{1,2}. However, because postsynaptic receptors are likely to be saturated by released transmitter, an increase in the number of active postsynaptic receptors may be a more efficient way of strengthening synaptic efficacy^{3–7}. But there has been no evidence for a rapid recruitment of neurotransmitter receptors to the postsynaptic membrane in the CNS. Here we report that insulin causes the type A γ -aminobutyric acid (GABA_A) receptor, the principal receptor that mediates synaptic inhibition in the CNS⁸, to translocate rapidly from the intracellular compartment to the plasma membrane in transfected HEK 293 cells, and that this relocation requires the β 2 subunit of the GABA_A receptor. In CNS neurons, insulin increases the expression of GABA_A receptors on the postsynaptic and dendritic membranes. We found that insulin increases the number of functional postsynaptic GABA_A receptors, thereby increasing the amplitude of the GABA_A-receptor-mediated miniature inhibitory postsynaptic currents (mIPSCs) without altering their time course. These results provide evidence for a rapid recruitment of functional receptors to the postsynaptic plasma membrane, suggesting a fundamental mechanism for the generation of synaptic plasticity.

We used quantitative electron-microscopic analysis of immunogold labelling to investigate translocation of GABA_A receptors in HEK 293 cells stably expressing the α 1, β 2 and γ 2 subunits of rat GABA_A receptors⁹, the most common subunit combination of native GABA_A receptors in the mammalian CNS¹⁰. We found that, under control conditions, only a small number of immunogold-labelled β 2 subunits could be observed at the membrane surface, and that most labelling was localized intracellularly (Fig. 1a,c). Treatment with insulin dramatically increased the number of gold-labelled GABA_A receptor subunits in the plasma membrane (Fig. 1b,c); the change could be detected as early as 10 min after insulin treatment (data not shown). An increase in receptor synthesis was not involved, as even 60 min of insulin treatment did not affect the total cellular expression of β 2 subunits (Fig. 1c). Pretreatment of cells with genistein, a membrane-permeable inhibitor of protein-tyrosine kinases^{11,12}, prevented the insulin-induced translocation without altering basal GABA_A receptor distribution (Fig. 1c), suggesting a requirement for activation of the insulin receptor tyrosine kinase¹³. To determine whether the insulin-induced receptor translocation leads to an increase in the number of functional GABA_A receptors on the plasma-membrane surface, we recorded GABA_A receptor-mediated whole-cell currents in these cells. Bath application of insulin (0.5 μ M) produced a significant increase in the amplitude of the currents, an effect not observed in cells pretreated with genistein (Fig. 1d). These results demonstrate a rapid increase in the number of functional GABA_A receptors on the plasma-membrane surface as a result of receptor translocation by activation of insulin-receptor tyrosine kinase.

To determine which subunit(s) is required for insulin-induced

receptor translocation, HEK 293 cells were transiently transfected with rat GABA_A receptor $\beta 2$, $\gamma 2$ and the epitope-tagged $\alpha 1^{\text{FLAG}}$ subunit complementary DNAs in various combinations. Confocal scanning sections double-stained for surface and for entire cell staining demonstrated that a large proportion of $\alpha 1\beta 2$ transfectants was surface-labelled (Fig. 2). However, among cells transfected with $\alpha 1\gamma 2$, or $\alpha 1$ or $\beta 2$ alone, few were surface-labelled. Insulin (0.5 μM , 10 min) increased the proportion of surface-labelled cells in $\alpha 1\beta 2$ and $\beta 2$ by 29% and 103%, respectively, but not in $\alpha 1\gamma 2$ or $\alpha 1$ transfected cells (Fig. 2). Thus the $\beta 2$ subunit seems to be required for the insulin-induced increase in receptor surface expression.

