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Growth Factor-Dependent Trafficking of Cerebellar NMDA Receptors Via Protein Kinase B/Akt Phosphorylation of NR2C

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Summary

NMDA receptor subunit composition varies throughout the brain, providing molecular diversity in NMDA receptor function. The NR2 subunits (NR2A-D) in large part dictate the distinct functional properties of NMDA receptors and differentially regulate receptor trafficking. Although the NR2C subunit is highly enriched in cerebellar granule cells and plays a unique role in cerebellar function, little is known about NR2C-specific regulation of NMDA receptors. Here we demonstrate that PKB/Akt directly phosphorylates NR2C on serine 1096 (S1096). In addition, we identify 14-3-3ε as a novel NR2C interactor, whose binding is dependent on S1096 phosphorylation. Both growth factor stimulation and NMDA receptor activity lead to a robust increase in both phosphorylation of NR2C on S1096 and surface expression of cerebellar NMDA receptors. Finally, we find that NR2C expression, unlike NR2A and NR2B, supports neuronal survival. Thus, our data provide a direct mechanistic link between growth factor stimulation and regulation of cerebellar NMDA receptors.

Introduction

NMDA receptors are widely distributed in the CNS where they regulate neuronal development and synaptic plasticity. NMDA receptors are tetramers composed of homologous subunits (NR1; NR2A-D; NR3A-B), but their precise subunit composition varies at excitatory synapses throughout the brain and during development (Cull-Candy and Leszkiewicz, 2004). Functional NMDA receptors require both NR1 and NR2 subunits; however, unlike NR1, there are multiple NR2 subunits, each with unique spatiotemporal expression patterns, ensuring molecular diversity in endogenous NMDA receptors. The NR2 subunit composition determines the functional properties of NMDA receptors as well as the coupling of NMDA receptors to distinct intracellular signaling pathways. Furthermore, at a given synapse, the subunit composition of NMDA receptors can change in response to experience and synaptic activity (Bellone and Nicoll, 2007). Ultimately, this precise regulation of NMDA receptor expression at excitatory synapses regulates synaptic function and plasticity of AMPA receptors.

The trafficking of NMDA receptors is tightly regulated. There is evidence that NMDA receptor trafficking is governed by a variety of mechanisms that regulate distinct intracellular trafficking pathways including ER-retention/egress, exocytosis, lateral diffusion in the plasma membrane, and endocytosis followed by either degradation or recycling (Groc and Choquet, 2006; Lau

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and Zukin, 2007). The precise molecular mechanisms regulating NMDA receptor trafficking depend on motifs located within the intracellular C-termini of receptor subunits, often within the NR2 subunits (Lau and Zukin, 2007; Wenthold et al., 2003). Protein phosphorylation is also an important mechanism regulating NMDA receptor trafficking, as well as channel function. NMDA receptors are directly phosphorylated by a variety of protein kinases, and the substrate specificity is often subunit-specific (Chen and Roche, 2007). A good example is the unique regulation of NR2B trafficking via direct phosphorylation. Casein kinase II phosphorylates S1480 on NR2B, which increases endocytosis of NMDA receptors (Chung et al., 2004), whereas the tyrosine kinase Fyn phosphorylates Y1472 on NR2B inhibiting NMDA receptor endocytosis (Lavezzari et al., 2003; Prybylowski et al., 2005). In contrast to NR2A and NR2B, the regulation of NR2C or NR2D by protein phosphorylation has not been explored in depth.

In cerebellar granule cells, the subunit composition of NMDA receptors changes dramatically during development (Akazawa et al., 1994; Monyer et al., 1994). NR2B is expressed early, but almost disappears by postnatal day 21 (Akazawa et al., 1994). In contrast, NR2A and NR2C are not expressed early in development, but replace NR2B in mature neurons. In contrast to NR2A and NR2B, which are highly expressed in many regions of the forebrain, NR2C is specifically enriched in cerebellar granule cells, suggesting a unique role for this subunit in the regulation of cerebellar neurons. NR2C-containing NMDA receptors have distinct characteristics including a reduced sensitivity to magnesium and a smaller conductance compared to NR2A- or NR2B-containing NMDA receptors (Cull-Candy and Leszkiewicz, 2004). In addition, mice expressing NR2C and NR2A with deleted C-terminal domains have impaired cerebellar synaptic plasticity (Rossi et al., 2002), and mice lacking NR2C subunit display impaired motor coordination (Sprengel et al., 1998), demonstrating a critical role for NR2C in cerebellar function. However, there has been little characterization of NR2C-specific regulation of glutamate receptor trafficking.

Cerebellar granule cells require growth factor signaling to survive during development (Contestabile, 2002). Insulin-like growth factor-1 (IGF-1), in particular, is essential to inhibit apoptosis (Blair et al., 1999; Dudek et al., 1997). IGF-1 upregulates PI3K and stimulates protein kinase B (PKB)/Akt, which phosphorylates many cellular substrates (Brazil et al., 2004; Datta et al., 1999). Although the IGF-1 signal transduction pathway has been implicated in both NMDA receptor regulation and synaptic plasticity (Man et al., 2003; Peineau et al., 2007; Sanchez-Perez et al., 2006; Sutton and Chandler, 2002; Yoshii and Constantine-Paton, 2007), there are no reports of PKB directly phosphorylating any subtype of glutamate receptor. In the current study, we now show that PKB phosphorylates NR2C on S1096, thus regulating NMDA receptor trafficking by promoting the association of 14-3-3ε with NR1/NR2C heteromeric receptors. Growth factor stimulation or receptor activity trigger PKB-dependent trafficking of cerebellar NMDA receptors to the cell surface. Furthermore, NR2C expression, unlike NR2A or NR2B, protects cerebellar granule cells in an NMDA toxicity assay. Thus, our findings provide a novel link between growth factor stimulation, NMDA receptor phosphorylation and trafficking, and neuronal survival.

Results

To identify novel NR2C interacting proteins, we screened a rat brain yeast two-hybrid library with the NR2C C-terminus (a.a. 1076 – a.a. 1250; Fig. 1A), and isolated 14-3-3ε (Fig. S1). The mammalian 14-3-3 proteins consist of seven highly homologous members that interact with a variety of proteins, regulating signal transduction and protein trafficking. The 14-3-3 family of proteins were originally isolated as abundant proteins in the brain and are highly conserved in a broad range of eukaryotic organisms.

To characterize the specificity of the NR2C-14-3-3ε interaction, we evaluated the binding of 14-3-3ε to the C-terminus of NR2A, NR2B or NR2C using yeast two-hybrid direct interaction assays. By evaluating growth on histidine-deficient plates (-HIS) as an assay for protein-protein interactions, we observed no specific binding when the C-terminus of NR2A or NR2B was co-expressed with 14-3-3ε (Fig. S1). In contrast, NR2C interacted strongly with 14-3-3ε, demonstrating that the interaction between 14-3-3ε and NMDA receptors is specific for the NR2C subunit.

