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NMDA Receptor Modulation by the Neuropeptide Apelin: Implications for Excitotoxic Injury

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Abstract

Excitotoxic neuronal damage via over-activation of the NMDA receptor has been implicated in many neurodegenerative diseases. *In vitro* modeling of excitotoxic injury has shown that activation of G-protein coupled receptors (GPCRs) counteracts such injury through modulation of neuronal pro-survival pathways and/or NMDA receptor signaling. We have previously demonstrated that the GPCR APJ and its endogenous neuropeptide ligand apelin can protect neurons against excitotoxicity, but the mechanism(s) of this neuroprotection remain incompletely understood. We hypothesized that apelin can promote neuronal survival by activating pro-survival signaling as well as inhibiting NMDA receptor-mediated excitotoxic signaling cascades. Our results demonstrate that (i) apelin activates pro-survival signaling via inositol trisphosphate (IP_3), protein kinase C (PKC), mitogen-activated protein kinase kinase 1/2 (MEK1/2), and extracellular signal-regulated kinase-1/2 (ERK1/2) to protect against excitotoxicity, and (ii) apelin inhibits excitotoxic signaling by attenuating NMDA receptor and calpain activity, and by modulating NMDA receptor subunit NR2B phosphorylation at serine 1480. These studies delineate a novel apelinergic signaling pathway that concurrently promotes survival and limits NMDA receptor-mediated injury to protect neurons against excitotoxicity. Defining apelin-mediated neuroprotection advances our understanding of neuroprotective pathways and will potentially improve our ability to develop therapeutics for excitotoxicity-associated neurodegenerative disorders.

Keywords

apelin; NMDA receptor; HIV; neuroprotection; excitotoxicity

Introduction

Excitotoxic neuronal damage via over-activation of the NMDA receptor has been implicated in many neurodegenerative diseases, and further understanding of endogenous mechanisms that regulate NMDA receptor signaling is critical for the development of effective

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therapeutics (Hardingham & Bading 2010). NMDA receptors include two glycine-binding NR1 subunits and two glutamate-binding NR2 subunits of four types (NR2A - NR2D; (Cull-Candy & Leszkiewicz 2004), and these subunits demonstrate well-defined developmental and regional distribution in rodent models and in humans (Monyer *et al.* 1994, Standaert *et al.* 1996, Conti *et al.* 1999, Law *et al.* 2003). Phosphorylation of these subunits, particularly within the C-terminus of NR2B, can differentially regulate receptor function and susceptibility to excitotoxicity (Waxman & Lynch 2005, Chen & Roche 2007). Specifically, Src family kinases phosphorylate NR2B tyrosine residues Y1336 and Y1472, and in general, potentiate NMDA receptor currents to enhance excitotoxicity (Salter & Kalia 2004). Casein kinase-2 (CK2) phosphorylates NR2B serine residue S1480 (Chung *et al.* 2004, Sanz-Clemente *et al.* 2010), which has been indirectly implicated in neuroprotection against excitotoxicity (Clapp *et al.* 2009). Our studies have focused on HIV infection of the CNS as an excitotoxicity disease model, and we have shown that neuronal vulnerability to HIV neurotoxicity is dependent upon developmentally-regulated expression of NR2B (O'Donnell *et al.* 2006). Additionally, NR2B phosphorylation has been implicated in susceptibility to excitotoxicity in various HIV neurotoxicity models (Haughey *et al.* 2001, Viviani *et al.* 2006, Eugenin *et al.* 2007).

Accordingly, G-protein coupled receptors (GPCRs) may protect against HIV-induced excitotoxicity via NMDA receptor modulation (Kaul & Lipton 1999, Meucci *et al.* 2000, Bruno *et al.* 2000). The GPCR APJ receptor and its endogenous neuropeptide ligand apelin are highly expressed in the CNS, especially in cortical, hippocampal, and hypothalamic neurons, with comparable distribution between rodents and humans (De Mota *et al.* 2000, Lee *et al.* 2000, Reaux *et al.* 2002, Kleinz & Davenport 2005). We have shown that treatment of rodent hippocampal neuronal cultures with several native apelin isoforms, including apelin-36, phosphorylates extracellular signal-regulated kinase-1/2 (ERK1/2) and protects against excitotoxicity (O'Donnell *et al.* 2007). However, the mechanism(s) of apelin-mediated neuroprotection remain largely unknown, including clear delineation of the neuronal apelinergic G-protein coupled signaling pathway and potential modulation of NMDA receptors. We hypothesized that apelin can promote neuronal survival by activating pro-survival signaling as well as inhibiting NMDA receptor-mediated excitotoxic signaling cascades. Our results demonstrate that apelinergic signaling concurrently promotes survival via inositol trisphosphate (IP_3), protein kinase C (PKC), mitogen-activated protein kinase kinase 1/2 (MEK1/2), and ERK1/2 activation and limits excitotoxicity by modulating NR2B S1480 phosphorylation and attenuating NMDA receptor-mediated ionic currents, Ca^{2+} accumulation, and calpain activation. Targeting apelinergic signaling may have therapeutic value for disorders involving excitotoxicity, including HIV-associated neurocognitive disorders, ischemia, epilepsy, Huntington's disease, Parkinson's disease and Alzheimer's disease (Waxman & Lynch 2005, Hardingham & Bading 2010).

Materials and Methods

Detailed information regarding materials, quantification of excitotoxicity in primary rat brain cultures, calcium imaging, electrophysiology and Western blotting can be found as Supplemental Methods.

Preparation of primary rat brain cultures

Primary rat cerebrocortical cultures were prepared from embryonic day 17 Sprague-Dawley rat pups as previously described (Brewer 1995, Wilcox *et al.* 1994). All procedures were within the ARRIVE guidelines for animal research, and in accordance with protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Cells were plated in tissue culture dishes pre-coated with poly-L-lysine (Peptides International, Louisville, KY) and maintained in neurobasal media plus B27 supplement

(Invitrogen, Carlsbad, CA) at 37 °C/5% CO_2. Half of the media was replaced every 7 days, and cultures were used between 14 days *in vitro* (DIV) and 18 DIV.

Preparation of human monocyte-derived macrophages and HIV infections

HIV infection of monocyte-derived macrophages was performed as previously described (Chen *et al.* 2002, O'Donnell et al. 2006), and in accordance with protocols approved by the University of Pennsylvania Committee on Studies Involving Human Beings. Briefly, macrophages were infected with a CSF-derived, macrophage-tropic HIV-1 isolate (HIV-Jago) from a patient with confirmed HIV-associated dementia. Viral stocks were prepared by the University of Pennsylvania Center for AIDS Research Virology Core. Supernatants from HIV-infected or non-infected (vehicle) macrophages were collected and monitored for HIV replication by quantifying viral reverse transcriptase activity as the amount of radiolabeled deoxythymidine incorporation (Ho *et al.* 1992).

Quantification of excitotoxicity in primary rat brain cultures

Cell survival was quantified by three methods: (i) cell counting of microtubule associated protein-2 (MAP-2)- and glial fibrillary acidic protein (GFAP)-reactive cells (O'Donnell et al. 2006); (ii) cell-based MAP-2 ELISA assay (Wang et al. 2007, White *et al.* 2011); and (iii) lactate dehydrogenase (LDH) assay.

