Brain Insulin Receptors and Spatial Memory

Correlated Changes in Gene Expression, Tyrosine Phosphorylation, and Signaling Molecules in the Hippocampus of Water Maze Trained Rats

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Evidence accumulated from clinical and basic research has indirectly implicated the insulin receptor (IR) in brain cognitive functions, including learning and memory (Wickelgren, I. (1998) Science 280, 517–519). The present study investigates correlative changes in IR expression, phosphorylation, and associated signaling molecules in the rat hippocampus following water maze training. Although the distribution of IR protein matched that of IR mRNA in most forebrain regions, a dissociation of the IR mRNA and protein expression patterns was found in the cerebellar cortex. After training, IR mRNA in the CA1 and dentate gyrus of the hippocampus was up-regulated, and there was increased accumulation of IR protein in the hippocampal crude synaptic membrane fraction. In the CA1 pyramidal neurons, changes in the distribution pattern of IR in particular cellular compartments, such as the nucleus and dendritic regions, was observed only in trained animals. Although IR showed a low level of in vivo tyrosine phosphorylation, an insulin-stimulated increase of in vitro Tyr phosphorylation of IR was detected in trained animals, suggesting that learning may induce IR functional changes, such as enhanced receptor sensitivity. Furthermore, a training-induced co-immunoprecipitation of IR with Shc-66 was detected, along with changes in in vivo Tyr phosphorylation of Shc and mitogen-activated protein kinase, as well as accumulation of Shc-66, Shc-52, and Grb-2 in hippocampal synaptic membrane fractions following training. These findings suggest that IR may participate in memory processing through activation of its receptor Tyr kinase activity, and they suggest possible engagement of Shc/Grb-2/Ras/mitogen-activated protein kinase cascades.

Because insulin and insulin receptors (IRs)¹ were found in the central nervous system, their role(s) in brain function has been a subject attracting many researchers’ interest. IR is widely distributed in the brain (1), with particularly high concentrations in neurons and much lower levels in glia (2, 3). Although IR mRNA is largely localized in neuronal somata, abundant IR protein is found in both cell bodies and synapses, including the dendritic field of the hippocampal CA1 pyramidal cells, the adrenergic terminals in hypothalamus, and membranes of dendrodendritic synaptosomes from the rat olfactory bulb (3, 4). Although the major molecular structure and most of the properties of IR in the central nervous system are indistinguishable from those in peripheral tissues, some differences exist between central nervous system IRs and peripheral IRs. Both the α and β subunits, for example, of IR in the central nervous system are slightly lower in molecular weight than those in the periphery (5). Unlike peripheral IR, the brain IR does not undergo down-regulation after exposure to high concentrations of insulin (6, 7). In addition, insulin appears to regulate glucose metabolism only in glia cells but not in neurons (3, 8, 9). These findings have led to speculation that the neuronal IRs mediate functions different from those regulated by their peripheral and glial counterparts. IR is a glycoprotein with an αβ₂ tetrameric structure. Binding of insulin to the extracellularly located α subunits results in autophosphorylation of the β subunits at tyrosine residues 1158, 1162, and 1163, located in the cell interior (10). Autophosphorylation stimulates the intrinsic tyrosine kinase activity of IR, which is believed to initiate the biological actions of insulin (10). Like other growth factor receptors, phosphorylated IR triggers subsequent biological responses by activating different cellular signal transduction cascades. These include interaction of the receptor with IR substrate proteins and a variety of Src homology domain 2- and 3-containing proteins (11–13). One of such pathways involves Shc, an adapter protein (14, 15) that mediates association of the receptor with the Grb-2/SOS protein complex (16). This process is known to bring about activation of Ras (16, 17) that in turn triggers the mitogen-activated protein kinase kinase/mitogen-activated protein kinase (MAPK) pathway leading to regulation of nuclear transcription (18). It is found that the phosphorytrosine binding domain of Shc is responsible for the IR-mediated mitogenic signaling (19).

