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Review

G protein-coupled receptors control NMDARs and metaplasticity in the hippocampus

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Abstract

Long-term potentiation (LTP) and long-term depression (LTD) are the major forms of functional synaptic plasticity observed at CA1 synapses of the hippocampus. The balance between LTP and LTD or "metaplasticity" is controlled by G-protein coupled receptors (GPCRs) whose signal pathways target the N-methyl-D-asparate (NMDA) subtype of excitatory glutamate receptor. We discuss the protein kinase signal cascades stimulated by Gαq and Gαs coupled GPCRs and describe how control of NMDAR activity shifts the threshold for the induction of LTP.

Keywords: NMDA receptor; G-protein coupled receptor; PKA; PKC; Src kinases; Synaptic efficacy; Longterm potentiation

Contents

1. Excitatory glutamatergic transmission

The primary function of ionotropic glutamate receptors is to respond to presynaptically released transmitter by generating depolarizing and excitatory postsynaptic potentials (epsps). As a result, rapid and high-resolution excitatory signals are passed from the presynaptic to the postsynaptic neuron. Spatial and temporal summation of these epsps at the level of a single postsynaptic neuron provides convergence and integration of a diversity of excitatory inputs from many presynaptic neurons.

The majority of epsps in the mammalian central nervous system are mediated by the α-amino-3-hydroxy-5-isoxazole-propionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) subtype of ionotropic (ion channel) glutamate receptors [1]. Almost all excitatory glutamatergic synapses possess both of these receptors, but the unitary epsp is almost entirely generated by current flow through AMPARs because of a voltage-

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dependent and rapid open channel block of NMDARs by extracellular Mg^{2+}. It is not until there is sufficient depolarization of the postsynaptic membrane and relief of the Mg^{2+} block via temporal and spatial summation of epsps that NMDARs contribute substantially to the generation of epsps [2]. At some specialized synapses, such as the calyx of Held synapse, AMPARs predominate and permit the postsynaptic cell to respond to presynaptic action potential frequencies of greater than 100 Hz [3].

Both AMPARs and NMDARs are tetrameric, non-selective cation channels (permeable to Na^+ and K^+) composed of GluR subunits (GluR1, 2, 3, 4) for AMPARs, and NR subunits ($NR1_{1\text{-}11 splice\ variants}$, NR2A, B, C, D and NR3A, B) in the case of NMDARs [4,5]. NMDARs provide a significant influx pathway for Ca^{2+} and the activity of these receptors is, as a consequence, coupled to a variety of Ca^{2+}-dependent intracellular signaling pathways. In contrast, the presence of the GluR2 subunit in most AMPARs dramatically reduces their permeability to Ca^{2+} [6]. Therefore, AMPARs are particularly well suited for generating unitary epsps and NMDARs serve as coincidence detectors in that the influx of Ca^{2+} via these channels is directly related to the degree of spatial and temporal summation of excitatory inputs to the postsynaptic cell [2]. Beyond simple direct signaling between neurons, NMDARs are important for the development of neurons and for the establishment of synaptic contacts [7]. They also play important roles in controlling the morphological and functional plasticity of synapses and contribute to the cellular mechanisms of learning and memory [8]. Paradoxically, overstimulation of NMDARs can lead to pathological conditions that may contribute to major degenerative diseases as well as to cell death in stroke and epilepsy [9]. Therefore, the basal level of NMDAR activity is pivotal to neuronal development, signaling and to the maintenance of neuronal viability.

2. Functional synaptic plasticity

Over the last several decades, considerable effort has been expended by researchers to determine the molecular and genetic basis of synaptic plasticity as well as its relationship to simple forms of learning and memory [10,11]. At many synapses in the CNS, long-term alterations of excitatory synaptic strength, induced by specific patterns of repetitive synaptic activity, serve as widely studied models of functional synaptic plasticity. For example, high-frequency tetanic stimulation typically elicits long-term potentiation (LTP) of epsps whereas repetitive low-frequency stimulation induces a long lasting depression (LTD) of these responses. These forms of synaptic plasticity are widely studied at excitatory synapses impinging upon CA1 pyramidal neurons where they are thought to underlie various forms of spatial learning and memory. The induction of LTP and LTD at these synapses paradoxically requires the activation of synaptic NMDARs [12,82]. Notably, NMDAR activation contributes to plasticity not only at high stimulus frequencies (>50 Hz), where relief of Mg^{2+} block is expected to be nearly complete, but also at very low frequencies of synaptic stimulation (~1 Hz). In other words, NMDARs can still contribute small but detectable Ca^{2+} signals in the dendritic spines of CA1 neurons even under conditions when their contribution to the current underlying the epsp is minimal. As a result of the different levels of NMDAR activation, LTP- and LTD-inducing stimulation protocols will consequently generate distinct Ca^{2+} signals. These differ in the absolute concentrations of Ca^{2+} achieved, especially within postsynaptic micro-domains, and in the kinetics of the resulting signal. Consequently, these distinct Ca^{2+} signals will recruit distinct signal transduction pathways that ultimately will determine whether LTP or LTD, expressed through changes in AMPAR function, is induced.

