SDF1α/CXCR4 Signaling, via ERKs and the Transcription Factor Egr1, Induces Expression of a 67-kDa Form of Glutamic Acid Decarboxylase in Embryonic Hippocampal Neurons*

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Stromal cell-derived factor α (SDF1α) and its cognate receptor CXCR4 play an important role in neuronal development in the hippocampus, but the genes directly regulated by SDF1α/CXCR4 signaling are unknown. To study the role of CXCR4 targeted genes in neuronal development, we used neuronal cultures established from embryonic day 18 rats. Hippocampal neurons express CXCR4 receptor proteins and are stimulated by SDF1α resulting in activation of extracellular signal-regulated kinase (ERK)1/2 and the transcription factor CAMP-response element-binding protein. SDF1α rapidly induces the expression of the early growth response gene Egr1, a transcription factor involved in activity-dependent neuronal responses, in a concentration-dependent manner. Gel-shift analysis showed that SDF1α enhances DNA binding activity to the Egr1-containing promoter for GAD67. Chromatin immunoprecipitation analysis using an Egr1 antibody indicated that SDF1α stimulation increases binding of Egr1 to a GAD67 promoter DNA sequence. SDF1α stimulation increases the expression of GAD67 at both the mRNA and protein levels, and increases the amount and neurite localization of γ-aminobutyric acid (GABA) in neurons already expressing GABA. SDF1α-induced Egr1/GAD67 expression is mediated by the G protein-coupled CXCR4 receptor and activation of the ERK pathway. Reduction of Egr1 gene expression using small interfering RNA technology lowers the level of GAD67 transcripts and inhibits SDF1α-induced GABA production. Inhibition of CXCR4 activation in the developing mouse brain in utero greatly reduced Egr1 and GAD67 mRNA levels and GAD67 protein levels, suggesting a pivotal role for CXCR4 signaling in the development of GABAergic neurons in vivo. Our data suggest that SDF1α/CXCR4/G protein/ERK signaling induces the expression of the GAD67 system via Egr1 activation, a mechanism that may promote the maturation of GABAergic neurons during development.

Chemokines are a family of small secreted proteins with diverse immune and neural functions, including control of leukocyte trafficking, organization of the hematopoietic/lymphopoietic system, and angiogenesis (1). Stromal cell-derived factor (SDF1α) belongs to the CXC subfamily and is a ligand for the cell surface, G protein-coupled receptor CXCR4 (2, 3). Binding of SDF1α to CXCR4 activates multiple signaling pathways, including extracellular signal-regulated kinase (ERK), cAMP/cAMP-dependent protein kinase, and phospholipase Cβ, resulting in an increase in intracellular calcium levels (4). In addition, CXCR4 has been shown to serve as a co-receptor-binding site for the HIV-1 virus (5).

Increasing evidence suggests that SDF1α/CXCR4 signaling plays important roles in brain development. CXCR4 mutant mice and SDF1α null mice exhibit similar phenotypes that include severe abnormalities in organogenesis, defects on neuronal precursor migration in the cerebellum, an abnormal developmental of the dentate gyrus, and defects in interneuron migration in the cortex (6–9). It has also been shown that CXCR4 is required for the development of retina, pallium, motor axons (10), and sensory neurons (11), as well as for migration of oligodendrocyte precursors (12). Effects of SDF1α/CXCR4 signaling on neuronal precursors and differentiating neurons include enhanced proliferation and survival (13), chemotraction, and regulation of axonal morphology and pathfinding (14, 15). Despite the importance of SDF1α/CXCR4 signaling in neuronal development, the genes targeted by CXCR4 signaling in developing neural cells are largely unknown.

γ-Aminobutyric acid (GABA), the major inhibitory neurotransmitter in the adult nervous system, also plays important roles in central nervous system development by regulating neurogenesis and synaptogenesis (16–18). In contrast to its inhibitory actions on adult neurons, GABA is capable of depolarizing neuronal progenitor cells and immature neurons (16, 19). GABA participates in formation of a primitive network-driven pattern of electrical activity called the giant depolarizing potentials (GDPs). This electrical circuit pattern is critical to generate large oscillations of intracellular calcium for activity-dependent modulation of neuronal growth and synapse formation (19). This developmental function of GABA is in part regulated by

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2 The abbreviations used are: SDF1α, stromal cell-derived factor α; ERK, extracellular signal-regulated kinase; GABA, γ-aminobutyric acid; CREB, cAMP-response element-binding protein; PTX, pertussis toxin; DIV, day in vitro; EMISA, electrophoretic mobility shift assay; RT, reverse transcription; PBS, phosphate-buffered saline; GDP, giant depolarizing potential; ChIP, chromatin immunoprecipitation; GAD, glutamic acid decarboxylase; CREB, cAMP-response element; siRNA, small interfering RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
GABA production, a process mediated by glutamic acid decarboxylases (GADs), the key rate-limiting enzymes for synthesis of GABA. Two GAD isoforms, GAD65 and GAD67, are expressed in the adult nervous system (20). An additional two alternatively spliced transcripts, l-80 and l-86, are generated from the GAD67 gene during embryonic development in rodents (21, 22). The expression sequence of GADs in the developing mouse brain is as follows: fetal GAD67, adult GAD67, and adult GAD65 (17, 22). Recently, it was reported that the HIV gp120 protein can promote GDPs through its co-receptor CXCR4 in the developing hippocampus. SDF1α also mimics the effects of gp120 on GDPs (23). However, association between SDF1α/CXCR4 signaling and GAD/GABA is not clear.

