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NMDA-Receptor Activation Induces Calpain-Mediated β-Catenin Cleavages for Triggering Gene Expression

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SUMMARY

The canonical Wnt-β-catenin signaling pathway is important for a variety of developmental phenomena as well as for carcinogenesis. Here, we show that, in hippocampal neurons, NMDA-receptor-dependent activation of calpain induced the cleavage of β-catenin at the N terminus, generating stable, truncated forms. These β-catenin fragments accumulated in the nucleus and induced Tcf/Lef-dependent gene transcription. We identified *Fosl1*, one of the immediate-early genes, as a target of this signaling pathway. In addition, exploratory behavior by mice resulted in a similar cleavage of β-catenin, as well as activation of the Tcf signaling pathway, in hippocampal neurons. Both β-catenin cleavage and Tcf-dependent gene transcription were suppressed by calpain inhibitors. These findings reveal another pathway for β-catenin-dependent signaling, in addition to the canonical Wnt-β-catenin pathway, and suggest that this other pathway could play an important role in activity-dependent gene expression.

INTRODUCTION

Activity-dependent gene expression in neurons is important for their development and survival as well as for synaptic plasticity (Deisseroth et al., 2003; Kandel, 2001; West et al., 2002). Several transcription mechanisms have been shown to be involved in this process (West et al., 2002). However, how neural activity is transduced to the gene-transcription machinery remains largely unclear.

One of the well-known signaling systems for regulating gene expression is the canonical Wnt-β-catenin pathway (Moon et al., 2004). In this signaling pathway, regulation of the cytosolic β-catenin level is a key event. In the absence of Wnt signals, a constitutively active kinase, GSK-3β, phosphorylates the N-terminal region of cytosolic β-catenin, leading to the subsequent ubiquitination and proteasome-mediated degradation of β-catenin (Aberle et al., 1997; Liu et al., 2002; Rubinfeld et al., 1996). Once Wnt signals are activated, on the other hand, these proteolytic processes are suppressed due to the inhibition of GSK-3β activity. Then, the stabilized β-catenin is translocated into the nuclei, where it associates with the transcription factor Tcf/Lef and thereby activates gene transcription. The β-catenin-Tcf system is essential for a wide variety of developmental phenomena (Logan and Nusse, 2004) and is involved in carcinogenesis (Polakis, 2000), but its physiological role in mature neurons is not well understood. Nevertheless, malactivation of β-catenin-Tcf-mediated gene regulation has been reported for various neurological diseases, including Alzheimer's disease (Chong et al., 2005; De Ferrari and Inestrosa, 2000) and bipolar disorder (Gould and Manji, 2002), implying that Tcf/Lef-mediated gene transcription plays a role in brain functions. The Wnt signaling system has alternative pathways, collectively called the noncanonical pathway (Montcouquiol et al., 2006), that are also important for various biological phenomena, such as cell polarity regulation; however, this pathway does not utilize β-catenin as a signaling mediator.

β-catenin works not only in the above signaling cascade but also in cadherin-mediated cell-cell adhesions. The cadherin-catenin complex is localized in synaptic junctions and is involved in synapse formation and stabilization (Salinas and Price, 2005; Takeichi and Abe, 2005). Synaptic localization of cadherins or catenins changes with synaptic activity (Abe et al., 2004; Murase et al., 2002; Okamura et al., 2004), and such changes may be important for the structural and functional plasticity of synapses (Huntley et al., 2002; Murase and Schuman, 1999; Okamura et al., 2004; Takeichi and Abe, 2005). However, how synaptic activity regulates the function of these adhesion proteins still remains an unresolved question.

The NMDA-R is an ionotropic glutamate receptor that plays an important role in synaptic plasticity (Malenka and Nicoll, 1993), a cellular mechanism for learning and memory (Nakazawa et al., 2004). Activation of the NMDA-R results in calcium influx. This NMDA-R-mediated calcium influx activates a variety of enzymes, including calpain, a calcium-dependent protease. In synapses, spectrin (Lynch and Baudry, 1987), NMDA-R2A and -R2B (Guttmann et al., 2001), GluR1 (Bi et al., 1996), PSD-95 (Lu et al.,

2000), and p35 (Kerokoski et al., 2004) have been demonstrated to be substrates of calpain-mediated cleavage. Moreover, inhibition of calpain activity was shown to suppress the formation of LTP (Staubli et al., 1988). These observations suggest that calpain plays an important role in both synaptic plasticity and neuronal degeneration (Chan and Mattson, 1999; Lynch and Baudry, 1984, 1987; Vanderklish et al., 1995, 2000).

In the present study, we examined whether neural activity had any effect on cadherin or catenins, focusing on NMDA-R-activity-dependent processes. When we activated the NMDA-R with glutamate in cultured hippocampal neurons, it resulted in N terminus truncations of β-catenin. This process was calpain dependent and caused β-catenin to become resistant to GSK-3β-mediated proteolysis. The stabilized β-catenin fragments accumulated in the nuclei, where they activated Tcf-mediated gene transcription. We further provide evidence that β-catenin-induced gene transcription also occurs in vivo. Thus, we found that two signaling pathways, the NMDA-R-dependent calpain activation and β-catenin-mediated gene regulation, merged together in neurons, forming a novel mechanism (to our knowledge) for activity-dependent gene expression.

RESULTS

Activity-Dependent Cleavages of β-Catenin by Calpain

We prepared hippocampal cultures and treated them with 10 μM glutamate for 30 min. Western blot analysis of cell lysates showed that this treatment caused the generation of two fragments of β-catenin (Figure 1A). These β-catenin fragments were recognized as 85 and 75 kDa bands by antibodies specific for the β-catenin C terminus but not by those raised against the N terminus, thus suggesting that the N terminus of β-catenin had been truncated. In contrast, glutamate treatment had no obvious effects on N-cadherin or αN-catenin. The 85 and 75 kDa β-catenin fragments represented ~5% of the total β-catenin in the cell lysate, as estimated by comparing the band intensities after serial dilutions of the samples.