We next investigated whether insulin can effect the translocation of native CNS GABA_A receptors *in situ* in cultured mouse hippocampal neurons. When native GABA_A receptors were stained under permeabilized conditions with anti- $\beta 2/\beta 3$ antibody, basal levels of plasma membrane-associated receptors were observed in all cells. However, large numbers of the receptors were found to be intracellularly localized through the cell soma (Fig. 3A,a). Insulin (0.5 μM , 10 min) increased the ratio of the receptor labelling on the plasma membrane to that in the cytoplasmic compartment (Fig. 3A, b and c), providing evidence for translocation of intracellularly localized receptors to the plasma membrane. Optical sections stained under non-permeabilized conditions demonstrated that insulin increased the labelling on the plasma-membrane surface (Fig. 3B), and that this increase in the surface labelling occurred preferentially on the proximate dendrites (Fig. 3B, c and d), sites where GABAergic synapses have been shown to be predominantly localized¹⁴. These results are consistent with a recruitment by insulin of GABA_A receptors to the postsynaptic domains near the sites of presynaptic release. This possibility was further examined, using electron microscopy, in the CA1 neurons of control and insulin-

treated hippocampal slices. Under control conditions, most immunogold-labelled $\beta 2/\beta 3$ subunits were found to be located along synaptic, dendritic and somatic membranes, with the highest concentration of receptors in the postsynaptic side of the synaptic junctions, consistent with the synaptic enrichment of the GABA_A receptors previously reported¹⁵. Treatment of slices with insulin (0.5 μM , 10 min) significantly increased the receptor density on both postsynaptic and dendritic membranes, but not on the somatic membrane (Fig. 3C and D, c). These results demonstrate that recruitment of GABA_A receptors to the postsynaptic domains by insulin is not unique to neurons in primary culture, but can also occur in mature neurons *in situ*.

To determine whether the newly recruited surface receptors are functional, we examined effects of insulin on currents evoked by exogenously applied GABA in cultured neurons. Bath application of insulin (0.5 μM) potentiated the currents at both near- and sub-saturated GABA concentrations ($26.4 \pm 5.3\%$ at 200 μM , $15.6 \pm 7.9\%$ at 10 μM , $n = 5$ neurons; Fig. 4A). Thus insulin increases the maximum response, providing support for an increase in the number of functional membrane GABA_A receptors. To provide evidence for recruitment of functional GABA_A receptors at postsynaptic junctions, we also investigated modulation by insulin of GABA_A receptor-mediated mIPSCs. Within 10 min, insulin caused an increase of approximately 30% in the amplitude of the mIPSCs (-37 ± 4.2 control versus -47 ± 2.9 pA after insulin, $P < 0.01$, $n = 5$; Fig. 4B, a), but caused no change in either the rise time (10% to 90% of the peak, 2.7 ± 0.1 versus 2.6 ± 0.2 ms, $P > 0.05$; Fig. 4B, b) or the decay time constant ($\tau = 28.3 \pm 3$ versus 26.8 ± 2 ms, $P > 0.05$; Fig. 4B, b). Thus the results are consistent with an increased number of active postsynaptic receptors. Insulin was also found to increase mIPSC

Figure 1 Insulin induces a rapid translocation of GABA_A receptors from intracellular compartments to the plasma membrane and potentiates GABA_A receptor-mediated currents in HEK 293 cells stably transfected with cDNAs encoding rat GABA_A receptor $\alpha 1$, $\beta 2$ and $\gamma 2$ subunits. Electron micrographs of ultrathin cryosections labelled with immunogold using a monoclonal antibody against the rat $\beta 2/\beta 3$ GABA_A receptor subunits show subcellular distribution of the $\beta 2$ subunits in control (a) and after a 60-min insulin (0.5 μM) stimulation (b). Immunogold-labelled receptor proteins are indicated by arrowheads; scale bars, 0.1 μm . c, Quantification of membrane and total cellular expression of $\beta 2$ subunits. Cells were incubated for 60 min in the extracellular solution (control) or in solution supplemented with either 0.5 μM insulin or 50 μM genistein (Gen.). For genistein pretreatment (Gen. + insulin), cells were first treated with 50 μM genistein for 10 min, and then incubated for 60 min in the genistein solution supplemented with 0.5 μM insulin. Values are means $\pm$ s.e. from 3 experiments (25 cells per experiment) and were analysed using the unpaired Student *t*-test (** denotes $P < 0.01$). d, Recordings of representative individual GABA_A receptor-mediated whole-cell currents induced by puffing GABA (100 μM , 100 ms) onto the transfected cells at a holding membrane potential of -60 mV before (control) and 10 min after addition of 0.5 μM insulin. For genistein treatment, cells were incubated in 50 μM genistein before the start of whole-cell recording and exposed to the drug throughout recording period. Insulin increased the amplitudes of the currents by 40% (-376.1 ± 106.6 pA for control versus -526.3 ± 145.3 pA 10 min after insulin, $P < 0.05$, $n = 8$), and this effect was prevented by genistein pretreatment (-263 ± 83.7 versus -261 ± 81.6 pA; $P > 0.05$, $n = 6$).

