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Decoding NMDA Receptor Signaling: Identification of Genomic Programs Specifying Neuronal Survival and Death

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SUMMARY

NMDA receptors promote neuronal survival but also cause cell degeneration and neuron loss. The mechanisms underlying these opposite effects on neuronal fate are unknown. Whole-genome expression profiling revealed that NMDA receptor signaling is decoded at the genomic level through activation of two distinct, largely nonoverlapping gene-expression programs. The location of the NMDA receptor activated specifies the transcriptional response: synaptic NMDA receptors induce a coordinate upregulation of newly identified pro-survival genes and downregulation of pro-death genes. Extrasynaptic NMDA receptors fail to activate this neuroprotective program, but instead induce expression of *Clca1*, a putative calcium-activated chloride channel that kills neurons. These results help explain the opposing roles of synaptic and extrasynaptic NMDA receptors on neuronal fate. They also demonstrate that the survival function is implemented in neurons through a multicomponent system of functionally related genes, whose coordinate expression is controlled by specific calcium signal initiation sites.

INTRODUCTION

A single neurotransmitter receptor, the N-methyl-D-aspartate (NMDA)-type of glutamate receptors, controls both the physiology and pathology of neurons (Hardingham and Bading, 2003). NMDA receptors promote neuronal survival, trigger changes in synaptic efficacy and neuronal network behavior, and are central to the process that leads to information storage and memory formation in the brain (Hardingham and Bading, 2003; Milner et al., 1998). However, the same receptor can also kill neurons: NMDA receptor activation is the primary cause of neuronal cell death associated with cerebral ischemia, head injury, and seizure. Moreover, NMDA receptors have been implicated in the etiology of several neurodegenerative disorders, including Huntington's disease and Alzheimer's disease (Hardingham and Bading, 2003; Choi, 1992; Lipton and Rosenberg, 1994; Bossy-Wetzel et al., 2004).

NMDA receptors are calcium-permeable ion channels that affect intracellular biochemical processes through the activation of calcium signaling pathways (Hardingham and Bading, 2003). It has been suggested that differences in the degree of receptor activation (giving rise to different size calcium transients) account for the directly opposing effects on cell fate, with low or moderate stimulation of the receptor promoting survival and overactivation inflicting neuronal damage (Hardingham and Bading, 2003; Choi, 1992; Lipton and Rosenberg, 1994; Bossy-Wetzel et al., 2004). However, other experiments indicate that survival-promoting signals derive from NMDA receptors that are localized to synaptic contact, whereas cell-death pathways are triggered by extrasynaptic NMDA receptors, suggesting that receptor localization rather than its degree of activation determines the cellular consequences (Hardingham et al., 2002). In addition to the controversy about the precise nature of the early signaling events that decides the neuronal fate, the downstream machinery that guides neurons toward survival or death is unexplored.

Because neuronal calcium transients activate signaling pathways (such as the ERK-MAP kinase and the CaM kinases pathways) that transduce information to the cell nucleus and modulate the activity of transcription factors (Bading and Greenberg, 1991; Bading et al., 1993; Bading, 2000; West et al., 2001), we reasoned that differences in genomic responses may account for the differences in the biological outcome. The method of choice to obtain a comprehensive picture of the transcriptome involves the use of DNA microarrays (DeRisi et al., 1997; Hughes et al., 2000). This technology has helped identify functional genetic modules in inflammatory responses (Calvano et al., 2005) and uncovered signature patterns of gene expression that, for example in cancer or diabetes, correlate

with disease progression, prognosis, or treatment responses (Lamb et al., 2003; Rhodes and Chinnaiyan, 2005; Segal et al., 2005; Mootha et al., 2003). Whole-genome profiling of hippocampal neurons revealed an unexpected difference in the transcriptional responses triggered by calcium flux through synaptic versus extrasynaptic NMDA receptors. The decoding of NMDA receptor signaling uncovered gene programs for neuroprotection and cell death that explain at the genomic level the opposing roles of synaptic and extrasynaptic NMDA receptors on cell fate.

RESULTS

The Type of Electrical Stimulation Specifies the Transcriptional Responses

Whole-genome transcriptional profiling was done using cultured hippocampal neurons derived from newborn mice that, after a culturing period of 10–12 days, form an elaborate and highly interconnected neuronal network. We focused on two stimulation protocols to electrically activate the neurons. One protocol leads to induction of bursts of action potential (AP) firing, which induces a long-lasting, transcription-dependent form of synaptic plasticity in the network (Arnold et al., 2005) and activates a robust neuronal survival program (Hardingham et al., 2002; Papadia et al., 2005); the second stimulation paradigm was bath-applied glutamate that can initiate cell-death pathways (Hardingham et al., 2002; see below). To trigger bursts of AP firing, we take advantage of the presence in the network of inhibitory (GABAergic) interneurons. GABAergic interneurons impose a tonic inhibition onto the network through activating $GABA_A$ receptors. Removal of $GABA_A$ergic inhibition with the $GABA_A$ receptor antagonist bicuculline results in the firing of bursts of APs (Hardingham et al., 2002; Arnold et al., 2005); this activates calcium entry through synaptic NMDA receptors, generates calcium transients in the cytoplasm and the nucleus, and stimulates expression of several CREB-regulated genes, including *c-fos* and brain-derived neurotrophic factor (*Bdnf*) (Hardingham et al., 2001, 2002). Changes in gene expression were assessed at 2 and 4 hr after exposure to bicuculline. Bath application of glutamate also activates calcium entry through NMDA receptors (Hardingham et al., 1999, 2002; Bading et al., 1995), but in contrast to the AP bursting, calcium enters the neurons also through extrasynaptic NMDA receptors (Hardingham et al., 2002). Glutamate treatment evokes global calcium transients and stimulates gene expression mediated, for example, by the serum response element (SRE) or by stimulating the coactivator CREB-binding protein (CBP) (Bading et al., 1993; Hardingham et al., 1999); it differs from bicuculline-induced AP bursting inasmuch as it triggers a CREB shut-off pathway (Hardingham et al., 2002; Sala et al., 2000). Glutamate stimulation of extrasynaptic NMDA receptors can also initiate cell-death pathways, which at high glutamate concentrations leads to failure of mitochondrial functions resulting in acute necrosis (Hardingham et al., 2002).

For the transcriptional profiling, we used a low concentration of glutamate (20 μM), which does trigger CREB shut off but does not cause acute necrotic cell death (data not shown). Changes in gene expression were assessed at 2 and 4 hr after exposure to glutamate. To identify genes controlled by NMDA receptor activation, the stimulations were done in the presence of the NMDA receptor blockers MK-801 and APV. MK-801 is a noncompetitive antagonist (Wong et al., 1986) and was chosen in those experiments in which synaptic NMDA receptors were activated by AP bursting (Hardingham et al., 2001). APV, a competitive NMDA receptor antagonist, was used to inhibit NMDA receptor stimulation following bath-glutamate application (Bading et al., 1993, 1995). For all experiments, the Affymetrix GeneChip Mouse Expression Set 430 (MOE 430A and MOE 430B) was used, which allows analysis of a total of 45,101 probe sets representing genes and ESTs belonging to 20,283 UniGene clusters (according to annotations from Affymetrix of July 12, 2006). Probe sets were classified as "upregulated" or "downregulated" if, compared to control, the sample mean of expression at a given time point is larger or smaller, respectively, by a factor of at least 1.5 ($p \leq 0.025$, two-tailed Welch t test). A 1.5-fold change was chosen as the threshold because a comparison of gene-expression data of known activity-regulated genes (i.e., *c-fos* and *Bdnf*) and newly identified activity-regulated genes obtained with either microarrays or quantitative reverse transcriptase (QRT)-PCR revealed that the microarray analysis underestimates the fold differences in gene expression (see Figure 4 and data not shown; Irizarry et al., 2003). Thus, by using a threshold of 1.5-fold for the microarray analysis, we are filtering out genes that are likely to undergo signal-induced changes in their expression that are in the range of at least 2- to 3-fold.