The above cleavage of β-catenin was NMDA-R dependent, because pretreatment of neurons with the NMDA-R antagonist D-2-amino-5-phosphonopentanoate (APV, 250 μM) inhibited the β-catenin truncation, whereas neither the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM) nor the mGluR antagonist (+)-α-methyl-4-carbocyphenylglycine (MCPG, 50 μM or 1 mM) showed any effects (Figure 1B). On the other hand, treatment of neurons with the NMDA-R agonist NMDA (25 μM) caused the same cleavage of β-catenin, whereas the AMPA-R agonist AMPA (2 μM) or the group I mGluR agonist 3,5-dihydroxyphenylglycine (DHPG, 10 μM) was not effective (Figure 1B), confirming that the above phenomenon was NMDA-R specific. Electrical stimulation of neurons also produced similar β-catenin fragments (see

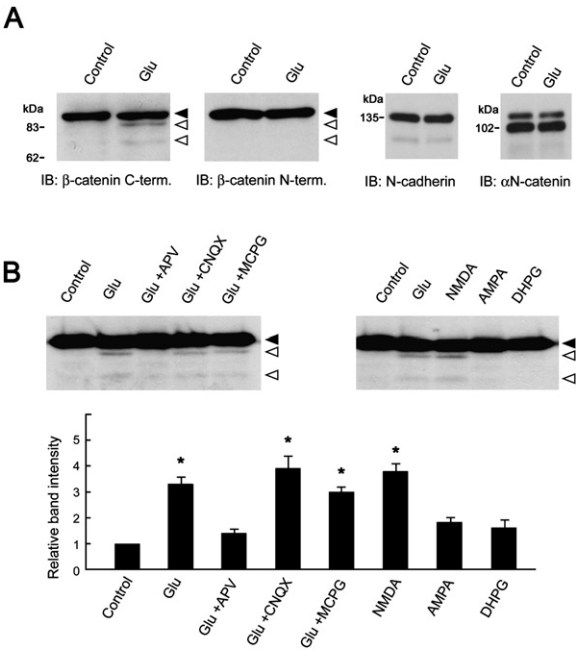

Figure 1. β-Catenin Cleavage by Glutamate Treatment

(A) Western blot detection of β-catenin, N-cadherin, and αN-catenin from the lysates of control or glutamate-treated neurons. Hippocampal cultures were treated with 10 μM glutamate for 30 min. Two different anti-β-catenin antibodies, which recognize the C terminus and N terminus, respectively, were used. Black arrowheads indicate full-length β-catenin, and open arrowheads indicate its 85 and 75 kDa fragments. Glu, glutamate (throughout the figures).

(B) Effects of glutamate receptor subtype-specific antagonists or agonists on β-catenin cleavage. Hippocampal cultures were pretreated with the indicated antagonists for 20 min and then stimulated with glutamate (10 μM) for 30 min. For agonist stimulation, the cultures were incubated with the reagents for 30 min. Bar graph, densitometric analysis of the relative band intensities of the 85 kDa fragment. Data are presented as the mean ± SEM. n = 4 independent experiments. Asterisks indicate a statistical difference against the control ($p < 0.01$, Tukey test).

Figure S1A in the Supplemental Data available online), although the efficacy was lower than that in the glutamate treatment, probably because we could stimulate only local portions of the culture, as contrasted with the case of glutamate experiments, in which all neurons could be exposed to glutamate.

Searching for proteases responsible for the cleavage of β-catenin, we identified calpain, a calcium-dependent protease, which is activated via NMDA-R activation (Vanderklish et al., 2000). Preincubation of neurons with the calpain inhibitor MDL28170 (20 μM) blocked β-catenin cleavage (Figure 2A). To test whether calpain directly cleaved β-catenin, we immunoprecipitated β-catenin and incubated the precipitate with recombinant μ-calpain. After incubation, we detected two β-catenin fragments with the same sizes as found in the glutamate-treated neurons (Figure 2B). The concentration of these fragments increased during prolonged incubation with μ-calpain,

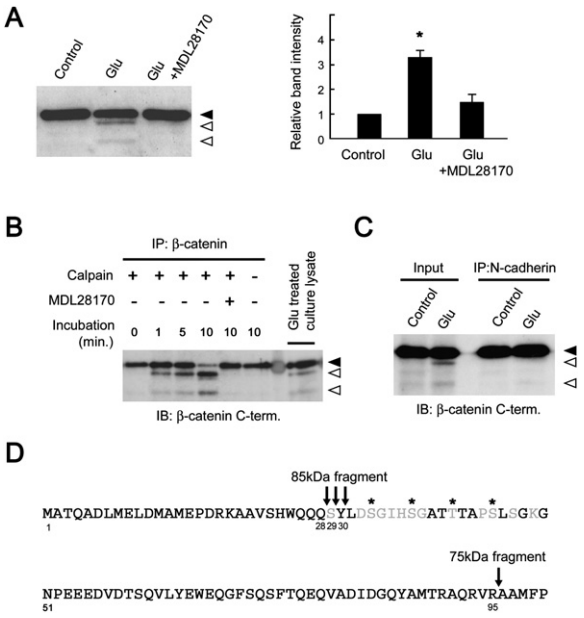

Figure 2. Calpain-Dependent Cleavage of β-Catenin

(A) Hippocampal cultures were treated with glutamate (10 μM) for 30 min in the absence or presence of the calpain inhibitor MDL28170 (20 μM). The inhibitor was added 20 min before the glutamate treatment. Bar graph, densitometric analysis of the relative band intensities of the 85 kDa fragment. Data are presented as the mean ± SEM. Asterisks indicate a statistical difference against the control ($p < 0.01$, Tukey test).

(B) β-catenin was immunoprecipitated with anti-β-catenin antibodies and incubated with μ-calpain during the indicated periods. The resulting fragments were detected with anti-β-catenin C terminus antibody.

(C) Hippocampal neurons were stimulated with glutamate (10 μM) for 30 min, and subsequently, N-cadherin was immunoprecipitated with anti-N-cadherin antibodies from their lysates. The resultant precipitates were analyzed with anti-β-catenin C terminus antibody.

(D) Cleavage sites (arrows) in the β-catenin N terminus identified by peptide sequencing. Sites phosphorylated by GSK-3β and CK1 are indicated by asterisks, and the amino acids that are mutated in some cancers are indicated in halftone (Polakis, 2000).

supporting the idea that calpain directly cleaves β-catenin. We also collected N-cadherin-catenin complexes by immunoprecipitation with anti-N-cadherin antibodies. Similar β-catenin cleavage fragments were observed when we incubated these complexes with the recombinant μ-calpain in vitro (data not shown), indicating that the cadherin-bound population of β-catenin is accessible to calpain. Interestingly, when the N-cadherin-catenin complexes were collected from the lysates of glutamate-treated neurons, they did not coprecipitate with the cleaved β-catenin fragments (Figure 2C). This observation suggests that these β-catenin fragments cannot stably associate with N-cadherin. While it remains possible that cadherin-associated β-catenin is resistant to calpain-mediated cleavage in vivo, the ability of μ-calpain to cleave N-cadherin-bound β-catenin in vitro supports the former possibility.