Figure 2 The β -subunit is required for insulin-mediated translocation of recombinant GABA_A receptors transiently expressed in HEK 293 cells. **A, B**, Confocal optical sections (1 μ m) of cells transfected with $\alpha 1^{\text{FLAG}}\beta 2$ (**A**) or $\alpha 1^{\text{FLAG}}\gamma 2$ (**B**) before (**a** and **b**) and after (**c** and **d**) 10-min insulin treatment (0.5 μ M). Double-immunofluorescence staining was done by first staining (under non-permeabilized conditions) for the FLAG epitope tag inserted in the extracellular domain (near the N terminus) of $\alpha 1$ (**b** and **d**, red) followed by permeabilized staining for the main intracellular loop of the same subunit (**a** and **c**, green). **C**, Effects of insulin on cell surface expression. For cells transfected with $\alpha 1^{\text{FLAG}}$,

$\alpha 1^{\text{FLAG}}\gamma 2$ or $\alpha 1^{\text{FLAG}}\beta 2$ subunit combinations, the rate of surface expression was calculated as the number of surface-labelled cells (labelled with anti-Flag antibody) as a percentage of total transfected cells (labelled with polyclonal anti- α antibody). For cells transfected with the β -subunit alone, surface-labelled cells and transfected cells were identified by staining with antibody to $\beta 2/\beta 3$ subunits under non-permeabilized and permeabilized conditions, respectively. Each combination was repeated in 3 experiments, and 500–1,000 transfected cells were counted in each combination in each experiment.

Figure 3 Insulin causes membrane translocation and clustering of native GABA_A receptors in CNS neurons. Control (**a**) and insulin-treated (**b**; 0.5 μ M, 10 min) cultured mouse hippocampal neurons (**A–C**) were stained with the $\beta 2/\beta 3$ monoclonal antibody. **A**, Optical sections (1 μ m) of the cell soma stained under non-permeabilized conditions show that insulin induces a relocation of intracellular GABA_A receptors to the plasma membrane. **B**, Optical sections scanned through the cell soma show that, under non-permeable conditions, GABA_A receptors expressed on the membrane surface are selectively labelled and insulin increases the surface labelling (**a** and **b**). The same neurons shown in **a** and **b** are below in stacked serial optic sections of the entire cell body. Under basal conditions (**c**), numerous small clusters of GABA_A receptors are distributed diffusely over the entire cell, including remote processes. After insulin treatment (**d**), receptors are concentrated in large clusters on the proximate dendrites. Scale bars, 10 μ m for **A** and **B**. **C**, Insulin increases synaptic GABA_A receptor density of

mature CNS neurons *in situ*. Electron micrographs show immunogold-labelled GABA_A receptor $\beta 2/\beta 3$ subunits in the postsynaptic junctions (arrowheads) in the hippocampal CA1 region from a control slice (**a**) and a slice treated with 0.5 μ M insulin for 10 min at 35 °C (**b**). Scale bars, 0.1 μ m. **D**, Effect of insulin on surface expression of native GABA_A receptors. **a**, Quantification of the ratio of fluorescence intensity of $\beta 2/\beta 3$ subunit staining on the membrane to that in cytoplasm from cultured neurons stained under permeabilized conditions ($n = 25$ neurons). **b**, Quantification of fluorescence intensity on the membrane surface from cultured neurons stained under non-permeabilized conditions ($n = 45$ neurons). **c**, Quantification of immunoparticle densities associated with the $\beta 2/\beta 3$ subunits of native GABA_A receptors in mature hippocampal CA1 neurons *in situ*, categorized by membrane region; $n = 25$, 25 and 100 for somatic, dendritic and synaptic membranes, respectively. * $P < 0.05$, ** 0.01 and *** 0.001.