Upon sequence examination, we found two regions (1093-R-H-A-S-L-P-1098 and 1141-R-L-P-S-Y-P-1146) within the NR2C C-terminus that are similar to previously identified 14-3-3 binding motifs, R-S-X-S^P-X-P and R-X-X-X-S^P-X-P (X can be any amino acid and S^P indicates a phosphorylated serine) (Yaffe et al., 1997). To determine whether these two regions of NR2C are required for 14-3-3ε binding, we tested the interaction of 14-3-3ε with NR2C containing a mutation in the critical serine residue within each putative binding site, S1096A (Fig. 1A) or S1144A. The NR2C S1096A mutation disrupted the 14-3-3ε binding, whereas NR2C S1144A mutant had no effect (Fig. 1B), indicating that S1096 is part of the 14-3-3ε binding motif on NR2C.

We also examined 14-3-3ε binding to NR2C using a co-immunoprecipitation assay in HEK-293 cells expressing FLAG-NR2C or FLAG-NR2C S1096A and Myc-14-3-3ε. Surprisingly, Myc-14-3-3ε was not co-immunoprecipitated efficiently with NR2C (Fig. 1C). Because NR2 subunits are ER-retained in the absence of NR1, we reasoned that perhaps the correct assembly of NR2C with NR1 is required for the 14-3-3 interactions. Indeed, there is evidence that 14-3-3 family members preferentially bind to assembled proteins in the ER-Golgi intermediate compartment thus facilitating export of properly assembled multimers from the ER (O'Kelly et al., 2002;Yuan et al., 2003). Consistent with this hypothesis, we observed robust binding of 14-3-3 to NR2C only upon co-transfection with NR1. Furthermore, the NR2C S1096A mutation disrupted the NR2C-14-3-3ε interaction (Fig. 1C). These results not only provided evidence that the R-H-A-S-L-P (S1096) motif of NR2C is responsible for 14-3-3 binding but also show that 14-3-3ε specifically binds NR1/NR2C heteromers.

To investigate the interaction between NR2C and 14-3-3ε in neurons, 14-3-3ε was immunoprecipitated from cerebellum homogenate and immunoblotted with an NR2C antibody, which also cross-reacted with NR2A and NR2B. Consistent with the results from yeast two-hybrid interaction assay, we found that NR2C, but not NR2A and NR2B, co-immunoprecipitated with 14-3-3ε (Fig. 1D), demonstrating that NR2C is a specific binding partner for 14-3-3ε *in vivo*.

NMDA receptors are dynamically regulated by protein phosphorylation, and many sites have been identified on the C-termini of NR2 subunits (Chen and Roche, 2007). Because 14-3-3 proteins typically bind to phosphorylated serine residues (Yaffe et al., 1997) and S1096 on NR2C is required for 14-3-3 binding, we investigated whether S1096 on NR2C is phosphorylated. Interestingly, sequence examination revealed a prototypical PKB consensus motif surrounding S1096 in the NR2C intracellular C-terminus, which coincides with the 14-3-3 binding motif (Fig. 1A). Although the serine is conserved in other NR2 subunits, the entire PKB recognition motif is not (Fig. 1A). We performed *in vitro* phosphorylation assays using GST fusion proteins (GST, GST-NR2C wild-type (WT) or GST-NR2C S1096A) incubated with recombinant PKB. The equal loading of GST-NR2C fusion proteins was confirmed by Coomassie blue staining (data not shown). GST-NR2C WT and GST-NR2C S1244A were efficiently phosphorylated by PKB *in vitro* (Fig. S2A), but reduced phosphorylation was observed for GST-NR2C S1096A (Fig. S2A), demonstrating the direct phosphorylation of S1096 by PKB *in vitro*. The residual phosphorylation of GST-NR2C

S1096A indicates that there is a second site on NR2C that PKB phosphorylates *in vitro*, although less efficiently than S1096.

We next raised a phosphorylation-state specific polyclonal antibody recognizing phosphorylated S1096 that specifically detected PKB phosphorylated GST-NR2C fusion protein (Fig. S2B). In contrast, the antibody did not recognize GST-NR2C S1096A even when the fusion protein was incubated with PKB (Fig. S2B), demonstrating the direct phosphorylation of S1096 by PKB *in vitro* and confirming the specificity of the antibody for NR2C phosphorylated on S1096.

To test if NR2C S1096 is phosphorylated by PKB activation in situ, we co-expressed NR2C with a constitutively active form of PKB (caPKB)(Cong et al., 1997) in HEK-293 cells. Co-expression of NR2C with either caPKB or a dominant negative form of PKB (dnPKB) in HEK-293 cells did not affect NR2C expression (Fig. 2A). The phosphorylation of S1096 increased dramatically upon co-transfection with caPKB, but no increase was observed for NR2C S1096A or for NR2C WT or NR2C S1096A co-expressed with dnPKB (Fig. 2A). Similar results were obtained when NR1 was co-transfected with NR2C and caPKB or dnPKB (Fig. S3A).

Various growth factors stimulate the phosphoinositide 3-kinase (PI3K) signal transduction cascade, which results in the activation of PKB (Datta et al., 1999). Because HEK-293 cells are insulin-sensitive, we investigated whether IGF-1 treatment, which activates PKB via PI3K, would stimulate NR2C phosphorylation on S1096. We observed a marked increase in NR2C S1096 phosphorylation in response to PKB activation that was blocked by incubation with the PI3K inhibitor wortmannin to prevent activation of the PI3K-PKB pathway (Fig. 2B). Taken together, these results demonstrate that IGF-1, through the activation of PI3K/PKB pathway, is able to induce NR2C S1096 phosphorylation. Similar results were obtained when NR2C was co-transfected with NR1 (Fig. S3B).

To evaluate the phosphorylation of endogenous NR2C, we analyzed NR2C isolated from mouse cerebellum slices (prepared from P12 animals). Immunoblots probed with the NR2C S1096 phosphorylation state-specific antibody revealed that endogenous NR2C was phosphorylated on S1096 (Fig. 2C). Furthmore, treatment of the membrane with lambda-phosphatase prior to immunoblotting eliminated the NR2C band recognized with the S1096 phosphorylation state-specific antibody (Fig. 2C), confirming that the immunoreactivity was specific for phosphorylated NR2C. NR2C is expressed at a very low level in hippocampus compared to cerebellum, accordingly in hippocampus we observed only a low level of NR2C expression (Fig. 2C).

