Permissive Role of Insulin in the Expression of Long-Term Potentiation in the Hippocampus of Immature Rats

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Abstract
Many studies indicate that impairment in insulin signaling leads to learning and memory deficits. However, previous studies failed to establish a clear role of insulin in long-term potentiation (LTP), the best cellular model of memory formation. Here we show that while insulin pretreatment did not affect LTP magnitude in the adult rat hippocampus, it facilitated LTP expression in the immature hippocampus. The tyrosine kinase inhibitor AG-1024 abolished the effect of insulin in young rats, suggesting the involvement of the insulin receptor. On the other hand, increasing extracellular glucose concentration failed to facilitate LTP and application of an insulin-responsive glucose transporter-4 inhibitor did not impair the effect of insulin. These results suggest that the facilitatory action of insulin on LTP is not an indirect effect on glucose homeostasis/utilization. Involvement of the MAPK/ERK pathway, a known downstream pathway of insulin signaling, was revealed by pretreatment with PD98059, which blocked the insulin-mediated LTP facilitation. Consistent with this, high-frequency stimulation induced a significant increase in the level of phosphorylated Erk-2 in insulin-treated hippocampus. Taken together, these results suggest that insulin may be an essential factor in the immature brain, allowing the expression of LTP to facilitate learning and memory.

Introduction
The role of insulin as a neuromodulator and neurotrophic factor in the brain has received much attention in recent years [1–4]. The interest in a central action of insulin was prompted by the early unexpected finding that the brain contains insulin and insulin receptors [5–8], and the increased awareness of a possible causal link between insulin resistance and cognitive deficits in diabetes [2, 9]. Furthermore, there is evidence that defective insulin signaling contributes to the pathophysiology of Alzheimer’s disease [10–13].

Experimental studies in humans and animals in general point to a facilitatory role of insulin in cognitive performance. For example, administration of insulin intranasally or by infusion in humans [14, 15] and via intracerebroventricular injection in rats [16] resulted in elevated insulin levels in the brain and enhancement of...
Insulin also exerts a restorative action on memory impairment in various settings, including drug-induced experimental model of diabetes [17, 18], models of stress [19] and also in Alzheimer’s disease patients [20]. These findings are consistent with the observation that insulin receptors are especially abundant in the hippocampus in both rats and humans [21–24] and that insulin receptors on pyramidal neurons are upregulated after training in the water maze task [25].

It is widely accepted that long-term synaptic plasticity in the hippocampus, especially long-term potentiation (LTP), is the mechanism that underlies some forms of learning and memory. In the hippocampus, insulin receptor immunoreactivity and insulin binding sites are particularly prominent in the CA1 subfield [26, 27]. However, although a role of insulin in inducing a long-term depression-like phenomenon has been well documented in the hippocampus [28], the effect of insulin on LTP remains unclear. In fact, it has been shown that insulin does not facilitate high-frequency stimulation (HFS)-induced LTP [29]. Also, although some studies show that in experimental diabetes, LTP is impaired and can be rescued by insulin [17, 30], there are reports showing that LTP is preserved in this condition [e.g. 31]. Thus, the exact relationship between insulin and LTP is still obscure.

In this study, we addressed the question of whether insulin application has any effect on LTP expression in the hippocampus of normal, healthy rats. Since it is known that insulin receptor expression in the brain is developmentally regulated, being highest in young age and decreasing with age [1, 32], we examined and compared the effect of insulin on LTP in both immature and adult rats. Our experiments reveal that insulin plays a critical permissive role in LTP expression in the immature brain via insulin receptors but not through its influence on glucose utilization/homeostasis. This finding suggests that insulin may be an indispensible endogenous factor allowing LTP expression in normal young animals for learning and memory, and provides an explanation for the impaired cognitive function in children with insulin-dependent type 1 diabetes [33].

Materials and Methods

All experimental protocols and procedures described were performed in compliance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Experimentation Ethical Committee of the Chinese University of Hong Kong. Efforts were made to minimize the number of animals used and their suffering.

Hippocampal Brain Slices

Sprague-Dawley rats, provided by the Laboratory Animal Services Center of the Chinese University of Hong Kong, were anesthetized with isoflurane, and then sacrificed by decapitation. The brains were immediately removed and cut into two halves in the sagittal plane and then immersed in ice-cold artificial cerebrospinal fluid (ACSF) of the following composition: 125 mM NaCl, 2.0 mM KCl, 1.2 mM MgSO4, 2.5 mM CaCl2, 1.2 mM KH2PO4, 11 mM glucose, and 26 mM NaHCO3, which was continuously bubbled with 95% O2 and 5% CO2 (pH 7.4; osmolarity 290–310 mosm). The brains were then glued with the lateral side down onto a platform in a chamber filled with oxygenated ice-cold ACSF, and 300-μm-thick parasagittal sections were cut using a vibrating microtome (Integralslice 7550MM, Campden Instruments Ltd., Loughborough, UK). Slices were preincubated in a holding chamber containing oxygenated ACSF at 34 ± 1°C for at least 1 h.