We previously reported that SDF1α/CXCR4 signaling is up-regulated as neuronal stem cells from embryonic spinal cord differentiate into more restricted precursors (24). In further characterizing SDF1α/CXCR4 signaling in neuronal precursors from embryonic spinal cord, we found that SDF1α activates the ERK pathway, cross-talks with Wnt signaling, and regulates early growth response gene 1 (Egr1) expression using both microarray and RT-PCR analysis (25, 26). Egr1 (also known as zif268, Krox-24, and NGF1-A) is a zinc finger–containing transcription factor that belongs to the category of immediate early genes (27). Egr1 is known to regulate genes associated with synaptic plasticity/memory by binding to specific Egr-response elements (ERE)s in genomic DNA sequences (28). Because the promoter of GAD67 contains an ERE consensus binding site (29, 30), we tested the hypothesis that SDF1α/CXCR4 signaling regulates the GAD67/GABA system via an Egr1-mediated mechanism in developing hippocampal neurons.

**EXPERIMENTAL PROCEDURES**

**Primary Rat Hippocampal Neuronal Cultures and Experimental Treatment**—Cultures of dissociated hippocampal neurons were prepared from embryonic day 18 (E18) Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) using methods described previously (31). Briefly, hippocampal cells were mechanically dissociated by trituration following 0.05% trypsin/EDTA treatment (Invitrogen) and were seeded into polyethyleneimine-coated dishes at a density of 20,000 cells/tray. The cultures were maintained at 37 °C in a humidified atmosphere (6% CO2, 94% room air). Growth medium consisted of Eagle's minimum essential medium (Invitrogen) in a humidified atmosphere (6% CO2, 94% room air). After a 4–6-h period to allow attachment to the substrate, the culture medium was replaced with Neurobasal medium containing B27 supplements (Invitrogen) in a humidified atmosphere (6% CO2, 94% room air) at 37 °C. All experiments were performed on cells that had been in culture for 2–8 days. To measure ERK and CREB activity, the cell cultures were stimulated with SDF1α (1–100 nM; PeproTech) for 5–60 min. To block activation of CXCR4 receptors, some cultures were pretreated with either 6 μM AMD3100, a specific CXCR4 antagonist, for 30 min or 0.01 μg/ml of Bordetella pertussis toxin V (PTX; Sigma) for 17 h and then treated with SDF1α. Preliminary tests showed that either AMD3100 or PTX alone at this concentration had no toxicity. To block activation of ERK enzymes, the cells were pretreated with 10 μM PD98059 or vehicle (0.1% dimethyl sulfoxide) for 0.5 h before SDF1α stimulation. In all time course studies, the end point time for cell harvesting was the same to ensure that the developmental stages on all dishes, including controls and treatments, were similar.

**Lysate Preparation and Immunoblot Analysis**—After treatment, the cells were washed with ice-cold PBS and lysed by adding 100 μl of ice-cold lysis buffer containing 25 mM Heps, pH 7.5, 300 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.5 mM diithiothreitol, 100 μg/ml phenylmethylsulfonyl fluoride, and 2 μg/ml leupeptin, followed by sonication for 10 s on ice. The cellular extracts were then centrifuged for 15 min at 12,000 rpm to remove debris. The supernatant was collected, aliquoted, and stored at −20 °C. Protein concentration was determined using a BCA protein assay kit (Pierce). For immunoblot analysis, equal amounts of supernatant protein (20 μg/lane) were run on 4–12% SDS-PAGE and electrophoretically transferred to PVDF membrane (Invitrogen). The membrane blots were first blocked with 5% nonfat dry milk in PBST buffer (10 mM sodium phosphate, 500 mM NaCl, and 0.01% Tween 20, pH 7.4) and then incubated overnight with different primary antibodies in PBST containing 1% BSA at 4 °C. The primary antibodies included the following: p-ERK1/2 (1:1000; Cell Signaling), p-CREB (1:1000; Upstate), CXCR4 (1:1000; Torrey Pines Biolabs, Inc.), GAD67 (1:1000; Chemicon), and Egr1 and β-actin (both at 1:1000; Santa Cruz Biotechnology). Immunoreactivity was detected by sequential incubation with horseradish peroxidase-conjugated secondary antibody (1:10,000; Jackson ImmunoResearch) and SuperSignal West Femto maximum sensitivity substrate (Pierce) following image acquisition using ChemiFluor<sup>TM</sup> 8900 (Alpha Innotech Corp.). To evaluate protein loading in the gel, the bound antibodies were stripped off by incubation in BlotFresh<sup>TM</sup> Western blot stripping reagent (version II) (SignaGen<sup>®</sup>Laboratories) for 10 min at room temperature, and the same membranes were then immunoblotted with polyclonal β-actin antibody.