To investigate if S1096 phosphorylation on endogenous NR2C is dynamically regulated in neurons, we treated cultured cerebellar granule cells (12 days in vitro) with IGF-1 for various periods of time to stimulate PKB activity. Immunoblot analysis with the NR2C S1096 phosphorylation state-specific antibody revealed that NR2C S1096 is phosphorylated even in the absence of IGF-1, indicating that there is a modest basal level of phosphorylation in cerebellar granule cells. However, IGF-1 treatment resulted in a dramatic increase in phosphorylation of NR2C S1096 (Fig. 2D), showing that endogenous NR2C S1096 is regulated by PKB *in vivo*. NMDA receptor activation has been shown to stimulate PKB activity (Lafon-Cazal et al., 2002); therefore, we examined if agonist exposure regulates phosphorylation of NR2C S1096 and found that brief NMDA treatment transiently increased phosphorylation of NR2C S1096 (Fig. 2E). In contrast, application of the NMDA receptor antagonist APV decreased NR2C S1096 phosphorylation over the same time course (Fig. 2F), revealing a tonic stimulation of NR2C S1096 phosphorylation by endogenous glutamate. These results

demonstrate that both IGF-1 and NMDA receptor activity regulate phosphorylation of NR2C on S1096 in cerebellar granule cells.

Phosphorylation of ionotropic glutamate receptors is an important mechanism modulating receptor trafficking (Lee, 2006). We therefore examined if PKB phosphorylation of NR2C affects surface expression of NR2C-containing NMDA receptors using fluorescence activated cell sorting (FACS) analysis. A bicistronic expression construct containing the coding sequence of FLAG-tagged NR2C and EGFP was used to monitor NR2C-transfected cells (GFP positive cells), and a second bicistronic expression construct containing the coding sequence of NR1-1a and DsRed was used to monitor NR1-1a-transfected cells (DsRed positive cells). Surface expression of FLAG-NR2C in live HEK-293 cells expressing both GFP and DsRed was measured. We found that caPKB dramatically increased wild-type NR2C surface expression, whereas expression of caPKB resulted in only a modest increase in the surface expression of NR2C S1096A (Fig. 3A), demonstrating that S1096 primarily mediates the PKB-dependent increase in NMDA receptor surface expression.

We also measured the effects of PKB on surface expression of NR2C/NR1 heteromers in HEK-293 cells using a cell surface biotinylation assay. We found that IGF-1 treatment robustly increased surface expression of NR2C WT with only a modest effect on NR2C S1096A (Fig. 3B). α-Tubulin, an intracellular protein, was used as a negative control for surface biotinylation.

To determine whether surface expression of endogenous NR2C is regulated by PKB phosphorylation in neurons, we treated cultured cerebellar granule cells with IGF-1 and performed a cell surface biotinylation assay. IGF-1 treatment dramatically increased surface expression of NR2C in cerebellar granule cells (Fig. 3C), and the increase was blocked by pre-incubation with the PI3K inhibitor wortmannin or LY294002 prior to IGF-1 treatment (Fig. S4A and S4C). We also treated cultured cerebellar granule cells with NMDA and evaluated receptor surface expression using the cell surface biotinylation assay. Interestingly, NMDA treatment also dramatically upregulated surface expression of endogenous NR2C (Fig. 3D), which can be blocked by pre-incubation with wortmannin or LY294002 (Fig. S4B and S4C). Taken together, these data demonstrate that PKB phosphorylation of NR2C S1096 driven by IGF-1 signaling or NMDA receptor activation facilitates NR2C trafficking to the plasma membrane.

The biotinylation results indicated that PKB phosphorylation of NR2C is required for receptor trafficking in neurons, but these experiments did not specifically address the role of 14-3-3ε in regulating surface expression of NR2C. To determine whether 14-3-3ε binding to NR2C is necessary for NR2C trafficking, we used a dominant negative form of 14-3-3ε (14-3-3ε dn) to functionally deplete 14-3-3ε in neurons (Czirjak et al., 2008; Thorson et al., 1998). We made a GFP-NR2C construct, which contains a GFP tag in the extracellular N-terminal domain of the receptor, to specifically label surface-expressed receptors. Cerebellar granule cells were co-transfected with GFP-NR2C WT or GFP-NR2C S1096A and Myc-14-3-3ε or Myc-14-3-3ε dn. We labeled the surface-expressed receptors with anti-GFP antibody. Surface expression of NR2C WT was dramatically decreased upon co-transfection with 14-3-3ε dn (Fig. 4), indicating that the interaction between NR2C and 14-3-3 is required for NR2C trafficking. In addition, surface expression of NR2C S1096A was much less than that of NR2C WT when 14-3-3ε was co-expressed and was further reduced upon co-expression with 14-3-3ε dn (Fig. 4), suggesting that S1096 is not the sole molecular determinant in 14-3-3ε-regulated NR2C trafficking.

Because IGF-1 has been shown to promote neuronal survival by activating PKB and increased surface expression of NR2C was driven by the same signaling pathway, we investigated whether NR2C is involved in neuronal survival. Cultured cerebellar granule cells were co-

transfected with vector, GFP-NR2A, GFP-NR2B or GFP-NR2C and Discosoma sp. red fluorescent protein (DsRed). At 6 DIV, the neurons were incubated with NMDA to induce excitotoxicity (Bonfoco et al., 1995). To closely monitor the effect of NMDA treatment, we used time-lapse microscopy of DsRed-labeled neurons. We found that neurons overexpressing NR2A or NR2B developed cell body swelling and dendritic varicosities within 5 minutes following NMDA treatment (Fig. 5A and Movie S1). Neurons expressing DsRed alone also showed swelling of the cell body and localized dendritic varicosities, although not as rapidly as neurons expressing NR2A or NR2B. In sharp contrast, NR2C expressing neurons were protected from the NMDA-induced toxicity (Fig. 5A and Movie S1).

To determine if PKB-dependent trafficking of NR2C played a role in cell survival, we evaluated toxicity in cerebellar granule cells expressing NR2C WT or NR2C S1096A and found no difference in cell survival upon acute NMDA treatment (data not shown). However, we analyzed older cultures (DIV8) and found that neurons expressing NR2C S1096A displayed increased dendritic fragmentation and varicosities compared to NR2C WT, indicating neuronal injury (Hasbani et al., 2001). Quantitation revealed that cerebellar granule cells expressing NR2C S1096A showed almost double the number of neurons with dendritic varicosities compared to NR2C WT-expressing cells (Fig. 5B). This is consistent with NR2C surface expression, and specifically S1096, playing an important role in regulating the cell survival effect of NR2C.

Discussion

In this study we have revealed a novel growth factor-dependent pathway for the subunit-specific regulation of cerebellar NMDA receptors. We demonstrate that NR2C specifically interacts with 14-3-3ε, that phosphorylation of S1096 on NR2C regulates NMDA receptor binding to 14-3-3, and that S1096 is phosphorylated by PKB. Furthermore, growth factor stimulation or NMDA receptor activation increases NR2C S1096 phosphorylation and surface expression of NMDA receptors in cerebellar granule cells. These data are all consistent with a model in which NR1 and NR2C oligomerize, PKB phosphorylates S1096, and 14-3-3ε binds to phosphorylated NR2C thereby promoting NR2C-containing NMDA receptor surface expression in cerebellar granule cells. Our findings therefore provide a direct link between growth factor signaling, which is essential for cerebellar granule cell survival, and NMDA receptor surface expression.