N terminus peptide sequencing of the calpain-cleaved β-catenin fragments revealed that the 85 kDa fragment was produced by the cleavage at one of the three contiguous sites corresponding to amino acids (aa) 28, 29, and 30 and that for the 75 kDa fragment the cleavage occurred at aa 95 (Figure 2D). Hereafter, the 85 kDa fragment is referred to as ΔN28, ΔN29, or ΔN30 β-catenin, and the 75 kDa fragment as ΔN95 β-catenin. The cleavage sites for generation of the larger three fragments reside next to sites of GSK-3β-mediated phosphorylation, and the ΔN95 β-catenin lost these sites, suggesting that their sensitivity to the GSK-3β-dependent proteolysis machinery might have been altered (Barth et al., 1997). We therefore tested the stability of these fragments by expressing them in L cells, in which the endogenous β-catenin is degraded due to the absence of any cadherin (Shibamoto et al., 1998). The results showed that GSK-3β-mediated phosphorylation (Liu et al., 2002) and subsequent ubiquitination (Aberle et al., 1997), which are generally observed in full-length β-catenin, were significantly reduced not only in ΔN95 but also in ΔN28 and ΔN30 β-catenins (Figure S2), suggesting that all these fragments had acquired a resistance to the GSK-3β-dependent degradation machinery.

Nuclear Translocation of Cleaved β-Catenin Fragments

β-catenin, stabilized by the canonical Wnt signaling, is known to move into the nuclei to participate in the transcriptional control of specific genes (Logan and Nusse, 2004; Moon et al., 2004). To test whether the NMDA-R-dependent stabilization of β-catenin would have a similar effect, we immunostained hippocampal neurons for β-catenin before or after glutamate treatment. In nontreated neurons, we did not detect any nuclear localization of β-catenin. However, shortly after the treatment, β-catenin became localized in the nuclei in more than 30% of pyramidal neurons in the culture (Figures 3A and 3B). This phenomenon was inhibited by preincubation of neurons with the NMDA-R antagonist, whereas neither AMPA-R nor mGluR antagonist was inhibitory (Figure 3B). The nuclear accumulation was also inhibited by preincubation of cells with the calpain inhibitor (Figure 2C). In addition, overexpression of an intrinsic calpain inhibitor, calpastatin (Goll et al., 2003), resulted in the complete suppression of nuclear β-catenin accumulation (data not shown). All these results demonstrate that NMDA-R and calpain were involved in the nuclear accumulation of β-catenin. Moreover, the nuclear β-catenin could not be recognized by the antibodies against its N terminus (Figure 3D), indicating that only the N terminus-truncated β-catenin had accumulated in the nuclei. Interestingly, the nuclear accumulation or the cleavage of β-catenin occurred even in the presence of a soluble Wnt antagonist, Dkk-1 (Glinka et al., 1998) (Figure S3). In addition, fragmentation of β-catenin could not be observed when neurons had been treated with Wnt3a, a Wnt expressed by the hippocampus (data not shown). These observations suggest that Wnt

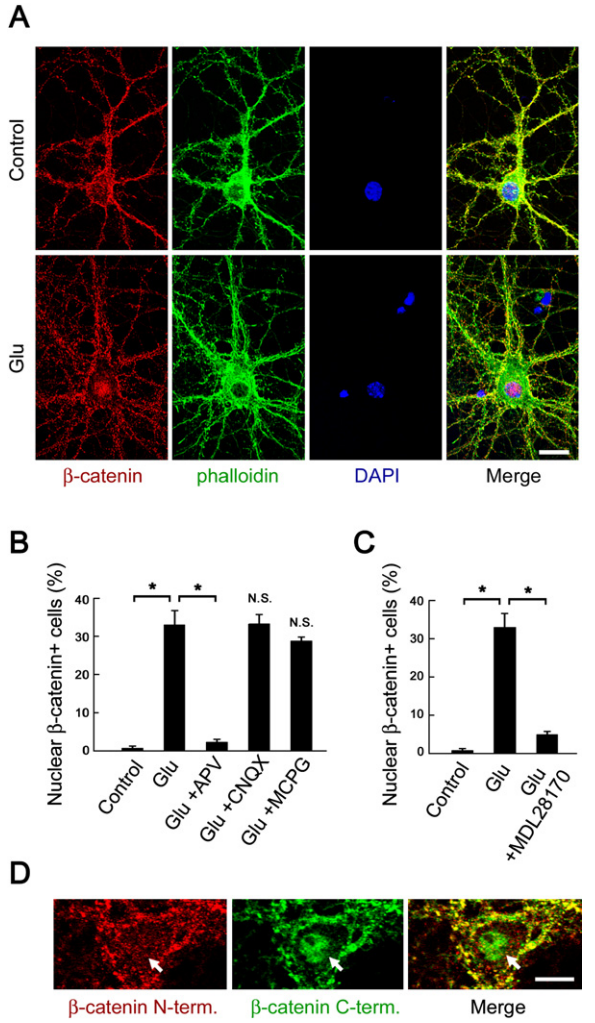

Figure 3. Nuclear Accumulation of β-Catenin after Glutamate Treatment

(A) Triple staining of control and glutamate-treated hippocampal neurons for F-actin (green), β-catenin (red), and DAPI (Blue). Cells were incubated for 30 min with 10 μM glutamate and fixed after 30 min of incubation in a new medium without glutamate. In nontreated cells stained for β-catenin, the nucleus appears dark contrasted with the surrounding positive signals, whereas, in glutamate-treated cells, nuclear signals have relatively increased. An anti-β-catenin C terminus antibody, 5H10, was used for detection.

(B and C) Quantification of the percentage of neurons showing nuclear accumulation of β-catenin. Using neurons immunostained as in (A), β-catenin immunofluorescence signals in the nucleus were compared with those in the cytoplasm, and the neurons showing higher fluorescence signals in the nucleus than in the surrounding cytoplasm were counted as "nuclear β-catenin-positive cells." Typical examples of the positive and negative cells are shown in (A), and cells exhibiting equal fluorescence intensity in the two regions are not included in the positive group. Data are presented as the mean ± SEM. n = 8 coverslips examined. Asterisks indicate a statistical difference ($p < 0.0029$, Dunnett test). Data for glu + CNQX- or MCPG-treated cultures were not significantly different (N.S.) compared with the value for the glutamate-treated culture ($p > 0.5$). Concentrations of the inhibitors used here are identical to those used in the experiments for Figures 1 and 2.