Calcium imaging

Primary rat cerebrocortical cells were plated at a density of 2×10^5 cells per 35mm glass bottom dish (MatTek Corporation, Ashland, MD), and intracellular neuronal Ca^{2+} concentration was determined as previously described (Wu *et al.* 2007, Lynch *et al.* 2001).

Electrophysiology

Primary rat cerebrocortical cells were plated at a density of $2–4\times10^5$ cells per 35mm dish with glass coverslips, and standard whole-cell voltage clamp techniques were used for recording NMDA-induced ionic currents. Treatments applications were performed under the following paradigm: 2sec agonist, 1min wash, 2min treatment, 2sec agonist, 3min wash.

Site-directed mutagenesis of NR2B

NR2B at serine 1480 was mutated to alanine (S1480A) in the mammalian expression vector pRK7-NR2B using the QuikChange II Site-Directed Mutagenesis kit (Agilent, Santa Clara, CA) as previously described (Wu et al. 2007) using published primers (Chung et al. 2004).

Transfection of HEK293 cells

HEK293 cells were plated in tissue culture dishes pre-coated with poly-D-lysine (Sigma) and maintained in minimum essential media plus 7.5% fetal bovine serum, 2.5% horse serum, 1% penicillin/streptomycin, and 1% L-glutamine at 37 °C/5% CO_2. Transfections were performed using calcium phosphate in the presence of ketamine (500μM, Sigma) to prevent NMDA receptor activation (Grant *et al.* 1997). Treatments were administered 16–18hr post-transfection.

Quantification of excitotoxicity in NMDA receptor-transfected HEK293 cells

HEK293 cells were plated in 24-well plates with glass coverslips, and following transfection and experimental treatments, cultures were incubated in Hoescht 33342 (5μg/ml) for 20min at room temperature and fixed in 4% paraformaldehyde in PBS for 20min at 4°C. The number of surviving GFP-positive cells was estimated by blinded counting of GFP- and Hoescht-positive cells from 3–5 randomly selected fields per coverslip at 20x magnification.

Western blotting

HEK293 cells or primary rat cerebrocortical cells were plated at a density of 1×10^6 cells per 60mm dish or 4×10^5 cells per 35mm dish. Following experimental treatments, whole cell lysates were subjected to SDS-PAGE as previously described (O'Donnell et al. 2006).

Statistical analysis

For quantification of excitotoxicity, Western blotting, and electrophysiology, values are expressed as mean ± standard error of mean, and statistical comparisons were made by Student's *t*-test or one-way ANOVA plus Newman-Keuls *post hoc* testing as indicated in the figure legends. For Ca^{2+} imaging, statistical comparisons for populations of individuals neurons were made by the Kruskal-Wallis test plus Dunn's Multiple Comparison *post hoc* testing, and correlations were made by Spearman's test. All graphs were generated and statistical analyses were performed using GraphPad Prism software (San Diego, CA), and values of $p<0.05$ were considered significant.

Results

Apelin protects cerebrocortical neurons against glutamate- or HIV-induced excitotoxicity via IP_3-, PKC-, MEK1/2, and ERK1/2-mediated signaling pathways

Several *in vitro* models have shown that HIV infection in the CNS causes release of soluble excitotoxins, especially glutamate, from productively infected macrophages (Kaul *et al.* 2005). Glutamate and supernatants from HIV-infected macrophages cause neurotoxicity in primary rat hippocampal and cerebrocortical cultures dependent upon developmentally-regulated expression of NR2B (14 days *in vitro* and older; (O'Donnell et al. 2006)). To define the mechanism(s) of apelin-mediated neuroprotection, we used 14–18 days *in vitro* fetal rat cerebrocortical neuronal/glial cultures exposed to glutamate or HIV-infected macrophage supernatants, each of which caused dose-dependent excitotoxicity (Fig. 1; Supplemental Fig. 1; Supplemental Fig. 2). Treatment with a native apelin peptide (apelin-36), but not a negative control peptide (apelin-36 scramble), showed robust, dose-dependent neuroprotection against both glutamate and HIV supernatant insults, while apelin in the absence of insult had no effect on neuronal survival (Fig. 1b, 1d). Interestingly, MK801 in the absence of insult induced some neurotoxicity (Fig. 1d), consistent with studies suggesting that elimination of all NMDA receptor activity can cause cell death (Papadia *et al.* 2005). Together, these results demonstrate that apelin protects cerebrocortical neurons against excitotoxicity in a dose-dependent manner.

We next sought to identify signaling pathways underlying apelin-mediated neuroprotection using specific pharmacologic inhibitors (Fig. 2). Apelin caused phosphorylation of ERK1/2 at the kinases' active sites (Fig. 2a). Apelin-induced ERK1/2 phosphorylation was blocked by 2-APB, which inhibits Ca^{2+} release from IP_3 receptors, and by MEK1/2 inhibitors U0126 and PD98059, consistent with Ca^{2+}-dependent GPCR activation of ERK1/2 (Fig. 2a, Fig. 8, (Gutkind 2000)). Apelin also caused PKC phosphorylation at its active site, which is required prior to Ca^{2+} and/or lipid second messenger binding for PKC activation (Gould & Newton 2008). As expected, apelin-induced PKC phosphorylation was blocked by GF109203X and chelerythrine chloride, which inhibit conventional and novel PKC isoforms, but not by 2-APB, as PKC phosphorylation occurs before its activation by Ca^{2+} (Fig. 2b). Notably, 2-APB, U0126, PD98059, GF109203X, and chelerythrine chloride all blocked apelin neuroprotection against glutamate, while a negative control epidermal growth factor tyrosine kinase inhibitor AG1478 did not (Fig. 2c). Notably, similar effects were seen at apelin concentrations of 20μM (Fig. 2c) and 2μM (data not shown). Together, these studies demonstrate that apelin promotes neuronal survival against excitotoxicity by activating IP_3, PKC, MEK1/2, and ERK1/2 intermediaries.

Additional studies investigated other pathways implicated in apelinergic signaling, including the phosphoinositide 3-kinase–Akt–mammalian target of rapamycin pathway (Masri *et al.* 2005, Japp & Newby 2008), but pharmacologic inhibitors caused significant neurotoxicity (data not shown), thereby preventing conclusive analysis.

Apelin attenuates NMDA receptor activity and calpain activation in cerebrocortical neurons

In addition to activation of pro-survival signaling pathways, some GPCR ligands can induce neuronal Ca^{2+} transients and inhibit NMDA receptor-mediated Ca^{2+} influx and accumulation to protect against excitotoxicity (Meucci et al. 2000, Deiva *et al.* 2004, Limatola *et al.* 2005, Yao *et al.* 2009). Similarly, apelin induced dose-dependent, oscillatory Ca^{2+} transients in cerebrocortical neurons (Fig. 3a-d), consistent with our previous findings in human NT2.N neuronal cell lines (Choe *et al.* 2000). Expanding on these findings, apelin-induced Ca^{2+} transients were below the maximum Ca^{2+} concentration, as determined by exposure to the Ca^{2+} ionophore ionomycin (Fig. 3a-d), and decayed within 10min post-treatment (data not shown), suggesting that apelin causes sub-maximal and reversible Ca^{2+} accumulation. Furthermore, apelin inhibited Ca^{2+} accumulation induced by NMDA plus glycine (Fig. 3e), and neurons with the largest Ca^{2+} response to apelin showed the greatest attenuation of NMDA receptor-mediated Ca^{2+} accumulation (Fig. 3f). This result directly links apelin induction of Ca^{2+} transients with attenuation of NMDA receptor-mediated Ca^{2+} accumulation. Additional studies demonstrated that apelin can attenuate Ca^{2+} accumulation at higher NMDA receptor agonist concentrations (200μM NMDA plus 100μM glycine), and that apelin did not affect potassium-depolarization-induced Ca^{2+} accumulation (data not shown), suggesting that apelin modulation of NMDA receptors is not due to non-specific inhibition of neuronal Ca^{2+} responses.