LTP Measurements

A planar multielectrode recording setup (MED64 system, Alpha Med Sciences Co., Ltd, Tokyo, Japan) was employed to record the field excitatory postsynaptic potential (fEPSP), and to study LTP. The methodology has been described in details elsewhere [34, 35]. Briefly, hippocampal slices were placed on special probes that were fabricated with 8 × 8 electrode arrays (20 × 20 μm, made of indium tin oxide and platinum black) and precoated with polyethyleneimine (Sigma). The P210A probes (Alpha Med Sciences) with an interelectrode distance of 100 μm were routinely used. Correct placement of the electrodes at the CA3-CA1 region was done manually, monitored by a microscope (MIC-D, Olympus Ltd., Japan). To increase the efficiency of the experiments and to minimize the variation in the results arising from differences in incubation times, a maximum of 4 slices were studied simultaneously by means of a splitter provided by the manufacturer. Each slice was submerged in and superfused with oxygenated ACSF at a flow rate of 1.3–1.5 ml/min. fEPSPs were recorded from the dendritic layer of CA1 neurons by choosing an electrode in the Schaffer collateral pathway as the stimulating electrode. Based on the stimulus-response curve, we chose a stimulation intensity that evoked the fEPSP with a magnitude of 30–40% of the maximum response. After allowing a stable baseline of 30 min in which the stimulus was delivered every 60 s, an LTP induction protocol consisting of 1 train of 100-Hz stimuli that lasted for 1 s was applied, and the field potential response after the tetanus was recorded for 1 h. The magnitude of LTP was quantified as percentage change in the average amplitude of the fEPSP taken at 50- to 60-min intervals after LTP induction. Drugs were applied to the slices for at least 30 min before the delivery of HFS.

Protein Analyses

After preparation of hippocampal slices from young rats, the CA1 regions from 5–7 slices were cut and incubated with radioimmunoprecipitation assay lysis buffer including Pimix (1:200 dilution) and 0.1 M phenylmethylsulfonyl fluoride (1:100 dilution) for 30 min. After centrifugation at 13,000 rpm for 15 min, the supernatant was measured for the concentration of protein and was then subjected to SDS–PAGE (10% polyacrylamide gel electrophoresis). The protein was transferred to nitrocellulose membrane using an electrophoretic transfer system. The membrane was blocked with Tris-buffered saline mixed with Tween-20 including 5% milk for 1 h followed by overnight incubation at 4°C.
with rabbit primary antibody against MAPK/ERK and phospho-MAPK/ERK (1:3,000). The membrane was stripped in 50 ml stripping buffer at 50°C and shaken for at least 30 min without signal could be detected. After incubation with goat anti-rabbit IRDye 800 CW secondary antibody (1:10,000, Li-Cor) for 1 h at room temperature, the membrane was scanned by an Odyssey scanner. The intensities of the specific bands were analyzed by Odyssey infrared imaging software at a resolution of 169 μm. Each experiment was performed three times.

**Chemicals and Data Analysis**

Insulin was purchased from Sigma (USA). Indinavir was obtained from TRC (Canada). PD98059 was purchased from Cayman (USA) and AG1024 was from Spectrum (USA). MAPK (Erk) antibodies and phospho-MAPK (p-Erk) antibodies were obtained from Cell Signaling Technology Company (USA). The fEPSPs obtained were analyzed using Prism 4.0 (GraphPad software, San Diego, Calif., USA). The data are expressed as means ± SEM and n stands for the number of brain slices used. An unpaired Student’s t test was used to compare LTP amplitude for multiple groups followed by a Newman-Keuls test.

**Results**

**Differential Effects of Insulin on LTP in Young and Adult Rats**

We first examined the ability of the hippocampal slices from 2-month-old (adult) and 11- to 12-day-old (young) rats to exhibit LTP. After baseline recording for at least 30 min, standard HFS (100 Hz, 1 s) stimuli were delivered to the Schaffer collateral pathway and fEPSPs were recorded in the CA1 dendritic layer for at least 60 min. As shown in figure 1a, in the adult group, the fEPSP amplitude was potentiated to 165.8 ± 13.0% of baseline (12 slices, 8 rats). On the other hand, HFS failed to induce potentiation of the fEPSP in the young rat measured at the end of 60 min (fig. 1b, 100.3 ± 6.4% of baseline, 8 slices, 4 rats).