**Histology and Immunocytochemistry**—Brains were fixed in 3% paraformaldehyde plus 1% glutaraldehyde in PBS (pH 7.4) overnight. Brains were cryoprotected by immersion first in 20% sucrose for 24 h and then in 30% sucrose for 24 h. The samples were embedded in Tissue-Tek (Sakura, Torrance, CA), and cryostat sections were cut in the coronal plane at a thickness of 7 μm. For immunohistochemical staining, the sections were first incubated with blocking solution (5% normal goat serum and 0.1% Triton X-100 in PBS, pH 7.4) for 1 h and then with anti-GAD67 (1:200; Chemicon) in blocking solution overnight at room temperature. After washing with PBS (0.1% Triton X-100 in PBS, pH 7.4), the sections were incubated with Alexa Fluor<sup>®</sup>488-conjugated secondary antibodies (1:200; Invitrogen) at room temperature for 1 h. Staining procedures for cells were similar to those described previously (32). Briefly, the cells were fixed using 3% paraformaldehyde plus 1% glutaraldehyde in PBS for 30 min. The fixed cells were then incubated with...
blocking buffer containing 5% normal goat serum, 0.1% Triton X-100, and 1% BSA in PBS, pH 7.4, for 1 h, and then incubated with primary antibodies in blocking buffer overnight at 4 °C followed by incubation with Alexa Fluor®488-conjugated secondary antibodies (1:500; Invitrogen) at room temperature for 0.5 h. The sources and dilutions of primary antibodies were as follows: anti-GABA (1:1000; Chemicon), anti-MAP2 (1:1000; Sigma), and anti-glial fibrillary acidic protein (1:1000; Cell Signaling Technology®). Stained tissues and cultured cells were examined under a fluorescence microscope, and images were acquired using an Olympus microscope with a digital camera attachment. Composites were prepared using Adobe Photoshop.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared using a nuclear extraction kit (Panomics). EMSA was performed using a commercially available kit (Panomics). The GAD67 promoter (TCCCCGCTTCCCCACTCCGCCCCGGCTTCCTCCCCAACGCC) contains an Egr1 consensus site (CGCCCCCGCC) and an Sp1 consensus site (CCCCGGCC) (33). The DNA duplex of GAD67 promoter oligonucleotides was synthesized by Integrated DNA Technologies, Inc., and labeled using a biotin 3’ DNA end-labeling kit (Pierce). For the EMSA, a biotin-labeled GAD67 promoter oligonucleotide was synthesized by Integrated DNA Technologies, Inc., and labeled using a biotin 3’ DNA end-labeling kit (Pierce). For the EMSA, a biotin-labeled specific consensus DNA sequence was incubated with 4 μg of nuclear extract protein for 30 min at 20 °C. A specific binding band was determined by adding excess unlabeled specific double strand DNA. The biotin-transcription factor-bound DNA complexes were separated from free probes in a 6% polyacrylamide gel and transferred to a Biodyne B® membrane. The biotin-labeled DNA complex was then detected by its reaction with streptavidin-horseradish peroxidase conjugate and its substrate, and images of the gels were acquired using ChemiFlure™ 8900 (Alpha Innotech Corp.).

RNA Preparation and Reverse Transcriptase PCR Amplification—Total RNAs from rat E18 hippocampal cultures were isolated using RNA STAT-60™ (Tel-Test, Inc). The cDNAs were synthesized using 1 μg of total RNA in the presence of Superscript II and (d1)12–18 (both from Invitrogen). The PCR was performed in a 20-μl reaction solution containing 2 μl of 10× PCR buffer, 150 nmol of MgCl2, 10 nmol of dNTP, 20 pmol of primer, 1 μl of 10× diluted cDNA, and 1 unit of RedTag DNA polymerase (Sigma). Primer sequences for Egr1, CXCR4, and GAPDH were described previously (25). The PCR cycle parameters were as follows: 35 cycles of 94 °C for 20 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension for 7 min at 72 °C. PCR products were separated by agarose gel electrophoresis.

RNA Interference—A chemically synthesized siGENOME ON-TARGET plus SMARTpool duplex for rat Egr1 (GenBank™ accession number NM_012551) was obtained from Dharmaco, Inc. (Chicago, IL.). The pool contains four RNA duplexes, and their antisense sequences are as follows: 5’-PAAUUAAGCGCAUGCAGAUUGU; 5’-PAUACCUGAGAGCGCUGCUUUU; 5’-PAUACCUGAGAGCGCUGCUUUU; 5’-PACAGUGACAGAUAUACCU; and 5’-PUAGUGACAGAUAUACCU. Both negative and positive GAPDH siRNA duplexes were from Ambion. Delivery of siRNAs into primary rat E18 hippocampal neurons was accomplished using the protocol described in siPORT™ siRNA electroporation kit (Ambion) using an electroporator Gene Pulser Xcell™ (Bio-Rad). Using fluorescent Cy3 siRNA, a negative control, and GAPDH siRNA, we optimized the conditions for the electroporation protocol as follows: a square pulse with 200 V and a 100-μs pulse in a 1-mm electroporation cuvette. The cell density was 7.5 × 104 in 75 μl of siRNA Electroporation Buffer (Ambion). This electroporation protocol resulted in a transfection efficiency of 74 ± 19% (mean ± S.D.) as determined using a Cy3-labeled siRNA duplex (Ambion) and 90% cell viability as determined by using trypan blue assay. This protocol also knocked down GAPDH expression at both the mRNA and protein levels compared with control cells. At 20 h after transfection with SMARTpool siEgr1 duplex at different concentrations, the cultures were treated with either 10 nm SDF1α or distilled water for 6 h. The cells were then used for extraction of total RNA or protein, and analysis of expression by RT-PCR, immunoblot, and immunocytochemistry as described above.

In Utero AMD3100 Injection—Intraventricular injections were done with approval from the NIA Institutional Animal Care and Use Committee using methods described previously (36). Briefly, pregnant female C57BL/6 mice at E17 were anesthetized with Ketamine and Xylazine and a midline laparotomy was performed exposing uterine horns. The lateral ventricle in the brain of each embryo was visualized with transillumination,
Primary rat E18 hippocampal cultures were maintained for the indicated DIV at which times cells were lysed, and samples of RNA and protein were prepared for RT-PCR and immunoblot analysis, respectively. At these times cells were lysed, and samples of RNA and protein were prepared for RT-PCR and immunoblot analyses, respectively. At which times cells were lysed, and samples of RNA and protein were prepared for RT-PCR and immunoblot analysis, respectively. At these times cells were lysed, and samples of RNA and protein were prepared for RT-PCR and immunoblot analysis, respectively.