As apelin effectively blocks NMDA receptor-mediated Ca^{2+} accumulation, we reasoned that apelin might also directly attenuate NMDA receptor-mediated ionic currents. Using whole-cell voltage clamp electrophysiology in cerebrocortical neurons, apelin demonstrated a dose-dependent inhibition of NMDA-induced currents, while apelin-36 scramble showed no effect (Fig. 4). These studies demonstrate that rapid apelin-mediated signaling events can directly inhibit NMDA receptor activity as measured by ionic currents and Ca^{2+} accumulation.

Excessive NMDA receptor activity can ultimately lead to Ca^{2+}-dependent activation of calpains (Goll *et al.* 2003), and calpain inhibitors can provide neuroprotection against glutamate- or HIV-induced excitotoxicity (Supplemental Fig. 3a, (O'Donnell et al. 2006, Wang *et al.* 2007)). As expected, treatment with apelin inhibited NMDA-induced calpain activation, as measured by calpain-specific cleavage of the cytoskeletal protein spectrin (Fig. 5). Moreover, apelin in the absence of NMDA did not modulate calpain activation (Fig. 5), again suggesting that apelin causes sub-maximal and reversible Ca^{2+} accumulation that is insufficient to either activate calpain or cause excitotoxicity. Together, these studies demonstrate that apelin attenuates excessive NMDAR activity and calpain activation, suggesting that apelin promotes neuronal survival by inhibiting excitotoxic NMDA receptor signaling.

Apelin-induced phosphorylation of NR2B S1480 mediates protection against excitotoxicity in cerebrocortical neurons and NMDA receptor-transfected HEK293 cells

NR2B-containing NMDA receptors are the primary mediators of excitotoxicity in our model system (Supplemental Fig. 3b–c, (O'Donnell et al. 2006)), and NR2B phosphorylation can differentially regulate susceptibility to excitotoxicity (Salter & Kalia 2004, Chen & Roche 2007). Thus, we sought to identify the effects of apelin on NR2B phosphorylation as a

mechanism for neuroprotection. Apelin increased NR2B phosphorylation at S1480 in the presence of NMDA (Fig. 6a) without altering NR2B phosphorylation at Y1336 or Y1472 (data not shown). Treatment with 2-APB or TBB, a competitive inhibitor of CK2, prior to apelin and NMDA exposure blocked S1480 phosphorylation (Fig. 6b), consistent with Ca^{2+}-dependent CK2 phosphorylation of S1480 (Chung *et al.* 2004, Sanz-Clemente *et al.* 2010). Neither PKC inhibitor GF109203X nor MEK1/2 inhibitor PD98059 blocked apelin-induced phosphorylation of S1480 (Fig. 6b). Additionally, TBB blocked apelin-mediated neuroprotection against glutamate, while the Src family kinase inhibitor PP2, which blocks phosphorylation at Y1336 and Y1472, had no effect (Fig. 6c). Similar effects were seen at apelin concentrations of 20μM (Fig. 2c) and 2μM (data not shown). Together, these studies demonstrate that apelin-induced phosphorylation of NR2B S1480 during NMDA receptor activation may contribute to apelin neuroprotection.

To further validate the role of NR2B S1480 phosphorylation in apelin-mediated neuroprotection, we transiently transfected HEK293 cells with expression plasmids containing green fluorescent protein (GFP), APJ, NR1, postsynaptic density-95 (PSD-95), and wild-type (WT) NR2B or NR2B with S1480 mutated to alanine (S1480A), which cannot be phosphorylated (Lim *et al.* 2002). Consistent with our studies in neurons, exposure to apelin and NMDA induced S1480 phosphorylation in WT NR2B-expressing cells, but not S1480A-expressing cells (Fig. 7a). Similarly, apelin protected WT NR2B-expressing cells, but not S1480A-expressing cells, against glutamate-induced excitotoxicity (Fig. 7b). These studies further suggest that apelin-induced phosphorylation of NR2B S1480 contributes to apelin neuroprotection.

Discussion

Previous work in our laboratory demonstrated that apelin can protect neurons against excitotoxicity (O'Donnell *et al.* 2007), and in this study, we sought to more fully define the mechanism(s) of this neuroprotection. We now propose a novel pathway in which apelin triggers signaling through IP_3, Ca^{2+}, CK2, PKC, MEK1/2, ERK1/2, and NR2B S1480 phosphorylation to concurrently promote survival and limit NMDA receptor-mediated excitotoxic activity (Fig. 8). Therefore, targeting the apelinergic system in the CNS may have significant and novel therapeutic value, especially for HIV and other neurodegenerative disorders in which excitotoxicity is implicated.

Apelinergic activation of pro-survival signaling

We have shown that apelin dose-dependently protects cerebrocortical neurons against HIV- or glutamate-induced excitotoxicity via IP_3-, PKC-, MEK1/2- and ERK1/2-mediated signaling pathways (Fig. 1–2). Although the G-protein coupling of APJ, apelin's receptor, is unknown in neurons, apelinergic signaling in heterologous systems and in non-neuronal cells occurs through Gi/o or Gq (Masri *et al.* 2005, Japp & Newby 2008). Gi/o and Gq can activate phospholipase C (PLC) to hydrolyze phosphatidylinositol bisphosphate (PIP_2) into IP_3 and diacylglcerol (DAG), causing Ca^{2+} release through endoplasmic reticulum IP_3 receptors and subsequent activation of several PKC isoforms, MEK1/2, and ERK1/2 (Fig. 8; (Gutkind 2000)). Thus, our findings are consistent with Gi/o- or Gq-coupling of apelinergic signaling in primary neurons (Fig. 2).

Apelin triggers neuronal Ca^{2+} transients typical of GPCR signaling, and our inhibitor studies indirectly implicate IP_3 receptor activation in these transients (Fig. 2–3). However, we have not ruled out the contribution of other Ca^{2+} channels in apelinergic signaling. Recent studies suggest that neuroprotective chemokine GPCR ligands that trigger IP_3 signaling can activate transient receptor potential canonical channels, a superfamily of Ca^{2+}-permeable channels

that shunt out intracellular Ca^{2+} during NMDA receptor activation (Yao et al. 2009). Apelin modulation of such channels remains a possibility for further investigation.

