entorhinal cortex layer IV, which projects to the basal forebrain and cortex (*14*).

In conclusion, our findings reveal a remarkably specific cellular pattern of pathology in Alzheimer patients. By giving rise to the major cortical input to the hippocampal formation, the cells affected in the entorhinal cortex constitute the major gateway of information from the association and limbic cortices. The cells damaged in the subiculum and CA1 field are equally critical since they are the major recipients of hippocampal output, and this constitutes the gateway of hippocampal influence on various parts of the neuraxis, such as the association cortices, the amygdala, the basal forebrain, and the diencephalon. Cellular damage to these key projection neurons of hippocampal circuitry isolates the hippocampal formation by disconnecting major input and output pathways, and it is difficult to conceive that the hippocampal formation in the brains of Alzheimer patients is functionally useful. This isolation of the hippocampal formation may be no less devastating (*15*) with regard to memory than removal or destruction of the entire structure, and contribute to the contextual memory defect that is a major component of the amnesia in Alzheimer's disease.

Our results are of further interest when viewed in relation to the cholinergic deficiency (*5*) in the cortex of Alzheimer patients and to reports of diminished cell density in the cholinergic neurons of the nucleus basalis of Meynert, both of which have been linked with memory impairments (*16, 17*). The basal forebrain has strong connections with medial temporal lobe structures, directly with neurons of the subiculum and layer IV of the entorhinal cortex (*14*) or indirectly via the amygdala (*18*).

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11. The perforant pathway arises from layers II and III of the entorhinal cortex, and courses through the subiculum. It terminates along the outer dendritic branches of the subicular and hippocampal pyramids, and on the outer two-thirds of the dendritic field of dentate gyrus granule cells. It initiates a multisynaptic excitatory sequence of intrinsic hippocampal circuits that terminate in the CA1 field and the subiculum.
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Insulin-Mediated Regulation of Neuronal Maturation

Abstract. *Exposure to insulin increased stimulus-evoked transmission at synapses formed in culture by cholinergic retinal neurons derived from fetal rats. This effect occurred at physiological concentrations and was long lasting. The findings support the hypothesis that insulin may serve as a developmental signal to regulate the emergence of effective neurotransmission across nascent synapses.*

Insulin and its receptors are found within the central nervous system (*1*). Although the significance of this discovery is unclear, one possibility is that insulin may play a role in brain development. Consistent with this possibility is the detection of insulin-like immunoreactivity, insulin receptors, and insulin-mediated effects on macromolecular synthesis in the fetal nervous system (*2*). However, a functional role for insulin in neuronal maturation has not been identified. Using a cell culture system, we examined the effect of insulin on the developmental step in which a presynaptic neuron becomes capable of information transfer. We found that insulin could precociously induce in cholinergic neurons derived from fetal rat retina the capability of releasing acetylcholine at synapses in response to an excitatory stimulus.

The cell culture system we used consisted of retinal and muscle cells (*3–4*). Cholinergic neurons dissociated from perinatal rat retinas rapidly form functional synapses in culture with rat striated muscle cells (*3*). Muscle cells are useful in studies of postsynaptic responses of cholinergic neurons because the membranes of muscle cells have areas with a high density of cholinergic receptors and because their physiological response to acetylcholine has been extensively studied in vivo and in culture (*5*). In addition, their relatively large size permits prolonged intracellular monitoring of postsynaptic responses.

The formation of functional retina-muscle synapses undergoes a sequence of developmental steps (*3, 4, 6*). Early in the maturational process, there is a period in which the neuronal release of acetylcholine occurs spontaneously but cannot be evoked by stimuli such as potassium or glutamate, a putative excitatory neurotransmitter. This "nontransmitting" phase is followed by the emer-

gence of the "transmitting" stage in which stimulation of cholinergic retinal neurons can evoke acetylcholine release by a mechanism dependent on extracellular calcium (*3, 7, 8*). Previous experimental findings (*4, 6*) have led to the hypothesis that the transition from the nontransmitting to the transmitting stage is due to the maturation of neuronal mechanisms which couple the ability to respond to a depolarizing stimulus with the capability of releasing acetylcholine.