frequency (Fig. 4B, c); although this could possibly be due to a higher rate of quantal release^{16,17}, it could also be fully accounted for by the recruitment of mIPSCs, previously below the threshold of detection, owing to an increase in the number of active receptors¹⁸. Finally, in CA1 hippocampal neurons in brain slices prepared from adult rats, insulin enhanced the amplitude of the mIPSCs by 23% (20.1 ± 1.9 versus 24.6 ± 2.8 pA, $P < 0.01$, $n = 4$) but not their time course ($\tau = 21.2 \pm 2.6$ versus 22.6 ± 4.1 ms, $P > 0.05$). These results suggest that the insulin-induced increase in the number of functional postsynaptic GABA_A receptors occurs in mature neurons *in situ*, indicating that the receptor relocation is likely to be of physiological importance.

Our results have demonstrated that insulin can regulate the surface expression of GABA_A receptors and thereby modulate GABA_A-receptor-mediated synaptic inhibition. Insulin and insulin receptors are expressed at high levels in discrete regions within the CNS, and insulin is released by depolarization in cultured CNS neurons¹³. Insulin has been shown to act as a neuromodulator of many brain functions, such as food intake¹³. The regulation of food intake by brain insulin may be mediated in part through GABA_A receptors, as insulin-induced hyperphagia in freely moving rats can be blocked by antagonists of GABA_A receptors applied in the ventromedial hypothalamic region¹⁹. The translocation of GABA_A receptors by insulin may also be important outside the CNS. In pancreatic islet, GABA is expressed at high levels in insulin-secreting β -cells²⁰, and functional GABA_A receptors are present in glucagon-secreting α -cells²¹. It has been reported that glucose inhibition of glucagon secretion is mediated through the activation of GABA_A

Figure 4 Potentiation of GABA_A receptor-mediated current responses by insulin in cultured hippocampal neurons. **A**, Insulin potentiates whole-cell GABA currents in perforated patch configuration at a holding membrane potential of -60 mV. Currents were induced in the same neuron by alternate perfusion of GABA at saturated and non-saturated concentrations through a multi barrel fast perfusion system. The doses were determined from full dose-response curves constructed for three neurons under control conditions; 10 and 200 μ M are doses producing approximately half and maximum responses, respectively (data not shown). **a**, Superimposed sample current traces before and 10 min after 0.5 μ M insulin application. **b**, Normalized peak currents ($I_{\text{insulin}}/I_{\text{control}}$) from 5 individual neurons. Insulin was applied in the bath during the period indicated by the bar. **B**, Whole-cell recordings of GABA_A receptor-mediated mIPSCs at a holding potential of -60 mV before (control) and 8 min after addition of insulin (0.5 μ M). **a**, Examples of consecutive traces of mIPSCs. **b**, Averages of 100 individual mIPSCs. The decay of mIPSCs before and after insulin were well fitted by a single exponential. **c**, Histograms showing amplitude distributions of a 3-min recording of mIPSCs. The histograms were fitted using an equation described previously³⁰. Bath application of insulin for 5–10 min increased the amplitude of mIPSCs without altering their time course. Note that insulin also increased the frequency of mIPSCs (0.94 ± 0.17 versus 1.6 ± 0.25 events s^{-1} , $n = 5$, $P < 0.01$)

receptors on the α -cells, and this does not seem to be associated with any detectable increase in GABA release²¹. Because secretion of insulin in the islet increases in the presence of high concentrations of glucose, our results suggest that insulin may mediate glucose feedback regulation of glucagon release by increasing the activity of GABA_A receptors in α -cells. Thus the observed modulation of GABA_A receptors by insulin may be important for the regulation of food intake and glucose homeostasis under physiological conditions, as well as in diseases such as diabetes and obesity.