Our inhibitor studies also indicate apelin activation of conventional (α, β_I/β_{II}, γ) and/or novel (δ, ε, η, θ) PKC isoforms (Fig. 2). PKC activation requires sequential phosphorylation at three sites followed by binding of Ca^{2+} and/or lipid second messengers, such as DAG. Conventional PKC isoforms require Ca^{2+} and DAG for activation, novel isoforms require DAG only, and atypical isoforms (ι/λ, ζ) require neither (Gould & Newton 2008). Apelin-mediated PKC phosphorylation was blocked by inhibitors of conventional and novel PKC isoforms, GF109203X and chelerythrine chloride, but not by 2-APB, since PKC phosphorylation occurs prior to its activation by Ca^{2+} (Fig. 2). However, 2-APB blocked apelin-mediated ERK1/2 phosphorylation (Fig. 2), suggesting that Ca^{2+}-dependent conventional PKC isoforms may underlie apelin activation of ERK1/2 (Fig. 8). These findings are inconsistent with studies in APJ-transfected cells in which an inhibitor of atypical PKCs blocked apelin-stimulated ERK1/2 activation (Masri *et al.* 2004), indicating that apelinergic signaling in primary neurons might be distinct from that observed in cell lines.

Apelinergic inhibition of excitotoxic signaling

We have also shown that apelin induces Ca^{2+} transients and attenuates excitotoxic NMDA receptor and calpain activity (Fig. 3–5). Furthermore, we correlate increased Ca^{2+} response to apelin with decreased response to NMDA, directly linking apelin-induced Ca^{2+} transients to NMDA receptor attenuation (Fig. 3f). Recent work has implicated apelin at lower concentrations than used in our study in modulation of NMDA-mediated Ca^{2+} accumulation and injury (Zeng *et al.* 2010). However, several key disparities suggest a substantially different mechanism of low-dose apelin neuroprotection, including no apelin-induced Ca^{2+} transients, greatly delayed kinetics of NMDA-mediated Ca^{2+} accumulation, decreased severity of the excitotoxic insult, and diminished rescue with apelin (Zeng et al. 2010). Moreover, neuronal signaling pathways activated by low-dose apelin have not been addressed, whereas our study outlines a detailed cascade linked to direct modulation of NMDA-induced Ca^{2+} accumulation as well as ionic currents, calpain activity and phosphorylation state (Fig. 8). Nonetheless, difference in dose-dependent apelin signaling can inform development of apelinergic therapeutics and remain an area of ongoing study in our laboratory.

Apelin can induce phosphorylation of NR2B S1480 through Ca^{2+}- and CK2-dependent mechanisms to provide protection against excitotoxicity (Fig. 6–7), and the phosphorylation of S1480 is of emerging importance in the regulation of synaptic plasticity and excitability (Chung *et al.* 2004, Clapp *et al.* 2009, Li *et al.* 2009, Sanz-Clemente *et al.* 2010). Recent work demonstrates that CK2 preferentially phosphorylates S1480, disrupting NR2B-PSD-95 interactions and causing decreased surface NR2B expression (Chung *et al.* 2004, Sanz-Clemente *et al.* 2010). Moreover, S1480 phosphorylation has been indirectly implicated in neuroprotection against excitotoxicity following ethanol withdrawal (Clapp *et al.* 2009). Therefore, S1480 phosphorylation may decrease NR2B surface expression to limit NMDA receptor-mediated injury, although the specific effects of S1480 phosphorylation on NMDA receptor and calpain activity are yet to be defined. An alternative hypothesis is that CK2 may have direct, PSD-95-independent effects on NMDA receptor-mediated Ca^{2+} permeability and conductance, as has been shown for other serine/threonine kinases like PKA (Skeberdis *et al.* 2006). Whether apelin-mediated S1480 phosphorylation modulates NR2B trafficking and/or activity is unknown, and our preliminary studies indicate no consistent effect of apelin on NR2B surface expression (Cook DR and Gleichman AJ, unpublished observations). Studies are currently underway in our laboratory to define the functional consequences of apelin-induced S1480 phosphorylation on NMDA receptors.

The relative contributions of increased pro-survival signaling and decreased excitotoxic NMDA receptor signaling are not clearly distinguished in our current study, and indeed they could be directly linked. Our proposed mechanism suggests that apelin-induced Ca^{2+} transients can activate canonical GPCR pro-survival signaling through PKC, MEK1/2, and ERK1/2 in conjunction with activation of CK2 and S1480 phosphorylation which may attenuate excitotoxic signaling (Fig. 8). Moreover, events such as PKC and ERK1/2 activation have pleiotropic effects on multiple signaling pathways, and these could certainly impact NMDA receptor modulation, either directly or indirectly. Excitotoxic NMDA receptor activity has also been shown to directly inhibit pro-survival signaling pathways, including ERK1/2 activation (Soriano & Hardingham 2007). Together, these findings suggest that increased pro-survival signaling and decreased excitotoxic NMDA receptor activity are overlapping pathways for apelin-mediated neuroprotection that combine to prevent neuronal injury.

Therapeutic considerations for apelinergic signaling

Our study focuses on apelin modulation of excitotoxic NMDA receptor activity, and we have no evidence for complete suppression of NMDA receptor activity by apelin. Indeed, NMDA receptor activity is essential for normal neuronal function and survival, and preservation of physiologic activity is a key therapeutic consideration (Hardingham & Bading 2010). Moreover, physiologic NMDAR activity can actually mitigate excitotoxic cell death. Referred to as preconditioning, a low dose of NMDA (~10μM) prior to an excitotoxic dose (≥20μM) promotes neuronal survival by preferentially activating synaptic NMDA receptors to cause ERK1/2 phosphorylation (Ivanov *et al.* 2006, Soriano *et al.* 2006) and attenuation of Ca^{2+} accumulation and calpain activity caused by the excitotoxic dose (Tauskela *et al.* 2001). NMDA preconditioning is contingent on the neuroprotective dose enhancing neuronal excitability, characterized by oscillatory, sub-maximal Ca^{2+} transients, while the toxic NMDA dose triggers non-oscillatory, maximum, and sustained Ca^{2+} accumulation (Soriano et al. 2006). These preconditioning mechanisms are strikingly similar to our findings with apelin (Fig. 2–5), including our observation that apelin only decreases calpain activity or increases NR2B S1480 phosphorylation in the presence of NMDA (50μM; Fig. 5–7). Furthermore, preliminary studies indicate that apelin increases excitatory postsynaptic potential frequency in a dose-dependent and reversible manner (Cook DR and Gleichman AJ, unpublished observations), suggesting that apelin may cause a large but transient increase in synaptic glutamate that preconditions neurons against excitotoxicity. Overall, our results suggest that apelin does not diminish NMDA receptor activity under physiologic conditions, but only under conditions of excitotoxicity. Further studies to determine the role of apelin in pro-survival NMDA receptor activity are crucial, as effective therapeutics must selectively inhibit excitotoxic signaling while preserving cell survival signaling.