(D) Immunostaining with antibodies against N terminus (7D11; red) or C terminus (rabbit polyclonal ones; green) of β-catenin. Arrows indicate the nucleus, in which accumulation of β-catenin was recognized only by the anti-C terminus antibodies.

Scale bars, 20 μm.

ligands were not involved in the glutamate-induced nuclear localization of β-catenin.

Activation of Tcf-Dependent Gene Transcription by the β-Catenin Fragments

In the canonical Wnt-β-catenin signaling pathway, β-catenin activates Tcf/Lef-dependent gene expression after its relocation into the nucleus (Logan and Nusse, 2004; Moon et al., 2004). We therefore tested whether the glutamate-induced nuclear accumulation of β-catenin fragments could also do so. First, we cotransfected HEK293 cells with expression plasmids of the TOP-flash reporter construct, which expresses the firefly luciferase under the control of 3xTcf binding sites, and full-length or truncated β-catenins and found that not only full-length but also ΔN28, ΔN30, and ΔN95 β-catenin were able to activate Tcf-dependent gene transcription (Figure S4). Then, we doubly transfected neurons with a DsRed expression plasmid and TOP-*EGFP* plasmid, which expresses EGFP instead of luciferase (Sakai et al., 2005). At 12 hr after the transfection, we treated the cells with glutamate and measured the intensity of TOP-EGFP fluorescence in individual DsRed-positive cells. The results showed that the glutamate treatment increased the average intensity of EGFP fluorescence per neuron and that ~20% of neurons displayed a higher level of the fluorescence in glutamate-treated cultures than in untreated (control) ones when compared by setting a given threshold (Figure 4A). Thus, the proportions of neurons with nuclear β-catenin (Figure 3B) and with upregulated Tcf activity in a culture fell into a similar range.

The above increase of TOP-EGFP expression was suppressed by preincubation of the cultures with the calpain or NMDA-R inhibitor. The glutamate-mediated TOP-EGFP expression was also suppressed by cotransfection of neurons with DN-Lef1, a dominant-negative form of Tcf/Lef (Kengaku et al., 1998) (Figure 4A). These results support the idea that glutamate treatment can activate the Tcf/Lef-dependent transcription system. Electrical-field stimulation also activated Tcf/Lef, as well as induced nuclear localization of β-catenin (Figures S1B and S1C). Similar results were obtained by using hippocampal neurons collected from TOPGAL mice (DasGupta and Fuchs, 1999), which can respond to the β-catenin signaling by β-galactosidase expression (Figure S5).

We then tested the effect of coexpression of TOP-*EGFP* and ΔN95 β-catenin in neurons. These neurons strongly expressed TOP-*EGFP* without glutamate treatment (Figures 3B and 3C). As a control, we used a αN-catenin construct lacking the β-catenin binding region (Abe et al., 2004) and found that this construct had no effect. These results confirmed that the expression of the N

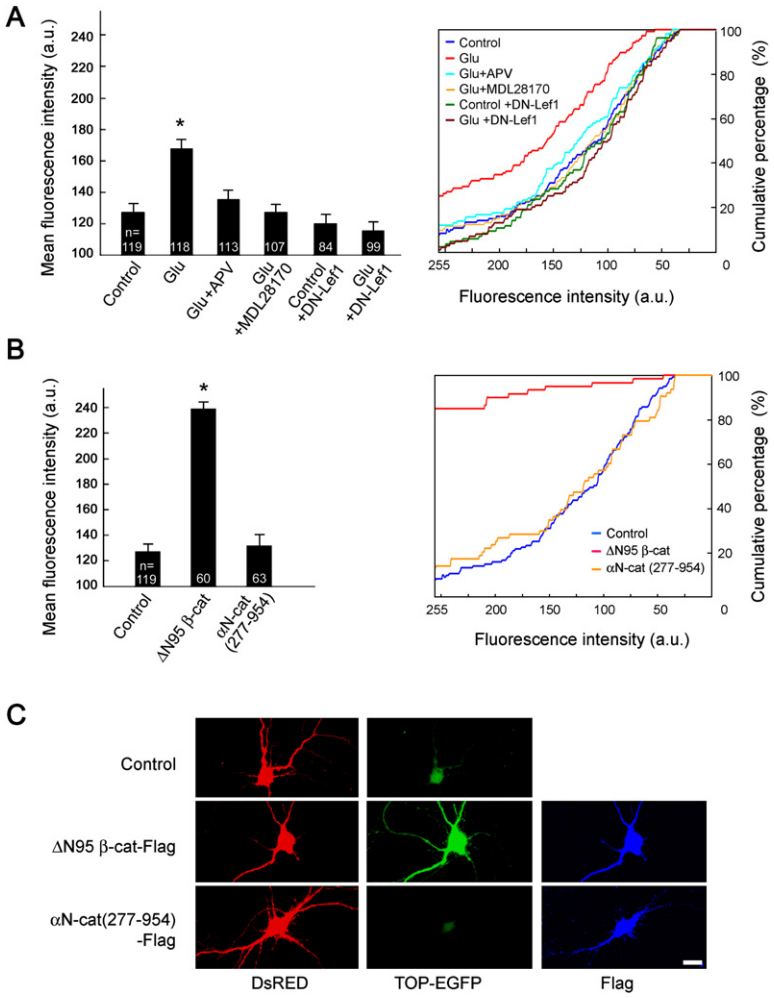

Figure 4. Activation of Tcf/Lef-Dependent Transcription by Glutamate

(A and B) Neurons were doubly transfected with DsRed expression plasmid and TOP-*EGFP* plasmid in a 2:1 ratio or were triply transfected with these two plasmids and DN-Lef1 or ΔN95 β-catenin expression plasmid in a 1:1:1 ratio, in which the amount of TOP-*EGFP* plasmid was equal in all the conditions. An αN-catenin construct lacking the β-catenin binding region (αN-277-954) (Abe et al., 2004) was used as a negative control. In (A), transfected cultures were treated with glutamate for 30 min and fixed after further incubation in a fresh medium without glutamate for 30 min. In (B), no glutamate treatment was performed. EGFP reporter was immunostained, and the fluorescence intensity of the EGFP in individual cells was measured. The average fluorescence intensity in each experimental condition and the cumulative percentage plot are shown. Data are presented as the mean ± SEM. n values from 4 independent experiments are indicated for each bar. Asterisks indicate a statistical difference against the control ($p < 0.01$, Tukey test in [A]; Steel test in [B]).

