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The secretogranin II gene is a signal integrator of glutamate and dopamine inputs

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Abstract
Cooperative gene regulation by different neurotransmitters likely underlies the long-term forms of associative learning and memory, but this mechanism largely remains to be elucidated. Following cDNA microarray analysis for genes regulated by Ca\(^{2+}\) or cAMP, we found that the secretogranin II gene (Scg2) was cooperatively activated by glutamate and dopamine in primary cultured mouse hippocampal neurons. The Ca\(^{2+}\) chelator 1,2-bis(2-aminophenoxy)ethane-N,N',N',N'-tetraacetic acid acetoxymethyl ester (BAPTA-AM) and the mitogen-activated protein kinase (MAPK) kinase (MEK) inhibitor PD98059 prevented Scg2 activation by glutamate or dopamine; thus, the Ca\(^{2+}\)/MEK pathway is predicted to include a convergence point(s) of glutamatergic and dopaminergic signaling. Unexpectedly, the protein kinase A inhibitor KT5720 enhanced Scg2 activation by dopamine. The protein-synthesis inhibitor cycloheximide also enhanced Scg2 activation, and the proteasome inhibitor ZLLLH diminished the KT5720-mediated augmentation of Scg2 activation. These results are concordant with the notion that dopaminergic input leads to accumulation of a KT5720-sensitive transcriptional repressor, which is short-lived because of rapid degradation by proteasomes. This repression pathway may effectively limit the time window permissive to Scg2 activation by in-phase glutamate and dopamine inputs via the Ca\(^{2+}\)/MEK pathway. We propose that the regulatory system of Scg2 expression is equipped with machinery that is refined for the signal integration of in-phase synaptic inputs.

Keywords: dopamine, glutamate, hippocampus, neurotransmission, secretogranin II, signal integrator.

converging signals, and can work as a signal integrator of distinct synaptic inputs. A typical model for the relevant mammalian signal transduction from the neuronal cell surface to the nucleus is as follows: the second messengers Ca\(^{2+}\) and cAMP activate multiple kinase pathways including calcium/calmodulin-dependent protein kinase (CaMK), protein kinase A (PKA), and extracellular signal-regulated protein kinase (ERK), which in turn phosphorylate transcription factors such as the cAMP responsive element (CRE)-binding protein (CREB). This results in the activation of target genes for growth factors such as brain-derived neurotrophic factor and transcription factors including Fos and Zif268/Egr1, which can further control a number of downstream genes (Hyman et al. 2006; Flavell and Greenberg 2008; Pape and Pare 2010). Given that this model is also applicable to the molecular mechanisms of associative learning and memory, fundamental questions are raised regarding these processes. What is the combination of synaptic inputs? What is the target gene that functions as a signal integrator? Surprisingly, to date, the knowledge of genes that are regulated cooperatively by different neurotransmitters is very limited.

To address this problem, we first screened genes regulated by Ca\(^{2+}\) and cAMP via complementary DNA (cDNA) microarray analysis. Subsequent investigations for related neurotransmitters revealed that the secretogranin II (SgII) gene (Scg2) is activated cooperatively by glutamate and dopamine, and repressed by GABA. SgII is a member of the granin family of acidic secretory proteins (Fischer-Colbrie et al. 1993; Taupenot et al. 2003), and is processed to a number of small peptides called secretoneurin (Kirchmair et al. 1993; Wiedermann 2000), BM66 (Anout et al. 1998), and manserin (Yajima et al. 2004). Various physiological roles have been assigned to SgII. In particular, the most well-characterized peptide, secretoneurin, has been found to be involved in hormone secretion (Nicol et al. 2002), angiogenesis (Kirchmair et al. 2004), neurotransmitter release (Saria et al. 1993; You et al. 1996), neurite outgrowth (Gasser et al. 2003), and neuroprotection (Shyu et al. 2008). Previous studies showed that Scg2 is activated by the cAMP pathway via a CRE in the Scg2 promoter (Cibelli et al. 1996; Scammell et al. 2000) and by the Ca\(^{2+}\) pathway depending on the voltage-gated Ca\(^{2+}\) channel (VGCC) (Fujita et al. 1999). However, the cognate extracellular agonists have not been reported, except for gonadotropin-releasing hormone, which activates Scg2 in pituitary gonadotrope lineage cells via the PKA/CREB pathway (Song et al. 2003) and the mitogen-activated protein kinase (MAPK)/activating transcription factor 3 pathway (Xie and Roberson 2008). This study indicates that Scg2 is activated cooperatively by representative fast (depolarizing) and slow (modulatory) neurotransmitters, that is, glutamate and dopamine, respectively. This finding, taken together with the characterization of the Scg2 regulatory mechanisms mediated by these neurotransmitters, leads us to postulate that Scg2 is a good candidate for the signal integrator required for activity-dependent plasticity such as associative learning and memory.