We assessed the effect of insulin on the development of evocable cholinergic neurotransmission at retina-muscle synapses. In all experiments reported here, the retina-muscle synapses examined under control conditions were in the nontransmitting phase of development (*9*). Figure 1A shows a penwriter record of synaptic activity detected by an intracellular micropipette in a postsynaptic muscle cell (*10*). Before exposure to insulin, application of glutamate did not evoke synaptic input. However, evoked synaptic input could be detected approximately 1 hour after the miniperfusion of medium containing insulin. By approximately 3 hours after exposure to insulin a relatively strong evoked response was observed. Repetitive applications of glutamate, without exposure to insulin, did not induce evocable transmission (sample size, 12).

The time course for the insulin-mediated induction of evocable synaptic transmission was quantified (Fig. 1B). Between 1 and 3 hours after exposure to insulin was begun, there was a marked increase in evocable transmission. In contrast, neither the incidence of innervated muscle cells nor the spontaneous rate of postsynaptic activity was significantly affected (*11*). Thus, insulin appears to influence selectively the evoked—not the spontaneous—transmission at these immature cholinergic synapses. If insulin had an effect such as increasing the half-life of acetylcholine or increasing the responsiveness of myotubes to the neurotransmitter, both spontaneous and evoked activity would be expected to be influenced. The induction of stimulus-dependent transmission required 30 minutes or less of exposure to medium supplemented with insulin. Specifically, combined cultures of retina and muscle cells were exposed to insulin (20 ng/ml) or vehicle for 30 minutes and then washed extensively. Twelve hours later, 46 ± 6 percent (mean ± standard deviation) (triplicate experiments; sample size, 23) of the innervated muscle cells in the experimental group had glutamate-evoked synaptic input. In the control group, the value was 13 ± 2 percent (triplicate experiments; sample size, 22). The finding of evocable transmission 12 hours after removal of the insulin-containing medium suggests that this hormonal effect is not rapidly reversible.

The effect of insulin was dependent on concentration (Fig. 1C). The half-maximally effective concentration of insulin was 0.6 ng/ml. This value is similar to that for insulin-mediated stimulation of lipogenesis in isolated adipocytes (*12*). Although one group of investigators has questioned the existence of insulin in the brain (*13*), most reported values of brain insulin are within the range 0.6 to 10 ng per gram of tissue (wet weight) (*14*). Thus, it appears that the insulin-mediated effect on neurotransmission found in our study occurs at physiological concentrations.

To help assess the specificity of the insulin effect on synaptic transmission, the potencies of bovine proinsulin, des-octapeptide insulin (DOP), and multiplication-stimulating activity (MSA) (an insulin-like growth factor derived from rat) were compared with the dose-response curve for insulin (Fig. 1C). The specificity of insulin relative to proinsulin, DOP, and MSA is consistent with a mechanism involving insulin receptors (*15*). This is in agreement with the biochemical evidence that there are specific insulin receptors in the rat retina (*1*).

An important issue was whether insulin acted primarily on the retinal cells or on the cells in the muscle culture to mediate the development of evocable neurotransmission. Dissociated retinal cells were incubated in medium supplemented with bovine insulin (20 ng/ml) or vehicle. After 7 hours, the retinal cells were centrifuged, washed extensively, and added to muscle cell cultures. When assayed 1 day later, 44 ± 10 percent (triplicate experiments; sample size, 24) of the myotubes innervated by the retinal neurons previously exposed to insulin had glutamate-evoked synaptic input. In contrast, only 5 ± 8 percent (triplicate experiments; sample size, 21) of the innervated muscle cells in the control group had evocable input. Additional experiments demonstrated that treatment of muscle cells with insulin (20 ng/ml) for 1 day before the addition of retinal cells had no effect on the development of evocable synaptic transmission.