FIGURE 1. Cultured hippocampal neurons express functional CXCR4 receptors and respond to SDF1α stimulation by increasing ERK and CREB activities. Primary rat E18 hippocampal cultures were maintained for the indicated DIV at which times cells were lysed, and samples of RNA and protein were prepared for RT-PCR and immunoblot analysis, respectively. A, CXCR4 mRNA is expressed from DIV1 to DIV4. B, CXCR4 protein levels are increased during the first 3 days in culture and are maintained at that level through DIV4 (Fig. 1A). C, application of SDF1α for 5 min induced ERK and CREB activation in a dose-dependent manner as indicated by enhanced phosphorylation of ERK and CREB detected by immunoblot analyses. D, AMD3100, a specific CXCR4 antagonist, inhibited SDF1α-induced ERK and CREB activation in a dose-dependent manner. The cells were pretreated with different concentrations of AMD3100 for 0.5 h and then stimulated with 10 nM SDF1α for 5 min. Activation of ERK and CREB by SDF1α (10 nM, 5 min) were completely inhibited at a concentration of 6 μM AMD3100. It was noticed that AMD3100 itself, at lower concentrations, also increased levels of phosphorylations of ERK and CREB. 

RESULTS

Cultured Hippocampal Neurons Express Functional CXCR4 Receptors—To establish the presence of the SDF1α/CXCR4 signaling pathway in developing hippocampal neurons, we employed hippocampal cell cultures established from embryonic day 18 rats (31). We evaluated the phenotypes of cells in our rat E18 hippocampal cultures at 8 using immunocytochemistry. The results showed that ~95% of cells were MAP2-positive neurons, and the remaining cells were glial fibrillary acidic protein-positive cells with a morphology typical of type I astrocytes. In these cultures, we examined CXCR4 expression at both the mRNA and protein levels at increasing time points during the development of hippocampal neurons in culture. The day when the cultures were prepared was defined as day in vitro (DIV) 0. RT-PCR results showed that CXCR4 mRNA was expressed at DIV1 and continued to be expressed at DIV4, although its levels gradually decreased (Fig. 1A). In contrast to its mRNA levels, CXCR4 protein levels, determined by immunoblot analysis, increased during the first 3 days in culture and were then maintained at that level through DIV4 (Fig. 1B).

We next determined if CXCR4 receptor signaling pathways were functional in the cultured embryonic hippocampal cells. Using immunoblot analyses with a phospho-specific ERK and CREB antibodies, which only recognizes the enzymatically activated form of ERK and CREB, we determined whether the ERK pathway and CREB activations are specifically mediated by CXCR4 receptors. To test if the SDF1α-induced ERK and CREB activations are specifically mediated by CXCR4 receptors, we pretreated the cells with AMD3100, a highly selective CXCR4 antagonist (37), at a series of concentrations as indicated in Fig. 1D, to determine inhibition of the SDF1α-induced functions. AMD3100 indeed blocked activation of ERK and CREB by SDF1α in a concentration-dependent manner (Fig. 1D). AMD3100 at a concentration of 6 μM completely inhibited SDF1α-induced ERK and CREB activation. This is consistent with the reported concentration in which AMD3100
can almost completely inhibit SDF1α/CXCR4 mediated increase of intracellular calcium, block the binding of anti-CXCR4 mAb 12G5 at the cell membrane, and prevent SDF1α-induced internalization of CXCR4 (37). However, it was noted that AMD3100 alone, at concentrations of 0.06 and 0.6 μM, slightly increased phosphorylations of ERK and CREB compared with controls, suggesting that AMD3100 alone at low concentrations may serve as a partial agonist, a possibility consistent with previous findings (38). We thus chose AMD3100 at 6 μM for specific effects of SDF1α in the future experiments. In addition, SDF1α-induced ERK and CREB activation could be completely inhibited by application of PD 98059 (10 μM), a specific inhibitor for ERK pathway, suggesting that the CREB activation was a downstream target of the ERK pathway. Collectively, the data described above demonstrate the presence of functional CXCR4 receptors that could mediate ERK/CREB activation in embryonic hippocampal neurons.

**ERKs Mediate the Induction of Egr1 Expression in Response to CXCR4 Activation**—Egr1 is a zinc finger-containing transcription factor and belongs to the category of immediate early genes (27). It can be activated by a variety of stimuli, including nerve growth factor, exposure of animals to a new environment, and ongoing neuronal plasticity (39, 40). The up-regulation of Egr1, at both the mRNA and protein levels, occurs rapidly in response to activation of the ERK pathway (40, 41). Because the ERK pathway is activated in response to SDF1α, we therefore examined the effect of CXCR4 signaling on Egr1 activation using RT-PCR and immunoblot methods.