**Materials and methods**

**Animals**

Pregnant C57BL/6 mice were obtained from CLEA Japan, Inc. (Tokyo, Japan) and housed at 24 ± 1°C in a 12-h light/12-h dark cycle with free access to food and water in the Laboratory Animal Center of Chiba University School of Medicine. All experimental procedures in this study were approved by the Animal Experiment Committee of Chiba University (Permit Numbers: 20000007, 20070018, and A25-229), and they were conducted in accordance with the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan.

**Cell culture**

Preparation of the mouse neuron/glia mixed culture was performed essentially as described previously (Fukunaga et al. 1992). The hippocampi were removed on postnatal day 1 and placed in growth medium, which consisted of a 1 : 1 mixture of Eagle’s minimum essential medium (Nissui Pharmaceutical Co., Tokyo, Japan) and Hank’s balanced salt solution, containing 1.2 g/L NaHCO\(_3\), 1 mM l-glutamine, 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 10% horse serum (Invitrogen), 2% B-27 supplement (Invitrogen), and 12 ng/mL nerve growth factor (Sigma Aldrich, St. Louis, MO, USA). Cells were mechanically dissociated by trituration with fire-polished Pasteur pipettes and seeded in 60-mm dishes or cover glasses coated with 0.2% polyethyleneimine (Sigma Aldrich). One day after plating cells, cultures were treated with 5 μM cytosine-β-arabinofuranoside to prevent the replication of non-neuronal cells for 24 h. Cultured cells were maintained in growth medium lacking 10% FBS at 37°C in a humified incubator with a 5% CO\(_2\) atmosphere for 1 week before use. Thirty minutes prior to stimulation with neurotransmitters, cells were placed in growth medium lacking FBS, horse serum, and B-27 supplement.

The mouse neuroblastoma cell line Neuro2a was obtained from the American Type Culture Collection (Manassas, VA, USA), and cells were cultured in Eagle’s minimum essential medium containing 1.5 g/L NaHCO\(_3\), 2 mM l-glutamine, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 10% FBS.

**Preparation of the cDNA library**

Primary cultured neuron/glia cells were stimulated by tetraethylammonium (TEA) or left unstimulated, and total RNA was prepared by the acid-guanidine-phenol-chloroform method (Chomczynski and Sacchi 1987). Since the amount of RNA obtained from the primary cultured cells was limited, we amplified total cDNA by PCR, as described previously (Ohtsuka et al. 2004; Adachi-Uehara et al. 2006) with slight modification. Briefly, poly(A)\(^{+}\) RNA derived from 0.5 μg of total RNA was absorbed on 50 μg of oligo(dT)\(_{25}\)c\(_{5}\) Dynal, Oslo, Norway, and used as a template for cDNA synthesis. The resulting double-stranded cDNA was trimmed to an estimated average length of 1024 bp from the 3′ terminus by digesting 3 aliquots of the bead-fixed cDNA with a restriction enzyme (BamHI, EcoO109I or HincII). The three 3′-trimmed cDNA-bead suspensions were combined, and the
restriction ends were blunted with T4 DNA polymerase, followed by ligation to a linker harboring the T7 promoter sequence. Sense-strand cDNA liberated by heat denaturation was subjected to antisense-strand cDNA synthesis-coupled introduction of the SP6 promoter sequence onto the 3′ terminus. The total cDNA was then amplified by PCR using the linker sequences at each end as primers. The exonuclease activity of T4 DNA polymerase generated 5′ AvaI and 3′ AccI ends, allowing for subsequent ligation of the cDNA into AvaI/AccI-cut pUC19 plasmids. The resulting plasmids were transformed into E. coli strain JM109.

cDNA microarray analysis

A microarray chip was prepared as described previously (Yoshikawa et al. 2000) with cDNA clones derived from the control cells and TEA-stimulated cells, in addition to other mouse brain cDNA clones. To prepare the hybridization target mixtures, 0.5 μL of oligo(dT), and 400 units of reverse transcriptase SuperScript II (Invitrogen), and was allowed to proceed for 1 h at 37°C before use. The reaction was performed in 30 μL of a solution containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, 0.5 mM each of dATP, dCTP and dGTP, 0.2 mM 5-(3-aminoallyl)-dUTP (Ambion, UK) according to the manufacturer’s protocol. The Cy3- and Cy5-labeled cDNAs were combined in a 40 μL solution containing 1.25 μg/μL yeast RNA, 1.25 μg/μL poly(A), 3.4 × sodium-saline citrate (SSC), and 0.3% sodium dodecyl sulfate (SDS). Hybridization with the microarray was performed at 65°C overnight under humidified conditions. After the hybridization, the array was washed twice for 5 min with 2 × SSC/0.1% SDS at 22 ± 3°C, twice for 5 min with 0.2 × SSC/0.1% SDS at 40°C, and finally rinsed with 0.2 × SSC. The array was centrifuged at 200 g for 1 min, and then scanned with a ScanArray4000 fluorescence laser-scanning device (GSI Lumonics, Bedford, MA, USA). The median intensity of the blank spots (337 spots on the chip) was subtracted from the intensity of each spot. The Cy3/Cy5 ratios or the Cy5/Cy3 ratios of all spots on the microarray were normalized by dividing each ratio by the median ratio.