For each stimulation paradigm, we obtained lists of upregulated and downregulated probes sets. We identified 478 unique genes (625 probe sets) that were induced and 399 unique genes (435 probe sets) that were repressed following bicuculline-induced AP bursting. The number of glutamate-regulated genes (induced 106 unique genes [120 probe sets]; repressed 51 unique genes [52 probe sets]) was slightly lower. A color-coded map, which provides an overview of all electrical activity-regulated unique genes (Figure 1), illustrates that many genes were induced or repressed specifically with one stimulation paradigm. Table S1 (see the Supplemental Data available with this article online) contains all genes regulated by AP bursting and/or bath-glutamate treatment. A comparison of the genes identified in this study with the genes identified in a study by Hong et al. (2004) revealed very limited overlap. This may be primarily due to the differences in the time points after stimulation at which gene expression was analyzed but may also result from differences in the methods, the stimulation paradigms, and cell types used.

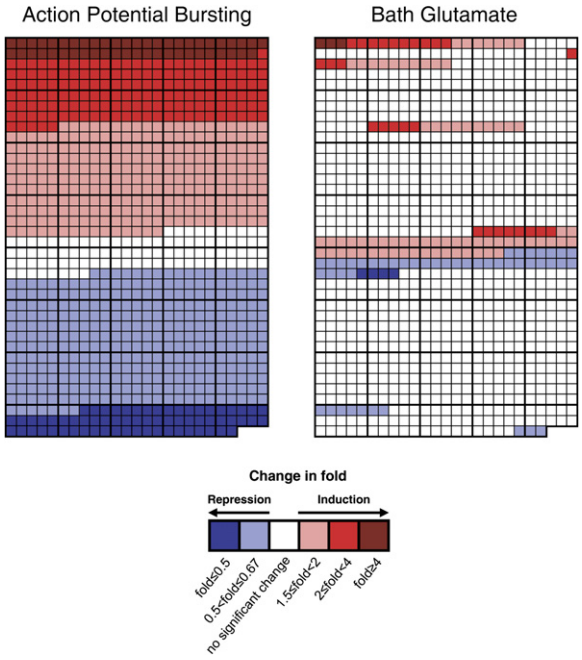

Figure 1. Color-Coded Map of All Genes Identified as Electrical Activity-Regulated Genes in Hippocampal Neurons

The maximum fold change in expression (induction or repression) based on Affymetrix microarray analysis at 2 or 4 hr after stimulation is color coded as indicated; for genes, which are represented with more than one probe set, only the probe set with the largest absolute effect is shown. White indicates no change upon stimulation. Genes within the AP-bursting group are sorted on the basis of their expression values in a "descending" order, i.e., from the highest levels of induction at the top (indicated in red colors) to the highest level of repression at the bottom (indicated in blue colors) of the map. Each box corresponds to one gene, the position of which within the two maps is identical; corresponding positions on the map indicate the regulation of a given gene by AP bursting and bath glutamate. The complete list of all electrical-activity-regulated genes is given in Table S1.

Calcium Signaling Pathways Induced by Synaptic and Extrasynaptic NMDA Receptors Target Different Gene Pools

We found that only 47 genes (i.e., 10%) out of 478 AP-bursting-induced genes were also induced by bath-glutamate treatment (Figure 2A). Similarly, 53 genes (i.e., 50%) out of 106 bath-glutamate-induced genes were also induced by AP bursting (Figure 2C). Although expression of many genes, which were induced specifically by one paradigm, changed only moderately (between 1.5- and 2-fold changes based on microarray analysis; see Figures 2A and 2C), for several dozen genes the differences between the two stimulation paradigms in terms of fold induction were dramatic. Examples are the genes *Btg2* and *Atf3*, which were dramatically induced by AP bursting but their expression virtually did not change upon bath-glutamate treatment (see Figure 4). Even less overlap of the gene pools was found for activity-dependent repression. Only ten genes (i.e., 3%) out of 399 AP-bursting-repressed genes were also repressed by bath-glutamate treatment (Figure 2B). Similarly, only ten genes (i.e., 20%) out of 51 genes downregulated following bath-glutamate treatment were also repressed by AP bursting (Figure 2D).

The induction (or repression) following AP bursting or bath-glutamate treatment of a large number of probe sets was dependent on NMDA receptor activation. The regulation of a probe set was considered dependent on NMDA receptor activation if the pharmacological blockade of NMDA receptors reduced its induction or repression at all time points analyzed by at least 50% and if the expression values with and without NMDA receptor blockers were found to be different according to a two-tailed Welch t test (p values $\leq$ 0.025). For 115 out of 478 genes (i.e., 24%), induction by AP bursting was inhibited by MK-801 (Figure 2A). In the case of bath-glutamate treatment, APV inhibited induction of 19 of 106 genes (18%) (Figure 2C). NMDA receptors were also important for activity-dependent gene repression. The downregulation of 34 out of 399 genes (in case of AP bursting) and 1 out of 51 (in case of bath-glutamate treatment) was inhibited by NMDA receptor blockers (Figures 2B and 2D). Given the selection criteria used for the microarray data analysis, calcium entry through NMDA receptors regulates a total of 167 genes (upregulation of 132 genes and downregulation of 35 genes).

We next investigated the possibility that calcium signaling pathways induced by synaptic and extrasynaptic NMDA receptors have different gene targets. Synaptic NMDA receptors are stimulated with the AP-bursting paradigm, whereas bath glutamate also activates extrasynaptic NMDA receptors. To obtain genes regulated by synaptic NMDA receptors only, we used the list containing those AP-bursting-regulated probe sets whose induction was inhibited by MK-801 and filtered out genes induced by bath glutamate. This identified 108 genes induced by synaptic NMDA receptor only (Figure 2A). Among those genes is a set of survival-promoting genes that mediate long-lasting neuroprotection afforded by synaptic NMDA receptors (see below). We also identified, using a similar procedure, genes regulated by extrasynaptic NMDA receptors only: genes induced by AP bursting (stimulating synaptic NMDA receptors) were removed from the list containing those bath glutamate-regulated genes whose induction was inhibited by APV. This revealed 11 genes induced by extrasynaptic NMDA receptors only (Figure 2C); this includes a putative calcium-activated chloride channel (*Clca1*) that promotes cell death (Elble and Pauli, 2001) (see below). The same filtering procedure was applied to the list of genes repressed by NMDA receptors. This identified a pool of 34 genes repressed selectively by synaptic NMDA receptors and one gene repressed selectively by extrasynaptic NMDA receptors (Figures 2B and 2D). These results indicate that target-gene induction and repression by calcium signaling pathways is specified by the localization of the site of calcium signal initiation. Genes selectively regulated by synaptic NMDA or extrasynaptic NMDA receptors are marked in Table S1.