NMDA receptors are directly phosphorylated by a variety of protein kinases, and in many cases phosphorylation of NMDA receptors regulates their trafficking and synaptic expression (Chen and Roche, 2007). The best-characterized examples of NMDA receptor regulation by protein phosphorylation are by PKC, casein kinase II, and Fyn (Chen and Roche, 2007; Lee, 2006). Our finding that the serine/threonine kinase PKB specifically phosphorylates NR2C expands the list of kinases regulating endogenous NMDA receptors and adds to the subunit-specific complexity of NMDA receptor regulation. PKB has been studied extensively vis a vis its role in cell cycle progression, apoptosis, and cell signaling. In addition, PKB phosphorylation of many distinct substrates can regulate protein-protein interactions and protein trafficking (Brazil et al., 2004). We now show that such a mechanism regulates NMDA receptor trafficking in the cerebellum. Interestingly, NR2C S1096 is conserved in NR2A (S1291) and NR2B (S1303), with the analogous serines being substrates for PKC and/or CaMKII (Jones and Leonard, 2005; Liao et al., 2001; Omkumar et al., 1996). However, the PKB recognition motif is specific for NR2C, as is the consensus sequence for 14-3-3 binding, suggesting a unique role for PKB phosphorylation and 14-3-3 binding in NR2C-containing NMDA receptor trafficking.

14-3-3 proteins act as molecular scaffolds or adapters to regulate intracellular signal transduction pathways (Berg et al., 2003). Furthermore, recent findings show that in many

cases 14-3-3 proteins mediate ER export of proteins (Shikano et al., 2006). We now show that 14-3-3ε preferentially associates with oligomerized NR1/NR2C, consistent with a role in promoting the forward trafficking of correctly assembled NR1/NR2C-containing NMDA receptors. 14-3-3 proteins favor consensus sequences containing phosphorylated serine residues (Yaffe et al., 1997), and S1096 on NR2C is part of a strong consensus motif for PKB phosphorylation. Because S1096 phosphorylation is required for the NR2C-14-3-3 interaction, our data suggest that NMDA receptor egress from the ER is regulated by a 14-3-3-dependent mechanism as shown previously for other multi-protein complexes (Shikano et al., 2006). However, this type of PKB-dependent mechanism has never been described for any glutamate receptor, and the specificity for NR2C suggests a unique role in cerebellar function.

Growth factors play a critical role in regulating neuronal survival during development (Pettmann and Henderson, 1998). Cerebellar granule cells, in particular, are known to undergo a period of extensive apoptosis during development, which is precisely regulated by growth factors (Contestabile, 2002). IGF-1 has been demonstrated to promote cerebellar granule cell survival both *in vitro* and *in vivo* by blocking apoptosis (Chrysis et al., 2001; D'Mello et al., 1997; Dudek et al., 1997). IGF-1 increases PI3 kinase activity leading to increased PKB activity and stimulation of downstream kinase/signaling cascades (Brazil et al., 2004; Datta et al., 1999). PKB phosphorylates a variety of substrates, many of which regulate mitochondrial function and apoptosis (Parcellier et al., 2008). Mice lacking various isoforms of PKB have an overall reduction in cell number in many tissues, and specifically in the brain when the PKBγ isoform is absent (Easton et al., 2005; Tschopp et al., 2005). The growth factor/PKB signaling pathway has been specifically implicated in regulating cerebellar granule cell survival (D'Mello et al., 1997; Dudek et al., 1997). Furthermore, there is evidence that PKB can potentiate NMDA receptor responses in cerebellar granule cells (Sanchez-Perez et al., 2006), suggesting a link between growth factor signaling and receptor activation in these neurons. However, until now, there was no mechanism described linking growth factor signaling, PKB activity, and NMDA receptor expression.

In this study, we found that growth factor signaling and PKB phosphorylation increase surface expression of NR2C-containing NMDA receptors. Importantly, we also showed that overexpression of NR2C protects neurons from NMDA-induced toxicity. Consistent with a role for phosphorylation of S1096 in receptor trafficking, NR2C S1096A was less effective at neuronal protection than NR2C WT, supporting a role for the PI3K/PKB pathway in neuronal survival. We found that overexpression of NR2C S1096A also has a protective effect compared to NR2A or NR2B (data not shown), which is probably due to the increased level of NR2C surface expression due to exogenous overexpression. For example, the S1096A mutation does not completely abolish the surface expression of NR2C when overexpressed in cerebellar granule cells (Fig. 5). Our results suggest that surface-expressed NR2C S1096A, even if decreased compared to that of NR2C WT, is sufficient to support neuronal survival. How does NR2C contribute to neuronal survival? The answer probably lies in the fact that NR2C-containing NMDA receptors have unique channel properties (Dravid et al., 2008) and that NR2C may mediate specific signaling pathway to activate downstream survival molecules. In conclusion, our findings that PKB directly phosphorylates cerebellar NMDA receptors and increases their surface expression reveals a novel molecular mechanism by which growth factor signaling can directly affect NMDA receptor activity.

Experimental Procedures

DNA constructs

The caPKB and dnPKB constructs were kindly provided by Michael Quon (NCCAM, NIH). GFP-NR2A and GFP-NR2B were kindly provided by Stefano Vicini (Georgetown Univeristy). Additional DNA construct information can be found in Supplementary Information.

Cell surface biotinylation assay

Cell surface biotinylation assay was performed as described previously (Chen et al., 2006).

FACS analysis

HEK-293 cells were transiently transfected with NR1-1a-IRES-DsRed, FLAG-NR2C-IRES-EGFP and vector, caPKB or dnPKB. FACS analysis were performed as described previously (Chen et al., 2006). Briefly, detached cells were labeled with anti-FLAG M2 monoclonal antibodies (100 ng/μl) for 30 min at 4°C. Cells were incubated with phycoerythrin (PE)-Cy5-conjugated anti-mouse secondary antibodies (100 ng/μl) for 30 min at 4°C, washed and suspended in PBS. The cells were immediately analyzed using a dual-laser FACSVantage SE flow cytometer (Becton Dickinson, San Jose, CA). EGFP, DsRed and PE-Cy5 signals were excited using a 488-nm laser light and the resulting emissions captured with bandpass filters set at 525±20 nm, 575±25 nm and 675±20 nm, respectively.

Immunoprecipitation

The P2 membrane fractions (Chen et al., 2006) from P12 mouse cerebellar and hippocampal slices was solubilized in PBS containing 1% SDS for 15 min at 37°C. Ten volumes of PBS containing 1% Triton X-100 were added to the lysate resulting in a final concentration of 0.1% SDS. Insoluble material was removed by centrifugation at 100,000g for 20 min. Immunoprecipitations were performed as described previously (Chen et al., 2006).