Evidence from functional studies demonstrates that insulin/IR may play a modulatory role in brain synaptic transmission. Consistent with its enriched distribution in adrenergic terminals, insulin is found to promote central catecholaminergic activities by releasing both epinephrine and norepinephrine (20), inhibiting synaptic reuptake of norepinephrine (21), and altering catecholamine kinetics (22). In the hippocampus, insulin is reported to enhance α₁ adrenergic receptor activity, leading to stimulation of membrane phosphoinositol turnover.

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¹ The abbreviations used are: IR, insulin receptor; MAPK, mitogen-activated protein kinase; RT, reverse transcription; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; IGF-1R, insulin-like growth factor-1 receptor; IGFR, insulin-like growth factor receptor.

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Spatial Training-induced Changes in Insulin Receptors and diacylglycerol production (23), two important second messengers involved in PKC activation. At the behavioral level, insulin/IR has been implicated in at least two major brain functions: feeding behavior (3, 24) and cognition, including learning and memory (25). A role for insulin/IR in learning and memory is supported by several findings. First, IR is highly concentrated in neurons of specific brain regions, such as hippocampus, amygdala, and some cortical areas (1, 2). Secondly, data from behavioral-pharmacological studies show that injection of streptozotocin, a diabetes-inducing compound, into the brain induces significant memory impairment (26). More importantly, defects in insulin action in both periphery and the brain have been found in Alzheimer’s disease (27, 28), and insulin has been shown to reduce phosphorylation of tau protein in human neuronal cultures by inhibiting activity of glycogen synthase kinase-3, hence promoting association of tau to microtubules (29). Finally, impairment of brain cognitive functions in diabetes mellitus, presumably due to disruption of glucose metabolism, is well documented (30–34).

Despite the large body of evidence from clinical and animal studies suggesting involvement of insulin/IR in learning and memory, actual correlated changes of IR during memory processing have never been reported. In this study, we investigate changes in IR expression, Tyr phosphorylation, and certain IR-associated signaling molecules in the rat hippocampal neurons following a water maze training experience.

**Experimental Procedures**

**Water Maze Training**—Male 60–90 day-old Wistar rats (200–250 g) were housed in standardized conditions as described elsewhere (35). To adapt rats to the experimental environment and behavioral activity, all rats were subjected, in the first day of experiments, to 2 min of swimming at 21 ± 1 °C. On the following day, rats were trained in a four-trial water maze task, each trial lasting up to 2 min. During training, rats learned to escape from water by finding an unseen rigid platform submerged about 1 cm below the water surface in a fixed location. The escape latency during each trial was measured as an indicator of learning. In order to assess short-term and long-term biochemical changes, rats were sacrificed at 1 and 24 h, respectively, after training. For the controls, rats were given four swimming trials, but without the platform present in the pool. The length of each swimming trial was 5 min in 4% formaldehyde, acetylated, and dehydrated in graded ethanol.

**In Situ Hybridization Histochemistry**—Brain slices were fixed for 5 min in 4% formaldehyde, acetylated, and dehydrated in graded ethanol. The IR riboprobe (1 × 10⁶ cpm in 50 μl) was applied to each slide for 30 min at 55 °C for 3 h in a mixture containing 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 300 mM NaCl, 50% formamide, 10% dextran sulfate, 1× Denhardt’s solution, 4 μg/ml salmon sperm DNA, 10 μg/ml yeast total RNA, 10 μg/ml yeast tRNA, 100 mM dithiothreitol, 0.1% SDS, 0.1% NTS. Slices hybridized with the sense riboprobe or pretreated with RNase before hybridization with the antisense probe were used as negative controls. Following high stringency posthybridization washes and RNase treatment, brain sections were dehydrated in graded ethanol and then subjected to film and liquid emulsion autoradiography.