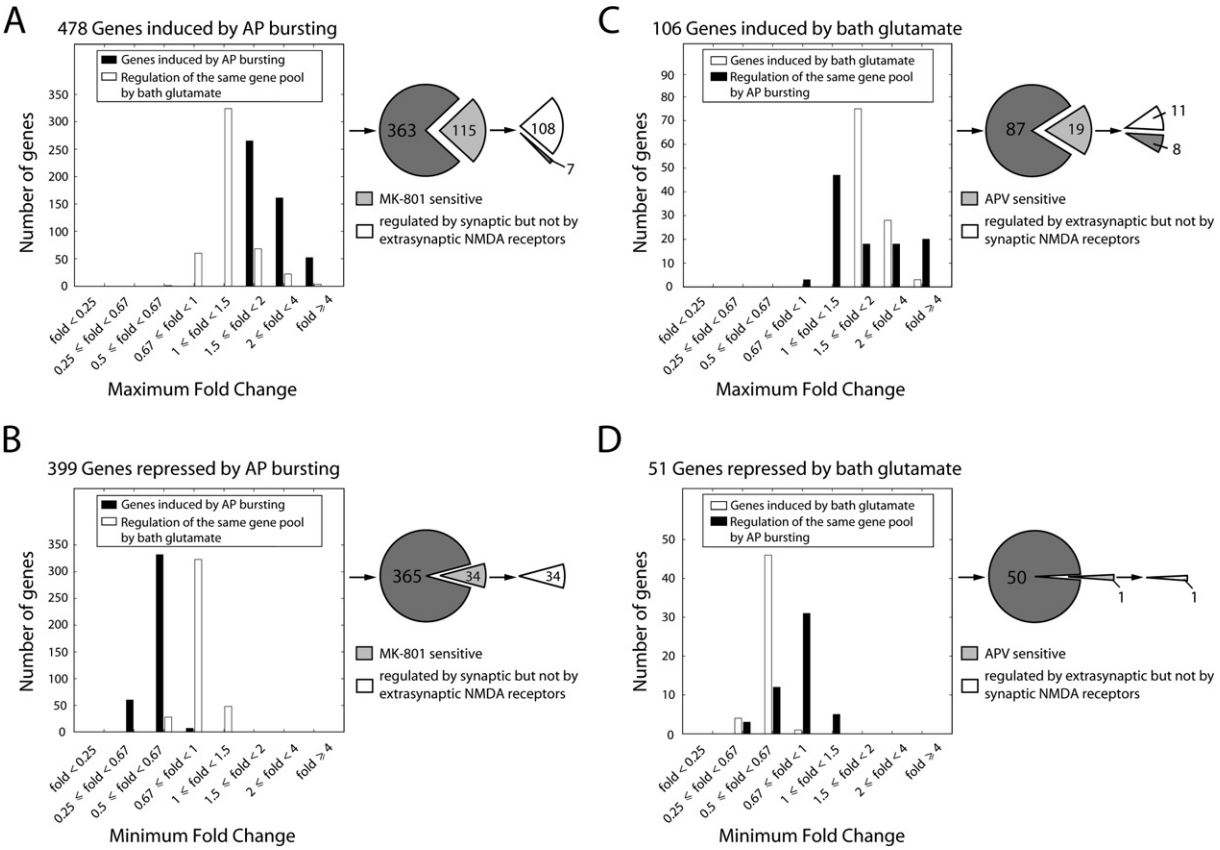

Figure 2. Transcriptional Responses Depend on the Type of Electrical Stimulation and the Site of Calcium Entry: Synaptic and Extrasynaptic NMDA Receptors Target Different Gene Pools

(A and B) The number of genes induced (A) and repressed (B) by AP bursting; the regulation of the same pool of genes by bath-glutamate treatment is also shown. The cake diagrams illustrate the number of genes regulated in an MK-801-sensitive manner and genes regulated by synaptic but not extrasynaptic NMDA receptors.

(C and D) The number of genes induced (C) and repressed (D) by bath-glutamate treatment; the regulation of the same pool of genes by AP bursting is also shown. The cake diagrams illustrate the number of genes regulated in an APV-sensitive manner and genes regulated by extrasynaptic but not synaptic NMDA receptors.

Transcription Is Required for Survival and Cell-Death Programs

We next investigated whether genomic responses are required for survival and cell-death programs induced by synaptic and extrasynaptic NMDA receptors, respectively. Activity-dependent survival was induced by a period of AP bursting stimulating synaptic NMDA receptors (Hardingham et al., 2002; Lee et al., 2005; Papadia et al., 2005). Hippocampal neurons were subsequently challenged by the withdrawal of growth factors, which induces apoptosis (Hardingham et al., 2002; Papadia et al., 2005). Synaptic NMDA receptor-induced survival blocked cell death induced by growth-factor withdrawal (Figure 3A; Hardingham et al., 2002; Papadia et al., 2005). This neuroprotective activity was dependent on gene transcription and was not generated when the period of AP bursting was done in the presence of the inhibitor of gene transcription, actinomycin D (Figure 3A). Gene transcription is also an important factor for glutamate-induced cell death, which had been shown previously in retinal ganglion cells (Dreyer et al., 1995). We found that, in hippocampal neurons, the increase in cell death triggered by 40 μM glutamate bath application was significantly reduced by actinomycin D (Figure 3B). These findings indicate that gene regulation induced by extrasynaptic NMDA receptors, which initiates death signaling (Hardingham et al., 2002), contributes to the degeneration of neurons.

Identification of a Multicomponent Genomic Survival Program

Gene ontology (GO) analysis (http://www.geneontology.org) helps to identify clusters of functionally related genes within the activity-regulated gene pools. Because calcium entry through synaptic NMDA receptors promotes neuronal survival (Hardingham et al., 2002; Ikonomidou et al., 1999; Lee et al., 2005), we were particularly interested in genes with a known or putative role in apoptosis or

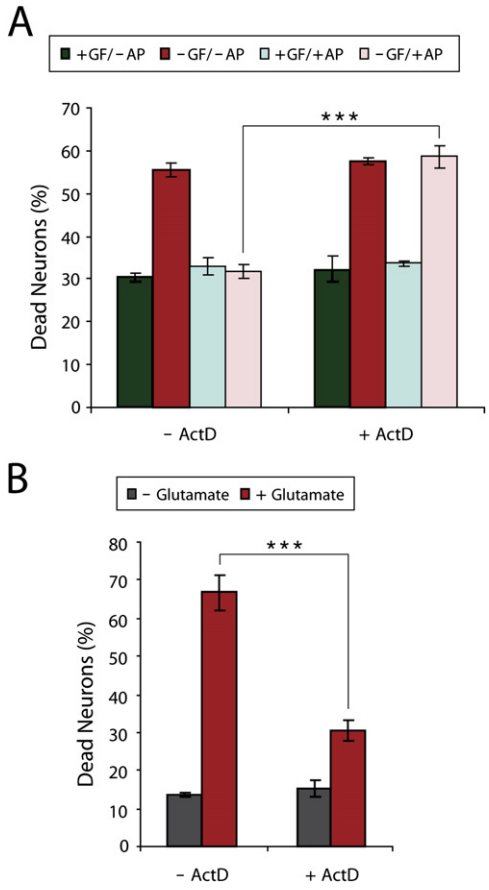

Figure 3. Gene Transcription Is Important for Synaptic NMDA Receptor-Induced Survival and Extrasynaptic NMDA Receptor-Induced Cell Death

(A) Analysis of growth-factor-withdrawal-induced (indicated as −GF) apoptosis in hippocampal neurons with or without subjecting the neuron to a period of AP bursting (AP) inducing synaptic NMDA receptor-induced survival in the presence or absence of actinomycin D (ActD). Actinomycin D was added 10 min before inducing AP bursting.

(B) Analysis of cell death induced by bath application 40 μM glutamate in the presence or absence of actinomycin D. Actinomycin D was added 10 min before glutamate bath application.

Bars represent means ± SEM [(A), n = 3; (B) n = 3]. Statistical significance was determined by analysis of variance (ANOVA); statistically significant differences are indicated with asterisks (***$p < 0.001$).

survival. GenMAPP analysis (http://www.GenMAPP.org) revealed that genes of the GO term "apoptosis" (GO 6915) were significantly enriched in the activity-regulated gene pool; the list of 44 activity-regulated genes of the GO term "apoptosis" is given in Table S2. The results of a comprehensive GO analysis including a statistical rating of the relative gene-expression activity for all GO terms using the standardized difference score (z score) are given in Table S3.