(C) Examples of neurons transfected with ΔN95 β-catenin or the control αN-277-954, which exhibit EGFP fluorescence near the average intensity, are shown. Scale bar, 20 μm.

terminus-cleaved β-catenin was sufficient to activate Tcf-dependent transcription in neurons.

To identify genes actually regulated by β-catenin in nuclei, we examined whether any of the genes known to be regulated by Wnt signaling (Nusse, 2006) responded to glutamate treatment. RT-PCR analysis of hippocampal cultures revealed that *Fosl1*, the gene encoding Fra-1, was upregulated by glutamate treatment and that this upregulation could be suppressed by pretreatment with the calpain inhibitor (Figure 5A). Furthermore, immunostaining for Fra-1 in hippocampal cultures showed that, after the glutamate treatment, some neurons exhibited increased levels of Fra-1 (Figure 5B). We measured immunofluorescence signals in individual neurons and found that the average intensity of fluorescence per neuron significantly increased in the glutamate-treated cultures (Figure 5C). Next, we separately quantified immunofluorescence signals in neurons with and without nuclear β-catenin, identified by double immunostaining for Fra-1 and β-catenin, in the glutamate-treated cultures and found that the former population of cells displayed significantly higher fluorescence intensities (Figure 5C), showing a correlation between Fra-1 upregulation and the nuclear relocation of β-catenin. Furthermore, preincubation with the calpain or NMDA-R inhibitors suppressed the upregulation of Fra-1 (Figures 5B and 5C), supporting the notion that calpain and NMDA-R-dependent nuclear translocation of β-catenin was involved in the observed upregulation of Fra-1.

In addition, we tested the effects of exogenous expression of ΔN95 β-catenin and found that it could increase the expression of endogenous Fra-1, even without glutamate stimulation (Figure 5D). Taken together, these results indicate that *Fosl1* may be one of the downstream genes regulated by calpain and β-catenin-dependent transcription in hippocampal neurons. Another early-responsive gene, *Fos*, the gene encoding c-*fos*, was also upregulated by glutamate treatment. However, this upregulation was not suppressed by the calpain inhibitor (Figure 5A), indicating that the upregulation of *Fosl1*, but not that of *Fos*, was specifically regulated by glutamate-dependent activation of calpain.

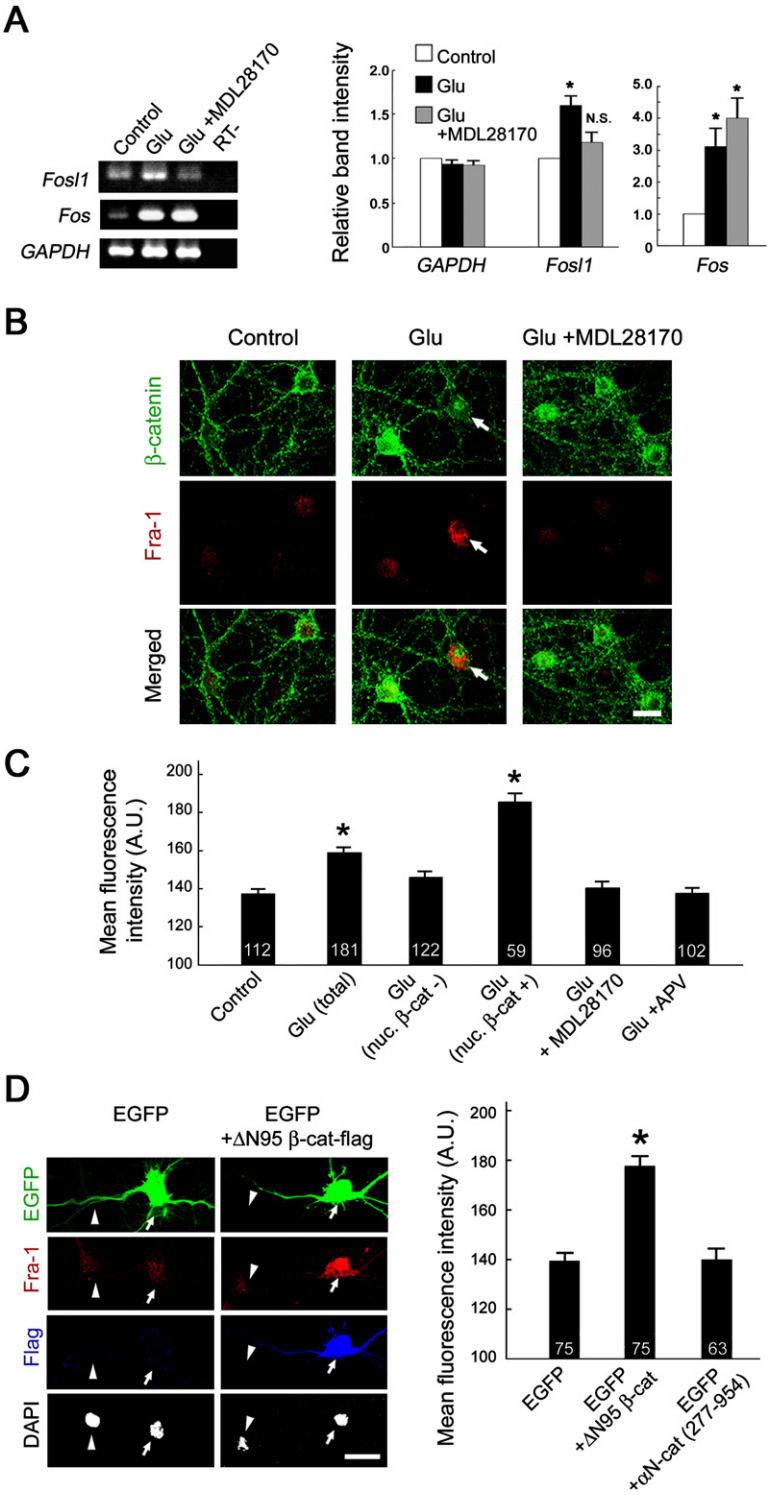

Figure 5. Induction of *Fosl1* Expression by Glutamate and Truncated β-Catenin

(A) RT-PCR analysis of hippocampal cultures treated as indicated. Bar graphs, quantification of the relative band intensity. n = 10 experiments for each.

(B) Immunostaining of control and glutamate-treated hippocampal cultures. Arrows indicate a neuron in which β-catenin (green) is localized in the nucleus, and simultaneously the expression of Fra-1 (red) is increased.