Northern analysis

Total RNAs (0.4–0.5 μg per lane) were electrophoresed in denaturing formaldehyde-1% agarose gels. RNAs were visualized by ethidium bromide staining, and blotted onto nylon membranes. Digoxigenin (DIG)-labeled RNA probes were synthesized using transcription kits (Roche Diagnostics, Mannheim, Germany) from cloned cDNAs for mouse Scg2 (accession NM_009129, nt. 671–2485) and rat glyceraldehyde-3-phosphate dehydrogenase (Gapdh; accession NM_017008, nt. 284–1087). Hybridization, washing, and chemiluminescent detection were performed as recommended by Roche Diagnostics. Chemiluminescent signals were quantified by a Light Capture system (ATTO, Tokyo, Japan) or by scanning X-ray films using Personal Density Scanning Imager (Molecular Dynamics, Sunnyvale, CA, USA). As needed, the quantified values of Scg2 mRNA were adjusted based on the levels of Gapdh on the same membrane.

Western analysis

Essentially as described previously (Iwase et al. 2000), cell extracts (10–20 μg of protein per lane) were subjected to SDS-8% polyacrylamide gel electrophoresis, and were electrotransferred to nitrocellulose membranes. As a primary antibody, the rabbit polyclonal anti-rat SgII antibody (1 : 5000 dilution; QED Bioscience, San Diego, CA, USA) was incubated with the membranes. After incubation with the horseradish peroxidase-conjugated secondary antibody (1 : 5000 dilution; DakoCytomation, Glostrup, Denmark) for 1 h, peroxidase activity was detected using the Immobilon Western kit (Merck Millipore, Darmstadt, Germany). The chemiluminescent signals were quantified as described for Northern analysis.

In situ hybridization

Primary cultured neuron/glia cells seeded onto cover glasses were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C overnight and then washed with PBS. The cells were subjected to digestion in 1 μg/mL proteinase K in PBS for 10 min, and then fixed with 4% paraformaldehyde in PBS for 10 min. Pre-hybridization, hybridization with DIG-labeled cRNA probes, washing, and color detection of the hybridized probes (with alkaline phosphatase-conjugated anti-DIG antibody and the chromogens nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate) were performed essentially as described (Iwase et al. 1998).

Immunocytochemistry

Primary cultured neuron/glia cells seeded onto cover glasses were fixed with methanol at −20°C, and were treated with 0.01% Triton X-100 in PBS for 10 min. After inhibition of endogenous peroxidase activity by treatment with 0.3% H2O2 in methanol for 30 min, cells were incubated with a primary antibody diluted with PBS containing 1.5% goat serum overnight at 4°C. The primary antibodies used were the mouse monoclonal anti-human microtubule-associated protein (MAP) 2B antibody (1 : 500 dilution; BD Bioscience, San Jose, CA, USA) and the mouse monoclonal anti-human glial fibrillary acidic protein (GFAP) antibody (1 : 20 dilution; American Research Products, Belmont, MA, USA). After incubation with the horseradish peroxidase-conjugated secondary antibody (1 : 200 dilution) for 1 h, peroxidase activity was visualized using diaminobenzidine as a substrate. Cells were slightly counter-stained with hematoxylin, and were mounted in Aquatex (Merck Millipore).

Statistical analysis

The data are presented as mean ± SEM unless otherwise noted. The statistical analyses were performed with ANOVA that were followed by post hoc comparisons with Tukey’s Honestly Significant Difference (HSD) tests for multiple samples by using SPSS software (IBM, Armonk, NY, USA) or with Student’s t-tests for selected pairs using Microsoft Excel (Microsoft, Redmond, WA, USA). Differences were considered statistically significant when p < 0.05.

Results

cDNA microarray analysis

To detect genes that are regulated by plasticity-related signaling in the neuron, we employed our in-house cDNA microarray system, which is suited for the analysis of small
quantities of RNA available from a limited number of cells (Ohtsuka et al. 2004; Adachi-Uehara et al. 2006). cDNA libraries were constructed with RNAs derived from mouse hippocampal primary cultured cells treated with TEA or left untreated. TEA is a K⁺ channel blocker, which causes elevation of cytosolic Ca²⁺ levels and can induce long-term potentiation (LTP) in the CA1 region of the hippocampus (Aniksztejn and Ben-Ari 1991; Huang and Malenka 1993). We determined the 5'-end sequences of about 3000 cDNA clones, integrated overlaps, and selected 1815 independent clones (1093 and 722 clones derived from TEA-treated and control cells, respectively). Microarray chips harboring these 1815 cDNAs and another 956 brain cDNAs (total 2771 cDNAs) were prepared and hybridized with target mixtures of Cy3-labeled cDNA derived from TEA- or dibutyryl (db)-cAMP-stimulated cells and Cy5-labeled cDNA derived from control cells. To correct for possible differences between the dyes (e.g., in the linearity of fluorescence intensity), the experiment was also performed with a target mixture of Cy5-labeled cDNA from treated cells and Cy3-labeled cDNA from control cells. Genes with at least a 1.5-fold change in both microarray experiments were considered candidates for regulation by TEA (Table S1) or db-cAMP (Table S2).