Inspection of the gene-expression profiles obtained with Affymetrix microarrays and QRT-PCR analysis of mRNA levels revealed that a subset of the genes listed in Table S2 are regulated by calcium entry through synaptic but not through extrasynaptic NMDA receptors and thus may play a key role in neuroprotection afforded by synaptic NMDA receptors (Figure 4). This subset contains several genes for which a function in the nervous system was previously unknown. One gene is *Btg2* (also known as *Tis21/PC3*) (Fletcher et al., 1991), which was first identified as a phorbol-ester-inducible gene in Swiss 3T3 cells (Lim et al., 1987). *Btg2* is a potentially antiapoptotic gene that can protect PC12 cells from cell death (el-Ghissassi et al., 2002; Corrente et al., 2002). A second gene is *Bcl6*, a transcriptional repressor that has been implicated in the pathogenesis of B cell lymphomas (Pasqualucci et al., 2003). It suppresses expression of *p53* and, in B cell lines, can protect from apoptosis induced by DNA damage (Phan and Dalla-Favera, 2004; Polo et al., 2004). Our experiments demonstrate that both genes, *Btg2* and *Bcl6*, confer robust neuroprotection, particularly when coexpressed in hippocampal neurons (see below; Figure 5). Additional components of the genomic pro-survival program controlled specifically by synaptic NMDA receptors are *Atf3*, a member of the ATF/CREB family of basic region/leucine zipper proteins that has been implicated in the survival of neuronal and nonneuronal cells (Nakagomi et al., 2003; Janz et al., 2006; S.-J.Z. and H.B., unpublished data); heat shock protein 70 (*Hsp70*, also termed *Hspa1b*), a chaperon with neuroprotective activity (Dong et al., 2005); the caspase 8-associated protein 2 (also known as *FLASH*) (Imai et al., 1999); *Tia1* (a member of the RNA recognition motif family of RNA-binding proteins [Forch and Valcarcel, 2001]). *FLASH* and *Tia1* are proapoptotic genes that in contrast to *Btg2*, *Bcl6*, and *Atf3* are repressed by synaptic NMDA receptor stimulation (Table S2 and Figure 4). The coordinate expression of proapoptotic and antiapoptotic genes indicates that the survival function is implemented in neurons through a multicomponent system of functionally related genes that are controlled by calcium signals initiated specifically at synaptic NMDA receptors. This core survival program (regulated by synaptic NMDA receptors) may be further supported by genes that are induced or repressed preferentially (but not exclusively) by the activation of synaptic NMDA receptors. Such genes include *Bdnf* and *Gadd45* (see Table S2).

Btg2 and Bcl6 Confer Neuroprotection

To investigate a possible neuroprotective potential of Btg2 and Bcl6, we determined their ability to protect hippocampal neurons from apoptosis induced by the withdrawal of growth and trophic factors or treatment with a low concentration of the general protein kinase inhibitor staurosporine (Hardingham et al., 2002). Hippocampal neurons were infected with a recombinant adeno-associated virus (rAAV) carrying myc-tagged Btg2 (rAAV-Btg2-Myc), myc-tagged Bcl6 (rAAV-Bcl6-Myc), or hrGFP (humanized Renilla reniformis green fluorescent) (rAAV-hrGFP) (Figure 5A). Expression of the proteins was assessed in immunoblots using antibodies to the myc-tag of Btg2 and Bcl6 or antibodies to hrGFP (Figure 5B); infection rates were

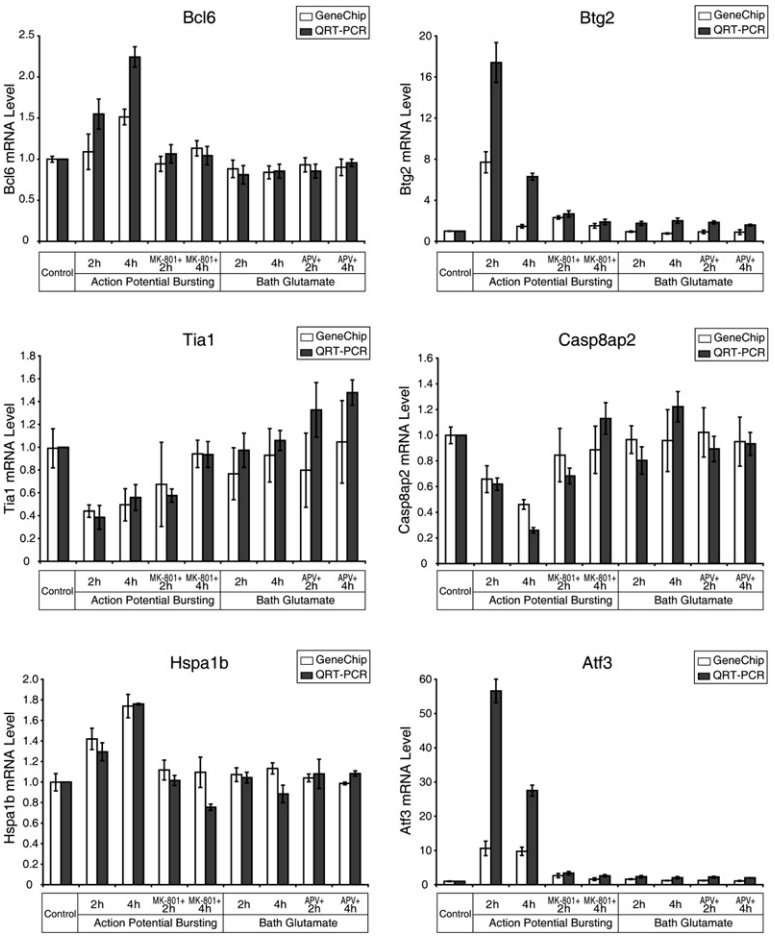

Figure 4. Differential Regulation by Synaptic and Extrasynaptic NMDA Receptors of Genes Involved in Cell Survival and Cell Death

Affymetrix gene chip profiles and QRT-PCR analysis are shown. Hippocampal neurons were left unstimulated or were treated for 2 and 4 hr either with 50 μM bicuculline (to induce AP bursting) in the presence or absence of MK-801 (10 μM) or with glutamate (20 μM) in the presence or absence of APV (500 μM). Bars represent means ± SEM (GeneChip, n = 3; QRT-PCR, n = 4).

determined immunocytochemically and ranged from 80%–95% of the viable neurons (data not shown). In uninfected hippocampal neurons and in neurons infected with rAAV-hrGFP, the percentage of apoptotic cells increased from 14% ± 1% and 17% ± 1%, respectively, in control condition to 53% ± 1% and 55% ± 2%, respectively, following 36 hr of staurosporine treatment (Figure 5C); a similar increase in the percentage of apoptotic cells (from about 25% in control condition to about 50%) was observed after 72 hr of growth-factor withdrawal (Figure 5D). Expression of Btg2 or Bcl6 alone reduced staurosporine-induced cell death to 30% ± 2% and 43% ± 2%, respectively, and reduced growth-factor-withdrawal-induced cell death to 28% ± 3% and 31% ± 1%, respectively (Figures 5C and 5D). The most dramatic neuroprotection was obtained when Btg2 and Bcl6 were coexpressed in the neurons; under these conditions, inhibition of apoptosis induced by staurosporine treatment and by growth-factor withdrawal was 77% ± 7% and 90% ± 7%, respectively (Figures 5C–5E). We also determined whether Btg2 and Bcl6 can block the activation of caspases, which is associated with apoptotic cell death (Yuan and Yankner, 2000). Immunoblot experiments using an antibody to the activated form of caspase 3 revealed that indeed in neurons infected with rAAV-Btg2-Myc or rAAV-Bcl6-Myc but not in neurons infected with rAAV-hrGFP, the activation of caspases following staurosporine treatment was inhibited (Figure 5F). Again, the strongest inhibition of caspase activation was observed in neurons expressing both Btg2 and Bcl6 (Figure 5F). These results indicate that Btg2 or Bcl6, particularly when coexpressed, can confer a robust neuroprotection.

