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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8. Both spontaneous and glutamate-evoked input to innervated muscle cells were blocked reversibly by 10 μM D-tubocurarine (sample size, 5).
9. Dissociated retinal cells from embryonic day 19 rats were added (5×10^6 cells per 35-mm dish) to muscle cultures and incubated for 1 day in medium A, which consisted of 90 percent basal medium of Eagle (BME) with Earle's salts (Gibco) and 10 percent dialyzed (cut off at 12,000 daltons) fetal bovine serum (MA Products). See (*3*) and (*4*) for details.
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).
19. Fraction III-2 of MSA was used. See A. C. Moses *et al.*, *Eur. J. Biochem.* **103**, 387 (1980).
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.

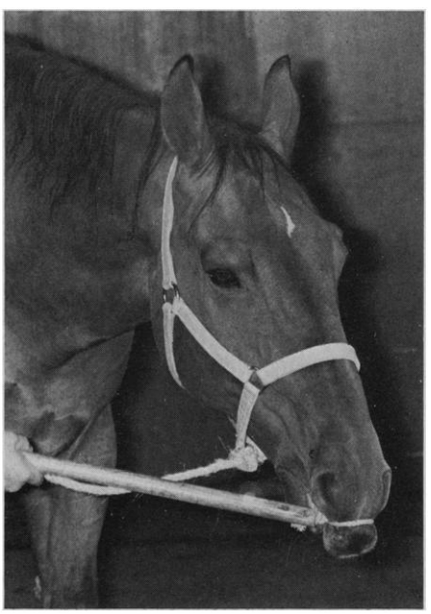

Fig. 1. The twitch procedure in horses.

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

twitch may, like acupuncture needles, result in activation of a pain-decreasing mechanism. The effect of acupuncture is partly explained by observations that this procedure results in release of endorphins (*6*). These substances may also be mediators for some or all effects of the twitch.

In order to test this suggestion we have determined the effect of painful stimuli in horses. The reaction of the animals was judged on a four-point scale (Table 1). Heart rate was monitored in order to have a more objective measure, since it is representative of the disposition of the animals and is also influenced by morphinelike substances.

The first experiment was carried out with 37 horses (19 males and 18 females; average age, 6.5 years). Heart rate was determined before, during, and after application of the twitch. During the 5-minute twitching procedure, ten painful stimuli were applied with a sharp needle to the skin alongside the spinal column and the shoulders at a frequency of one per second. The reaction of the horses was recorded and heart rate was measured immediately after the stimuli were delivered. After 40 minutes, this procedure was repeated without the use of the twitch. In ten of the horses this sequence was reversed. As no statistically significant differences in the two sequences were observed with respect to test scores (Table 1) and heart rate, the results were combined.

The test scores revealed that the twitch procedure significantly lowered the reaction of the animals to painful stimuli (Table 1). When using the twitch, heart rate decreased by 8 percent. The rate returned to basal values after application of the painful stimuli. Without the twitch the heart rate increased by 22 percent (Fig. 2A) under the influence of the painful stimuli. The difference in heart rate in the presence and absence of the twitch was statistically significant (Table 1). The percentage changes in heart rate (y) due to the painful stimuli in the absence of the twitch was related to the reaction of the horses (x) as assessed with the four-point grading system [linear regression analysis: $y = a + bx$: $a = 13.8 \pm 2.7$ (standard deviation); $b = 5.6 \pm 1.6$ (standard deviation); $r = 0.51$, $P < 0.01$]. Thus, heart rate appears to be a reliable index for measuring the reaction of horses to painful stimuli.

Table 1. Number of horses responding in each behavioral category and heart rate in response to painful stimuli in the presence or absence of the twitch. The behavioral response was judged on a four-point scale, on which 0 indicated no reaction; 1, local muscular twitching and a slightly rigid posture; 2, avoidance of the painful stimuli, looking around, and moving away; and 3, fierce reaction, lashing out, or trying to escape. S.E.M., standard error of the mean.