To test the effect of insulin on LTP, some hippocampal slices from both age groups were pretreated with insulin (0.08 or 0.8 ng/ml, equivalent to 13.7 and 137 nM, respectively) for at least 1 h before delivery of HFS. Insulin at these concentrations did not affect the baseline fEPSPs. As shown in figure 1a, in the adult group, the magnitude of LTP was not altered by insulin at both 0.08 ng/ml (158.5 ± 14.4%, 8 slices 5 rats, p = 0.64 compared with control) and the higher concentration of 0.8 ng/ml (149.3 ± 4.3%, 10 slices 5 rats, p = 0.59 compared with control). Strikingly, however, in the young rats in which HFS could not induce LTP, pretreatment with insulin induced potentiation of the fEPSP in a dose-dependent manner (fig. 1b). At a concentration of 0.08 ng/ml, the fEPSP measured at the end of 60 min was 102.6 ± 3.4% of baseline (fig. 1b, 9 slices from 6 rats, p = 0.77 compared with control). Significant potentiation of the fEPSP, i.e., LTP, was enabled at an insulin concentration of 0.8 ng/ml. The amplitude of the fEPSP at 60 min after HFS was 120.4 ± 4.4% of baseline (fig. 1b, 10 slices from 6 rats, p < 0.01, compared with control). These results indicate that under the experimental conditions of the present study, insulin plays a permissive role in the LTP expression in young rats while it is without effect on the LTP in the adult hippocampus.

**The Facilitatory Effect of Insulin Is Mediated by the Insulin Receptor**

To determine the involvement of the insulin receptor in mediating the effect of insulin on synaptic plasticity, we studied the effect of AG-1024, a tyrosine kinase inhibitor known to inhibit the insulin receptor [36], on the observed insulin-dependent LTP in the young rats. Incubation of the hippocampal slices with AG-1024 (80 nM) did not affect the basal synaptic transmission. However, as shown in figure 2a, pretreatment of the slices with AG-1024 for 30 min before HFS and throughout the experiment largely abolished the insulin-dependent LTP. The magnitude of LTP in the presence of AG-1024 was 105.8 ± 2.5% (15 slices from 9 rats), which was significantly lower than that of the insulin-alone group (fig. 2a, 130.4 ± 5.7%; 14 slices from 7 rats, p < 0.001).

We also tested the effect of AG-1024 on the LTP expressed by the adult hippocampus. In the control group, the LTP magnitude was 143.8 ± 9.4% (fig. 2b, 12 slices from 6 rats). Treatment with AG-1024 did not affect the LTP (148.6 ± 8.3%, 11 slices 6 rats, p = 0.71; fig. 2b). These results indicate that AG-1024 itself does not have a direct effect on LTP and is in line with the notion that the LTP expressed in the adult hippocampus is not dependent on insulin.