Much current effort is directed towards targeting ubiquitous GPCRs for therapeutics against both CNS and systemic diseases, and similar efforts are underway for targeting the apelinergic system (Lee *et al.* 2006, Sorli *et al.* 2006, Ladeiras-Lopes *et al.* 2008). Because of the remarkable redundancy of GPCR/ligand expression and function, ‘off target effects’ for any such pharmacologic activation of GPCRs are being considered, but are not seen as deterrents for investigating APJ agonists as therapeutics. Further studies *in vitro* and *in vivo* are needed to determine the effects of longer-term APJ activation, and we hypothesize that chronic APJ activation would provide persistent neuroprotection via upregulation of pro-survival genes without prolonged and potentially detrimental induction of ERK1/2 signaling or suppression of NMDA receptor signaling due to ligand-induced APJ internalization (Zhou *et al.* 2003, Lee *et al.* 2010). Additionally, a neuroactive, nonpeptidic APJ agonist has recently been identified (Iturrioz *et al.* 2009), and our studies suggest that such small

molecule agonists should be considered as potential neuroprotectants for excitotoxicity-associated neurodegenerative disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

Ca^{2+}	calcium
CK2	casein kinase-2
DAG	diacylglcerol
DIV	days *in vitro*
ERK1/2	extracellular signal-regulated kinase-1/2
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GPCR	G-protein coupled receptor
HEK293	human embryonic kidney cells
HIV	human immunodeficiency virus
IP_3	inositol trisphosphate
LDH	lactate dehydrogenase
MAP-2	microtubule associated protein-2
MEK1/2	mitogen-activated protein kinase kinase 1/2
PIP_2	phosphatidylinositol bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PSD-95	postsynaptic density-95
UT	untreated
WT	wildtype

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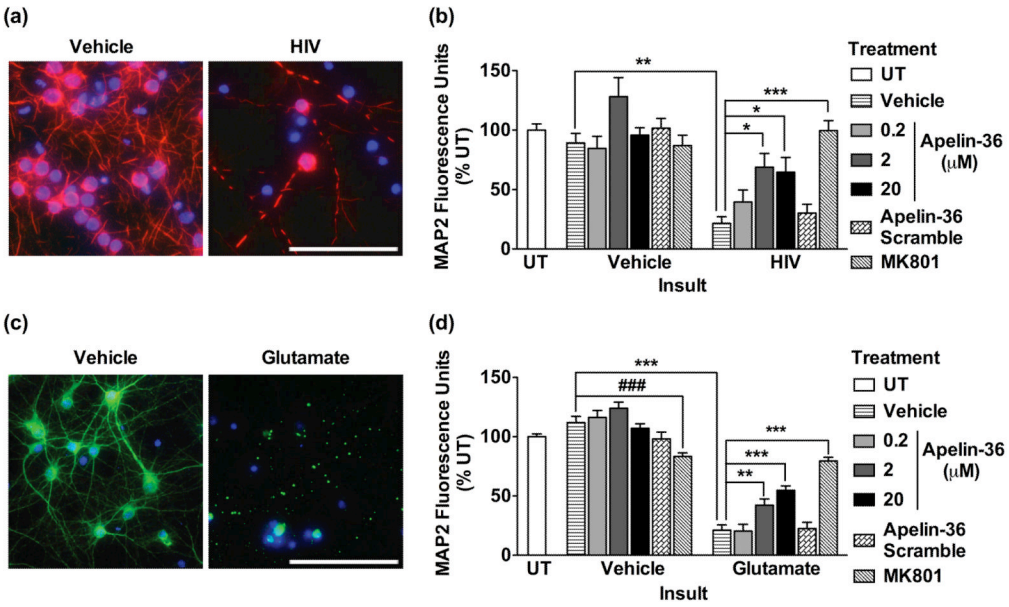

Fig 1.

Apelin protects cerebrocortical neurons from HIV-and glutamate-induced excitotoxicity in a dose-dependent manner. (a) Cultures were exposed to HIV-infected macrophage supernatant for 24h (1:20 dilution). Cell survival was assessed by immunofluorescent staining for neuronal marker MAP-2 (red) and nuclear marker Hoescht 33324 (blue). Magnification for fluorescent micrographs is 20x (scale bar: 20μM). (b) Cultures were treated with apelin-36 (0.2–20μM), apelin-36 scramble (20μM), or MK801 (10μM) for 45 minutes, followed by exposure to HIV supernatant for 24hr. Cell survival was quantified by MAP-2 ELISA and expressed as a percentage of untreated (UT) cultures (n = 12; *p<0.05, **p<0.01, ***p<0.01 vs. HIV). (c) Cultures were exposed to glutamate for 24h (50μM). Cell survival was assessed by immunofluorescent staining for MAP-2 (green) and Hoescht 33324 (blue). (d) Cultures were treated as in (b), followed by exposure to glutamate for 24hr. Cell survival was quantified by MAP-2 ELISA (n = 11–17; ###p<0.001 vs. vehicle; **p<0.01, ***p<0.001 vs. glutamate). All statistical comparisons were made by one-way ANOVA plus Newman-Keuls *post hoc* testing.

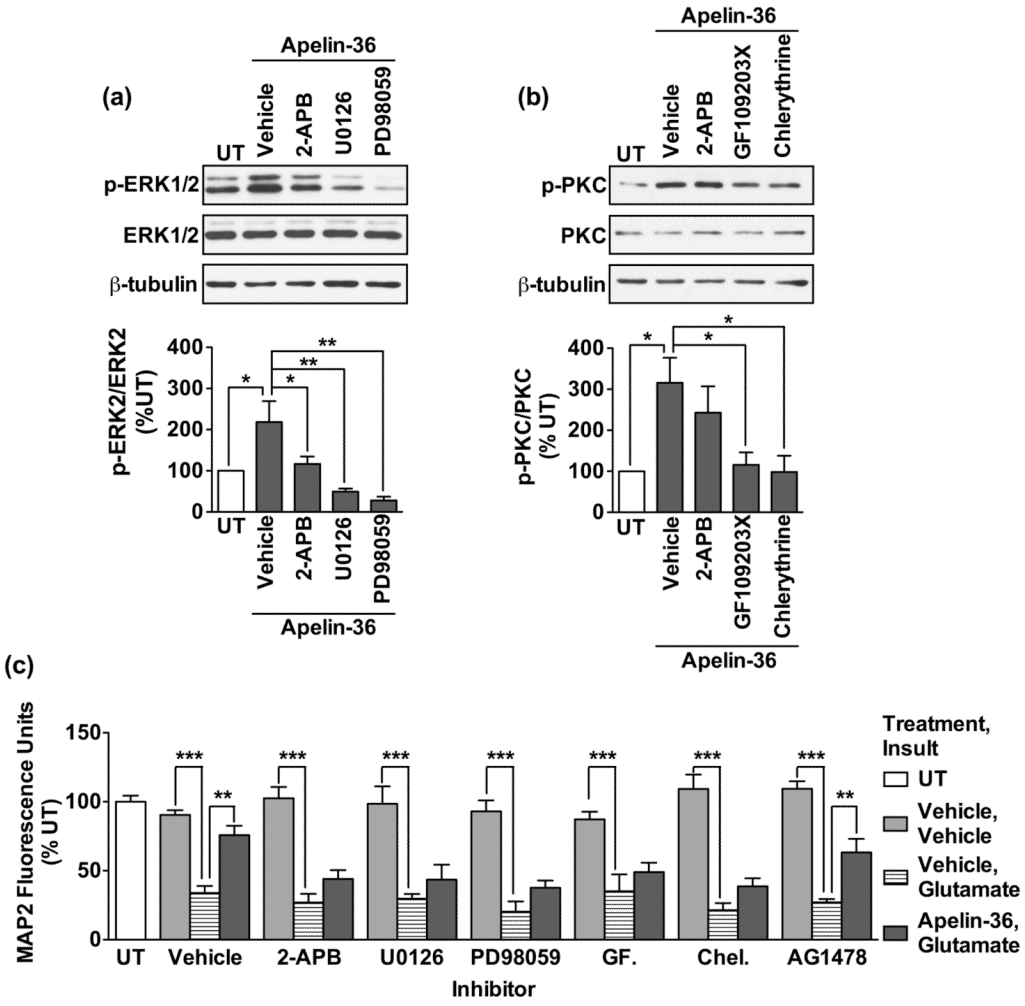

Fig 2.