Exposure of cells to SDF1α resulted in a rapid increase in Egr1 mRNA levels within 0.5 h and a subsequent maintenance of Egr1 mRNA levels above base line for at least 6 h (Fig. 2A). Immunoblot analysis of nuclear extracts showed that SDF1α increases levels of Egr1 protein with a time course pattern similar to that of the Egr1 mRNA (Fig. 2B). The SDF1α-induced increase in nuclear Egr1 activity was dose-dependent at concentrations from 0.01 to 10,000 pM (Fig. 2C). To investigate if the G protein-coupled CXCR4 receptor mediated SDF1α-induced ERK activation and Egr1 expression, cells were pretreated with AMD3100 (6 μM, CXCR4-antagonist) or PTX (0.01 μg/ml; an inhibitor of PTX-sensitive G proteins), or PD98059 (10 μM, a specific inhibitor of the ERK pathway) and were then treated with 10 nM SDF1α for 0.5 h. Levels of Egr1 mRNA and protein levels were measured by RT-PCR and immunoblot analysis, respectively. SDF1α-induced Egr1 expression was inhibited by AMD3100, PTX, and by PD98059 (Fig. 2, D and E). Thus, SDF1α induces Egr1 expression via a PTX-sensitive G protein-coupled CXCR4 receptor and activation of the ERK pathway.

**SDF1α/CXCR4 Signaling Enhances GAD67 Promoter Binding Activity**—It is known that Egr1 regulates its target genes through binding to specific Egr-response elements (EREs) in genomic DNA sequences (28). In developing neurons, several targets whose promoters contain EREs have been identified, including synapsin I and II (42, 43), and a 67-kDa isoform of glutamic acid decarboxylase (GAD) (29, 30). GAD is an enzyme essential for the synthesis of the neurotransmitter γ-aminobutyric acid (GABA) from the substrate glutamate (44). In the developing immature brain, GABA is excitatory and plays...
important roles in neuronal maturation and synapse formation. These functions include depolarizing neuronal progenitor cells and inhibiting their proliferation (16), promoting neuronal fate specification and differentiation by enhancing NeuroD expression (16), regulating neuronal migration (45), and participating formation of a primitive network-driven pattern of electrical activity called the GDPs, a process that may be critical for synapse formation (19). We hypothesized that SDF1α-induced Egr1 activity may promote ERE binding in GAD promoters. We therefore designed a probe that contains Egr1 and Sp1 consensus binding sites from the 5’-flanking region of the rat GAD67 gene (33) and labeled this probe using a biotin 3’ end DNA labeling kit. We then examined the effects of SDF1α on GAD67 promoter binding using an EMSA.

As expected, SDF1α rapidly stimulated GAD67 promoter binding activity within 5 min and maintained its activity for at least for 60 min (Fig. 3A). The EMSA analysis revealed multiple bands that may represent different transcription factor complexes binding to the DNA, because the probe contained both Egr1- and Sp1-binding sites. The results of an experiment in which cells were exposed for 15 min to increasing concentrations of SDF1α revealed that SDF1α induces GAD67 activity in a concentration-dependent manner (Fig. 3B). SDF1α at 10 pm also enhanced GAD67 promoter binding activity.

We next performed a ChIP assay using an Egr1 antibody to enrich Egr1-binding DNA sequences in the GAD67 promoter. We designed three primer pairs for amplifications of sequence from +107 to −934 in the rat GAD67 promoter as shown in Fig. 3C. Based on GAD67 promoter structure and functions (33), the ERE-containing region 1 (−154 to +107) contains the most important and essential transcription factor binding domains for GAD67 promoter activity. These include Sp1.1, Sp1.2, Sp1.3, Egr1, one Pbx1, E-box, and PEA3. The non-ERE-containing region 2 (−359 to −653) covers PDX1.1, PDX1.2, and another Pbx1. The non-ERE-containing region 3 (−685 to −934) lacks any known transcription factor-binding site. The cells were incubated with either 10 nm SDF1α or vehicle (water) for 30 min, and ChIP assays were performed. SDF1α stimulation enriched GAD67 promoter sequences containing both ERE region 1 and non-ERE region 2, but not the region 3 sequence (Fig. 3C). Because region 3 is far away from the ERE-binding site, and has no changes between the control and SDF1α stimulation, this may truly reflect the background precipitation for the Egr1 antibody. Nonimmune control rabbit IgG itself did not pull out the GAD67 promoter sequence. However, it was noted that in samples from SDF1α-treated cells region 2 was precipitated with the Egr1 antibody to an extent greater than region 1. The major difference was in the control group, where the region 1 was precipitated much more relative to the region 2. One possible explanation of these results is that in the basal control condition, the ERE-containing region 1 may contain a high activity because of endogenous stimuli for cell growth relative to the region 2. The DNA precipitated by anti-Egr1 should include more of region 1 compared with region 2. Another possibility is that the Egr1 complex in cells stimulated with SDF1α may have a different configuration relative to the basal condition. Although the molecular components for the Egr1 complex in the GAD67 promoter are unknown, it was reported that Egr1 can interact with the transcription factors Sp1 and CREB to up-regulate chromogranin A expression in response to gastrin (46). It was also observed that co-expression of Egr1 with Sp1 and/or CREB resulted in additive enhancement of chromogranin A promoter activity, suggesting that different configurations of the Egr1 complex occur in the gastrinstimulated and control conditions. In our system, SDF1α stimulated CREB and Egr1 activity (Figs. 1 and 2) and binding activity (Fig. 3) to the GAD67 promoter that contains binding sites for ERE and Sp1. Although there is no cAMP-response element in the rat GAD67 promoter, Egr1 may interact with the Sp1 element in response to stimulation with SDF1α. This is also indicated by multiple bands in the EMSA using ERE- and Sp1-containing GAD67 promoter probes. The SDF1α-stimulated Egr1 complex configuration may interact with other not yet identified transcription factors resulting in more non-ERE region 2 DNA being precipitated by anti-Egr1. Thus, our ChIP data not only support the idea that SDF1α enhances GAD67 promoter binding via Egr1 activation, but also suggest that a complex regulatory machinery may exist in the GAD67 promoter.