**Immunohistochemistry**—Frozen brain sections from naive, swimming control, and trained rats were fixed for 5 min in 4% formaldehyde. After washing with phosphate-buffered saline (PBS), and preincubation with 0.5% bovine serum albumin in PBS for 1 h, the slices were incubated overnight at room temperature. Following a wash process, signals were visualized with the avidin-biotin-peroxidase technique (Elite kit, Vector Laboratories), in which 3% hydrogen peroxide was used as chromogen.

**RT-PCR**—Adult rats were killed by decapitation, and their cerebral cortex, hippocampus, and cerebellar cortex were rapidly dissected and frozen on dry ice. The total RNA from each above region was extracted using RNA Isolator (Genosys). Following removal of genomic DNA with DNase treatment, the RNA samples were subjected to a reverse transcription reaction to synthesize single strand cDNAs using the first strand cDNA synthesis mix (Novagen). Amplification of a 372-base pair IR cDNA fragment was performed on a DNA thermal cycler 480 (Perkin-Elmer) through a 25-cycle PCR (94 °C 1 min, 50 °C 1 min, and 74 °C 2 s) with primers 5′-CTCGTGTCCAGCCCTGGAAA-3′ (forward) and 5′-CCACACACGAGCCTCACT-3′ (reverse). To control for any variability in sample processing, primers (forward, 5′-AGGTGCTCAAACATG-3′; reverse, 5′-TACCAAGGGGCCATGACT-3′) synthesizing a 183-base pair rat phosphoglycerate kinase (PGK) cDNA fragment were included in the PCR reaction.

**Preparation of Homogenate, S3, and P2M Fractions**—The general cytosolic and crude synaptic membrane fractions were prepared by centrifugation according to the procedure of Rostas et al. (36) with minor modifications. Briefly, the frozen tissues from different brain regions were homogenized respectively in precooled Buffer A (0.25 m sucrose, 20 m Tris-HCl, pH 7.4, 1 mM EDTA, 2 mM PMSF, 1% Triton X-100, 0.1 m mercaptoethanol, 1000 mCi/mmol, NEN Life Science Products). The homogenate was diluted to 1:400. In control slices, the primary antibody was omitted. Slices were washed with PBS, followed by incubation with biotinylated anti-rabbit IgG diluted to 1:400 for 1 h at room temperature. Following a wash process, signals were visualized with the avidin-biotin-peroxidase technique (Elite kit, Vector Laboratories), in which 3% hydrogen peroxide was used as chromogen.

**Preparation of Brain Sections**—Brains from naive, trained, and swimming control rats were sectioned at 12 μm in a cryostat at −20 °C. Sections were collected on silanated glass slides (Digene) and dried at room temperature before being returned to −80 °C for storage. In Situ Hybridization Histochemistry—Brain slices were fixed for 5 min in 4% formaldehyde, acetylated, and dehydrated in graded ethanol. The IR riboprobe (1 × 10⁶ cpm in 50 μl) was applied to each slide for 30 min at 55 °C for 3 h in a mixture containing 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 300 mM NaCl, 50% formamide, 10% dextran sulfate, 1× Denhardt’s solution, 4 μg/ml salmon sperm DNA, 10 μg/ml yeast total RNA, 10 μg/ml yeast tRNA, 100 mM dithiothreitol, 0.1% SDS, 0.1% NTS. Slices hybridized with the sense riboprobe or pretreated with RNase before hybridization with the antisense probe were used as negative controls. Following high stringency posthybridization washes and RNase treatment, brain sections were dehydrated in graded ethanol and then subjected to film and liquid emulsion autoradiography.
lected as cell lysates. Protein concentrations were assessed using the BCA reagent (Pierce).