Apelin protects cerebrocortical neurons from glutamate-induced excitotoxicity in an IP_3-, PKC-, and MEK1/2-dependent manner. (a–b) Cultures were exposed to 2-APB (50μM, 5min), U0126 (500nM, 1hr), PD98059 (20μM, 1hr), GF109203X (1μM, 1hr), or chelerythrine chloride (5μM, 30min), then treated with apelin-36 (20μM) for 5min. Phosphorylation of ERK1/2 (threonine 202/ tyrosine 204; (a)), or PKCγ (threonine 514 and homologous residues; (b)) was assessed by Western blot and quantified by densitometry of phospho- over total kinase normalized to β-tubulin (n = 3; *p<0.05, **p<0.01 vs. vehicle). (c) Cultures were exposed to inhibitors as in (a–b) or AG1478 (250nM, 30min), then treated with apelin-36 (20μM) for 45min, followed by glutamate (50μM) for 24hr. Cell survival was quantified by MAP-2 ELISA (n = 6–30; **p<0.01, ***p<0.001 vs. glutamate). All statistical comparisons were made by one-way ANOVA plus Newman-Keuls *post hoc* testing.

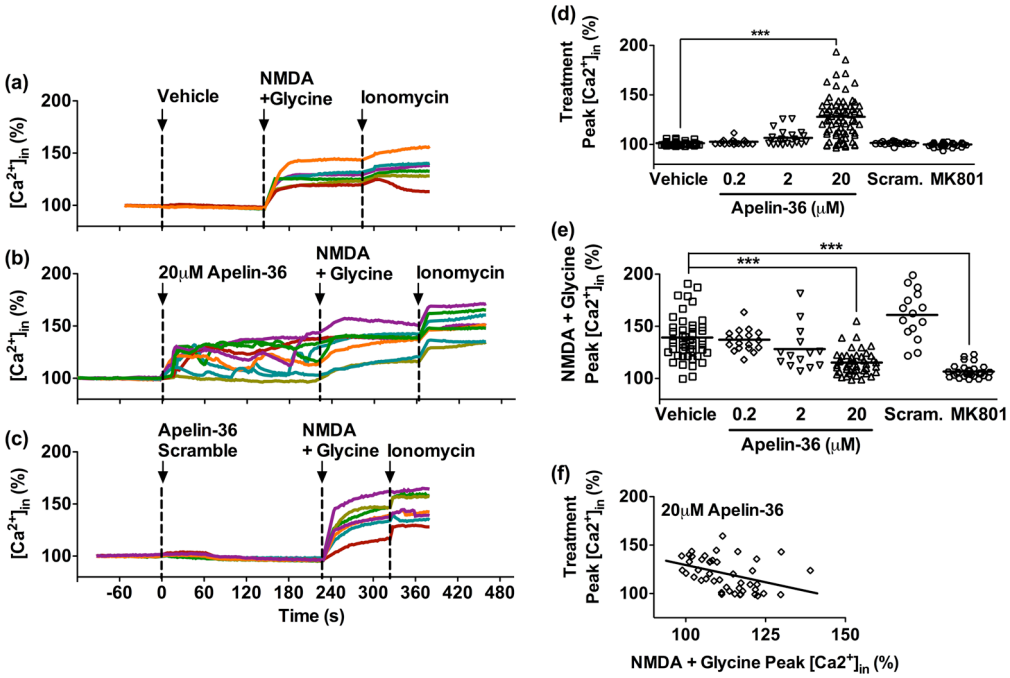

Fig 3.

Apelin induces Ca^{2+} transients and attenuates NMDA-induced Ca^{2+} accumulation in cerebrocortical neurons. (a–c) Cultures were loaded with Fura-2/AM and intracellular Ca^{2+} concentration ($[Ca^{2+}]_{in}$) was recorded during treatment with apelin-36 as in Fig. 1, followed by exposure to NMDA (50μM) + glycine (10μM), then ionomycin (1μM). Representative recordings from individual neurons are shown for treatment with vehicle (a), apelin-36 (20μM; (b)), or apelin-36 scramble (20μM; (c)), expressed as a percent change from baseline. (d–e) Quantification of peak $[Ca^{2+}]_{in}$ after each treatment (d) and after NMDA + glycine ((e); n= 3–5 cultures per condition, 6–10 neurons per culture; ***p<0.001 vs. vehicle by Kruskal-Wallis test plus Dunn's *post hoc* testing). (f) Correlation between peak $[Ca^{2+}]_{in}$ induced by treatment with 20μM apelin-36 and by NMDA + glycine (n = 5 cultures, 40 neurons total; r = −0.4891, p = 0.0007 by Spearman's test).

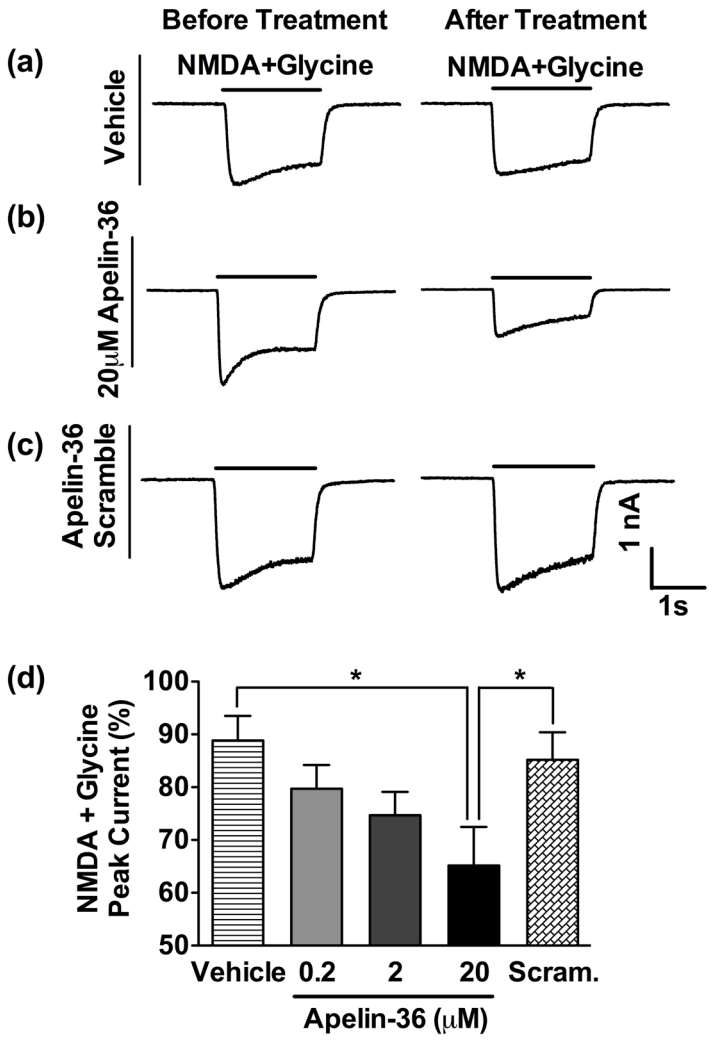

Fig 4.