Receptor translocation has important implications for synaptic function. Evidence from both experimental and modelling studies supports the view that GABA released from a single vesicle nearly saturates postsynaptic GABA_A receptors at CNS synapses^{3,6,7}. Therefore, an increase in quantal transmitter release would have little effect on the amplitude of the inhibitory synaptic potential. In contrast, the insertion of additional functional GABA_A receptors into the postsynaptic membrane would serve as an effective means of enhancing inhibitory synaptic transmission. Thus the rapid insulin-induced translocation of GABA_A receptors to the postsynaptic domain of inhibitory synapses provides a mechanism for the generation of synaptic plasticity in these synapses²², and may also have implications for the proposed recruitment of silent synapses following the long-term potentiation of glutamate-receptor-mediated synaptic transmission^{18,23–25}. □

Methods

Immunogold staining and electron-microscopic examination. HEK cells stably transfected with cDNAs encoding the $\alpha 1$, $\beta 2$ and $\gamma 2$ subunits of rat

GABA_A receptors (4D4 cells)⁹ were provided by D. Carter. Transcription of all three subunit mRNAs in the cell line was confirmed by reverse transcription–polymerase chain reaction (RT–PCR) (data not shown). Cells of passages 14–19 were plated in 100-mm culture dishes and used at 80% confluence. Cells were rinsed in PBS and incubated in the extracellular solution (control) or in the extracellular solution supplemented with 0.5 μM insulin for 10–60 min. Vibratome hippocampal slices (300 μm) were prepared from adult Sprague–Dawley rats. After a recovery period of 1 h artificial cerebrospinal fluid (ACSF)²⁶ at 35 °C, slices were incubated in ACSF containing 0.5 μM insulin for 10 min. After treatment, cells or slices were immediately fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 h. Pelleted cells were then embedded in gelatin. Embedded cells or dissected CA1 region of the slices were infused with 2.3 M sucrose for several hours and frozen in liquid nitrogen. Sections (100 nm) were then cut and immunogold-labelled using the monoclonal antibody bd-17 against rat β2/β3 subunits and 10-nm gold particles conjugated to goat anti-mouse IgG. In some cases, primary antibody was omitted, or replaced with a monoclonal antibody (bd-24) that recognizes human, but not rat, GABA_A-receptor α-subunit²⁷, to show the specificity of β2/β3 labelling. Non-transfected 293 cells were also stained with anti-β2/β3 antibody as a negative control. Images were captured with a CCD camera in the transmission electron microscope, and gold particles were quantified using the morphometric program NIH Image 1.59.

Cell transfection and neuronal cultures. The rat GABA_A-receptor subunit cDNAs, pcDNA1-β2 and the pcDNA3-γ2 (short form of γ2), were provided by C. Kaufman and D. Gunnersen (Laboratory of Neuroscience, NIDDK). The Flag-tagged rat α1 subunit cDNA (pcDNA3-α1^{FLAG}) was provided by J. H. Steinbach. This was generated by inserting the Flag sequence (KDYKDDDDKL) in the extracellular N-terminal domain between amino acids 33 and 34 of the translated sequence. This epitope tag did not alter either expression or function of the recombinant GABA_A receptor, and the tagged α1 subunit protein was recognized on the surface of intact cells using the M2 monoclonal anti-FLAG antibody²⁸. HEK 293 cells were plated onto collagen-coated 22-mm glass coverslips set in standard 35-mm culture dishes and transfected with 2 μg of each GABA_A-receptor subunit plasmid using Lipofectamine (GIBCO) according to the manufacturer's protocol. Cultured mouse hippocampal neurons were grown as previously described²⁹.