Immunoblotting and Immunoprecipitation Processes—Proteins from each subcellular fraction were separated by 4–20% gradient SDS-PAGE. The resolved proteins were transferred to a 0.45 μm nitrocellulose membrane. Blocked with 5% milk powder in 0.01 M PBS, pH 7.5, the membrane was incubated with a given primary antibody (such as antibody against IR, Sch, Grb-2, MAPK, or phosphotyrosine) at 4 °C overnight with constant shaking. On the following day, the membrane was washed and incubated with a secondary antibody conjugated with horseradish peroxidase at room temperature for 1 h. The immunoreactive signal was then revealed with the enhanced chemiluminescence reagent (Pierce). If the protein of interest was expected to lose membrane. Blocked with 5% milk powder in 0.01 M PBS, pH 7.5, the membrane was incubated with a given primary antibody (such as antibody against IR, Sch, Grb-2, MAPK, or phosphotyrosine) at 4 °C overnight with constant shaking. On the following day, the membrane was washed and incubated with a secondary antibody conjugated with horseradish peroxidase at room temperature for 1 h. The immunoreactive signal was then revealed with the enhanced chemiluminescence process. Alternatively, a target protein was immunoprecipitated from subcellular fractions with its specific antibody and then detected with immunoblotting procedures. If the protein of interest was expected to have a molecular mass similar to that of the heavy chain of IgG (around 55 kDa), the primary antibody was covalently cross-linked to agarose beads in a cross-link reaction using the CarboLink™ kit (Pierce). This process retained IgG molecules on agarose beads during sample denaturing, thereby preventing the target protein signal(s) from being masked by IgG in immunoblotting.

In Vitro Tyrosine Phosphorylation of Hippocampal Proteins—In vitro phosphorylation of hippocampal S3 and P2M proteins was carried out in a total volume of 50 μl of reaction mixture containing 50 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 2 mM sodium vanadate, 2 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/μl leupeptin, ± 1 mM CaCl₂ ± 0.1 μM insulin, and 50 μg of S2 or P2M proteins. Although Mn³⁺ has been known to be a potent effector for in vitro tyrosine phosphorylation, it was eliminated from the present reactions, as it reacted with sodium vanadate to form yellow precipitates. The phosphorylation reaction was initiated by addition of ATP to a final concentration of 100 μM. After incubating at 37 °C for 3 min, the reaction was terminated by adding 50 μl of SDS reducing sample buffer. The mixture was boiled for 10 min followed by separation by a 4–20% gradient SDS-PAGE. The extent of tyrosine phosphorylation was detected in Immunoblotting using a monoclonal anti-phosphotyrosine antibody (PY20, Upstate).

Data Analysis—All biochemical experiments were repeated at least three times for each pool of hippocampal tissues. In situ hybridization histochemistry images and signals from immunoblotting were analyzed by measuring the optic mean density using the NIH Image program. Values from the swimming controls and trained animals were normalized against values from naive animals. Data from three pooled samples were subjected to one- or two-way analysis of variance.

RESULTS

Distribution of IR mRNA and Protein in the Rat Brain—Consistent with previous findings, our in situ hybridization histochemistry results showed that IR mRNA is widely expressed in the rat brain (Fig. 1A). The highest density of IR mRNA signals was revealed in the cerebellar cortex and choroid plexus of the third and lateral ventricles. Concentrated IR mRNA signals were also detected in forebrain areas, such as anterior olfactory nucleus, pyriform cortex, thalamic and hypothalamic nuclei, hippocampal formation, amygdaloid nucleus, and the cerebral cortex. IR mRNA levels in the hippocampus, cerebral, and cerebellar cortex were also measured using RT-PCR, which again revealed highest levels of IR PCR product in the cerebellum (Fig. 2A), indicating a high concentration of IR mRNA in this area.

When IR protein levels in the rat brain were measured with immunoblotting and immunohistochemistry, the polyclonal anti-IR-β antibody detected a 95-kDa protein band from subcellular preparations of the rat brain that migrated slightly faster on SDS gel than the human IR transfected in NIH 3T3 cells.
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Variability showed significant training (F(1,8) = 14.04, p = 0.006), but not time effects. Similarly, 30–40% increases were found in dentate gyrus after training (F(1,8) = 14.51, p = 0.005), whereas no significant changes were found in CA3 region.