Treatment with TEA and db-cAMP increased the mRNA levels of 31 and 34 genes, respectively; both treatments up-regulated the genes for SgII (Table S1, No. 20; Table S2, No. 1) and transcriptional elongation regulator 1 (Table S1, No. 3; Table S2, No. 24). In addition, TEA and db-cAMP treatment decreased the mRNA levels of 33 and 20 genes, respectively; both treatments down-regulated the genes for tenascin C (Table S1, No. 48; Table S2, No. 53) and tubulin β5 (Table S1, No. 60; Table S2, No. 40). Here, we focused on Scg2, since a number of physiological roles in the nervous system have been assigned to this gene (You et al. 1996; Gasser et al. 2003; Li et al. 2008; Shyu et al. 2008).

Elevation of neuronal Scg2 mRNA levels in response to Ca²⁺ -mobilizing reagents and/or db-cAMP

To confirm the results of the microarray assays, we performed Northern blot analysis (Fig. 1a), which

![Fig. 1](image-url)

**Fig. 1** Induction of Scg2 mRNA by tetraethylammonium (TEA), db-cAMP, or ionomycin.
(a) Northern blot analysis was performed with total RNAs isolated from mouse hippocampal primary cultured cells. Cells were stimulated with 25 mM TEA 6 h before harvesting, or with 1 mM db-cAMP or 2 µM ionomycin (Iono) twice, 6 h and 24 h before harvesting. In controls (Con), cells were treated with vehicles. Representative chemiluminesograms for Scg2 and Gapdh mRNAs are shown at the top, with the quantified relative Scg2 mRNA levels shown graphically below, presented as mean ± SEM (n = 3–4). **p < 0.01, *p < 0.05 (Student’s t-test). (b–d) In situ hybridization analysis for Scg2 mRNA. The mouse hippocampal primary cultured cells treated with TEA (c and d) or vehicle (b) were probed with antisense-strand cRNA (b and c) or sense-strand cRNA (d). (e and f) Immunodetection of microtubule-associated protein (MAP) 2B as a marker for neurons (e) and glial fibrillary acidic protein (GFAP) as a marker for astrocytes (f).
demonstrated that Scg2 mRNA levels were increased by TEA and db-cAMP, with a stronger effect exerted by the latter. TEA is known to cause neural plastic changes such as LTP through activation of VGCCs (Aniksztejn and Ben-Ari 1991; Huang and Malenka 1993); therefore, we examined the effects of ionomycin, a Ca\textsuperscript{2+} ionophore that raises intracellular Ca\textsuperscript{2+} levels, in a more direct manner. Ionomycin caused an increase in Scg2 mRNA levels to an extent comparable to that of TEA.

To determine which cell species were expressing Scg2 in the hippocampal neuron/glia mixed cultures, in situ hybridization was carried out (Fig. 1b-d). The signals detected in the control culture (Fig. 1b) increased in response to TEA (Fig. 1c), while no obvious signal was detected with the sense-strand probe (Fig. 1d). The Scg2 mRNA-positive cells resembled neurons immunostained with the marker protein MAP2B (Fig. 1e) rather than astrocytes stained with the marker protein GFAP (Fig. 1f), suggesting that neuronal expression of Scg2 mRNA was TEA-inducible. Concomitantly, Scg2 mRNA induction by TEA was not observed in the mouse hippocampal astrocyte culture (data not shown).

Cooperative induction of Scg2 mRNA by ionomycin and db-cAMP

To examine whether Ca\textsuperscript{2+} and cAMP exhibit cooperative effects, we treated hippocampal primary cultured cells with 2 \( \mu \)M ionomycin and 1 mM db-cAMP (Fig. 2a). Compared to the individual treatments, the co-administration of these reagents significantly augmented the elevation of Scg2 mRNA levels. To verify the effects of Ca\textsuperscript{2+} and cAMP more precisely, we employed the mouse neuroblastoma cell line, Neuro-2a, and found that ionomycin (2 \( \mu \)M) and db-cAMP (0.2–5 mM) cooperatively induced Scg2 mRNA (Fig. 2b), in a synergistic manner (\( p = 0.010 \) interaction effect, two-way ANOVA). On the other hand, in the hippocampal primary cultured cells, the effects of ionomycin and db-cAMP (Fig. 2a) were additive rather than synergistic (\( p = 0.57 \) interaction effect, two-way ANOVA) as was also the case for the effects of neurotransmitters (see below). The experimental conditions that caused the synergism in Neuro-2a cells but not in the primary cultured cells remain to be investigated. As shown in Fig. 2c, SgII protein also seemed to be cooperatively induced by ionomycin and db-cAMP.

Regulation of Scg2 mRNA levels by neurotransmitters

The induction of Scg2 mRNA in response to several reagents such as db-cAMP, forskolin, and KCI has been reported (Scammell et al. 1995; Cibelli et al. 1996; Fujita et al. 1999), but their cognate neurotransmitters have not yet been identified. To address this point, we treated hippocampal primary cultured cells with monoamine and amino acid neurotransmitters (Fig. 3). Scg2 mRNA levels were elevated by glutamate, dopamine, and noradrenaline, lowered by GABA, and not significantly affected by serotonin or histamine. Previously, it was reported that GABA induced
Scg2 mRNA in the goldfish pituitary (Blazquez et al. 1998); thus, the effects of GABA seem to differ among brain regions or species.