(C) Mean fluorescence intensity of Fra-1 immunostaining per neuron under different culture conditions. Glutamate-treated neurons (total) were subdivided into two groups, neurons with (nuc. β-cat +) and without (nuc. β-cat −) nuclear β-catenin accumulation. n values are indicated for each bar.

(D) Expression of endogenous Fra-1 (red) is upregulated in a neuron expressing ΔN95 β-catenin-Flag (blue), but not in the one not expressing this construct. Bar graph, mean fluorescence intensity of Fra-1 immunostaining per neuron. αN-277-954 was used as a negative control. n values are indicated for each bar. Arrows, transfected neurons; arrowheads, nontransfected neurons.

The conditions for glutamate or inhibitor treatments were identical to those in other experiments. Data are presented as the mean ± SEM in all panels. Asterisks indicate a statistical difference against the control ($p < 0.01$, Tukey test in [A]; $p < 0.0001$, Dunnet test in [C] and [D]). Scale bars, 20 μm.

Calpain-Dependent β-Catenin Cleavage and Tcf Activation In Vivo

We next asked whether the above system operates in vivo. Novelty exploration by animals is known to induce the expression of activity-dependent genes within 30 min (Guzowski et al., 1999; Ramanan et al., 2005; Vazdarjanova et al., 2002). We examined whether transferring adult mice, 8–12 weeks old, into a novel and environmentally enriched cage would alter β-catenin profiles. By 30 min after the transfer, we could detect the generation of β-catenin fragments, at least the ΔN28-29-30 form, by Western blotting of hippocampal (Figure 6A) and cortical (data not

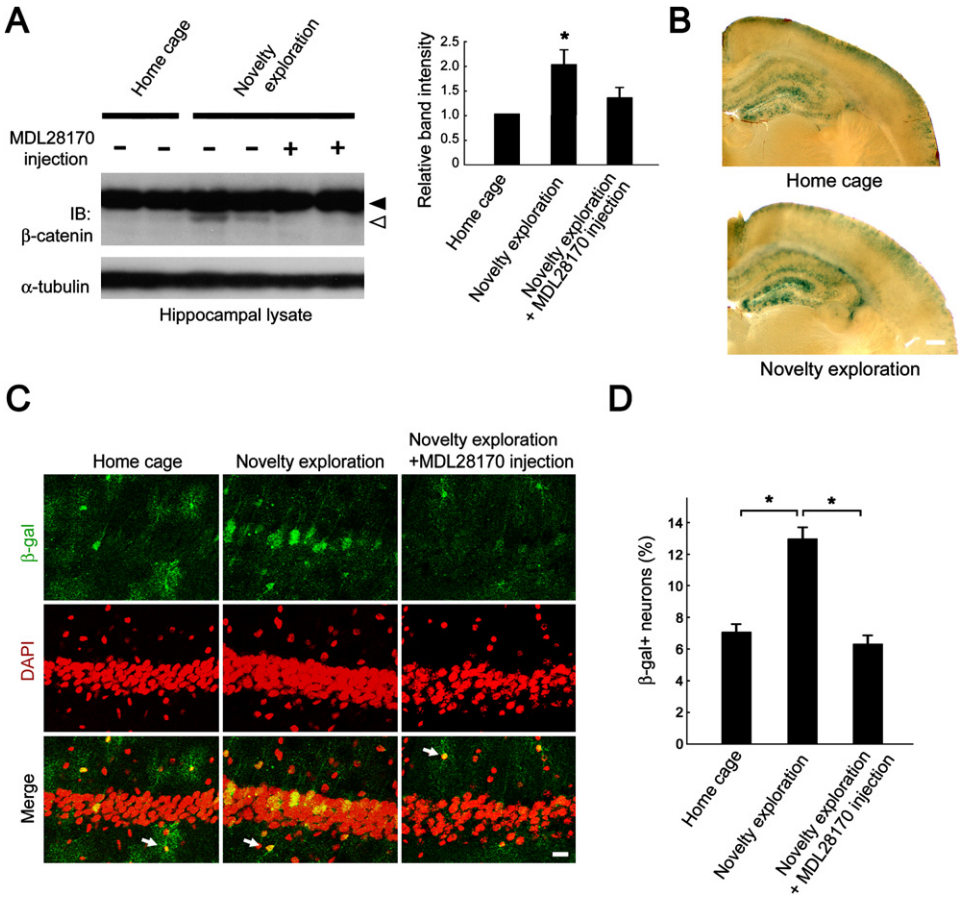

Figure 6. Neural Activity-Dependent Activation of Tcf-Mediated Transcription In Vivo

(A) Western blot analysis of hippocampal lysates collected from home-caged or novelty-exploring mice, injected or not with the calpain inhibitor MDL28170 2 hr before the novelty exploration test. α-tubulin was blotted as a loading control. For each condition, 16 animals in total were examined through eight independent experiments. The truncation of β-catenin was observed in at least four out of the 16 animals, only in the novelty-exploring group without the calpain inhibitor. Black arrowhead indicates full-length β-catenin, and open arrowhead indicates the 85 kDa β-catenin fragment. The latter band increased only after novelty exploration in the absence of MDL28170. Bar graph, densitometric analysis of the relative band intensities of the 85 kDa fragment. Data are presented as the mean ± SEM. n = 16 animals. Asterisk indicates a statistical difference against the control ($p < 0.019$, Steel test).

(B) X-gal staining of brain sections obtained from home-caged or novelty-exploring TOPGAL mice.

(C) Immunostaining for β-gal (green) and nuclei (DAPI, red) in the CA1 pyramidal layer of the hippocampus of TOPGAL mice. Arrows point to some of the β-gal-positive glial cells, which are present equally in both home-cage and novelty-exploration conditions.

(D) Quantification of the percentage of β-gal-positive neurons in the CA1 region. Data are presented as the mean ± SEM. n = 12 animals for each. Asterisks indicate a statistical difference between experimental groups ($p < 0.00025$, Dunnett test).

Scale bars, 200 μm in (B), 20 μm in (C).

shown) lysates. When mice had been intravenously injected with the calpain inhibitor MDL28170 2 hr prior to the novelty exploration test, these fragments did not appear. We then assessed whether the expression of a β-galactosidase reporter could be induced in TOPGAL mice in the novel environment. Adult TOPGAL mice were transferred to a novel and enriched cage or kept in their home cage as a control and sacrificed after 30 min. In both groups we could detect the expression of β-galactosidase in several regions of the brain, including the cortex and the hippocampus (Figure 6B). However, in the CA1 region of the hippocampus, the number of β-galactosidase-positive pyramidal cells had significantly increased after the exploratory behavior (Figure 6C). This induction was suppressed by injection of the calpain inhibitor 2 hr before the test (Figure 6C), although the injection of calpain inhibitor had no obvious effects on the locomotor activity of the mice (Figure S6). Taken together, these data indicate that activity-dependent stimulation of Tcf-dependent gene transcription also occurred in vivo, at least in CA1 pyramidal neurons.