Changes in IR protein levels after training were determined with immunoblotting (Fig. 4C). No significant changes in IR protein were found in the total homogenate fraction (Fig. 4C, a). A decrease (19%) in IR immunoreactivity in the S3 fraction was apparent 1 h after training (F(1,8) = 18.55, p = 0.0026, Fig. 4C, b). On the other hand, a significant increase in IR protein was detected in the P2M fraction at 1 and 24 h after training (F(1,8) = 11.36, p < 0.01, Fig. 4C, c). Similar results were also obtained following immunoprecipitation of IR from P2M fractions from each group of animals (data not shown).

Fig. 4D illustrates immunocytochemical changes in IR protein in hippocampal CA1 pyramidal cells after training. The brown staining indicates immunoreactivity of IR in the CA1 neurons. For swimming controls, the majority of cells (~80%) in the CA1 area showed moderate IR immunostaining, with a distribution pattern similar to that of naive animals (see Fig. 3). A small population of cells (<20%) showed weak staining. After training, however, an increase in IR immunostaining (dark brown) was observed in some of these cells. Table I shows a semiquantitative analysis of training-induced IR immunoreactivity changes. Although the proportion of the cells with weak staining was not changed, 30–38% of the CA1 neurons showed strong immunostaining after training, compared with 3–5% in the swimming controls. In addition, IR immunostaining appeared to be clustered within specific intracellular compartments after training, compared with an even distribution in the cytoplasm of the cell in swimming controls. Increased staining was also seen in dendritic areas.

Changes in Tyrosine Phosphorylation of Hippocampal Proteins—To investigate a possible involvement of the IR tyrosine protein kinase activity and its downstream signal cascades in learning and memory, we first measured changes in overall tyrosine phosphorylation of the hippocampal proteins. Protein concentrations of the tissue preparations across each group were equalized to 1 mg/ml, of which 15 µl was resolved on SDS-PAGE. Tyr phosphorylation was measured in immunoblots with a monoclonal antibody against phosphotyrosine (Py20). As shown in Fig. 5, more than 10 Tyr-phosphorylated major protein bands were revealed in hippocampal homogenates. One of these bands (P60) showed a decrease in phosphorylation at 1 and 24 h after training, whereas increasing in phosphorylation in the swimming controls. A two-way analysis of variance indicated significant training (F(1,8) = 37.57, p < 0.001) effects. In the S3 fraction, dephosphorylation of P60 also occurred at 1 h after training (F(1,8) = 34.5, p < 0.001). A significant increase in Tyr phosphorylation was found in a P36 band in trained animals at both 1 and 24 h after training (F(1,8) = 28.5, p < 0.001). In the P2M fraction, the most obvious training-specific changes occurred to the P66 and P52 bands, both of which showed significant increases in Tyr phosphorylation at 1 and 24 h after training (P66: F(1,8) = 10.55, p = 0.01; P52: F(1,8) = 16.6, p < 0.01). In addition, several other P2M proteins showed marked increases in Tyr phosphorylation at 24 h after training. These included P120, P95, P75, P32, P25, and P10. The higher intensity of those protein bands was not due to greater amounts of protein loaded on the gel, as protein staining after electrophoresis showed similar amount of proteins across each lane (data not shown).