SDF1α Increases GAD67 Expression, but Not Numbers of GABA-immunoreactive Neurons—Two different GAD isoforms (GAD65 and GAD67), which are encoded by different genes, are expressed in the adult nervous system (20). During embryonic development, two additional alternative transcripts, termed I-80 and I-86, are produced from the rodent GAD67 gene (21, 22). I-80 encodes an N-terminal 25-kDa “leader peptide” (GAD25) and a C-terminal 44-kDa “truncated GAD” (GAD44). I-86 encodes only GAD25, a protein that corresponds to the putative regulatory domain of the full-length protein. GAD44 contains the cofactor-binding site and enzymatic activity for synthesis of GABA. GAD25 is relatively abundant early in mouse central nervous system development (E10.5–12.5), whereas GAD44 is detected in the mouse brain from E11 to P21. GAD67 is undetectable in mice at E11, and then its expression increases considerably through P0 and reaches adult levels at 4 weeks postpartum (22, 47). Adult GAD65 can be found in mature neurons after birth (47). Thus, the developmental pattern of expression of the different GAD protein in the mouse central nervous system follows the order GAD25, GAD25/GAD44, GAD67, and GAD65 (17, 22). Because SDF1α stimulated GAD67 promoter activity, we used primer pairs specific for either fetal GAD67 (GAD67F) or adult GAD67 (GAD67A) and for GAD65 (34) to examine their expression patterns in rat E18 hippocampal neuronal cultures and the effects of SDF1α on their expression.

To investigate expression of GAD isoforms during the process of neuronal maturation, we collected hippocampal cells at different time points in culture from DIV1 to 8. Total RNAs were extracted, and RT-PCRs were performed. Embryonic hippocampal cells expressed both GAD67F and GAD67A, but not GAD65, through DIV8 (Fig. 4A). GAD65 was barely detectable, even at DIV8.

We next evaluated the effects of SDF1α on transcription of GAD isoforms in hippocampal cells at DIV2. SDF1α stimulated an increase in the expression of both the fetal and adult forms of GAD67; increased levels of the GAD67 mRNA were evident
FIGURE 3. SDF1α/CXCR4 signaling enhances GAD67 promoter binding activity in embryonic hippocampal neurons. A, EMSA was performed to measure GAD67 promoter binding activity stimulated by SDF1α (10 nM). SDF1α rapidly stimulated GAD67 promoter binding activity within 5 min, and the increased activity was maintained for at least 60 min. Cold unlabeled excess GAD67 promoter probes were added as a control. B, cell cultures were stimulated with vehicle (control) or the indicated concentrations of SDF1α for 15 min. Nuclear extracts were prepared, and their GAD67 promoter binding activities were measured by EMSA. SDF1α stimulated GAD67 activity in a dose-dependent manner. C, hippocampal cells were incubated with 10 nM SDF1α (S) or vehicle (C) for 30 min. The samples were prepared for ChIP assay as described under “Experimental Procedures.” The amplified regions in the GAD67 promoter are diagramed in the top panel. SDF1α stimulation increased the amounts of bound GAD67 promoter DNA sequences of both the ERE region 1 and non-ERE region 2 but not non-ERE region 3.
SDF1α/CXCR4 Regulates GAD67

A RT-PCR, GAD expression

| Developing stage (mice) | E10.5 | E12.5 | P0 | P21 | Adult |
|-------------------------|-------|-------|----|-----|-------|
| GAD65                   | GAD64 |       |    |     |       |
| GAD67A                  |       |       |    |     |       |
| GAD65                   |       |       |    |     |       |

DIV 1 2 3 8
GAD67F GAD67A GAD65 GAPDH

B RT-PCR

SDF1α (10 nM) hr 0 1 3 6
GAD67F GAD67A GAPDH

C IB

| SDF1α | hr | 0 | 1 | 3 | 6 | 24 |
|-------|----|---|---|---|---|----|
| β-Actin |    |   |   |   |   |    |

D AMD effect

| SDF1α | AMD | RT-PCR |
|-------|-----|--------|
| -     | -   | +      |

E PTX and PD effects

| SDF1α | PTX | PD |
|-------|-----|----|
| -     | +   | +   |

F Quantification

GABA+ Cells (%)

GAPDH

H Ctrl SDF1α

Merged

DAPI GABA
within 3 h of exposure to SDFα and were maintained through 24 h (Fig. 4B). We could not detect GAD65 transcripts in control or SDF1α-treated cultures. After 7 days of SDF1α treatment, there was no apparent difference in the expression levels of GAD67F and GAD67A between control and SDF1α-treated neurons. Immunoblot analysis using a specific GAD67 antibody showed that GAD67 protein levels were increased within 3 h of SDF1α treatment and were maintained at an elevated level through 24 h (Fig. 4C). To test if SDF1α-induced GAD67 expression is mediated by G protein-coupled CXCR4 receptors linked to ERK activation, we pretreated the cells with PTX (0.01 µg/ml) for 17 h, or AMD3100 (6 µM) and PD98059 (10 µM) for 0.5 h, and then treated the cells with 10 nM SDF1α for 6 h. The expression of the GAD67F and GAD67A isoforms was reduced by CXCR4 antagonist AMD3100 (Fig. 4D) as well as by either PTX or PD98059 (Fig. 4E). It was noted that application of AMD3100 alone also showed a slight increase in GAD67 expression. This may be because of the partial agonist activity of AMD3100 reported previously (38). Thus, SDF1α induced the expression of GAD67F and GAD67A transcripts via ERK-mediated pathway.