Condition	Behavioral reaction					Heart rate (beats per minute)
	0	1	2	3	$\bar{X} \pm$ S.E.M.	
Without twitch	2	17	14	4	1.54 ± 0.13†	47.9 ± 0.8‡
With twitch	19	17	1	0*	0.51 ± 0.09§	38.5 ± 0.9**‖

*$\chi^2(3) = 29.0$, $P < 0.001$. **$t(36) = 11.6$, $P < 0.001$. †Twitch first, 1.56 ± 0.14; twitch second, 1.50 ± 0.27. ‡Twitch first, 48.4 ± 1.0; twitch second, 46.8 ± 1.5. §Twitch first, 0.44 ± 0.10; twitch second, 0.70 ± 0.21. ‖Twitch first, 38.5 ± 1.1; twitch second, 38.4 ± 1.4.

To determine whether endorphin systems are concerned in the mechanism of the twitch, the morphine anatagonist naloxone (0.3 mg per kilogram of body weight) and a placebo (saline) were injected intravenously 5 minutes before the application of the twitch in a double-blind test of seven horses. Apart from the intravenous injection, this experiment was done exactly as before. The effect of the twitch on heart rate was completely absent in horses treated with naloxone. Moreover, in these horses the painful stimuli increased heart rate to a point that did not differ significantly from the values in the first experiment without the twitch (Fig. 2B). This result indicates that the effect of the twitch on heart rate is mediated by endorphins in the body and that these endorphins can influence the heart rate of horses.

To determine whether endorphins are released when the twitch is applied, blood samples were taken from nine horses before application, 3 minutes after application, and 30 minutes after removal of the twitch. Plasma was extracted with acid-acetone (*7*). Endorphins in the extract were separated from other material by chromatography over Sep-Pak C-18 columns. Subsequently, the immunoreactive β-endorphin was measured by radioimmunoassay. The plasma concentrations of immunoreactive β-endorphin ranged from 100 to 500 pg/ml. During the twitch procedure, the plasma concentrations increased with 81 ± 33 percent (mean ± standard error of the mean) of basal values [$t(8) = 2.46$, $P < 0.05$]. Thirty minutes after removal of the twitch, they had returned to baseline values (2 ± 31 percent higher than basal values). Repeated sampling did not significantly change the plasma concentrations of immunoreactive β-endorphin (decrease with 8 ± 18 percent of basal values at the second sampling). Thus, shortly after applying the twitch immunoreactive β-endorphin was elevated in plasma, indicating that the twitch procedure may indeed activate the endorphin systems in the body. Whether plasma β-endorphin is responsible for the effectiveness of the twitch procedure remains to be shown.

Concerning the mode of action of the twitch procedure, our data indicate that divertive pain cannot serve as a plausible explanation for this phenomenon, be-