DISCUSSION

Our results revealed a novel (to our knowledge) signaling mechanism controlling activity-dependent gene

expression. In cultured hippocampal neurons, NMDA-R-dependent activation of calpain resulted in the N terminus cleavage of β-catenin. Activation of calpain triggered by calcium influx is known to degrade a number of proteins, including pre- and postsynaptic components, and this process has been implicated not only in neurodegenerative processes but also in modulating synaptic plasticity (Chan and Mattson, 1999). We showed that β-catenin can be added to the list of calpain substrates localized in neurons. The resultant β-catenin fragments became resistant to the GSK-3β-dependent degradation machinery and accumulated in the nuclei. This process, in turn, activated Tcf-dependent gene transcription.

In the canonical Wnt signaling pathway, Wnt and GSK-3β act upstream of the β-catenin-Tcf-dependent gene regulation machinery, and this pathway is widely used for the regulation of gene expression required for developmental and carcinogenetic processes (Logan and Nusse, 2004; Moon et al., 2004; Polakis, 2000). The signaling mechanism described in the present study appears to have resulted from the substitution of the Wnt-GSK-3β signaling mechanisms by the NMDA-R-calpain system, leading to downstream activation of the β-catenin-Tcf-dependent pathway. This substitution allows neurons to utilize β-catenin as a mediator of activity-dependent gene expression, since the NMDA-R is a critical sensor of physiological stimuli that can induce plastic changes in neurons. On the other hand, we do not see any relations between this newly recognized β-catenin-dependent cascade and the noncanonical Wnt pathway because the latter system does not require β-catenin (Montcouquiol et al., 2006).

We identified *Fosl1* as a target gene of the NMDA-R-mediated β-catenin signaling system. This gene is a known target of the canonical Wnt signaling pathway (Mann et al., 1999), but our results indicate that *Fosl1* activation can also occur through the NMDA-R dependent β-catenin pathway. Notably, *Fosl1* was shown to be upregulated in the rodent brain following learning (Faure et al., 2006), supporting the idea that the NMDA-R-β-catenin signaling system is physiologically relevant. Other genes are likely activated by this system, and identifying such genes remains an important goal to aid our understanding of which neural processes are controlled by this signaling pathway. Interestingly, a recent study suggested that Wnt secretion might also be involved in the NMDA-R-dependent neural activity (Chen et al., 2006). Thus, the two β-catenin-dependent signaling systems, the canonical Wnt-dependent and NMDA-R-dependent ones, might represent two parallel mechanisms utilized in a larger signaling network, although additional studies are necessary to clarify how these different systems are coordinated to regulate neuronal functions. Of note, the 75 kDa β-catenin fragment was initially identified in some cancers (Rios-Doria et al., 2004), and thus, calpain-dependent activation of the β-catenin-Tcf signaling pathway may also operate in other cellular systems.

We provided evidence that the calpain-mediated β-catenin-Tcf signaling pathway also operates in vivo. Novelty exploration by mice resulted in calpain-dependent cleavage of β-catenin, as well as activation of the Tcf signaling pathway in hippocampal neurons. The overall amount of cleaved β-catenin detected after the novelty exploration appears small; however, the tissue lysates used for this analysis should have contained a large excess of cells that were nonresponsive to physiological stimuli and resulted in a high proportion of noncleaved β-catenin in these other cells. Although our preliminary analyses did not detect any behavioral changes in calpain-inhibitor-injected mice, it would be intriguing to perform more detailed analyses of brain functions in these mice, as it was reported that inhibition of calpain (Staubli et al., 1988) or β-catenin signaling (Chen et al., 2006) impairs long-term potentiation. In addition, although the details of the mechanisms involved have not yet been well elucidated, drugs that affect β-catenin stability, such as lithium and valproic acid, have been prescribed as mood stabilizers (Gould and Manji, 2002). Further, calpain malactivation (Chong et al., 2005; Zatz and Starling, 2005) and aberrant Wnt signaling (Chong et al., 2005; De Ferrari and Inestrosa, 2000; Gould and Manji, 2002, 2005; Moon et al., 2004) have been implicated in various neurological disorders, implying that dysfunction of β-catenin-dependent gene expression may be involved in neurological pathologies.

In addition to its critical role in gene regulation, β-catenin is also a component of the cadherin-catenin complex that is essential for the stability of synaptic junctions (Takeichi and Abe, 2005). We found that β-catenin in the cadherin-catenin complex could be cleaved by calpain, but β-catenin fragments were not detectable in the immunoprecipitated cadherin-catenin complexes. This suggests that the cleaved β-catenin is unable to stably associate with cadherin, resulting in the observed translocation into the nuclei. If calpain-mediated release of β-catenin from cadherin occurs excessively, it might reduce the amount of the functional cadherin-catenin complexes that are required to maintain normal synaptic junctions (Takeichi and Abe, 2005). Thus, the NMDA-R-dependent β-catenin cleavage could result in bidirectional effects, i.e., stimulation of Tcf-dependent gene transcription and structural modulation of synaptic junctions. Recent studies showed that ADAM10 and presenilins could cleave the cytoplasmic domain of cadherin and release it from the cell membrane in an activity-dependent manner (Marambaud et al., 2003; Reiss et al., 2005; Uemura et al., 2006). Such cadherin degradation may also enhance the translocation of β-catenin into the nuclei. Determining how these various activity-dependent cleavages of cadherin and catenins ultimately regulate neuronal function is an important subject for future study.

EXPERIMENTAL PROCEDURES

Cell Culture

Hippocampal cultures were prepared from E17 wild-type or TOPGAL mice (DasGupta and Fuchs, 1999) (Jackson Laboratory) as described (Abe et al., 2004) and analyzed at 20–24 DIV. For reporter assays and

endogenous *Fosl1*-induction experiments, neurons were transfected at 10 DIV using Effectene (Qiagen) as described (Abe et al., 2004) and analyzed after 12 hr. For glutamate treatment, 10 μM glutamate was applied to the medium and incubated for 30 min. After the incubation, the whole medium was replaced with fresh medium lacking glutamate, and the cultures were further incubated prior to analyses. For calpain inhibition, MDL28170 (20 μM, Sigma) was added to the medium 20 min before glutamate treatment. L cells were maintained in DMEM/F-12 medium (Iwaki) supplemented with 10% fetal-calf serum and 2.5 mM glutamine.