In Vitro Phosphorylation of IR and Its Changes after Training—Because the above immunoblotting results indicated a low basal level of IR Tyr phosphorylation, we then examined in vitro regulation of IR Tyr phosphorylation in hippocampal P2M...
fractions in the absence and presence of Ca2+. In the presence of ATP, Mg2+, and Na3VO4, a 95-kDa band was heavily phosphorylated (Fig. 6a, 3). This band co-migrated on SDS-PAGE with the human IR (Fig. 6a, lane 5) and IGF-1R (Fig. 6a, 1). The presence of Ca2+ (1 mM) in the reaction markedly reduced its Tyr phosphorylation (Fig. 6a, lane 4). To distinguish IR and IGF-1R, both of which migrated to a similar position on the SDS gel, immunoprecipitation was carried out with anti-Tyr(P) antibody following in vitro phosphorylation reactions, and the precipitates were detected by immunoblotting with anti-IR (Fig. 6b) and IGF-1R (Fig. 6c) antibodies. There was little cross-reaction between IR antibody and IGF-1R (Fig. 6b, lane 1), or between IGF-1R antibody and IR (Fig. 6c, lane 5), indicating that these antibodies were target-specific. A major IR-β protein was detected from the immunoprecipitates after phosphorylation (Fig. 6b, lane 3). The amount of precipitated IR-β was markedly reduced when Ca2+ was added to the phosphorylation reaction (Fig. 6b, lane 3). The phosphorylated 95-kDa band was found to also include IGF-1R (Fig. 6c, lane 3), the phosphorylation of which, however, was not affected by Ca2+ (Fig. 6c, lane 4). Results from lane 2 in all three panels of Fig. 6 indicated that IR and IGF-1R were predominantly in a de-phosphorylated state in untreated synaptic membrane fractions.

We next measured whether in vitro phosphorylation of IR was changed as a function of training, because it may reflect alterations of the properties and sensitivity of the receptor to its ligand during memory processing. A significant increase of in vitro Tyr phosphorylation was found in P180 (F1,8 = 12.04, p < 0.01) but not P95 after training (Fig. 7a1). The presence of insulin in the reaction stimulated the overall phosphorylation of P180, with significantly stronger signals (F1,8 = 29.12, p < 0.001) detected in the trained animals (Fig. 7a2). Insulin treatment did not change Tyr phosphorylation of P95 from naive and swimming control rats, but it significantly enhanced that from trained animals (F1,8 = 17.48, p < 0.001). In the presence of Ca2+, the overall phosphorylation of P180 was markedly increased (Fig. 7b1), but no significant differences were detected among the trained and control rats. The overall Tyr phosphorylation of P95, on the other hand, was markedly reduced in the presence of Ca2+. Samples from the swimming controls showed particularly weaker phosphorylation signals (Fig. 7b1) compared with that from other groups (F1,8 = 5.3, p = 0.05). When Ca2+ and insulin were both present in the reaction (Fig. 7b2), increases in phosphorylation of P180 (F1,8 = 20.11, p = 0.002) and P95 (F1,8 = 18.37, p = 0.003) were shown only in trained animals. In addition, phosphorylation of P66 was also increased after training (Fig. 7, b1 and b2). These results suggest that sensitivities of P180 and P95 to insulin were increased after water maze training.

Changes in Shc Protein and Its Interaction with IR—To identify the 66- and 52-kDa proteins in P2M fractions that showed increased Tyr phosphorylation after training (Fig. 5, P2M), freshly prepared S3 and P2M samples from trained and control rats were separated on SDS gels and immunoblotted with anti-Shc antibody. This antibody detected Shc-66 and -52, localized mainly in the cytosolic fractions of the hippocampus. Although no apparent change was seen in the S3 fraction, significant increases in amounts of Shc-66 (F1,8 = 31.8, p < 0.001) and Shc-52 (F1,8 = 14.42, p = 0.005) were found in the P2M fractions from trained animals (Fig. 8A).

A immunoprecipitation experiment was performed to determine possible in vivo interactions between IR and Shc. Equal amounts of P2M proteins from each group were subjected to immunoprecipitation with a goat anti-IR-β antibody cross-linked to agarose beads. The immunoprecipitate was then blotted with anti-Shc antibody following SDS-PAGE and transfer processes. Shc-66 was co-precipitated with IR by the IR-β antibody from trained animals (Fig. 8B). Similarly, P2M proteins were immunoprecipitated with anti-Shc antibody followed by immunoblotting with anti-IR-β antibody. A strong immunoreactive band was only detected by IR-β antibody in samples from trained animals (Fig. 8B). The co-immunoprecipitation of Shc with IR suggests that these two proteins may be associated in vivo after training.