We next investigated whether Btg2 and Bcl6 contribute to activity-dependent survival afforded by AP bursting and activation of the synaptic NMDA receptor (Hardingham et al., 2002; Lee et al., 2005; Papadia et al., 2005). RNA interference (RNAi) was used to inhibit expression of *Btg2* and *Bcl6*. Short hairpin RNA (shRNA) designed to target the mouse *Btg2* and *Bcl6* mRNA was inserted downstream of the U6 promoter of a rAAV vector. The resulting rAAV, termed rAAV-Btg2-RNAi and rAAV-Bcl6-RNAi, also harbors an expression cassette for hrGFP. To control for nonspecific effects of infections with rAAVs carrying an expression cassette for shRNAs, a rAAV was used that contains a negative control shRNA (rAAV-control-RNAi), which has no significant sequence similarity to the mouse, rat, or human genome. Analysis of hrGFP expression allowed us to assess infection rates, which ranged from

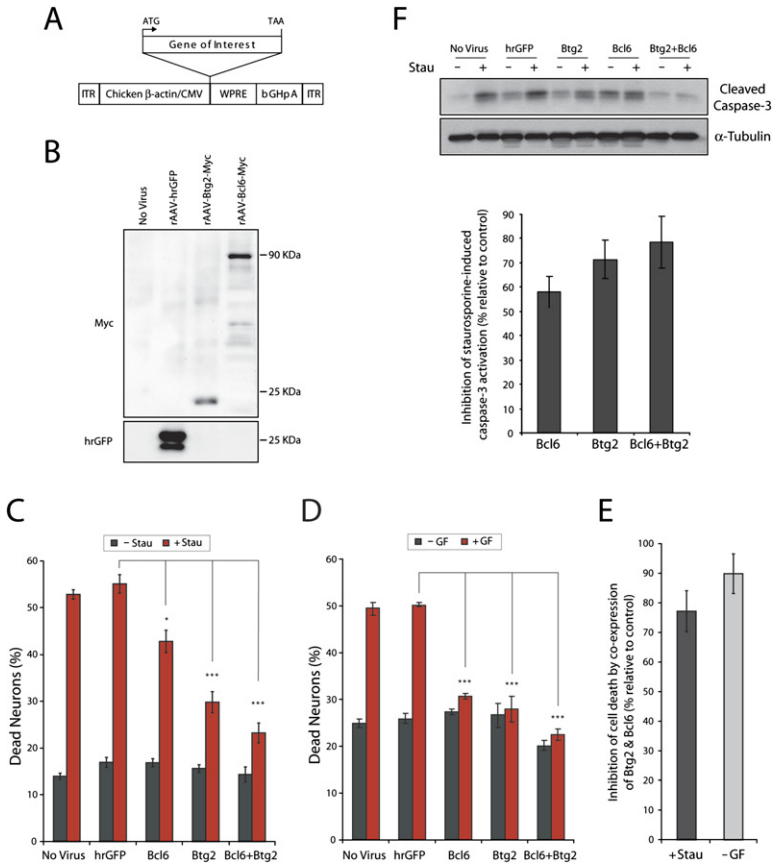

Figure 5. Expression of Btg2 and Bcl6 in Hippocampal Neurons Promotes Survival

(A) Schematic drawing of the structure of the viral vector used to generate recombinant adeno-associated virus.

(B) Immunoblot analysis of uninfected hippocampal neurons or hippocampal neurons infected with rAAV-hrGFP, rAAV-Btg2-Myc, or rAAV-Bcl6-Myc. Btg2 and Bcl6 are myc-tagged and were detected with the 9E10 antibody; hrGFP was detected with an antibody to hrGFP. A representative example of three independent experiments is shown.

(C–E) Growth-factor-withdrawal-induced (−GF) (C) and staurosporine-induced (D) apoptosis in uninfected hippocampal neurons and in hippocampal neurons infected with rAAV-hrGFP, rAAV-Btg2-Myc, rAAV-Bcl6-Myc, or with a mixture of rAAV-Btg2-Myc and rAAV-Bcl6-Myc. Stau, staurosporine. The inhibition by Btg2 and Bcl6 overexpression of growth-factor-withdrawal- and staurosporine-induced apoptosis is summarized in (E).

Bars represent means ± SEM (A), n = 3; (B) n = 3. Statistical significance was determined by analysis of variance (ANOVA); statistically significant differences are indicated with asterisks (*$p < 0.05$, ***$p < 0.001$).

(F) Immunoblot analysis using antibody to the activated form of caspase 3 in uninfected hippocampal neurons or hippocampal neurons infected with rAAV-hrGFP, rAAV-Btg2-Myc, or rAAV-Bcl6-Myc. A representative example is shown in the upper panel; quantitative analysis of three independent experiments is shown in the lower panel.

80% to 95% of the neuron population (data not shown). QRT-PCR analysis revealed that in neurons infected with rAAV-Btg2-RNAi or rAAV-Bcl6-RNAi but not in neurons infected with rAAV-control-RNAi, induction of *Btg2* and *Bcl6*, respectively, was inhibited (Figure 6A). Given the neurotropism of rAAVs used in the study (Xu et al., 2001), the results also indicate that the induction of *Btg2* and *Bcl6* occurs in hippocampal neurons and not in glial cells.

To assess activity-dependent survival, apoptotic cells were counted after treatment with staurosporine. In neurons without previous periods of neuronal activity, staurosporine increased the percentage of apoptotic cells to about 50%–60% (Figure 6B). Upon subjecting the neurons to a period of 16 hr of AP bursting prior to staurosporine exposure, the induced cell death is almost completely blocked (Figure 6B). This neuroprotection, which is activated by the synaptic NMDA receptor (Hardingham et al., 2002; Lee et al., 2005; Papadia et al., 2005), was observed in uninfected neurons and in neurons infected with rAAV-control-RNAi but not in neurons infected with rAAV-Btg2-RNAi. This indicates that *Btg2* is an important component of the synaptic NMDA receptor-induced genomic survival program. In cells infected with rAAV-Bcl6-RNAi, we observed cell-death rates that even in control condition (i.e., without staurosporine) already reached levels higher than normally seen only after exposure to death-inducing stimuli (Figure 6B). Staurosporine did not further increase the percentage of dead neurons (Figure 6B). This finding indicates that Bcl6 is also an important player in the survival of neurons. Since Bcl6 is a transcriptional repressor (Phan and Dalla-Favera, 2004), one function of Bcl6 in neurons may be to repress proapoptotic genes.

Nuclear Calcium Signaling Controls Survival Gene Expression

Recent experiments revealed that nuclear calcium may be a key signal in the induction of survival programs (Hardingham et al., 2002; Papadia et al., 2005). We therefore investigated the possibility that *Btg2* and *Bcl6* belong to the class of nuclear calcium-regulated genes involved in activity-dependent survival. To interfere with nuclear calcium signaling, we expressed in hippocampal neurons the calmodulin (CaM)-binding peptide, CaMBP4 (Wang et al., 1995). CaMBP4 is a nuclear protein that consists of four repeats of the M13 calmodulin-binding peptide derived from the myosin light chain kinase; it binds to and inactivates the nuclear calcium/CaM complex (Wang et al., 1995).