Generation of phosphorylation-state specific antibody and immunoblotting

Rabbits were immunized with a synthetic phosphopeptide Ac-RPRHA[pS]LPSSVC-amide corresponding to amino acids 1091-1102 of NR2C by QCB (Hopkinton, MA). Sera were collected and affinity-purified using the antigen phosphopeptide. Anti-NR2C antibody was purchased from Chemicon International (Temecula, CA), and FLAG and Myc antibody was purchased from SIGMA. HEK-293 cells were transfected with full-length NR2C WT or NR2C S1096A using the calcium phosphate method (BD Biosciences, San Jose, CA) and incubated with IGF-I for 20 min at 37°C. Cell membranes were prepared as described previously (Chen et al., 2006). Proteins were resolved by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with relevant antibodies.

Cerebellar granule cell culture

Primary cultures of cerebellar granule neurons were prepared as described previously (Chen et al., 2006).

Immunocytochemistry of neurons

Cultured cerebellar granule cells were transfected by the calcium phosphate method at 4 DIV. After transfection, the cultures were maintained in the original medium containing 25 mM KCl. At 6 DIV, immunocytochemistry was performed as described previously (Lavezzari et al., 2004). Briefly, transfected neurons were incubated with polyclonal anti-GFP antibody (Invitrogen) for 20 min at room temperature to label surface-expressed protein. The cells were fixed and incubated with Alexa 647-conjugated (blue) anti-rabbit secondary antibody (molecular Probes) for 30 min. The cells were permeabilized, incubated with 10% normal goat serum, and labeled with monoclonal anti-myc antibody (9E10) for 30 min at room temperature. Following extensive washing, cell were incubated with Alexa 568-conjugated (red) anti-mouse secondary antibody for 30 min, and mounted with a ProLong Antifade kit (Molecular Probes). Images were collected with a 63× objective on a Zeiss LSM 510 confocal microscope. Series of optical sections collected at intervals of 0.5 μm were used to create maximum projection images. For quantitative analysis, images from three dendrites per neuron (three neuron per

experiment) were collected and quantitated using Metamorph 6.0 software. Blue fluorescence intensity indicative of surface expression was divided by green fluorescence intensity to control for the GFP-NR2C protein expression level.

Neurotoxicity Assay

At DIV4, cerebellar granule cells were transfected with DsRed and pRK5, GFP-NR2A, GFP-NR2B or GFP-NR2C. After transfection, the cultures were maintained in the original medium containing 25 mM KCl. At DIV6, Time-lapse imaging was performed for 25 minutes following NMDA treatment, and collected every 2.5 minutes. Confocal images were acquired using 40× objective. Serial optical sections were obtained at 1 μm intervals. Movies were created using Metamorph 6.0 software. Neurotoxicity was determined by the cell morphology visualized by DsRed fluorescence.

Analysis of dendritic morphology

At DIV4, DsRed and GFP-NR2C WT or S1096A were transfected at a ratio of 1:5. After transfection, the cultures were maintained in the original medium containing 25 mM KCl. The experiments were terminated by fixing the cultures at 8 DIV. The assessment of dendritic varicosities in older culture (DIV8, not treated with NMDA) was determined as described previously (Hasbani et al., 2001). Briefly, the presence of dendritic varicosities in DsRed-expressing neurons was examined 400× under epifluorescence illumination. For each experimental condition, 100 DsRed-expressing neurons were scored for the presence or absence of dendritic varicosities. The resulting values were averaged for three independent experiments.

Yeast two-hybrid assay

Yeast two-hybrid assay was performed as described previously (Chen et al., 2006). For yeast expressing LexA-NR2A or LexA-NR2B, 3-Aminotriazole (3-AT) (10 mM) was included in the medium to reduce or eliminate the background transactivation. All plates were photographed after 3 days of incubation at 30°C.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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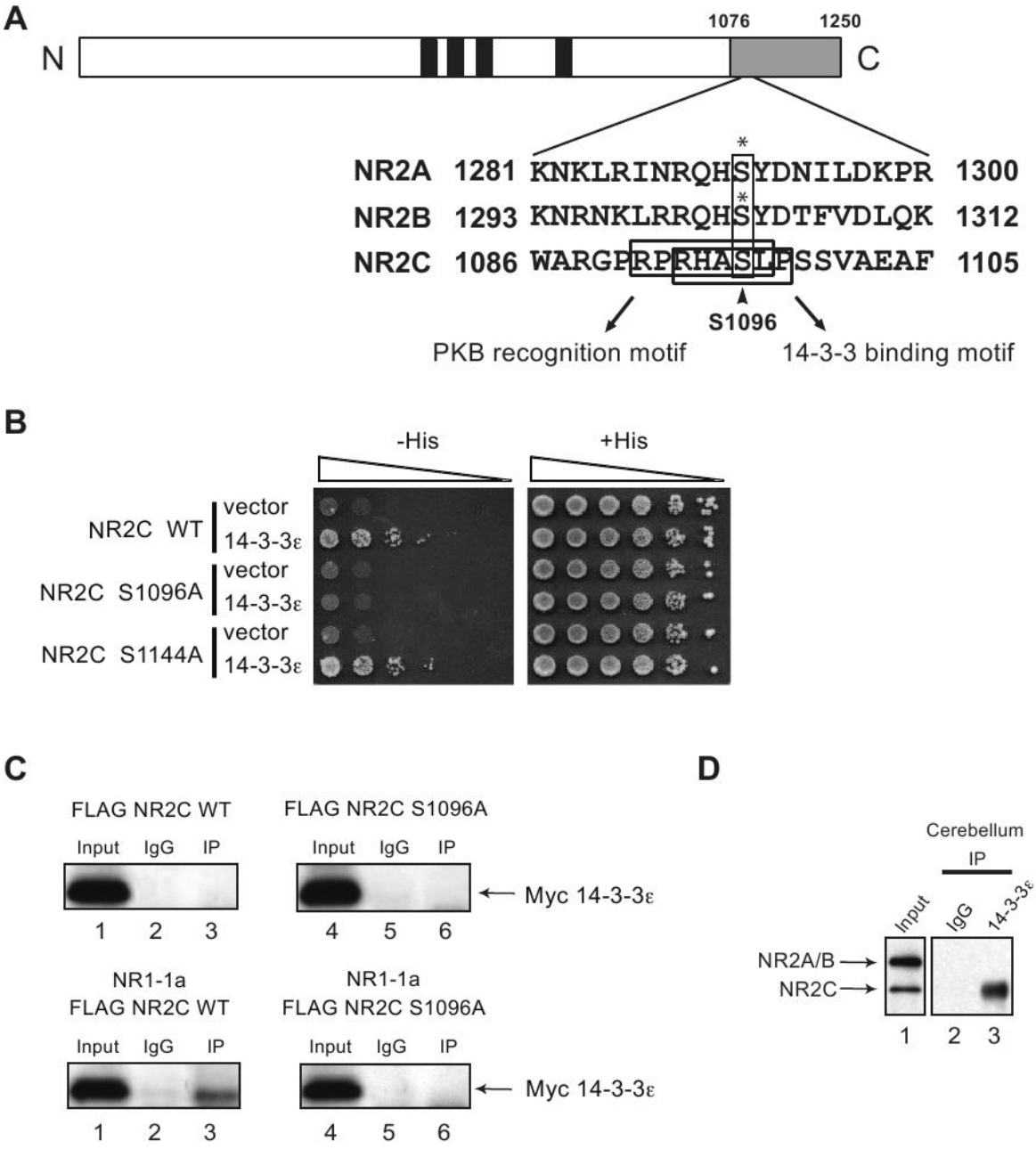

Fig. 1.