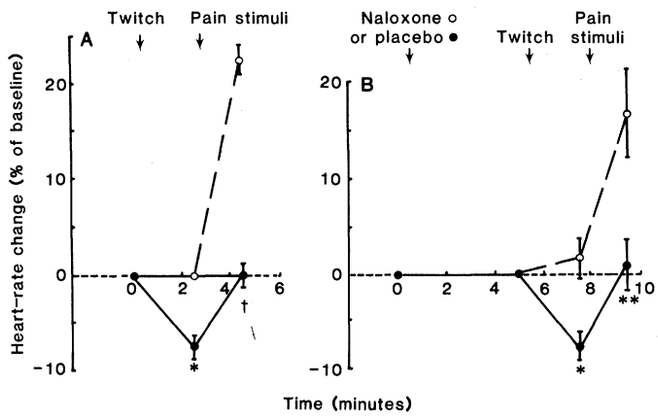

Fig. 2. Changes in heart rate (means ± standard error of the mean) produced by painful stimuli in the absence or in the presence of the twitch in horses. Values are expressed as percentages of individual basal values (0 minutes), (A) Heart rate was determined in 37 horses before and after painful stimuli in the absence (○) for presence (●) of the twitch; basal heart rate was 39.2 ± 0.8 and 38.8 ± 0.9 beats per minute, respectively. *Different from basal values [$t(36) = 6.69$, $P < 0.001$]. †Different from heart rate without the twitch [$t(36) = 11.6$, $P < 0.001$]. (B) Naloxone (○) or placebo (●) was injected into seven horses (cross-over design) before the twitch was applied and painful stimuli delivered. *Different from naloxone treatment [$t(6) = 2.71$, $P < 0.05$]. **Different from naloxone treatment [$t(6) = 2.96$, $P < 0.025$].

cause there was no sign that this procedure indeed leads to pain. The horses appeared to be sedated and heart rate was reduced by the twitch, whereas painful stimuli activated horses and increased heart rate. It is more likely that mechanisms involved in pain relief, such as endorphin systems, are activated by the twitch procedure. Because both analgesia and sedation are observed after applying the twitch, this procedure may be comparable to neuroleptic-analgesia and may be regarded as a Western example of acupuncture.

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7. Blood was collected in chilled tubes containing EDTA (1.45 mg per milliliter of blood), immediately centrifuged to remove cells, and frozen. Portions (1 ml) were spiked with ^{125}I-labeled β_h-endorphin (approximately 1000 count/min) to monitor recovery of the subsequent procedure and agitated 30 minutes at 4°C with five volumes of acetone in 1*N* HCl (100:3 by volume) to extract endorphins. The precipitate was removed by centrifugation and the extract was dried at 60°C. The residue was dissolved in 5 ml 0.01*M* ammonium acetate buffer (*p*H 4.15) and passed three times over a Sep-Pak^R C-18 column. The column was then washed with 10 ml of methanol and ammonium acetate buffer (20:80 by volume), and the endorphins were eluted with 5 ml of methanol and ammonium acetate buffer (80:20 by volume). The endorphin fraction was evaporated to dryness at 60°C, and the residue was dissolved in 400 μl of 125 m*M* phosphate-buffered saline (*p*H 7.5) containing 0.1 percent Triton X-100 and 0.25 percent bovine serum albumin (radioimmunoassay buffer). Portions of 375 μl were counted for recovery (40 to 60 percent) and then diluted for β-endorphin radioimmunoassay. Assays were performed in duplicate on three different dilutions of each sample, using an antiserum (B4) directed at the midportion of the β-endorphin molecule, ^{125}I-labeled β_h-endorphin as a tracer, and synthetic β_h-endorphin as a standard. Samples (100 μl) were incubated for 24 hours at 4°C with antiserum (50 μl; final dilution 1:20,000) and further incubated for 16 hours in the presence of tracer (50 μl; 10,000 count/min). The bound fraction was precipitated with polyethylene glycol (1 ml; 20 percent weight to volume), pelleted, and pg of β_h-endorphin at 10 percent displacement. Cross-reactivity (on a mass base) in this system was β_h-lipoprotein, 100 percent; γ-endorphin, 450 percent; α-endorphin, 250 percent; [Met]enkephalin, 0.2 percent. Radioimmunoassay data were corrected for recovery.