Next, we studied the combinatorial effects of neurotransmitters on Scg2 expression (Fig. 4). Compared to the individual treatments, the co-administration of glutamate and dopamine significantly augmented the elevation of Scg2 mRNA levels (Fig. 4a, left panel). The co-administration of glutamate and noradrenaline (middle panel) augmented the Scg2 mRNA increase compared to glutamate alone with significance, and compared to noradrenaline alone with tendency \( (p = 0.09) \). In contrast, no cooperative effects were observed with the co-administration of dopamine and noradrenaline (right panel). As shown in Fig. 4b, SgII protein levels were also elevated by the co-administration of glutamate and dopamine, while the individual treatments showed no apparent effect after treatment for 6 h.

We further examined the effects of GABA co-administered with glutamate or dopamine (Fig. 4c), and found that GABA almost completely suppressed the induction of Scg2 mRNA by glutamate or dopamine.

**Involvement of Ca\(^{2+}\) and ERKs in Scg2 mRNA induction by neurotransmitters**

To elucidate the downstream pathways of neurotransmitter stimulation, we assessed the effects of specific inhibitors of the relevant signaling pathways. Pre-administration of the membrane-permeable Ca\(^{2+}\) chelator, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester (BAPTA-AM), almost completely inhibited Scg2 mRNA induction by glutamate, dopamine or noradrenaline (Fig. 5a), suggesting that intracellular Ca\(^{2+}\) increase is commonly essential for Scg2 activation by these neurotransmitters. We further examined the effects of NMDA that stimulates the Ca\(^{2+}\)-mobilizing NMDA receptor (NMDAR) (Fig. 5b). NMDA up-regulated Scg2 mRNA levels, while a stereoisomer of NMDA, N-methyl-L-aspartate, did not. In addition, the selective NMDAR antagonist MK-801 reduced Scg2 mRNA induction by glutamate (Fig. 5c). These results are concordant with the notion that glutamate induces Scg2 mRNA through the stimulation of NMDAR followed by Ca\(^{2+}\) influx.

An increase in intracellular Ca\(^{2+}\) leads to the activation of ERKs and CaMKs as canonical kinases that in turn activate the transcription of target genes in neurons (LeDoux 2000; Kandel 2001; Kelley 2004). We examined the effects of PD98059, a specific inhibitor of MAPK kinase (MEK), which blocks ERK activation (Fig. 6). Pre-administration of PD98059 completely disrupted Scg2 mRNA induction by glutamate or dopamine, but did not significantly inhibit the induction by noradrenaline. On the other hand, the CaMK inhibitor KN-93 had no apparent effect on Scg2 activation by any of these neurotransmitters (Figure S1). Therefore, the activation of ERKs, rather than CaMKs, appears to transduce Ca\(^{2+}\) signaling in Scg2 induction by glutamate or dopamine.

**KT5720-sensitive Scg2 repression in response to dopamine**

We also examined the effects of the selective PKA inhibitor KT5720 on Scg2 activation by the neurotransmitters (Fig. 7). Unexpectedly, KT5720 enhanced the Scg2 mRNA induction by dopamine or noradrenaline, but did not enhance the induction by glutamate. These results suggested that the activation of the KT5720-sensitive kinase in response to dopamine or noradrenaline repressed Scg2 expression. While KT5720 is widely used as a PKA inhibitor, various non-specific effects of KT5720 on other protein kinases have been reported (Murray 2008). Thus, we examined if other PKA inhibitors, such as myristoylated protein kinase inhibi-
itor peptide 14–22 amide (10 μM) and Rp-8-Br-cAMPS (50 μM), enhanced Scg2 induction by dopamine, but we could not detect any obvious effects of these reagents (data not shown). As for inhibitors of other candidates of KT5720 target kinases, in addition to PD98059 for MEK and KN-93 for CaMK described above, (2′Z,3′E)-6-bromo-indirubin-3′-oxime (5 μM) for glycogen synthase kinase 3β, 1α,6-hydroxymethyl-chiro-inositol-2-(R)-2-O-methyl-3-O-octadecyl-sn-glycerocarbonate (10 μM) for Akt/PKB, and dorsomorphin (10 μM) for AMP kinase did not enhance the Scg2 induction by dopamine in our preliminary results (data not shown). Therefore, further investigations are required to confirm if the KT5720-sensitive kinase is PKA or another kinase.