NR2C specifically interacts with 14-3-3ε, and binding is regulated by S1096. A, Schematic diagram of NR2C including an alignment of NR2A (amino acids 1281-1300), NR2B (amino acids 1293-1312) and NR2C (amino acids 1086-1105). NR2C S1096 is indicated with an arrowhead, and NR2A S1291 and NR2B S1303 are indicated with asterisks. The shaded area indicates a.a. 1076 to a.a. 1250 of NR2C, which was used as bait in the yeast two-hybrid screen. B, Yeast were co-transformed with LexA-NR2C WT, LexA-NR2C S1096A or LexA-NR2C S1144A and either Gal4 vector or Gal4-14-3-3ε and growth was evaluated on appropriate yeast selection medium. Results shown are 10-fold serial dilutions of yeast cells. C, HEK-293 cells were transiently transfected with FLAG-NR2C or FLAG-NR2C S1096A and Myc-14-3-3ε

with or without NR1-1a. Receptors were immunoprecipitated from cell lysates with anti-FLAG antibodies or IgG antibodies as a negative control. Immunoprecipitates were resolved by SDS-PAGE and immunoblotted with anti-c-Myc antibodies. Input = 2% of total lysate. D, adult rat cerebella were solubilized, and 14-3-3ε was immunoprecipitated from lysates with anti-14-3-3 antibodies or IgG antibodies as a control. Immunoprecipitates were resolved by SDS-PAGE and immunoblotted with anti-NR2C antibodies. Input = 5% of total lysate.

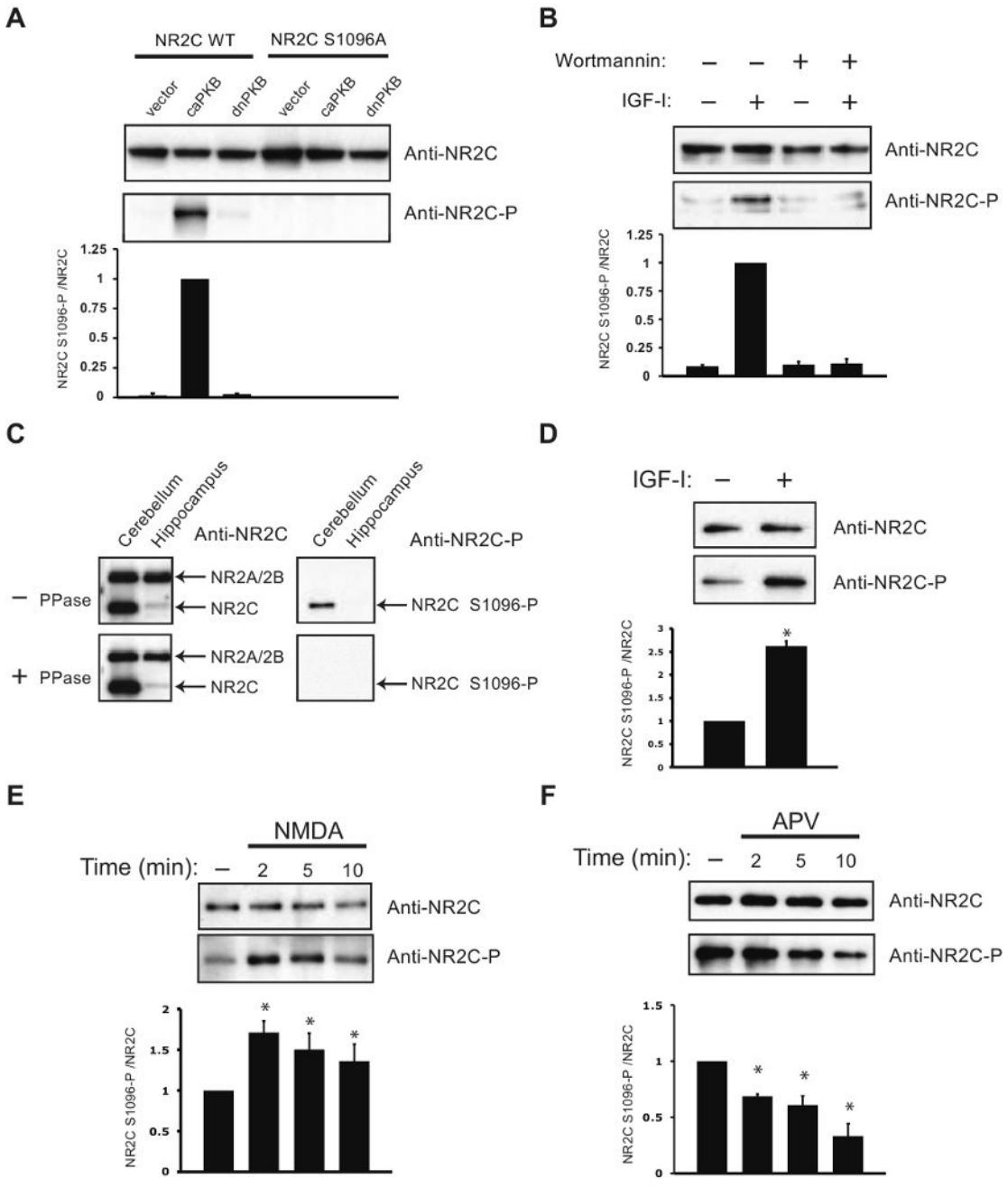

Fig. 2.

NR2C is phosphorylated on S1096 by PKB and phosphorylation is regulated by IGF-I and NMDA receptor activity. A, HEK-293 cells were co-transfected with NR2C WT or NR2C S1096A and vector, a constitutively active form of PKB (caPKB), or a dominant-negative form of PKB (dnPKB), and immunoblots of cell lysate were probed with either the NR2C antibody or NR2C S1096 phosphorylation-state specific antibody. B, HEK-293 cells expressing NR2C were treated for 10 min +/− wortmannin, and the cells were stimulated for 10 min +/− IGF-I in the continued presence or absence of wortmannin. The immunoblots of cell lysate were probed with either the NR2C antibody or NR2C S1096 phosphorylation-state specific antibody. A-B, The data were quantified by measuring phosphorylated NR2C/NR2C band

intensity ratios using ImageJ software and normalizing to either NR2C with caPKB (A) or NR2C with IGF-I (B). Data represent means +/− SEM (n=3). C, cerebellum and hippocampus from a P12 mouse were solubilized, and NR2C was immunoprecipitated from lysates, resolved by SDS-PAGE and immunoblotted with antibodies as indicated. Recognition of phosphorylated NR2C was prevented following λ phosphatase treatment of immunoblots. D-F, Cultured cerebellar granule cells were treated +/− IGF-I (50 ng/ml), NMDA (50 μM) or APV (50 μM) for various times as indicated. NR2C was immunoprecipitated from lysates, resolved by SDS-PAGE and immunoblotted with either the NR2C antibody or the NR2C S1096 phosphorylation-state specific antibody. The data were quantified by measuring phosphorylated NR2C/input NR2C band intensity ratios using ImageJ software and normalizing to untreated NR2C WT control cultures. Data represent means +/− SEM (n=3) (*, $P < 0.05$).