Changes in Grb-2 Protein after Training—Because Grb-2
protein is known to be involved in IR-Shc signaling, its change in learning was assessed in the S3 and P2M fractions with immunoblotting. Like the Shc proteins, Grb-2 is largely a cytosolic localized protein (Fig. 8C). Although no changes were seen in S3 fraction, a significant training-induced increase in Grb-2 immunoreactivity was shown in the P2M fraction (Fig. 8C).

Changes in MAPK after Training—Finally, we measured the training-induced changes in MAPK, a protein known to be a downstream molecule to the IR/Shc/Grb-2/SOS cascade (16–19). No apparent changes in the total amount of MAPK were detected in the S3 and P2M fractions by the Regular-MAPK
A significant increase, however, in the active form of MAPK (P-MAPK) was observed in the S3 fraction at 1 h after training ($F_{1,8} = 15.32, p < 0.01$) and in the P2M at both 1 and 24 h after training ($F_{1,8} = 42.25, p < 0.001$), whereas the P2M from swimming controls showed a decreased MAPK phosphorylation (Fig. 9B). When in vitro phosphorylation was performed, the overall P-MAPK signal in the S3 was elevated (Fig. 9C, left panel) and further enhanced by addition of insulin to the reaction (Fig. 9D, left panel). In the P2M, insulin treatment during in vitro phosphorylation only increased the P-MAPK signal in the samples from trained animals (Fig. 9D, right panel).

**DISCUSSION**

The present findings from in situ hybridization, immunohistochemistry, and immunoblotting experiments confirm that IR is abundantly distributed in the brain. Specific regional concentrations of IR may reflect different IR functions associated with particular brain regions. An abundance of IR, for example, in areas such as olfactory bulb and thalamic nuclei is consistent with its involvement in regulation of food intake. High concentrations of IR in the hypothalamus and limbic system including the hippocampus, pyriform cortex and amygdala areas that reciprocally connect and communicate among each other, suggests its role in emotion and higher cognitive functions, particularly learning and memory. The very high density of IR in the choroid plexus suggests that it may be required for transport of glucose and peripheral insulin across the blood-brain barrier.

Although levels of IR protein match that of its mRNA in the forebrain areas, a striking disassociation between IR mRNA and protein levels was revealed in the cerebellar cortex. These results suggest differences in IR mRNA translational efficiency and/or IR protein stability in different brain regions. For example, IR in the cerebral cortex and hippocampus may possess a high efficiency of translation, or enhanced stability, whereas in the cerebellar cortex very high levels of IR mRNA may be required to supply a rapid turnover of the receptor. We believe that the distribution patterns of IR mRNA and protein reflect
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the significance of IR functions in different brain regions.