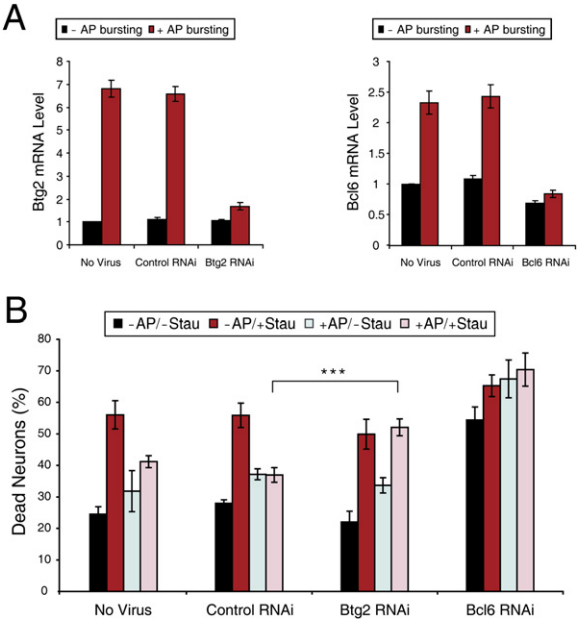

Figure 6. Btg2 and Bcl6 Are Necessary for Neuronal Survival

(A) QRT-PCR analyses illustrating the blockade of activity-dependent induction of *Btg2* and *Bcl6* expression using RNAi. Uninfected hippocampal neurons or hippocampal neurons infected with rAAVs expressing either a control shRNA (rAAV-control-RNAi) or shRNAs that target *Btg2* (rAAV-Btg2-RNAi) or *Bcl6* (rAAV-Btg2-RNAi) were stimulated for 4 hr with 50 μM bicuculline to induce AP bursting or were left unstimulated.

(B) Analysis of the role of Btg2 and Bcl6 in neuronal survival. Uninfected hippocampal neurons or hippocampal neurons infected with rAAV-control-RNAi, rAAV-Btg2-RNAi, or rAAV-Bcl6-RNAi were left untreated or were treated for 16 hr with bicuculline (50 μM) in the presence of 4-AP (250 μM) to induce AP bursting and synaptic NMDA receptor-dependent neuronal survival. Subsequently, apoptosis induced by staurosporine (Stau) treatment was analyzed. Expression of rAAV-Btg2-RNAi but not rAAV-control-RNAi eliminates neuroprotection afforded by synaptic activity. Expression of rAAV-Bcl6-RNAi dramatically increased cell death in control condition.

Bars represent means ± SEM [(A), n = 3; (B) n = 4]. Statistical significance was determined by ANOVA; statistically significant differences are indicated with asterisks (***$p < 0.001$).

Expression of CaMBP4 in hippocampal neurons blocks synaptic activity-evoked CREB-mediated transcription and prevents the induction by neuronal activity of a genomic neuroprotective program (Papadia et al., 2005). Using rAAV-mediated gene transfer, we obtained expression of CaMBP4 or hrGFP in 80%–95% of the viable neurons (Figures 7A and 7B). The regulation of expression of *Btg2* and *Bcl6* was assessed using QRT-PCR analysis (Figure 7C). We found that compared to control (i.e., noninfected neurons or neurons infected with rAAV-hrGFP), induction of *Btg2* transcription by AP bursting was dramatically reduced in neurons infected with rAAV-CaMBP4 (Figure 7C). In contrast, the expression of several other genes, which are not regulated by calcium signaling pathways in hippocampal neurons, was not affected by infection of the hippocampal neurons with AAV-CaMBP4. These genes include fibroblast growth factor 1, peroxisome proliferator-activated receptor γ, coactivator 1 α, DNA (cytosine-5-)-methyltransferase 1, and glucuronidase, β (data not shown). These results indicate that nuclear calcium signaling is critical for activity-dependent regulation of *Btg2* expression. Nuclear calcium signaling is also important for *Bcl6* induction by AP bursting, although compared to *Btg2*, *Bcl6* was only moderately induced by synaptic activity (Figure 7C).

Extrasynaptic NMDA Receptors Induce Expression of CLCA1 that Kills Neurons

Extrasynaptic NMDA receptors fail to activate the nuclear calcium-regulated core survival program but instead couple to expression of a pro-death gene. Gene ontology queries have drawn our attention to *Clca1*. CLCA1 is a putative calcium-activated chloride channel (Elble and Pauli, 2001), although the precise nature of this protein remains to be determined (Gibson et al., 2005; Pawlowski et al., 2006). CLCA1 shows a very unusual, unprecedented mode of regulation: *Clca1* is induced by calcium entry through extrasynaptic NMDA receptors, but not by calcium entry through synaptic NMDA receptors (Figure 8A. In tumor cells, expression of CLCA1 has been linked to cell-cycle arrest and the induction of proapoptotic pathways (Elble and Pauli, 2001). This raises the possibility that an increase in CLCA1 expression following activation of extrasynaptic NMDA receptors promotes neuronal cell death. To test this hypothesis, we transfected hippocampal neurons with an expression vector for CLCA1 and followed the fate of the neurons. We found that CLCA1-expressing neurons undergo cell death (Figures 8B and 8C). We also attempted loss-of-function experiments using *Clca1* RNAi. Although we succeeded in generating an shRNA that inhibited *Clca1* expression following bath-glutamate stimulation by about 75%, this reduction of *Clca1* expression did not protect the neurons from glutamate toxicity (data not shown). Therefore, an essential role for CLCA1 in excitotoxicity remains to be shown. Nevertheless, *Clca1*, the first gene identified that is induced specifically by extrasynaptic NMDA receptors, is sufficient to kill neurons and is thus a likely part of the genomic death program activated by extrasynaptic NMDA receptors.

DISCUSSION

Neuronal Activity-Regulated Genes

The development of DNA microarrays and bioinformatics tools makes it possible to obtain whole-genome expression profiles and to relate them to specific biological functions. This approach has been successful in the study of various human diseases including cancer and diabetis mellitus (DeRisi et al., 1997; Hughes et al., 2000; Lamb et al., 2003; Mootha et al., 2003). Here we used expression profiling to survey the genome for transcripts whose levels are altered in neurons after electrical activation and stimulation of the calcium signaling pathway. We focused on

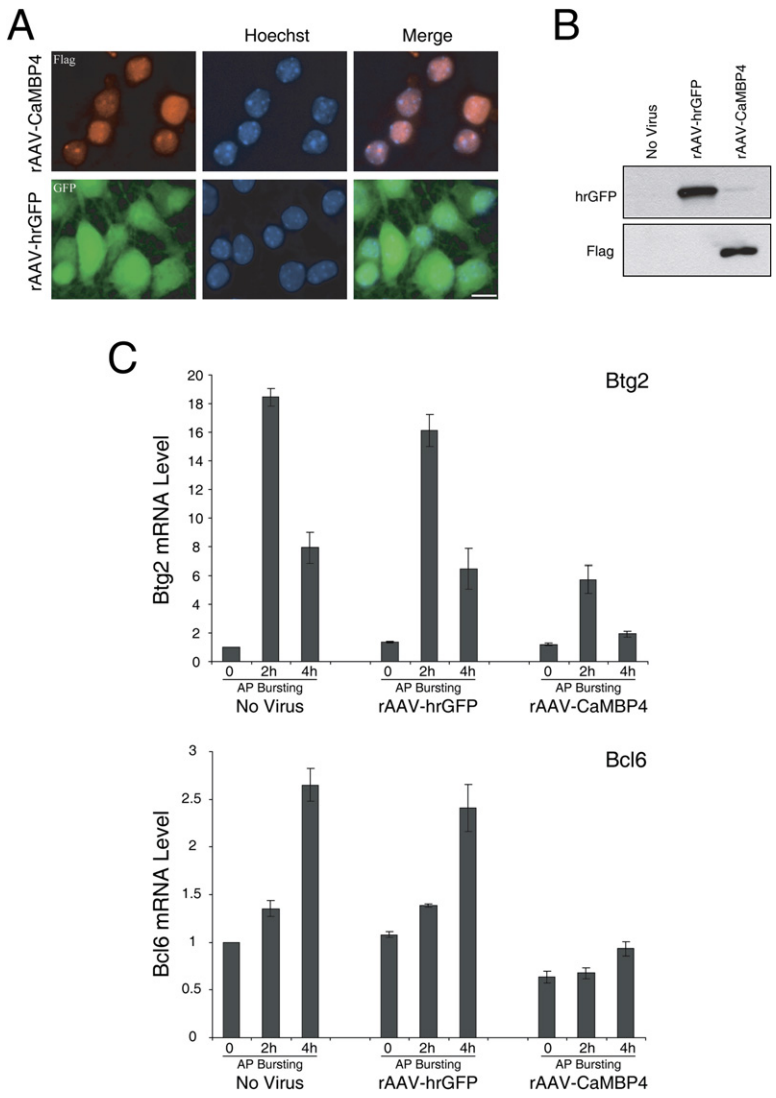

Figure 7. Nuclear Calcium Signaling Controls Activity-Dependent Regulation of Btg2 and Bcl6

(A and B) Immunocytochemical (A) and immunoblot analysis (B) of hippocampal neurons infected with rAAVs expressing CaMBP4 or hrGFP. CaMBP4 is Flag-tagged and was detected with an antibody to the Flag; hrGFP was detected with an antibody to hrGFP. (A) A representative example of three independent experiments is shown. (B) Nuclei of cells were counterstained with Hoechst 33258. Representative images are shown. Scale bar is 15 μm.