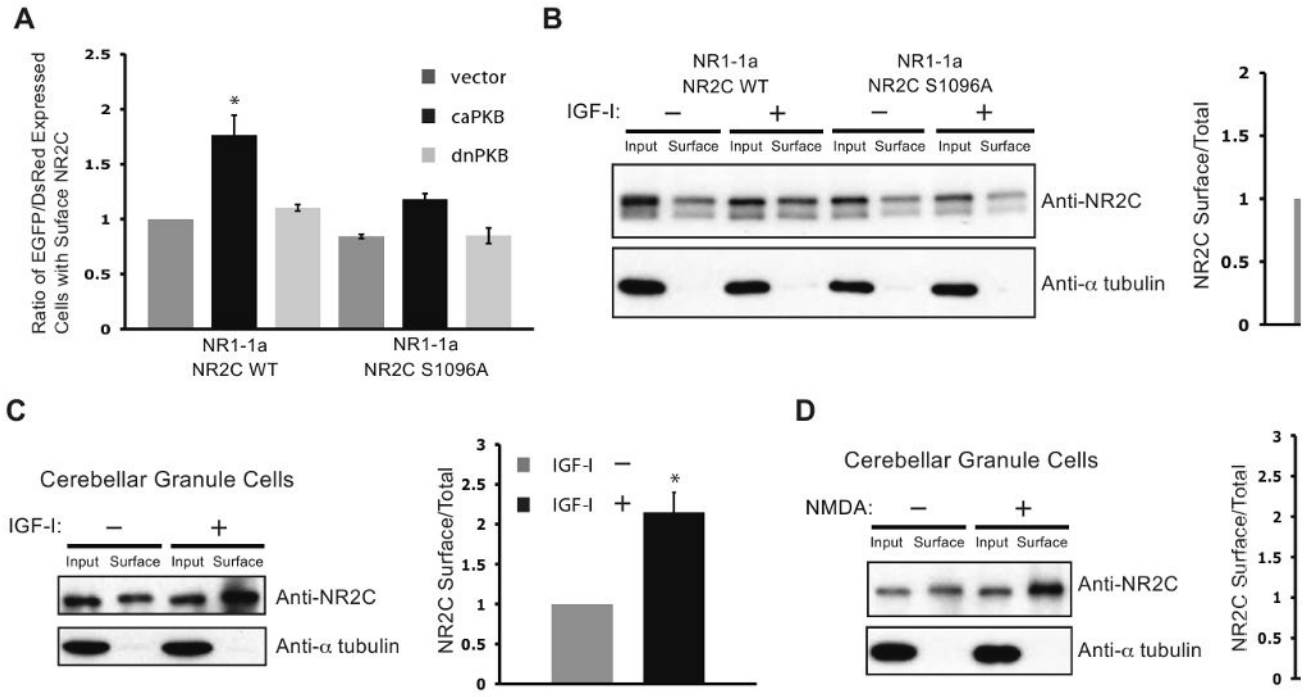

Fig. 3.

PKB phosphorylation of NR2C on S1096 increases NMDA receptor surface expression. A, HEK-293 cells were co-transfected with NR1-1a-IRES-DsRed, FLAG-NR2C-IRES-EGFP and vector, caPKB or dnPKB. Cells were collected and analyzed by FACS analysis (see Experimental Procedures). Bar plot showing the normalized ratio of NR2C, EGFP and DsRed expressed cells versus EGFP and DsRed expressed cells. Data represent means +/− SEM (n=3) (*, $P < 0.05$). B, HEK-293 cells expressing NR1-1a and NR2C WT or NR2C S1096A were incubated +/− IGF-I for 20 min before biotinylating surface proteins. Total lysates were compared to surface pools of proteins by immunoblotting with the NR2C antibody. The data were quantified by measuring surface NR2C/input NR2C band intensity ratios using ImageJ software and normalizing to untreated NR2C WT control cultures. Data represent means +/− SEM (n=3) (*, $P < 0.05$). C and D, Cultured cerebellar granule cells were treated +/− IGF-I (50 ng/ml) (C) or NMDA (50 μM) (D) before biotinylating surface proteins. Total lysates (lanes 1 and 3) were compared with surface pools of proteins (lanes 2 and 4) by immunoblotting with the NR2C antibody. Data was quantified using ImageJ software. Data represent means +/− SEM (n=3) (*, $P < 0.05$).