We also examined GABA production induced by SDF1α using immunocytochemistry. Embryonic hippocampal cultures at DIV2 were treated with either 10 nM SDF1α or its vehicle (water) for 6 h and immunocytochemistry was performed using an antibody against GABA. Neurons treated with SDF1α exhibited increased GABA immunoreactivity in the cell body and neurites (Fig. 4F). There was no significant difference in the percentage of GABA immunoreactive cells in the total cell population in control and SDF1α-treated cultures (Fig. 4G), suggesting that SDF1α may have a role in promoting maturation of GABAergic neurons in the developing hippocampus.

Depletion of Endogenous Egr1 by RNA Interference Decreases GAD67 Expression—We have provided evidence that SDF1α-induced Egr1 activity is associated with increased GAD67 promoter activity, induction of GAD67F and GAD67A transcripts, and increased GABA production. To establish whether Egr1 is essential for SDF1α-induced GABA production, we employed siRNA-mediated gene silencing to knock down Egr1 levels in embryonic hippocampal neurons. We used a chemically synthesized siGENOME ON-TARGET plus SMARTpool RNA duplex complementary to the rat Egr1 mRNA (GenBank™ accession number NM_012551; Dharmacon Inc., Chicago, IL). The siRNA pool included four RNA duplexes to maximize their silencing efficiency. Using our optimized protocol of electroporation, we successfully delivered the RNA duplexes into embryonic rat hippocampal neurons with a transfection efficiency of 74 ± 19% (mean ± S.D.), as determined using a Cy3-labeled siRNA duplex (Ambion), and ~90% cell viability as determined using the trypan blue assay. This protocol also decreased the expression of GAPDH at both the mRNA and protein levels by more than 70%. The results of an Egr1 siRNA dose-response experiment showed that siRNA concentrations of 0.5, 1.5, and 3.0 µg greatly reduced Egr1 mRNA levels (Fig. 5A). The reduction of Egr1 expression was accompanied by a decrease in the levels of endogenous GAD67 RNA levels, particularly GAD67F. At 72 h, Egr1 protein levels were also significantly decreased in neurons transfected with Egr1 siRNA (Fig. 5B).

We also examined effects of Egr1 siRNA on SDF1α-induced GABA synthesis. Embryonic rat hippocampal cultures were first electroporated with either negative or siEgr1 RNAs for 20 h and then treated with 10 nM SDF1α for 6 h. The cells were fixed and immunostained with a GABA antibody. In the control group, SDF1α increased levels of GABA immunoreactivity in neurites (Fig. 5C). In the Egr1 siRNA group, SDF1α-induced GABA production and its accumulation in neurites were significantly reduced. These results suggest that Egr1 activation is essential for induction of GAD67 expression and GABA production in response to SDF1α.

In Utero Intraventricular Administration of CXCR4 Antagonist AMD3100 Decreases GAD67 Expression—From the in vitro cell culture, it is clear that SDF1α promotes GAD67 expression, thus resulting in an enhanced GABA synthesis through CXCR4/Egr1-mediated pathway. To look for an in vivo role of SDF1α in enhancement expression of GAD67, we performed in utero intraventricular injection of CXCR4 antagonist AMD3100 (14 pmol, i.e. 11 µg/ml per embryo at E17 stage). As shown in Fig. 6A, mouse brain at E18 stage (control group) expressed Egr1, GAD67F, and GAD67A detected by whole brain RT-PCR. Immunohistology showed that GAD67 proteins were mainly localized in the ventricular zone (Fig. 6B, PBS control group). Injection of AMD3100 significantly decreased Egr1 and GAD67 mRNA expression and GAD67 protein levels relative to PBS control groups (Fig. 6). Thus, SDF1α/CXCR4 signaling may play a role in promotion of GAD67 expression with an involvement of Egr1 in brain neuronal development.

**FIGURE 4. SDF1α promotes GAD67 expression and GABA levels without affecting the number of GABA-immunoreactive neurons.** A, diagram for expression of GAD isoforms during mouse brain development is shown in the top. GAD67F is a fetal form of GAD67 that include two spliced transcripts I-80 and I-86. I-80 mRNA encodes both GAD25 and GAD44, whereas I-86 encodes only GAD25 protein. The primer used for amplification of GAD67F does not distinguish between I-80 and I-86. GAD67A and GAD65 are two isoforms that are known to be expressed in the adult nervous system. See details in the text. Rat E18 hippocampal cultures were prepared, and cells were collected at different DIV for total RNA extraction for RT-PCR. The rank order of relative expression of different GAD isoforms was GAD67F > GAD67A > GAD65. B, cultures were stimulated with 10 nM SDF1α for the indicated time periods, and RT-PCR was performed on RNA samples to evaluate the expression of GADs. SDF1α increased expression of both GAD67F and GAD67A but not GAD65. C, control; S, 10 nM SDF1α; C, immunoblot (8) analysis results showing that SDF1α increases GAD67 protein levels relative to the control. D, application of AMD3100 (6 µM) for 30 min prevented SDF1α-induced GAD67 expression at both RNA and protein levels although AMD3100 treatment alone caused a small increase in GAD67 expression. E, PTX and PD98059 also inhibited SDF1α-induced GAD67 RNA expression. Hippocampal cells were pretreated with either PTX (0.01 µg/ml) or PD98059 (10 µM) and then incubated with 10 nM SDF1α for 6 h. Total RNAs were isolated, and RT-PCRs were performed. SDF1α induced an increase in the levels of GAD67F and GAD67A, an effect that was inhibited by preincubation with either PTX or PD98059. F, SDF1α enhanced GABA immunostaining, particularly in neurites. Hippocampal cultures were treated with either 10 nM SDF1α or its vehicle (water) for 6 h and then fixed in 3% paraformaldehyde and 1% glutaraldehyde for 30 min. Immunocytochemistry was performed using an antibody against GABA. DAPI, 4’,6-diamidino-2-phenylindole. G, percentage of cells that were GABA-positive was determined. Note that in all time course studies, the time point for harvesting was same to ensure that the developmental stages of all dishes were similar. Values are the mean and S.D. Ctrl, control.