(C) Blockade of activity-dependent induction of *Btg2* and *Bcl6* expression in neurons expressing CaMBP4. Uninfected hippocampal neurons or hippocampal neurons infected with rAAVs expressing either CaMBP4 or hrGFP as control were treated for 2 and 4 hr with bicuculline (50 μM) to induced AP bursting or were left unstimulated. Expression of *Btg2* and *Bcl6* was analyzed by QRT-PCR; bars represent means ± SEM (n = 3).

the importance of the site of calcium signal initiation, in particular the NMDA receptor, in the specification of the genomic responses. A comprehensive list of 959 activity-regulated genes was generated. A large fraction of the genes were regulated in an NMDA receptor-dependent manner. However, several hundred genes were induced or repressed by electrical activity even when NMDA receptors were blocked pharmacologically. The transcriptional responses of those genes may be triggered by calcium signals generated by opening of L-type voltage-gated calcium channels. Calcium flux through L-type calcium channels is known to stimulate transcription both via the SRE and the CRE (Bading et al., 1993); target genes of calcium signaling pathways include many immediate-early genes and the neurotrophin *Bdnf* (Hardingham et al., 2002; Bading et al., 1995; Ghosh et al., 1994). It is conceivable that also the activation of metabotropic glutamate receptors contributes to gene regulation upon AP bursting or glutamate bath application. Further expression profiling studies are required for a detailed analysis of NMDA receptor-independent transcription regulatory mechanisms.

Calcium Entry Site-Specific Genomic Responses

Our finding that calcium entry through synaptic and extrasynaptic NMDA receptors evokes different transcriptional responses may not be unexpected given the known differences of these spatially distinct entry sites in the control of CREB function. However, the strikingly small degree to which the target gene pools overlap is surprising. Moreover, even those genes that are not exclusively regulated by one calcium entry site (i.e., 7 out of 149 genes in the case of synaptic NMDA receptors and 8 out of 20 genes in the case of extrasynaptic NMDA receptors; see Figure 2) showed large quantitative differences in their regulation by synaptic and extrasynaptic NMDA receptors. These

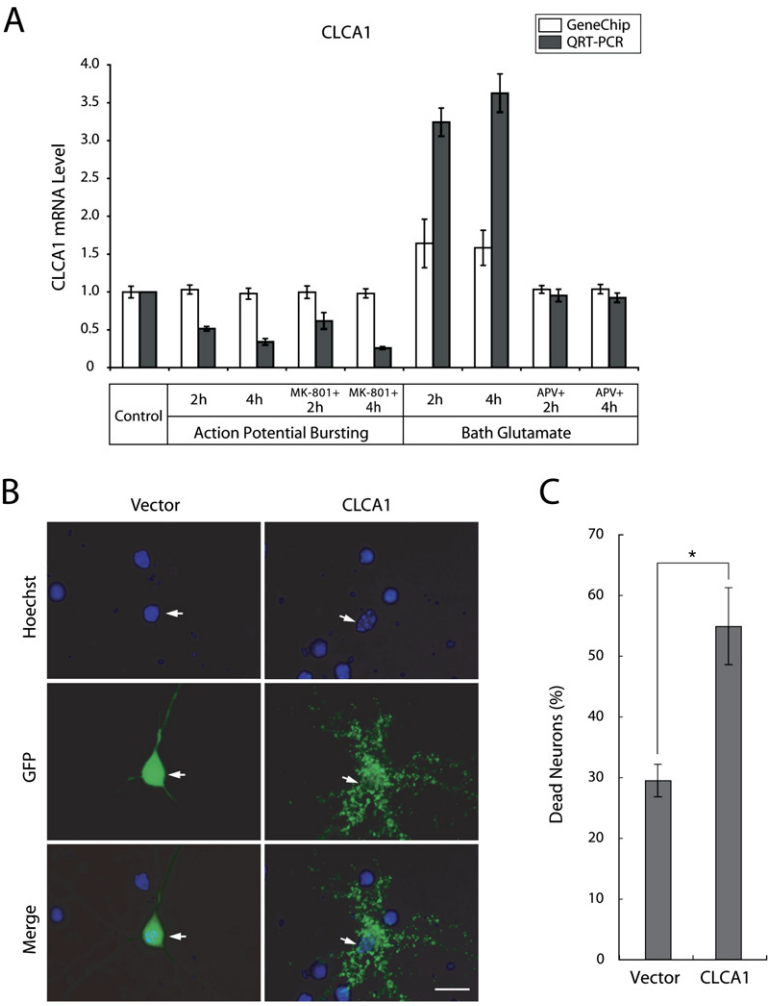

Figure 8. *Clca1*, a Pro-Death Gene Induced by Extrasynaptic NMDA Receptor

(A) Extrasynaptic NMDA receptors induce expression of *Clca1*; Affymetrix gene chip profiles and QRT-PCR analysis are shown. Hippocampal neurons were left unstimulated or were treated for 2 and 4 hr either with 50 μM bicuculline (to induce AP bursting) in the presence or absence of MK-801 (10 μM) or with glutamate (20 μM) in the presence or absence of APV (500 μM).

(B) Overexpression of CLCA1 induces hippocampal neuron cell death. Examples of photomicrographs of hippocampal neurons transfected with an expression vector for EGFP together with either an expression vector for CLCA1 or vector control are shown. Hippocampal neurons expressing CLCA1 showed typical signs of cell death; vector-transfected neurons remained healthy. The arrows indicate healthy (left) and apoptotic (right) neurons. Scale bar is 50 μm.

(C) Quantitative analysis of cell death of hippocampal neurons transfected with an expression vector for EGFP together with either an expression vector for CLCA1 or vector control. The percentage of apoptotic nuclei within the population of EGFP-positive neurons was measured 3 days after transfection. Bars represent means ± SEM (n = 3). Statistical significance was determined by analysis of variance (ANOVA); statistically significant differences are indicated with asterisks (*$p < 0.05$).

results underscore the ability of calcium, the universal messenger in activity-dependent transcription, to act as a versatile carrier of information that, depending on its site of action, initiates qualitatively and quantitatively distinct transcriptional programs.

Differential gene regulation by synaptic and extrasynaptic NMDA receptors is most likely due to differences in the local, near-NMDA receptor signaling. Many components of the NMDA receptor signaling complex have been identified (Husi et al., 2000; Sheng and Kim, 2002), yet it is unclear which proteins associate specifically with synaptic versus extrasynaptic NMDA receptors. It is possible that one difference is in the control of protein phosphatase (PP) 1 function. PP1 is the principal CREB phospho-serine 133 phosphatase (Hagiwara et al., 1992) and thus a likely mediator of the CREB shut-off pathway triggered by extrasynaptic NMDA receptors (Sala et al., 2000). In addition, the action of a phosphatase (perhaps PP1) may also be responsible for nuclear localization of class II HDACs that is promoted by bath-glutamate treatment stimulating extrasynaptic NMDA receptors (Chawla et al., 2003). PP1 is a known target of a calcium signaling pathway acting via calcineurin and inhibitor 1 (Bito et al., 1996). Alternatively, a possible regulation of PP1 by extrasynaptic NMDA receptors could involve a PP1 regulatory subunit (Bollen, 2001) that targets the enzyme to specific substrates such as CREB.