Fig. 4 Effects of co-administration of neurotransmitters on Scg2 expression. (a) Northern blot analysis was performed with total RNAs isolated from mouse hippocampal primary cultured cells treated with 100 μM glutamate (Glu), 50 μM dopamine (Dop) or 100 nM noradrenaline (NA) alone or in pairwise combinations for 6 h. In controls (Con), cells were treated with vehicles. Representative chemilumograms for Scg2 and Gapdh mRNAs are shown at the top. Below, the quantified relative Scg2 mRNA levels are presented as mean ± SEM (n = 3–14). **p < 0.01; n.s., not significant (Tukey’s HSD test). (b) Western blot analysis for SgII protein (SCG2) was performed with cell lysates isolated from mouse hippocampal primary cultured cells treated with glutamate (Glu), dopamine (Dop), or both (Glu + Dop) for 6 h. In controls (Con), cells were treated with vehicles. Below the chemilumogram, the quantified values of SCG2 signal are indicated. (c) Northern blot analysis was performed with total RNAs isolated from mouse hippocampal primary cultured cells treated with glutamate (Glu), dopamine (Dop) or control vehicle (Con) in the presence of 10 μM GABA or vehicle (−) for 6 h. Representative chemilumograms for Scg2 and Gapdh mRNAs are shown on the left. On the right, the quantified relative Scg2 mRNA levels are presented as mean ± SEM (n = 5–9). The solid and hatched bars show the absence and presence of GABA, respectively. **p < 0.01, *p < 0.05 (Student’s t-test).

One well-characterized transcriptional repressor downstream of the cAMP pathway is the inducible cAMP early repressor (ICER) (Sassone-Corsi 1998; Mioduszewska et al. 2003). Therefore, we aimed to examine the dopamine-induced changes in the ICER mRNA and protein levels; however, ICER expression was below detectable levels, even after dopamine treatment (data not shown). To characterize the putative KT5720-sensitive transcriptional repressor, we examined the effects of the protein synthesis inhibitor cycloheximide. As shown in Fig. 8a, cycloheximide raised the basal Scg2 mRNA level (lane 9), suggesting the presence of a short-lived protein(s) that repressed basal Scg2 expression. The co-administration of cycloheximide with dopamine (lane 11) or noradrenaline (lane 12) induced Scg2 mRNA in a
Fig. 5 Contribution of intracellular Ca\(^{2+}\) and the NMDA receptor to Scg2 mRNA induction. Northern blot analysis was performed with total RNA that was isolated from mouse hippocampal primary cultured cells. In each panel, representative chemiluminograms for Scg2 and Gapdh mRNAs are shown at the top. Below, the quantified relative Scg2 mRNA levels are presented. (a) Cells were treated with glutamate (Glu), dopamine (Dop), or noradrenaline (NA) for 6 h. Thirty minutes prior to neurotransmitter stimulation, cells were treated with vehicle. Thirty minutes prior to stimulation with neurotransmitters, cells were treated with 50 \(\mu\)M 1,2-bis(2-aminophenoxo)-ethane-N,N,N,N-tetraacetic acid acetoxymethyl ester (BAPTA-AM) in Ca\(^{2+}\)-free medium (BAPTA) or with vehicle in normal medium (–). The quantified data are presented as mean ± SEM (\(n = 4–6\)). The solid and hatched bars show the absence and presence of BAPTA-AM, respectively. **\(p < 0.01\); *\(p < 0.05\); n.s., not significant (Student’s t-test). (b) Cells were treated with 50 \(\mu\)M NMDA (\(n = 3\)), 50 \(\mu\)M N-methyl-D-aspartate (NMLA) (\(n = 2\)), or control vehicle (Con, \(n = 3\)) for 6 h. The quantified data are presented as mean ± SEM (NMDA) or as mean ± range (NMLA). *\(p < 0.05\) (Student’s t-test). (c) Cells were treated with glutamate (Glu) or control vehicle (Con) for 6 h. Thirty minutes prior to neurotransmitter stimulation, cells were treated with 10 \(\mu\)M MK801 or vehicle (–). The quantified data are presented as mean ± SEM (\(n = 4\)). The solid and hatched bars show the absence and presence of MK801, respectively. **\(p < 0.01\); n.s., not significant (Student’s t-test).

Fig. 6 Effects of the extracellular signal-regulated protein kinase (ERK) activation inhibitor PD98059 on Scg2 mRNA induction. Northern blot analysis was performed with total RNAs isolated from mouse hippocampal primary cultured cells treated with glutamate (Glu), dopamine (Dop) or noradrenaline (NA) for 6 h. In controls (Con), cells were treated with vehicles. Thirty minutes prior to neurotransmitter stimulation, cells were treated with 20 \(\mu\)M PD98059 (PD) or vehicle (–). Representative chemiluminograms for Scg2 and Gapdh mRNAs are shown at the top. Below, the quantified relative Scg2 mRNA levels are presented as mean ± SEM (\(n = 3–4\)). The solid and hatched bars show the absence and presence of PD98059, respectively. *\(p < 0.05\); n.s., not significant (Student’s t-test).

strongly synergistic manner. Therefore, it seems that the signaling pathways from dopamine or noradrenaline to Scg2 repression also involve a short-lived and/or newly synthesized protein(s). KT5720 exhibited no additive effect on the Scg2 activation by cycloheximide (lanes 13–16). Thus, the KT5720-sensitive Scg2 repression in response to dopamine or noradrenaline essentially requires a protein(s) sensitive to cycloheximide. It remains to be clarified whether the KT5720-sensitive repressor is identical to the basal Scg2 repressor.