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Our results suggest that SDF1α/CXCR4 signaling enhances GAD67 enzyme expression and subsequent GABA production through Egr1 activation in rat embryonic hippocampal neurons. This conclusion is based on data showing the following. 1) Embryonic hippocampal neurons express CXCR4. 2) SDF1α stimulates ERK/CREB activities and induces the expression of Egr1. 3) SDF1α induces the expression of GAD67 (both fetal and adult isoforms) but not GAD65 transcripts. 4) SDF1α increases the levels of GABA in neurites. 5) SDF1α-induced GAD67 expression is inhibited by pretreatment of the neurons with AMD3100 (a CXCR4 antagonist). 6) SDF1α enhances endogenous GAD67 promoter binding activity. 7) ChIP assay analysis using an Egr1 antibody showed that an Egr1-specific GAD67 promoter DNA sequence is enriched in response to SDF1α stimulation. 8) Depletion of Egr1 from hippocampal neurons using RNA interference methods results in a suppression of SDF1α-induced GAD67 expression and GABA production. 9) SDF1α rapidly stimulates ERK and CREB activation, presumed pathways for Egr1 activation. 10) Intraventricular infusion of AMD3100 into developing mouse embryos resulted in reduced expression of GAD67 mRNA levels and GAD67 protein levels compared with PBS control groups.

Collectively, these findings suggest that SDF1α/CXCR4 signaling may play important roles in the development of the GABAergic phenotype in developing neurons prior to and during the period of synaptogenesis.

Increasing evidence suggests that GABA plays important roles in regulating neurogenesis, and the growth and synaptogenesis of newly generated neurons, in the developing and adult brain.
hippocampus. Newly generated neurons in the hippocampus exhibit strong GABAergic evoked and spontaneous synaptic currents, and may initially receive only GABAergic inputs prior to the formation of glutamatergic synapses (50). In the adult hippocampus, GABA regulates the synaptic integration of newly generated neurons (51). Previous cell culture studies have provided evidence that signals from glial cells influence development of the GABAergic phenotype of hippocampal neurons during early postnatal development (52). Although the identity of the glial cell-derived factor is unknown, SDF1α is one possibility as it is known to be produced by astrocytes (53, 54).

The signaling pathways that regulate the GABAergic phenotype of neurons in the hippocampus are beginning to be elucidated. The expression of GAD65 and GAD67 is increased by estrogen (55); levels of GAD65 are increased in response to the brain-derived neurotrophic factor (56), and stress increases GAD67 expression in hippocampal interneurons (57). Our data demonstrate that activation of the SDF1α-CXCR4 signaling pathway up-regulates the expression of GAD67 and the production of GABA in embryonic hippocampal neurons. In the developing hippocampus, SDF1α may play an important role in regulating neurogenesis, gliogenesis, and the migration of dentate granule cells and synaptogenesis (26, 58, 59). Additional regulation of GABAergic neurons by SDF1α might function in the fine-tuning of synaptic circuitry. Our data showing that Egr1 mediates SDF1α-induced expression of GAD67 provides the first evidence for the involvement of this transcription factor in regulating the GABAergic phenotype. ERKs and CREB appear to function upstream of Egr1 in the SDF1α-CXCR4 pathway. Previous studies have suggested that signaling pathways that activate ERKs, such as the glutamate–N-methyl-D-aspartate receptor pathway, can increase the expression of GAD67 in neurons (60). Therefore, ERKs and Egr1 appear to be important regulators of the GABAergic phenotype.

In the adult brain, both neurons and astrocytes express SDF1α (53), and the SDF1α-CXCR4 pathway is therefore poised to regulate development of the GABAergic phenotype during adult hippocampal neurogenesis and the synaptic integration of newly generated neurons. Because perturbations in GABAergic neurons are associated with several different neurological disorders that affect the hippocampus, a possible role for altered SDF1α/CXCR4/ERK/Egr1 signaling in the pathogenesis of such neurological disorders should be considered. The expression of GAD67 is altered in several different neurological disorders, including epileptic seizures (34, 61) and schizophrenia and bipolar disorder (62). Levels of Egr1 are increased in the hippocampus in response to plasticity-related synaptic activity (63) and are decreased in the hippocampus of cognitively impaired aged rats (64). In addition, disruption of GABAergic regulation of hippocampal neurogenesis is associated with abnormal anxiety- and depression-like behaviors in mice (65). Further studies will be required to elucidate the involvement of perturbed SDF1α/CXCR4/ERK/Egr1 signaling and GABA expression in the pathogenesis of such neurological disorders.

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