Immunofluorescent confocal microscopy. HEK 293 cells at 48 h post-transfection or neurons following 8–20 days in culture were fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with 0.25% Triton X-100 in PBS for 10 min, or fixed without permeabilization. GABA_A receptors were stained with bd-17 monoclonal antibody recognizing rat β2/β3 subunits (15 μg ml⁻¹) and with FITC-conjugated secondary antibody. For double-labelling experiments, cells were first stained with monoclonal anti-FLAG epitope (16 μg ml⁻¹) and rhodamine-conjugated anti-mouse antibody without permeabilization. After a further 10 min fixation and 10 min permeabilization, cells were stained with a polyclonal antibody recognizing the rat α-subunit (1:200) and with FITC-conjugated anti-rabbit antibody. Specific staining was tested by either replacing the monoclonal primary antibody with an unrelated antibody, the monoclonal anti-α1 subunit of the human GABA_A receptor (clone bd-24)²⁷, or by omitting the primary antibody in the case of the polyclonal antibody. Subcellular localization of immunofluorescently labelled receptors was examined with a Leica TCS-4D confocal microscope, and fluorescence intensity was quantified using the Scion ImagePC software.

Electrophysiology. Whole-cell recordings of stably transfected HEK 293 cells (4D4 cells) and cultured neurons were made using an Axopatch-1B or -1D amplifier (Axon Instruments, Foster City, CA) at room temperature (20–22 °C). Culture medium was exchanged completely with standard extracellular solution 1–2 h before recording. The extracellular solution contained (in mM): 140 NaCl, 1.3 CaCl₂, 5.4 KCl, 1 MgCl₂, 25 HEPES, 33 glucose, 0.0005 TTX (pH 7.4 using NaOH, 320–335 mosm). The intracellular solution contained (in mM): 120 CsCl, 35 CsOH, 2 MgCl₂, 1 CaCl₂, 11 EGTA, 2 TEA, 10 HEPES, 4 ATP, pH 7.3. For perforated-patch recording, 100 μg ml⁻¹ nystatin (Sigma) was added to the intracellular solution. Standard whole-cell recordings of CA1 neurons were performed in adult rat hippocampal slices perfused with ACSF at 35 °C in a submerged recording chamber. For recording of mIPSCs, 2 mM Mg²⁺ (plus AP5 for neurons in slices) and 20 μM CNQX were added to the

extracellular solution to inhibit NMDA and AMPA receptor-mediated excitatory synaptic currents. The mIPSCs were abolished by the addition of 10 μM GABA_A receptor antagonist bicuculline. mIPSCs were first recorded on videotape and later acquired and analysed off-line using the SCAN program (Strathclyde Software).

Drugs and materials. Insulin (purified pancreatic bovine, Zn²⁺-free) was provided by C. C. Yip (Banting & Best Institute, Toronto, Canada). Monoclonal antibody bd-17, which recognizes extracellular epitopes on both β2 and β3 subunits of the rat GABA_A receptor²⁷, was purchased from Boehringer Mannheim Biochem (Canada). The polyclonal antibody against the main intracellular loop between transmembrane domains III and IV of rat α1 subunit of the GABA_A receptor was purchased from PharMingen (San Diego). The monoclonal antibody to the Flag epitope (M2) was from Eastman Kodak Scientific Imaging System (New Haven).

Received 27 February; accepted 12 May 1997.

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Acknowledgements. We thank M. Wojtowicz and F. Dorri for assistance with slice experiments; C. Kaufman and D. Gunnersen for GABA_A receptor β2 and γ2 subunit cDNAs; J. H. Steinbach for the α1^{FLAG} cDNA; D. Carter for the 4D4 cells; C. C. Yip for insulin; and M. W. Salter for helpful comments on the manuscript. This work was supported by the Medical Research Council of Canada and Neuroscience Network of Centers of Excellence of Canada. Y.T.W. is a research scholar of the Heart and Stroke Foundation of Canada/Ontario; Z.G.X. is a MRC-NCE fellow and W.Y.L. is a NCE fellow. H.Y.M. is supported by a studentship from the Canadian Foundation for the Study of Infant Death.

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