The transitory nature of the putative repressor prompted us to examine whether its degradation is proteasome-dependent, using the proteasome inhibitor ZLLLH (Fig. 8b). Most remarkably, ZLLLH completely diminished the effects of KT5720 in augmenting the induction of Scg2 mRNA by dopamine (compare lane 15 with lanes 3 and 7) or noradrenaline (compare lane 16 with lanes 4 and 8), suggesting that the inhibition of proteasomes resulted in an accumulation of the Scg2 repressor up to the levels that were obtained in the
In this study, we showed that Scg2 mRNA levels in primary cultured hippocampal neurons were elevated by glutamate, dopamine, and noradrenaline, and lowered by GABA. The converse regulatory effects of the fast neurotransmitters, that is, glutamate (activating or depolarizing) and GABA (inhibiting or hyperpolarizing), are concordant with the previously suggested notion that Scg2 expression is regulated in a neuronal activity-dependent manner (Nedivi et al. 1993; Konopka et al. 1995; Fujita et al. 1999), and are also concordant with the present finding that TEA, a K+ channel blocker causing depolarization, activates Scg2. On the other hand, the activation of Scg2 by the slow neurotransmitters dopamine and noradrenaline suggests that the modulation of Scg2 expression is complex. Interestingly, Scg2 was cooperatively activated by glutamate and dopamine; therefore, we characterize Scg2 as a candidate for the signal integrator of these synaptic inputs. However, it remains to be confirmed that the cooperative activation of Scg2 by glutamate and dopamine is executed in each individual neuron. While it cannot be definitively ruled out that glutamate and dopamine stimulate separate neurons, the possibility that all the neurons in the culture respond to only glutamate or dopamine is minor.

Interaction of the intracellular signals induced by glutamate and dopamine inputs seems to underlie a number of plastic changes in the CNS; however, it is a major challenge to demonstrate definitively the causal relationship between cellular biochemistry and behavioral physiology in a single neuron. Medium spiny neurons (MSNs) in the dorsal striatum and the ventral striatum (the nucleus accumbens) are characterized by dense corticostriatal/thalamostriatal glutamatergic and mesostriatal dopaminergic inputs (for review, see David et al. 2005), and have served as a model system for investigation of the consequences of these in-phase inputs. One group of MSNs predominantly expresses D1-type dopamine receptors (D1Rs), while the other group expresses D2Rs. Murine D1R and D2R MSNs in the dorsal striatum (Bateup et al. 2010; Hikida et al. 2010; Kravitz et al. 2010), as well as in the nucleus accumbens (Beutler et al. 2011; Pascoli et al. 2012), play distinctive roles in physiological and addictive behaviors. In striatal D1R and D2R MSNs, dopaminergic inputs are required for the induction of NMDAR-dependent LTP and mGluR5-dependent LTD, respectively (Shen et al. 2011). In the CA1 region of the hippocampus, the NMDAR-dependent LTP can be separated into a protein synthesis-independent transient early LTP and a protein synthesis-dependent prolonged late LTP; the latter requires dopaminergic inputs on D1/D5Rs (Navakkode et al. 2007; for review, see Lisman et al. 2011). It remains to be determined whether Scg2 activation by glutamate and dopamine is involved in these plasticities. Intracellular glutamatergic and dopaminergic signals can converge at multiple steps in their signaling pathways. First, D1R directly interacts with the NMDAR subunits NR1 and NR2A (Lee et al. 2002; Fiorentini et al. 2003), and D2R interacts with the NMDAR subunit NR2B (Liu et al. 2006).
Second, the activation of ERK, a MAPK critical for long-term synaptic changes, requires the simultaneous stimulation of D1R and NMDAR in the striatum (Valjent et al. 2005). D1R stimulation leads to cAMP/PKA pathway activation followed by inactivation of the phosphatase cascade of protein phosphatase-1 and striatal-enriched tyrosine phosphatase, allowing effective phosphorylation of ERK by MEK in response to elevation of intracellular levels of Ca\(^{2+}\) channeled via NMDAR. Third, in the regulation of genes that are activated during plastic changes, such as bdnf and c-fos (Flavell and Greenberg 2008; Day and Sweatt 2011), multiple transcriptional and epigenetic regulators can be targets of both glutamatergic and dopaminergic inputs. In this study, the activation of the Scg2 gene by either glutamate or dopamine was inhibited by the Ca\(^{2+}\) chelator BAPTA-AM or the MEK inhibitor PD98059. These results suggest that at least one converging point of glutamatergic and dopaminergic signals is located within the functional realm of the second messenger Ca\(^{2+}\) and its downstream Ras/Raf/MEK/ERK pathway. Ca\(^{2+}\) mobilization can exhibit differential effects on gene expression, depending on the mobilization route (Greer and Greenberg 2008). Binding of glutamate to NMDAR and \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA receptor) causes respectively direct and indirect Ca\(^{2+}\) influx via depolarization-triggered opening of L-type VGCC, while GluR2-lacking AMPAR can also conduct direct Ca\(^{2+}\) influx (Jonas et al. 1994). The present study suggests that NMDAR is involved in Scg2 activation (Fig. 5b), although the roles of other glutamate receptors remain to be examined.