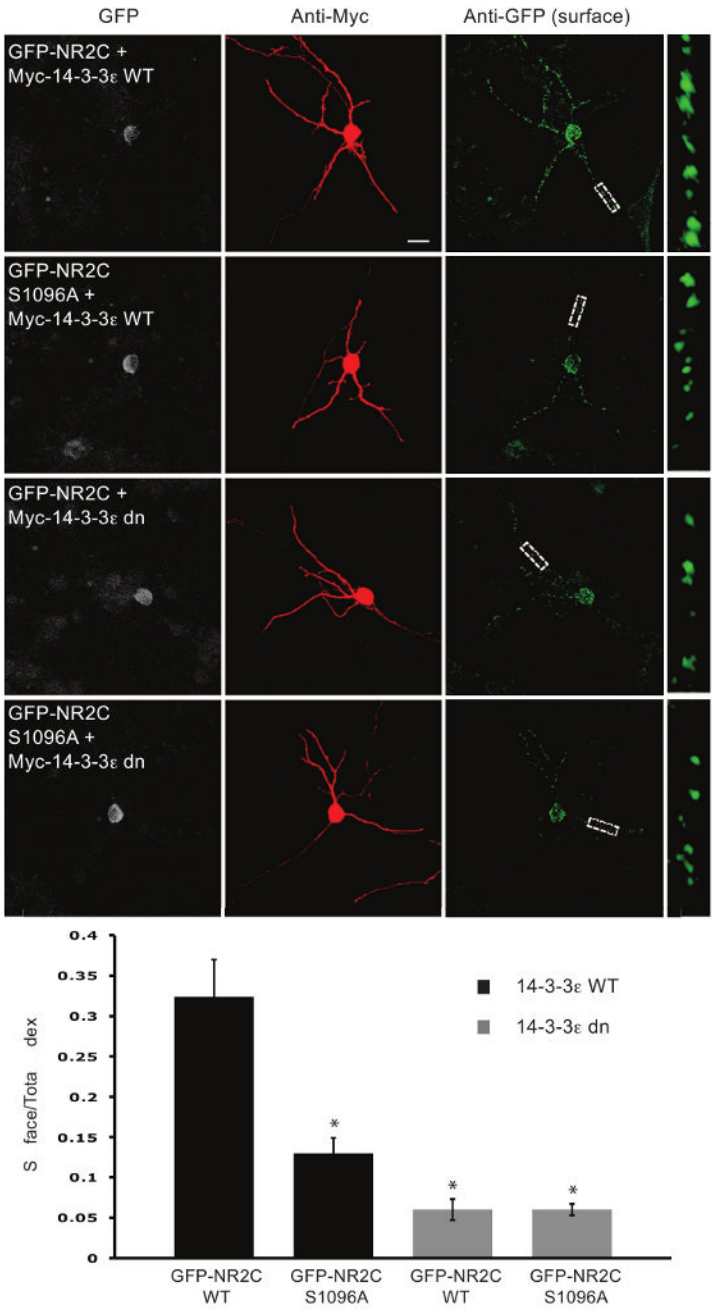

Fig. 4.

Disruption of 14-3-3ε binding to NR2C reduces NR2C surface expression. Cerebelluar granule cells were co-transfected with NR2C containing an extracellular GFP protein tag (GFP-NR2C) or GFP-NR2C S1096A and Myc-14-3-3ε WT or Myc-14-3-3ε dn. Two days after transfection, cells were incubated with anti-GFP antibody for 30 min at room temperature. Cells were fixed and incubated with Alexa 647-conjugated (blue) anti-rabbit secondary antibody to visualize the surface receptors. Cells were then washed, permeabilized, and labeled with anti-Myc monoclonal antibody and Alexa 568-conjugated (red) anti-mouse secondary antibody to visualize the 14-3-3ε transfected cells. The Alexa 647 fluorescence is shown in green for visual effects. The right panels show higher magnification images of boxed regions. Scale bar = 10

μm. The data were quantified by measuring ratios of surface NR2C/total NR2C using Metamorph software. Data represent means +/− SEM (n=3) (*, $P < 0.05$).

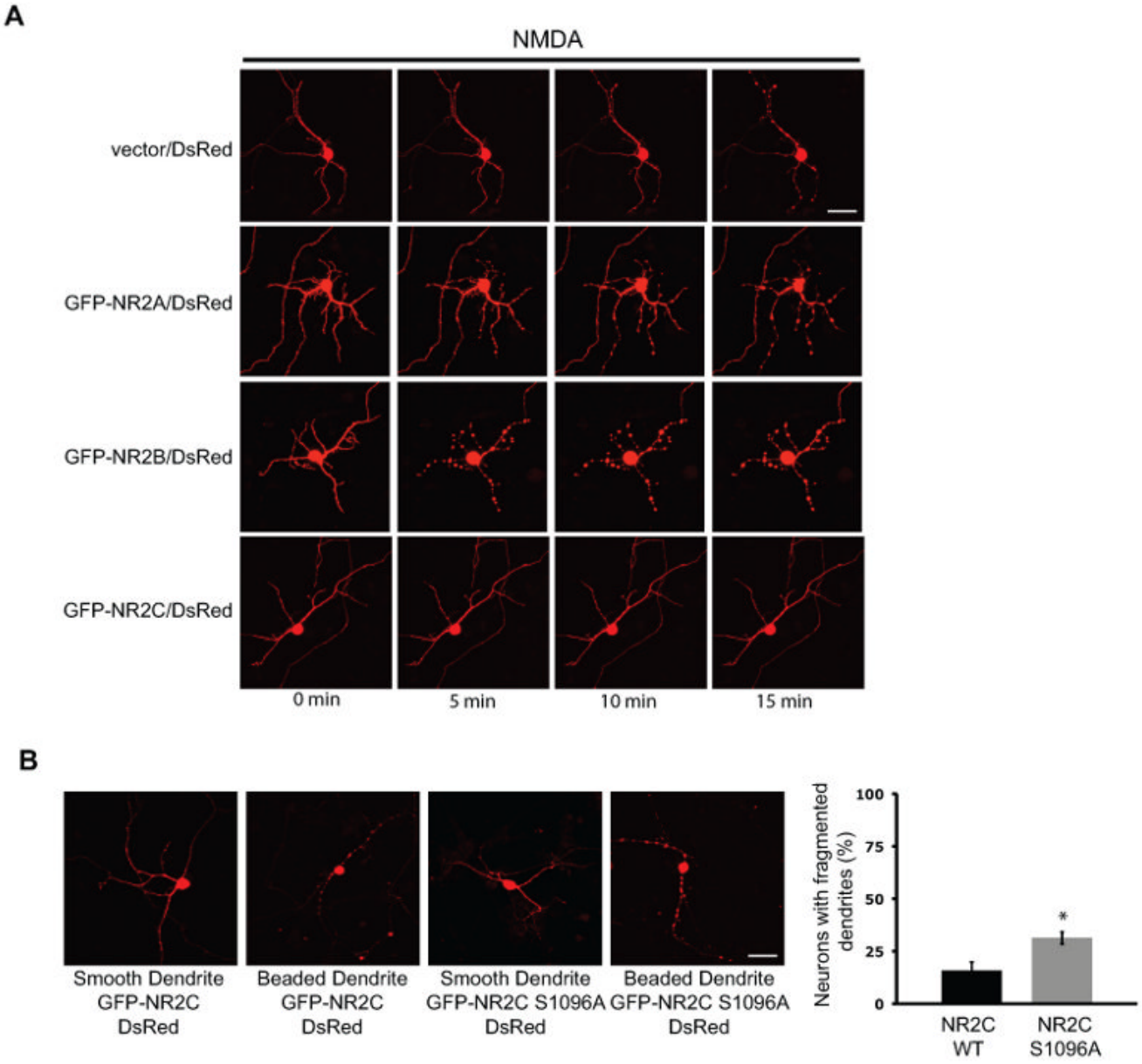

Fig. 5.

NR2C surface expression is neuroprotective. A, Cerebellar granule cells (DIV4) were co-transfected with DsRed and vector, GFP-NR2A, GFP-NR2B or GFP-NR2C. On DIV 6, time-lapse imaging was performed during exposure to NMDA (200 μM). DsRed was used to visualize the changes in cell morphology of transfected neurons. NMDA treatment resulted in cell body swelling and dendritic varicosities of neurons expressing DsRed alone, NR2A or NR2B. Scale bar = 20 μm. Data shown are representative of four independent experiments. B, Cerebellar granule cells (DIV4) were co-transfected with DsRed and GFP-NR2C WT or GFP-NR2C S1096A. Cells were fixed on DIV 8. The images shown are representative of healthy and sick neurons. The data were quantified by measuring percentage of NR2C-expressing neurons with dendritic fragmentation or varicosities. Data represent means +/− SEM (n=3) (*, $P < 0.05$).