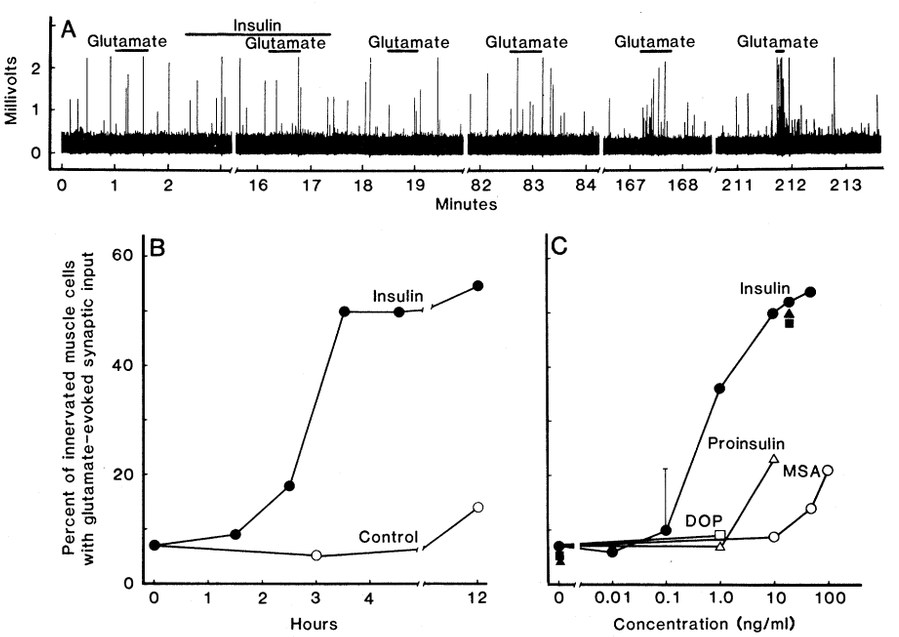

Fig. 1. (A) Effect of insulin on stimulus-dependent synaptic transmission (*17*). Bars show the times for the microiontophoresis (55 nA) of glutamate or the miniperfusion of bovine insulin (50 ng/ml) (*18*). (B) Time course for the development of evocable neurotransmission. At time zero the medium was replaced with medium A supplemented with bovine insulin (20 ng/ml) or vehicle. Each point represents the mean of two to four experiments (mean sample size, 25). Standard deviations were 10 or less. (C) Dose-response curves for the induction of evocable synaptic transmission by insulin and some naturally occurring and chemically modified insulin-like molecules. At the seventh hour of culture, medium A was replaced with medium A containing bovine insulin (●), proinsulin (△), or DOP (□); or with BME supplemented with 10 percent nondialyzed fetal bovine serum (Gibco) (■) and bovine insulin; or with BME supplemented with bovine serum albumin (2 mg/ml) (Sigma) and MSA (*19*) (○) or bovine insulin (▲). At 1 day after culture, the electrophysiological assay was performed. One hour before the assay, the medium with bovine serum albumin was replaced with medium A. Each point represents the mean of assays from two to five cultures with a mean of eight innervated myotobes sampled per culture (*20*). The vertical bar shows the largest standard deviation observed.

Taken together, these experiments indicate that the effect of insulin on neurotransmission at retina-muscle synapses can be explained by an action of the hormone on retinal cells. Whether insulin acts directly on cholinergic neurons or indirectly via other types of retinal cells, such as noncholinergic neurons or glial cells, remains to be determined. Also, the possibility that insulin may influence transmission at mature synapses has yet to be explored.

In summary, insulin can regulate the timing of the developmental step in which cholinergic neurons derived from the rat retina acquire the ability to transmit excitatory information across synapses formed in culture. Earlier studies indicate that glucocorticoid hormones (*4*) and dopamine (*16*) also are regulatory signals for the maturation of cholinergic retinal neurons. Although the regulation of neuronal development is a complex process, it appears possible, with the use of a cell culture system, to dissect and analyze certain ontogenetic steps.