Immunoprecipitation and In Vitro Calpain Assay

P0 brains were lysed with an immunoprecipitation buffer (50 mM Tris-HCl [pH 7.5], 10% glycerol, 150 mM NaCl, 1% NP-40, and protease inhibitor cocktail [Roche]), the supernatants were subjected to immunoprecipitation with anti-β-catenin antibody, and the immunoprecipitates were collected with Protein G Sepharose (GE Healthcare). Immunoprecipitation of N-cadherin from cultured hippocampal cell lysates was done using the same solutions and reagents as above. For in vitro cleavage by calpain, the immunoprecipitates were incubated at room temperature with purified μ-calpain (Calbiochem) in the immunoprecipitation buffer supplemented with 5 mM DTT and 1 mM $CaCl_2$. For determination of the cleavage sites, bacterially expressed recombinant GST-β-catenin was constructed and incubated with μ-calpain under the same conditions. The resultant products were separated by SDS-PAGE, and the bands were cut out and then subjected to N terminus peptide sequencing (Aproscience). Immunoprecipitation from L cell lysates was carried out by using RIPA buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail, and phosphatase inhibitor cocktail [Sigma]).

Immunostaining

Immunostaining of cells was performed as described earlier (Abe et al., 2004). For the immunostaining of brain slices, mice were sacrificed, and their brains were then immediately fixed with ice-cold 2% PFA for 90 min. Coronal sections (60 μm thick) were made with a vibratome and postfixed for 60 min at 4°C. Free-floating sections were blocked in TBS with 5% BSA and 0.3% Triton X-100 at 4°C overnight and incubated sequentially with first antibodies at 4°C for 48 hr and with secondary Alexa-conjugated antibodies (Invitrogen) at 4°C overnight. The sections were mounted with Fluosave (Calbiochem) supplemented with 4′,6′-diamidino-2-phenylindole (DAPI). Images were acquired with the LSM510 META multiphoton confocal system (Zeiss), and fluorescence intensity was analyzed by using LSM510 software. For X-gal staining, coronal sections (180 μm thick) were fixed with an X-gal fixative (1× PBS, 1% PFA, 0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM EGTA, 0.05% NP-40) for 20 min and washed three times with a washing buffer (1× PBS, 2 mM $MgCl_2$, 0.1% NP-40). The sections were then stained with an X-gal staining solution [1× PBS, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 1 mg ml^{-1} X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside)] for 24 hr at 37°C. Images were acquired with a Leica M420 microscope, equipped with a Leica DC500 CCD.

Antibodies and Reagents

We used mouse monoclonal antibodies against β-catenin N terminus region (7D11; Calbiochem), β-catenin C terminus (5H10; a gift from M.J. Wheelock, University of Nebraska, Omaha, NE), α-tubulin (DM1A; Sigma), β-galactosidase (Promega), Flag-tag (M2; Sigma), and ubiquitin (6C1.17; BD Bioscience); and rabbit polyclonal antibodies against β-catenin C terminus (SIGMA), Fra-1 (Santa Cruz), GFP (Chemicon), and phospho-Ser33/37/Thr41-β-catenin (Cell Signaling). F-actin was visualized with Alexa-488-conjugated phalloidin (Invitrogen), and nuclei were stained with DAPI. APV, NMDA, AMPA, DHPG, MDL28170, and MG132 were purchased from Sigma; CNQX and MCPG were from Calbiochem; and recombinant Wnt3a and Dkk-1 were from R&D systems. TOP-flash and FOP-flash reporter plasmids were from Upstate.

Plasmid Construction

For the construction of the expression plasmid for ΔN28 β-catenin, amino acids 29–781 of the β-catenin coding region were amplified by PCR with a primer set of 5′- gcggccgccaccatgtcttacttggattctggaat-3′ and 5′-gtcgaccaggtcagtatcaaaccag-3′ (βcat-C primer), and subcloned into pCA-Sal-flag, as described (Abe et al., 2004). Expression plasmids for ΔN30 and ΔN95 β-catenin were constructed in the same way with primer sets of 5′-gcggccgccaccatgttggattctggaatccatt-3′ and the βcat-C primer for ΔN30 β-catenin; and 5′-gcggccgccaccatggctgcc atgttccctgagac-3′ and the βcat-C primer for ΔN95 β-catenin. All the constructs were checked by sequencing.

RT-PCR

From hippocampal cultures (at 18–21 DIV) treated with glutamate for 60 min, total RNA was extracted by using RNAeasy (Qiagen). cDNAs were synthesized by using a SuperScript III kit (Invitrogen). One nanogram of the resultant first-strand cDNAs were subsequently used for PCR analysis with the following primers: *Gapdh*, 5′-agaaggtggtgaagc aggca-3′ and 5′-cgaaggtggaagagtgggag-3′; *Fosl1*, 5′-cacctagacagaa ggtgcccttt-3′ and 5′-ctcctgcgttgtgccatt-3′; *Fos*, 5′-acctcccgctctgtgcc agatgtg-3′ and 5′-ttgctgctgctgccctttcggtgg-3′. PCR was carried out under 25 cycles at 94°C, 60°C, and 72°C, each for 30 s. The number of the cycles was determined so that the resultant products were amplified within the linear range. The intensity of PCR and Western blot bands was analyzed by using Image-J.

Novelty Exploration Assay

Adult TOPGAL mice, 8–12 weeks old, were habituated to the laboratory overnight and transferred into new, enriched cages or kept in their home cages as a control. The mice were sacrificed 30 min after the transfer (Ramanan et al., 2005). For injection of the calpain inhibitor, MDL28170 was dissolved in PEG300/EtOH (9:1) and diluted 1:1 in saline. Mice were anesthetized and subsequently injected via a tail vein with 30 mg kg^{-1} of the solution (Neumar et al., 1998). For behavioral analysis, at 2 hr after calpain-inhibitor injection, mice were transferred to novel cages (45 cm × 25 cm), and their locomotion was recorded by using a video camera for 30 min. The moving distances of the mice were analyzed automatically with a Scion Image macro.

Statistical Analysis

Statistical analyses used in this study are indicated in each figure legend.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/53/3/387/DC1/.

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