Coordinate Expression of Functionally Related Genes

Our finding that calcium entry through synaptic NMDA receptors induced several antiapoptotic genes and represses several proapoptotic genes indicates that the known prosurvival activity of the synaptic NMDA receptor results from a coordinate change in expression of a set of functionally related genes. In this study we have analyzed two of these genes, *Btg2* and *Bcl6*. Btg2 may function as a nuclear protein involved in the regulation of gene transcription and inhibition of cell-cycle progression at the G1 checkpoint (Prevot et al., 2000; Tirone, 2001; Park et al., 2004). Btg2 expression can also be induced by a p53-dependent mechanism that may be relevant to

the cellular response to DNA damage (Rouault et al., 1996). The regulation by electrical activity and calcium signaling pathways in postmitotic neurons was previously not known, although transient expression of Btg2 has been reported during neurogenesis in precursor cells of the telencephalic ventricular/subventricular zone (Iacopetti et al., 1994, 1999; Haubensak et al., 2004) and following neuroprotective ischemic preconditioning in the spinal cord (Carmel et al., 2004). Its pro-survival activity was first demonstrated in NGF differentiated PC12 cells (el-Ghissassi et al., 2002; Corrente et al., 2002). Our experiments indicate that the induction of *Btg2* expression following calcium entry through synaptic NMDA receptors is a significant part of a transcriptional program that mediates activity-dependent survival.

Bcl6, a transcriptional repressor, has been studied in the immune system, where it is necessary for germinal-center formation and involved in the pathogenesis of B cell lymphomas (Phan and Dalla-Favera, 2004; Polo et al., 2004). Among the targets of Bcl6 are the cell-cycle-arrest gene *Cdkn1a* and the tumor suppressor *p53* (Phan and Dalla-Favera, 2004; Phan et al., 2005). Through suppression of *Cdkn1A*, Bcl6 appears to facilitate the proliferative response of germinal center cells during the normal immune response (Phan et al., 2005). The reduction of p53 expression by Bcl6 in germinal center B cells enables those cells to tolerate physiological DNA breaks required for the recombination of immunoglobulin genes and somatic hypermutation without activating an apoptotic response (Phan and Dalla-Favera, 2004; Polo et al., 2004). One possible function of the here described upregulation of *Bcl6* expression following the stimulation of synaptic NMDA receptors in neurons may be to antagonize increases in p53 production associated with DNA damage or other forms of cellular stress (Culmsee and Mattson, 2005), thereby preventing or attenuating p53-induced apoptosis. In addition, DNA strand breaks and recombination events could conceivably take place in neurons physiologically (Chun et al., 1991; Gao et al., 1998) as a result of neuronal activity and NMDA receptor activation, and similar to the situation in B cells, Bcl6 may suppress the apoptotic response triggered by those events.

Genomic Events Linked to Extrasynaptic NMDA Receptors

Compared to the large number of genes induced (and repressed) by synaptic NMDA receptors, a set of only 12 genes was specifically regulated by glutamate bath application and the stimulation of extrasynaptic NMDA receptors. This was not unexpected because extrasynaptic NMDA receptors, in contrast to the synaptic NMDA receptors, couple to a CREB shut-off pathway and lead to import of class II HDACs (that cause repression of gene transcription) into the nucleus (Hardingham et al., 2002; Sala et al., 2000; Chawla et al., 2003). The mechanism through which extrasynaptic NMDA receptors activate this set of genes remains to be investigated. It may involve the coactivator CBP, whose transcription-activating function is robustly induced by bath-glutamate treatment of hippocampal neurons (Hardingham et al., 1999; Hu et al., 1999), and/or transcription factors that interact with the SRE (Bading et al., 1993). However, both CBP and the SRE can also be activated by calcium entry through synaptic NMDA receptors, leaving unexplained the observed differences in the genomic responses triggered by synaptic versus extrasynaptic NMDA receptors. A more complicated scenario (e.g., a decrease of one or several CREB-regulated genes leads to upregulation of other genes) or a posttranscriptional process may underlie the gene-expression events specifically induced by extrasynaptic NMDA receptors.

The stimulation of extrasynaptic NMDA receptors can initiate cell death (Hardingham and Bading, 2003; Hardingham et al., 2002), which is at least in part mediated by extrasynaptic NMDA receptor-induced transcription. A genomic death program may involve *Clca1*, a pro-death gene specifically induced by extrasynaptic NMDA receptors.

Nuclear Calcium: On-Switch for Neuroprotective Genomic Program

CREB, a key target of the nuclear calcium signaling pathway (Hardingham et al., 1997), is a robust activator of gene expression following calcium entry through synaptic NMDA receptors (Hardingham et al., 2001) and an important mediator of a nuclear calcium-regulated neuroprotective genomic program (Hardingham et al., 2002; Papadia et al., 2005; Walton and Dragunow, 2000; Lonze and Ginty, 2002). Mice containing targeted disruptions of both the *Creb* gene and its close relative *Crem* show extensive cell death in the brain, underscoring a central role of these transcription factors in neuronal survival (Mantamadiotis et al., 2002). In this study we identified a set of survival-promoting genes that are specifically induced by calcium entry through synaptic NMDA receptors. We could show that at least two of these genes, *Btg2* and *Bcl6*, are controlled by nuclear calcium signaling and when overexpressed in hippocampal neurons confer robust neuroprotection. Transcription factors and pathways controlling the *Bcl6* promoter have not been characterized in detail, although analysis of the *Bcl6* 5′ regulatory region revealed the presence of a CRE (5′-CGACGTCA-3′) in the 5′ UTR. However, the functional importance of this site remains to be investigated. *Btg2* is a known CREB target: it contains a CRE in its proximal promoter (Fletcher et al., 1991), and chromatin immunoprecipitation assays indicate that CREB occupies the *Btg2* promoter in vivo (Impey et al., 2004). Inspection of the CREB regulon (Impey et al., 2004) and CREB target gene database queries (http://natural.salk.edu/creb/) (Zhang et al., 2005) revealed that many genes of the NMDA receptor-regulated survival program are known or putative CREB targets (see Table S2). Although it remains to be studied precisely how calcium signaling controls the expression of those genes, given the possible involvement of CREB, it seem likely that an increase in the nuclear calcium concentration serves as the on-switch for many of them. Thus, nuclear

calcium may prove to be the master regulator of a neuroprotective genomic program.

EXPERIMENTAL PROCEDURES

See Supplemental Experimental Procedures for details of whole-genome transcription profiling, QRT-PCR, production of rAAV, and assessment of cell death.

Hippocampal Neurons

Hippocampal neurons from new-born C57Black mice were cultured in Neurobasal media (Invitrogen, Gaithersburg, MD) containing 1% rat serum and B27 (Invitrogen). The procedure used to isolate and culture hippocampal neurons has been described (Bading and Greenberg, 1991; Bading et al., 1993). Stimulations were done after a culturing period of 10–12 days.

Whole-Genome Transcription Profiling

DNA microarray analysis was done using Affymetrix GeneChip Mouse Expression Set 430 (MOE 430A and MOE 430B). See Supplemental Experimental Procedures for details.

Recombinant Adeno-Associated Virus

The vectors used to construct and package rAAVs have been described previously (Klugmann et al., 2005). Hippocampal neurons were infected with rAAVs at 4 days in vitro (DIV). See Supplemental Experimental Procedures for details.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/53/4/549/DC1/.

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