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Control of Extracellular Potassium Levels by Retinal Glial Cell K^+ Siphoning

Abstract. *Efflux of K^+ from dissociated salamander Müller cells was measured with ion-selective microelectrodes. When the distal end of an isolated cell was exposed to high concentrations of extracellular K^+, efflux occurred primarily from the endfoot, a cell process previously shown to contain most of the K^+ conductance of the cell membrane. Computer simulations of K^+ dynamics in the retina indicate that shunting ions through the Müller cell endfoot process is more effective in clearing local increases in extracellular K^+ from the retina than is diffusion through extracellular space.*

Local changes in extracellular potassium ion concentration, $[K^+]_o$, are produced within the central nervous system as part of normal neuronal activity (*1*). These changes can affect neuronal activity by altering cellular resting potentials. Astrocytic glia are thought to attenuate changes in local $[K^+]_o$ by a process known as "K^+ spatial buffering" (*2*, *3*). In this process, local increases in $[K^+]_o$ are accompanied by K^+ influx into astrocytes. An equal amount of K^+ exits from these cells or from cells electrically coupled to them in regions where $[K^+]_o$ is lower, thus transferring K^+ away from the sites of initial increase.

We have suggested that the retinal Müller cell, a specialized astrocyte that spans nearly the entire width of the retina, buffers changes in retinal $[K^+]_o$ (*4*, *5*). We have shown that amphibian Müller cells are almost exclusively permeable to K^+ (*6*) and that 94 percent of the total K^+ conductance in these cells occurs in the Müller cell endfoot, a process lying adjacent to the vitreous humor (*4*). This highly asymmetric K^+ conductance distribution may make the process of K^+ spatial buffering more powerful than has been recognized. For example, nearly all of the K^+ current entering Müller cells from regions of increased $[K^+]_o$ within the retina may leave the Müller cell endfoot process at the vitreo-retinal border. Thus, the vitreous would function as a large potassium sink.

We now present experimental evidence of extracellular K^+ buffering by Müller cells which utilizes this asymmetric conductance distribution. Dissociated Müller cells from the salamander *Ambystoma tigrinum* were prepared and maintained as described (*4*). The distal end of the Müller cell surface was exposed to increased $[K^+]_o$ by pressure-ejecting an 85 m*M* KCl-Ringer solution from an extracellular pipette (approximately 3 μm in tip diameter). Perfusate near this ejection pipette was drawn into a suction pipette (30 μm in diameter) to limit the spread of K^+ from the ejection site to other areas of the Müller cell membrane. Single-barreled (*7*) K^+-selective microelectrodes [8 μm in diameter, filled with Corning resin 477317 (*8*)] were used to measure $[K^+]_o$ near different regions of the dissociated Müller cell surface (Fig. 1A, sites a through d).

The results of one experiment are shown in Fig. 1B. At the distal end of the dissociated Müller cell (Fig. 1A, site a), the site of K^+ ejection, we measured an increase in $[K^+]_o$ to 40 m*M* (Fig. 1B, trace a). When the K^+-selective microelectrode was moved from the ejection site to sites b and c (Fig. 1A), much

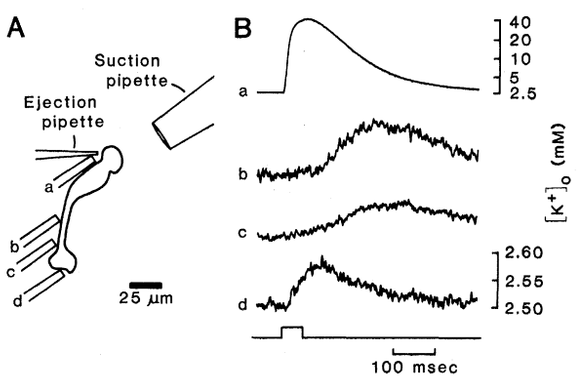

Fig. 1. Measurement of K^+ efflux from a dissociated *Ambystoma tigrinum* Müller cell. (A) Schematic of a dissociated cell showing location of K^+ ejection and suction pipettes and the four positions of the ion-selective microelectrode (a through d). (B) Voltage records from the ion-selective microelectrode made at the locations indicated in (A). The onset and duration of a 50-msec pressure pulse applied to the ejection pipette is indicated at the bottom. Traces b, c, and d are expanded vertically relative to trace a. The concentration scales (m*M*) were determined by calibrating ion-selective pipettes in a series of K^+ solutions.