Fig. 8 Effects of cycloheximide and the proteasome inhibitor ZLLLH on Scg2 mRNA induction. Northern blot analysis for Scg2 mRNA was performed with total RNAs isolated from mouse hippocampal primary cultured cells treated with neurotransmitters in the presence of 10 \(\mu\)g/mL cycloheximide (CHX) (a) or 1 \(\mu\)M ZLLLH (b) combined with 10 \(\mu\)M KT5720 (KT). The cells were treated with glutamate (G), dopamine (D), or noradrenaline (N) for 6 h. In controls (C), cells were treated with vehicles. Thirty minutes prior to stimulation with neurotransmitters, cells were treated with the indicated inhibitors or vehicles (−). Representative chemiluminograms for Scg2 and Gapdh mRNAs are shown at the top. Below, the quantified relative Scg2 mRNA levels are presented as mean ± SEM (n = 3–8). **p < 0.01, *p < 0.05; n.s., not significant (Tukey’s HSD test).
postulating a candidate mechanism underlying the signal integration of glutamatergic and dopaminergic inputs. Further investigations are required to clarify how dopamine mobilizes Ca\(^{2+}\) in our experimental system.

Characteristically, Scg2 seems to be under the control of a transcriptional repressor(s) that is potentiated by the KT5720-sensitive pathway in response to dopamine and noradrenaline (Fig. 7). Previous studies (Scammell and Valentine 1994; Jones and Scammell 1998) that examined the rat pituitary cell line GH\(_4\)C\(_1\) have also suggested the presence of a cycloheximide-sensitive repressor for basal and forskolin-induced Scg2 expression, and its relationship with the putative repressor of this study remains to be examined. The present KT5720- and cycloheximide-sensitive repressor seems to be different from ICER (Sassone-Corsi 1998; Mioduszewska et al. 2003), which had an abundance that was below the detectable level in our experimental system (data not shown). The putative repressor(s) is apparently short-lived and/or requires de novo protein synthesis (Fig. 8a), and is degraded at least in part by proteasomes (Fig. 8b), thus exhibiting properties suitable for a sensitive regulatory point in neuronal responses to the synaptic inputs. It is tempting to speculate that the putative repressor(s) ceases the Scg2 activation caused by in-phase glutamate and dopamine inputs via the Ca\(^{2+}\) pathway. Accumulation of the repressor protein to an effective level may require a lag time compared to the Ca\(^{2+}\)-mediated immediate Scg2 transcriptional activation by protein modifications such as phosphorylation. Thus, repressor accumulation may determine the time window permissive to Scg2 activation in response to the in-phase glutamate and dopamine inputs. Without such a programmed repression mechanism, sporadic glutamate or dopamine inputs after the simultaneous inputs might cause unexpected Scg2 reactivation, thus disturbing the signal integration system. The identification and characterization of this repressor will help to uncover the unique regulatory mechanisms of Scg2.

Taken together, the results of this study led us to propose the working model illustrated in Fig. 9. In this model, glutamate inputs mobilize Ca\(^{2+}\) via NMDAR and/or other routes. Dopamine inputs also activate the same and/or distinct Ca\(^{2+}\) mobilization pathway(s). Thus, Ca\(^{2+}\) itself, or its downstream factor leading to the Ras/Raf/MEK/ERK pathway, constitutes a part of the signal integration system of glutamate and dopamine inputs directed to Scg2 activation. The involvement of cAMP in the mobilization of Ca\(^{2+}\) in response to dopamine remains to be confirmed. We propose that the dopamine-activated cAMP signaling diverges to the KT5720-sensitive kinase pathway leading to the accumulation of repressor protein(s), limiting the time window open to Scg2 activation by the in-phase glutamate and dopamine inputs. This programmed repression system prevents accidental Scg2 activation by sporadic glutamate or dopamine inputs, and increases the signal-to-noise ratio for specific activation by the simultaneous inputs. Thus, the regulatory system of Scg2 expression is equipped with machinery that is refined for the signal integration of in-phase synaptic inputs. Further studies are required to confirm this hypothetical mechanism, and to clarify its physiological roles in neuronal plasticity such as associative learning and memory.

**Acknowledgements**

This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Science, Sports, and Technology of Japan, and by a grant from the Kashiwado Memorial Foundation for Medical Research. We thank Ayano Oohira for help in sequence analysis and members of our laboratories for their advice. The authors declare no conflicts of interest.

**Supporting information**

Additional supporting information may be found in the online version of this article at the publisher’s web-site:
Figure S1. Effects of the CaMK inhibitor KN-93 on Sceg2 mRNA induction.

Table S1. Genes whose mRNA levels changed in response to TEA in cDNA microarray analysis.

Table S2. Genes whose mRNA levels changed in response to db-cAMP in cDNA microarray analysis.

References