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10. Electrophysiological methods, assays, and controls are detailed elsewhere (*3*). For miniperfusion a micropipet (tip size, 5 μm) and a pneumatic ejection system (1.2 psi, Medical Systems) were used. Miniperfusion of 2 nM HCl (the concentration of HCl in medium containing insulin at 50 ng/ml) did not influence the membrane potential of myotubes nor the evocability of synaptic input.
11. At 4 to 8 hours, the percentage of innervated muscle cells was 48 ± 10 (standard deviation) and 45 ± 13 in control and experimental groups, respectively. The rate of spontaneous postsynaptic activity was 8.5 ± 3.5 (standard deviation) responses per minute in control cultures and 7.9 ± 3.1 in the experimental group.
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17. This a-c coupled recording permitted considerable amplification while maintaining a relatively fixed baseline. The vertical scale is limited to 2.25 mV. During the evoked response at 212 minutes, two of the responses were greater than 10 mV in amplitude.
18. With miniperfusion of insulin, the membrane potential of the myotube increased by 2 mV within 1 minute. When insulin perfusion was stopped, the membrane potential returned to −54 mV within 2 minutes. A hyperpolarizing effect of insulin on mature skeletal muscle has been observed by others (see K. Zierler and E. M. Rogus, *Biochim. Biophys. Acta* **640**, 687 (1981).
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20. In control cultures, 50 ± 11 percent (four experiments; sample size, 30) of the sampled myotubes had spontaneous synaptic activity. Over the range of concentrations shown, insulin or the insulin-like molecules did not affect the incidence of innervated muscle cells.
21. We thank C.-D. Agardh and H. Yeh for helpful discussion and R. Chance and S. P. Nissley for gifts of insulin-like substances.
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The Twitch in Horses: A Variant of Acupuncture

Abstract. *The twitch procedure in horses attenuates the increase in the heart rate evoked by pain-inducing stimuli and the reaction of the animals to such stimuli. Endorphin systems are probably involved in the effectiveness of the twitch, since its action is blocked by naloxone and its application increases plasma concentrations of immunoreactive β-endorphin. The mode of action of the twitch cannot be explained by the generally accepted theory of divertive pain and may resemble that of classical acupuncture.*

Techniques for restraining horses have been used for many years by farmers and veterinarians who wanted to perform minor procedures such as shoeing, medical examinations, and injections. One of the most popular techniques is twitching, which is still generally used. The twitch is a 20- to 100-cm long wooden stick with a loop of rope at the end. This loop is twisted rather tightly around the upper lip of the horse (Fig. 1). After application of the twitch, the horse becomes quieter, appears somewhat sedated, the eyelids drop, and its hostile attitude decreases. The horse's interest in its surroundings diminishes and it becomes difficult to stimulate it to walk. The tolerance and acceptance of pain increases. Conversely, if the loop is twisted too tightly the horse strikes at the twitch with its forelegs, which suggests a reaction to an aversive stimulus.

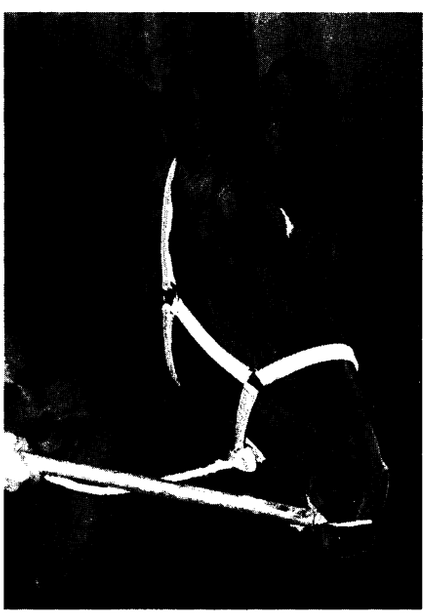

Fig. 1. The twitch procedure in horses.

Information concerning the mechanism underlying the effectiveness of the twitch is scarce. Several hypotheses have been proposed to explain its mode of action: (i) the attention of the horse is distracted, (ii) the pain induced by the pressure on the upper lip leads to a decreased perception and awareness of pain produced by treatments performed on another part of the body, and (iii) the horse becomes insensible to painful stimuli (*1*). The generally accepted explanation is based on a combination of the first two and is described as "divertive pain" (*1–5*). It is suggested that the twitch activates pain receptors evoking a feeling of pain with the result that the perception of other painful stimuli decreases or is even absent. However, horses do not react to the twitch as they do to painful stimuli, but are instead more or less sedated and quiet. Moreover, wounds in the upper lip have less influence on food intake than wounds in other parts of the body.

For these reasons, we propose that the effect of the twitch is not due to divertive pain but to activation of a system that is capable of decreasing pain perception, awareness, or both. The mechanism of the twitch seems to resemble that of classical acupuncture in that it stimulates mechano-receptors in the skin. The