**The Facilitation of LTP Expression Is Not via Regulation of Glucose Utilization**

The effect of insulin on glucose metabolism in the brain is uncertain, and is still debated [37, 38]. To address the possibility that the facilitatory action of insulin on LTP expression in the young animals is via its effect on glucose utilization, we performed two sets of experiments. In the first experiment, we studied the effect of indinavir, an insulin-sensitive glucose transporter-4 (GLUT-4) inhibitor. In the insulin-treated group, the magnitude of LTP was 122.2 ± 4.9% (fig. 3a, 10 slices from 5 rats). Preincubation of the slices with indinavir
Fig. 1. Different effects of insulin on hippocampal LTP in mature and immature rats. a Left panel: fEPSPs recorded from the hippocampal CA1 region of 2-month-old rats showed that LTP was induced by conventional HFS delivered to the Schaffer collateral pathway at 30 min after stable baseline recording. The presence of insulin in the ACSF (0.08 and 0.8 ng/ml) did not affect the magnitude of the LTP. The results are summarized in the right panel. Representative recordings are shown in the insets, sampled during the baseline and at the time indicated by the arrow. b The same HFS failed to induce LTP in the hippocampus of young (11–12 days) rats, but insulin at 0.8 ng/ml facilitated the expression of LTP. Statistical analysis shown in the right panel shows that the averaged amplitude of fEPSPs measured in the last 10 min of recording in the 0.8 ng/ml group was significantly higher than those of the control and the low-concentration (0.08 ng/ml) group. Insulin was applied for at least 1 h before the delivery of HFS and was present throughout the experiment, ** p < 0.01.
Fig. 2. The facilitatory effect of insulin on LTP in immature rats is mediated by insulin receptor. a Incubation of the hippocampal slices with AG-1024 (80 nM), an inhibitor of insulin receptor, significantly decreased the magnitude of insulin-mediated LTP in the young rats. Representative traces are shown in the insets, sampled during the baseline and at the time indicated by the arrow. Statistical analysis shown in the right panel shows that the averaged amplitude of fEPSPs measured in the last 10 min of recording in the AG-1024-treated group was significantly lower than the insulin group. b In the mature rats, AG-1024 failed to alter the magnitude of the LTP. AG-1024 was applied for 30 min before HFS delivery and was present throughout the experiments, *** p < 0.001.
(100 μM) for over 30 min did not affect the magnitude of LTP observed (118.7 ± 9.1%, 11 slices from 6 rats, p = 0.75). In the brain, uptake of glucose is mainly dependent on facilitated glucose transport, which is driven by the glucose concentration gradient across the cell membrane. Thus, to assess the impact of increased glucose uptake per se, we tested the effect of raising extracellular glucose concentration on the expression of LTP in the young rats.

As shown in figure 3b, increasing the glucose concentration from 11 to 22 or 33 mM did not affect the magnitude of LTP in young rats (104.6 ± 3.2%, 9 slices from 6 rats, p = 0.79 and 106.7 ± 2.2%, 10 slices from 6 rats, p = 0.87, respectively). Taken together, these data strongly suggest that the facilitatory action of insulin on LTP expression is not an indirect effect via the regulation of glucose availability to the neurons.

Fig. 3. The facilitatory action of insulin on LTP is not via its effect on glucose utilization. a Application of indinavir (100 μM), an inhibitor of the insulin-sensitive GLUT-4, did not affect the magnitude of insulin-dependent LTP in young rats. b Doubling and tripling the concentration of glucose from 11 to 22 and 33 mM, respectively, did not facilitate the expression of LTP. Representative traces are shown in the insets, sampled during the baseline and at the time indicated by the arrow. Indinavir was applied for 30 min prior HFS delivery and was present throughout the experiment.

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We next explored the intracellular signaling pathway of the insulin-dependent LTP. Specifically, we tested the involvement of the MAPK/ERK pathway, which has been shown to be a major signaling pathway mediating the effect of insulin in the peripheral organs [39]. Figure 4a shows that in the insulin treatment group, the average magnitude of fEPSP obtained in 8 slices from 4 rats was 119.4 ± 3.2%. When PD98059 (20 μM), an inhibitor of MEK1, was applied before and continued throughout the experiment, the LTP magnitude was reduced to 106.6 ± 3.3% (10 slices from 5 rats, p < 0.05). To further confirm the involvement of the MAPK/ERK pathway in the effect of insulin, we examined the expression of phosphorylated Erk-2 (p-Erk-2, or p42 MAPK) in the hippocampus of young rats after HFS stimulation by Western blot analysis. As shown in figure 4b, in the presence of insulin, the expression of phosphorylated Erk-2 (p42 MAPK) in the hippocampal slices was increased 8 min after the delivery of HFS. Analysis showed that the ratio of phosphorylated Erk-2 versus total Erk-2 increased significantly in the presence of insulin compared with that in control group (fig. 4c, 0.077 ± 0.025 vs. 0.045 ± 0.019, n = 3, p < 0.05). However, the ratio of phosphorylated Erk-1 (p44 MAPK) versus total Erk-1 was not significantly different from that of the control group (fig. 4c, 0.038 ± 0.017 vs. 0.041 ± 0.017, n = 3, p > 0.05).