The hippocampus, which contains high levels of IR, is critically involved in spatial memory processing. IR mRNA was clearly up-regulated in the hippocampal CA1 and dentate gyrus areas shortly following water maze training, suggesting that synthesis of IR may be increased in these areas as a result of learning. An increased IR in the P2M was accompanied by a reduction of IR in the S3, suggesting a possible translocation of IR to the synaptic membrane after training. Changes in the distribution pattern of IR were also observed in the immunohistochemical studies, in which IR became more concentrated in certain cellular compartments such as the nucleus and dendrites of the CA1 neurons after training. It is of interest that this change is only seen in a subpopulation of the pyramidal cells in the CA1 area, suggesting that only in specific neurons was IR activated by training. This subpopulation specificity of learning-induced changes of IR are consistent with numerous previously published biophysical and biochemical studies. Voltage-dependent K⁺ currents, for example, were found in single identified neurons of the mollusk Hermissenda after Pavlovian conditioning (39). Related changes of K⁺ currents were found in a subpopulation of CA1 pyramidal cells (40), and a subpopulation of H6 cerebellar cortical Purkinje neurons (41) after Pavlovian conditioning of the rabbit eyelid response. Translocation of PKC was demonstrated in a subpopulation of cells with these same paradigms within these same regions, as well as with rat spatial learning (42, 43). Finally, in a very recent study, a subset of CA1 pyramidal cells was found to have reduced inhibitory postsynaptic potentials only after spatial maze learning (44). These findings, together with those of other reports that changes in expression of neuronal cell adhesion molecule (45) are found in a subset of hippocampal neurons after learning, support the interpretation that learning of a particular task involves only a subpopulation of neurons within a relevant region such as the hippocampus.

Given that IR is a receptor tyrosine kinase, autophosphorylation of which is essential for its activation, changes in autophosphorylation of IR after learning would be expected if the receptor is actively involved in memory formation. Although there was a low level of in vitro phosphorylation of IR, an insulin-stimulated in vitro phosphorylation of IR was detected in the synaptic membrane fraction only from trained animals. Although these results did not necessarily reflect the in vivo phosphorylation status of IR under trained or swimming control conditions, they provide evidence that the molecular properties of some component of the IR signaling pathway may be altered by training. Interestingly, the in vitro phosphorylation of IR by not IGF-1R was markedly inhibited by presence of 1 mM Ca²⁺ in the reaction. It is unclear how changes in intracellular Ca²⁺ due to increased synaptic activity during learning might influence the IR phosphorylation. The complexity of both the relevant pre- and postsynaptic biochemical cascades, however, precludes a straightforward interpretation at this time, but it will certainly motivate intensive follow-up studies.

The activated IR signaling cascade after training appeared to involve She protein. Levels of shc-66 and -52 were increased, and both proteins were significantly phosphorylated in vivo in the P2M only in trained animals. Co-immunoprecipitation of She with IR from trained animals suggests that activation of She during learning may be associated with IR PTK activity. Because Tyr phosphorylation of She leads to its specific association with the Grb-2-SOS complex, our detection of a training-induced accumulation of Grb-2 in the P2M fraction suggests that such an event may occur in water maze training. Finally,
a training-specific activation of MAPK was also detected. Activation of MAPK was also previously reported following increases in intracellular Ca\(^{2+}\) (46) and retrieval of spatial memory (47). Although no evidence, either from previous work or the present study, directly identifies the upstream events associated with activation of MAPK, our results showed that the training-induced MAPK phosphorylation was further enhanced by \textit{in vitro} insulin treatment of the P2M fraction, suggesting an increased sensitivity of this signaling pathway to insulin. Although it is tempting to speculate that during learning IR may have triggered a Ras/MAPK cascade mediated by Shc and Grb-2, further studies are needed to identify the precise links among changes in IR, Shc/Grb-2, and MAPK during spatial memory formation.

Apart from Shc, a 180-kDa P2M protein, which co-migrated with the insulin receptor substrate-1 (data not shown), also showed an increased insulin-sensitive \textit{in vitro} Tyr phosphorylation after training. More detailed studies of this protein following training are under investigation.

Taken together, our results reveal, for the first time, that spatial training induces a series of changes in IR of the hippocampus, including IR gene expression, protein translocation, and Tyr phosphorylation. Because most of these changes were detected in synaptic membrane fractions, IR may play a role in regulation of synaptic activities (such as neurotransmission and/or synaptic plasticity) during memory formation.

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Brain Insulin Receptors and Spatial Memory: CORRELATED CHANGES IN GENE EXPRESSION, TYROSINE PHOSPHORYLATION, AND SIGNALING MOLECULES IN THE HIPPOCAMPUS OF WATER MAZE TRAINED RATS
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