Apelin attenuates NMDA-induced whole cell currents in cerebrocortical neurons. (a–c) Individual neurons were recorded in response to NMDA (50μM) + glycine (10μM) before and after 2min treatment with apelin-36 as in Fig. 1. Representative recordings are shown for treatment with vehicle (a), apelin-36 (20μM; (b)), or apelin-36 scramble (20μM; (c)). (d) Quantification of peak current after each treatment, expressed as a percent change from before treatment (n=6–11; *p<0.05 vs. 20μM apelin-36 by one-way ANOVA plus Newman-Keuls *post hoc* testing).

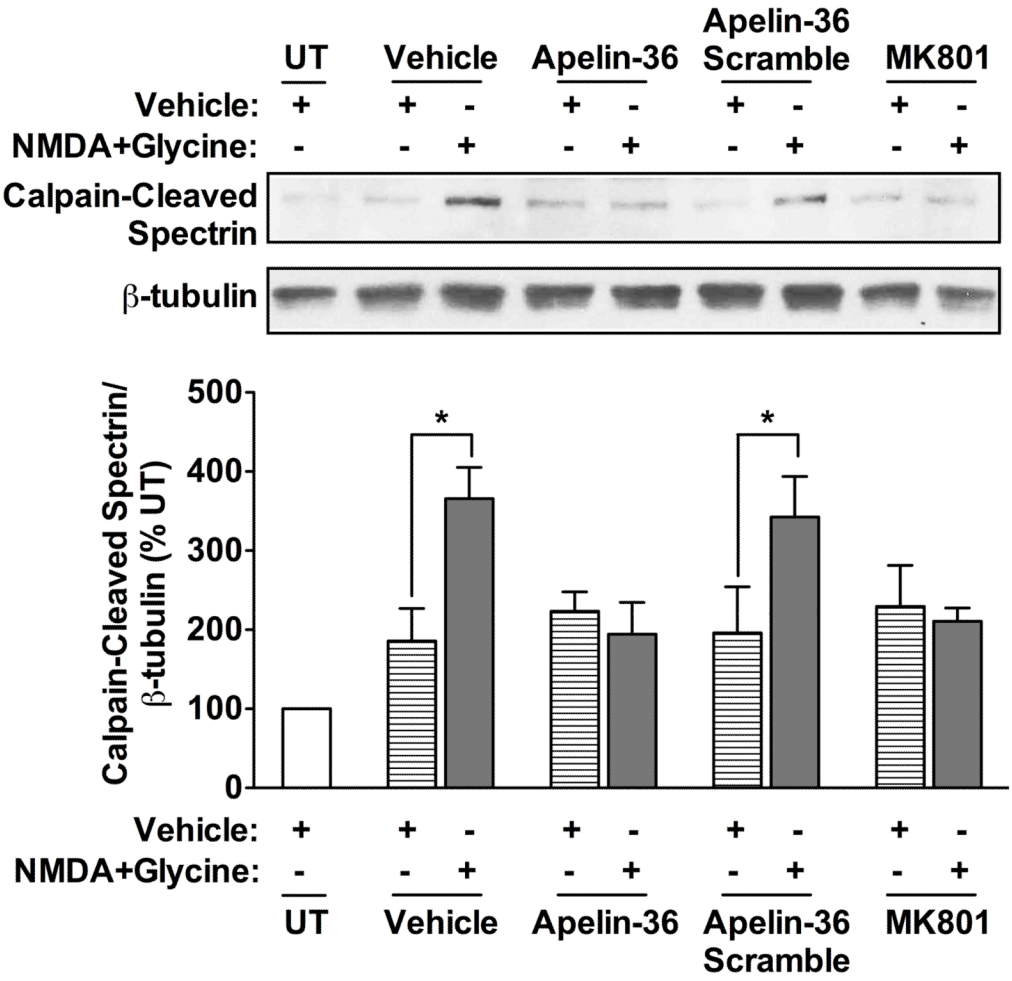

Fig 5.

Apelin inhibits NMDA-induced calpain activation in cerebrocortical cultures. Cultures were treated with apelin-36 (20μM), apelin-36 scramble (20μM), or MK801 (10μM) for 5min, followed by exposure to NMDA (50μM) + glycine (10μM) for 5min. Expression of calpain-cleaved spectrin was assessed by Western blot and quantified by densitometry normalized to β-tubulin ($n = 3$; *$p<0.05$ vs. vehicle by Student's t-test).

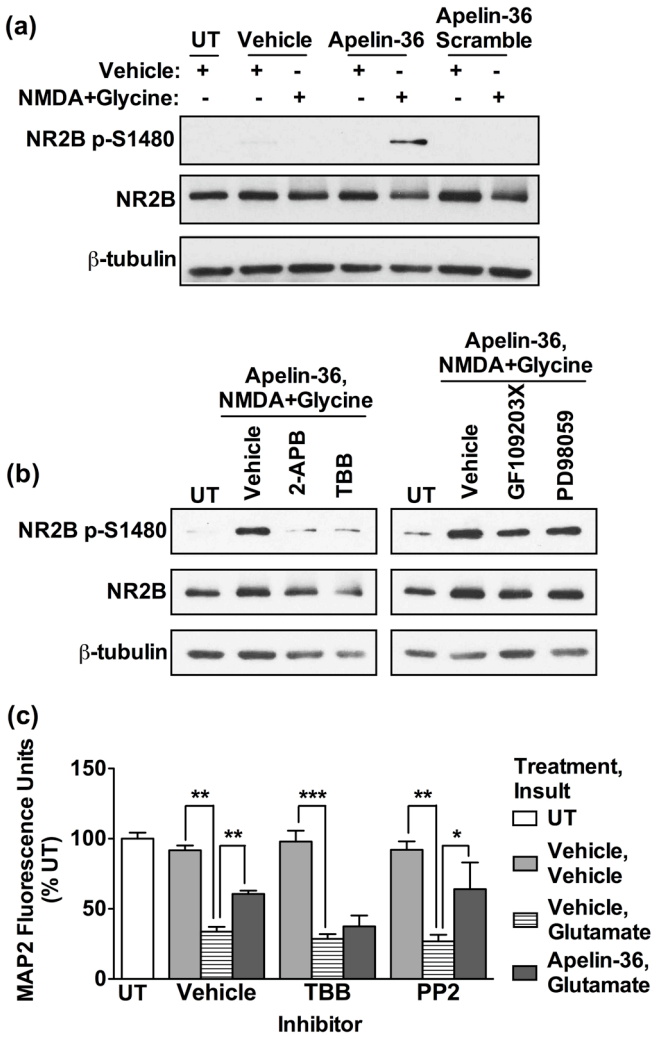

Fig 6.