**Involvement of the MAPK/ERK Pathway**

Despite different lines of evidence supporting a role of insulin in facilitating learning and memory, the relationship between insulin and LTP, the best cellular candidate underlying memory formation, remains elusive up to date. By investigating the action of insulin on the expression of hippocampal LTP in healthy adult and young rats, we show that insulin plays a permissive role in the expression of LTP in young rats but has a minimal effect in adult rats. This conclusion is based on the fact that the hippocampus from 11- to 12-day-old rats, in response to the conventional HFS paradigm, produced a small increase in fEPSP magnitude that was not sustained. However, addition of 0.8 ng/ml (or 137 nM) of insulin allowed the expression of a decent LTP, which amounted to over 20% of potentiation in fEPSP. On the other hand, insulin application had no effect on the magnitude of the LTP in the adult hippocampus, which is in agreement with previous studies [29, 40].
These findings lead to some interesting speculations. First, it has been shown by others [41–43] and also in the present study that hippocampal slices from rats younger than 2 weeks are much weaker in the ability to express LTP, and therefore are regarded as developmentally immature. Our study suggests that the hippocampus at 11–12 days is equipped with the machinery to express LTP in response to the standard one-train HFS. The fact that they could not express LTP could be due to the removal of endogenous factors like insulin from the incubation fluid rather than to an inherent deficit. Second, insulin as a neuromodulator is particularly important for learning and memory in young subjects. This speculation is in line with the observation that the expression of insulin receptor is developmentally regulated, being highest in early postnatal stages and decreasing with age [1, 32], and can explain the cognitive dysfunction found in children with type 1 diabetes in which there is deficiency in insulin production [33, 44, 45]. In the adult hippocampus, it is possible that other factors, e.g. brain-derived neurotrophic factor, or mechanisms serve to ensure the expression of LTP which as a result relies less on insulin.

Our results imply the involvement of the insulin receptor in mediating the insulin-dependent LTP. This is based on the fact that AG-1024 and the MAPK/ERK pathway inhibitor PD98059 significantly suppressed the effect of insulin. However, due to the similarity between the insulin receptor and that of insulin-like growth factor-1 (IGF-1), and that both insulin and IGF-1 can act on these two types of receptors, we cannot rule out a role of IGF-1. In fact, the tyrosine kinase inhibitor AG-1024 inhibits both receptors. However, since the expression of the insulin receptor and the IGF-1 receptor within the hippocampus is different, with the insulin receptor being higher in the CA1 region [21], it is likely that insulin is the more important endogenous factor in permitting the expression of LTP in the CA3-CA1 pathway in vivo. What is the mechanism enabling insulin to facilitate the expression of LTP? It is well known that glucose is the main nutrient and energy source of neurons. The brain has traditionally been regarded as an insulin-insensitive organ [46] because the main glucose transporters expressed centrally are glucose transporter-1 (or GLUT-1) and glucose transporter-3 (or GLUT-3), both of which are insulin insensitive. However, recent studies suggest the presence of the insulin-responsive GLUT-4 in the brain, and insulin can induce GLUT-4 translocation to the plasma membrane in neurons, including those in the hippocampus [47–51]. Therefore, an insulin-induced increase in glucose utilization and therefore an increase in energy supply is a possible mechanism underlying the facilitatory effect of insulin on LTP. Our study, however, is not in favor of this scenario because neither elevating the concentration of glucose in the ACSF nor incubation with the GLUT-4 inhibitor indinavir [52, 53] affected the magnitude of the insulin-dependent LTP. It remains possible that GLUT-4 plays a role under high metabolic requirement, which is not reached in the in vitro slice condition. Alternatively, GLUT-4-mediated glucose uptake may be related to growth and development rather than long-term synaptic plasticity.

There are other potential mechanisms by which insulin can facilitate the expression of LTP. For example, it has been found that insulin promotes cell surface expression of N-methyl-D-aspartic acid (NMDA) receptors [54] and the GluR1 α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor subunit [55]; both can lead to enhanced synaptic transmission. Insulin also enhances NMDA receptor-mediated current in hippocampal neurons [56]. These effects could lower the threshold for the induction of LTP in the hippocampus and allow its expression in response to the HFS. Interestingly, both the activity of the NMDA receptor and the expression of the GluR1 AMPA receptor subunit can be increased in an MAPK-dependent manner [57, 58]. Consistent with these observations, our results revealed that activation of Erk-2 (p42 MAPK) might be involved in the facilitatory effect of insulin on LTP. Insulin may also facilitate the expression of LTP by increasing the protein expression of the dendritic scaffolding protein PSD-95 [59] and the recruitment of the insulin receptor substrate IRSp53, which has been shown to be translocated to the synapses in response to neuronal activity [60]. Obviously, further work is needed to dissect the detailed mechanism.

In conclusion, our results identify an acute, restorative action of insulin on LTP expression in young animals, suggesting its importance in the synaptic plasticity, and therefore learning and memory, of the immature brain. This finding highlights a new facet of insulin signaling in addition to the growing belief that this protein is important for synapse maturation and maintenance during development [1]. Since cognitive impairment is a common feature in children suffering from insulin-dependent diabetes, elucidating the role of insulin in the developing nervous system is important both scientifically and clinically.

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