Apelin induces phosphorylation of NR2B S1480, and inhibition of NR2B S1480 phosphorylation blocks apelin-mediated neuroprotection in cerebrocortical cultures. (a) Cultures were treated with apelin-36 (20μM), or apelin-36 scramble (20μM) for 5min, followed by exposure to NMDA (50μM) + glycine (10μM) for 5min. Phosphorylation of NR2B at S1480 was assessed by Western blot (n = 3). (b) Cultures were exposed to 2-APB (50μM, 5min), TBB (20μM, 1hr), GF109203X (1μM, 1hr), or PD98059 (20μM, 1hr), then treated with apelin-36 (20μM) for 5min, followed by NMDA + glycine for 5min. Western blots were performed as in (a) (n = 3). (c) Culture were exposed to TBB as in (b) or PP2 (10μM, 1hr), then treated with apelin-36 (20μM) for 45min, followed by glutamate (50μM) for 24hr. Cell survival was quantified by MAP-2 ELISA (n = 12–18; *p<0.05, **p<0.01, ***p<0.001 vs. Veh by one-way ANOVA plus Newman-Keuls *post hoc* testing).

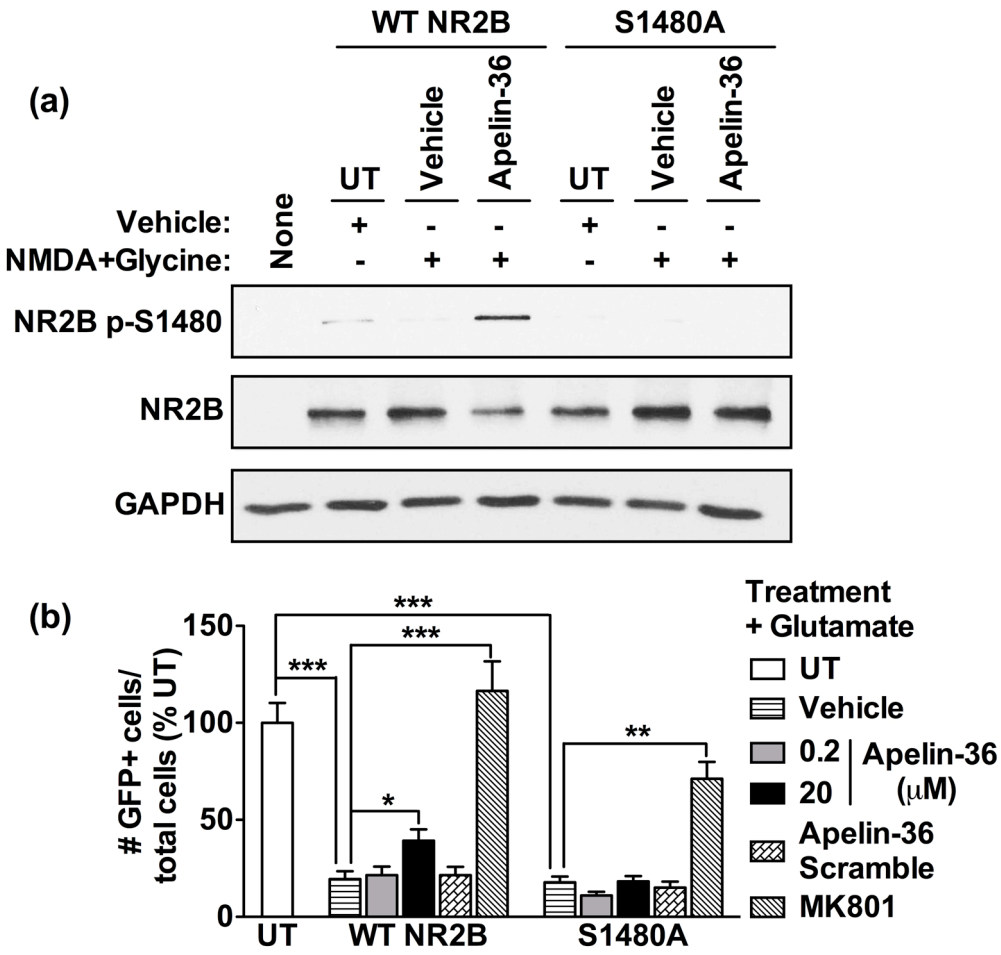

Fig 7.

Apelin-induced phosphorylation of NR2B S1480 mediates protection against excitotoxicity in HEK293 cells. (a) Cultures transfected with APJ, NR1, PSD-95, GFP, and wild-type (WT) NR2B or NR2B S1480A-expressing plasmids were treated with apelin-36 (20μM) for 5min, followed by NMDA (50μM) + glycine (10μM) for 5min. Phosphorylation of NR2B at S1480 was assessed by Western blot (n = 3). (b) Cultures were treated with apelin-36 (0.2, 20μM), apelin-36 scramble (20μM), or MK801 (10μM) for 45min, followed by glutamate (50μM) for 24hr. Transfected cell survival was quantified by counting GFP-positive cells and expressed as the ratio of GFP-positive cells to the total number of cells (n = 5–6 coverslips per treatment condition, 25–30 fields total; *p<0.05, **p<0.01, ***p<0.001 vs. vehicle by one-way ANOVA plus Newman-Keuls *post hoc* testing).

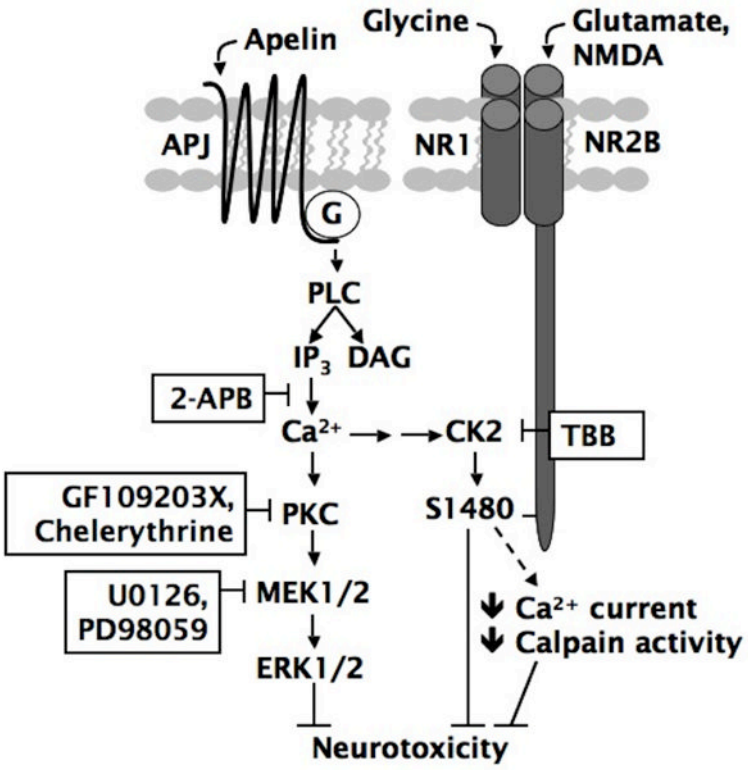

Fig 8.

Proposed mechanism of apelin-mediated neuroprotection. Apelin binds to APJ and triggers a GPCR signaling cascade through IP_3, Ca^{2+}, PKC, MEK1/2, and ERK1/2 to prevent neurotoxicity. Apelin/APJ signaling can also prevent neurotoxicity through Ca^{2+}-dependent CK2 phosphorylation of NR2B S1480. NR2B S1480 phosphorylation may provide the molecular mechanism for apelin-mediated attenuation of NMDA receptor and calpain activation. Inhibitors used in these studies are shown in boxes.