Although a large number of molecules and signal transduction pathways have been implicated in synaptic plasticity, not all of these are likely to be both "sufficient and necessary" for either the induction and/or maintenance of LTP and LTD [13,14] . The signal components that have emerged as leading candidates include the activation and autophosphorylation of CamKII necessary for the establishment of LTP and the activation of protein phosphatases, including protein phosphatases 1 (PP1) and calcineurin, necessary for the establishment of LTD. These kinases and phosphatases alter the phosphorylation of AMPARs resulting in changes in single-channel conductance as well as changes AMPAR surface expression [15,16]. Ultimately however, the sustained maintenance of a resultant plastic change must involve changes in gene expression. In the case of LTP, this may occur, for example, through PKA-induced stimulation of CREB [17,18] or through NMDAR-dependent stimulation of MAPK signaling cascades [19,20].

3. Metaplasticity

The threshold for the induction of LTP and LTD can be influenced by prior activity within the network which does not itself alter the amplitude of the basal epsp or the efficacy of synaptic transmission. This type of "plasticity of plasticity" has been termed "metaplasticity" [21]. The potential mechanism(s) of metaplasticity are numerous and include changes in the effectiveness of GABA-mediated synaptic inhibition [22]. However, given the ability of NMDARs to function as a gate for the induction of several forms of synaptic plasticity, changes in NMDAR function are clearly the most direct way to alter the thresholds of synaptic plasticity without detectably altering basal epsps. An early example was reported by Coan et al. [23] which showed that depriving hippocampal slices of extracellular Mg^{2+} caused a subsequent inhibition of the induction of LTP (increase in threshold). This was entirely unexpected because the reduction in Mg^{2+} would lead to enhanced NMDAR activation and would be predicted to cause a reduction in the threshold for LTP. Indeed relatively weak pre-stimulation of NMDARs by a variety of mechanism leads to alterations in the relative balance between the induction of LTP and LTD [12,21].

One means by which pre-stimulation may alter NMDAR function is by modifying tyrosine phosphorylation through the activities of specific kinases and phosphatases [12]. Many of these enzymes are in fact highly localized within NMDAR micro-domains or even intimately associated with the receptors themselves. For example, the Src family kinases, including Src

and Fyn, and the phosphatase STEP, regulate NMDAR gating by promoting tyrosine phosphorylation and dephosphorylation respectively (see extensive reviews by Salter [24–26]). Interestingly, Src is both "sufficient and necessary" for the induction of LTP at CA1 synapses [27]. It is noteworthy that basal AMPAR-mediated epsps are unaltered by applications of Src-specific inhibitors even though they block the subsequent induction of LTP [27]. These results illustrate that Src is not actively up-regulating NMDAR activity under basal conditions. The LTP-inducing period of high frequency stimulation (the tetanic stimulation) leads to the entry of Na^+ and Ca^{2+} and activates Src [28,29] that in turn facilitates NMDAR channel activity providing a biochemical mechanism of positive feedback on NMDARs. This means that synaptically located Src family kinases are an ideal target to underlie mechanisms of metaplasticity.

Consequently, prior activity need not necessarily cause substantial NMDAR activation in order to induce metaplasticity. Such activity could alternatively cause the activation of several GPCRs capable of regulating the function of NMDARs and thus the ability to subsequently induce plasticity. Indeed, a wide variety of G-protein coupled receptors (GPCRs), including those for glutamate, acetylcholine and dopamine, converge via their signal pathways to alter the gating and/or the trafficking of NMDARs by regulating the activities of Src family kinases and tyrosine phosphatases. GPCRs are also known to regulate receptor tyrosine kinases (RTKs), such as epidermal growth factor receptors (EGFRs) and platelet derived growth factor receptors (PDGFRs) [30]. These RTKs can in turn alter NMDAR expression and function [31].

There are a variety of ways that GPCR signaling could converge to alter NMDAR activity at CA1 synapses and thereby contribute to metaplasticity. For example, the GPCR or its related G-protein components can:

(1) Bind directly to a subunit of the NMDAR and alter its activity or trafficking [32].
(2) Activate a second messenger cascade linked to the serine–threonine kinases (e.g. PKC, CAMK or PKA) which leads to a direct increase or decrease in phosphorylation of NMDAR subunits [7].
(3) Activate a signal cascade that increases or decreases the phosphorylation of an accessory protein that brings the NMDAR into or out of the appropriate proximity of a given signaling enzyme [33].
(4) Activate signals that target a RTK which in turn regulates the activity of NMDARs [31].
(5) Activate signals that target the proteins responsible for regulating actin polymerization which in turn alters NMDAR activity or possibly their trafficking [34].

In this review we will examine how some GPCRs act via PKA- and PKC-dependent signaling to regulate NMDARs and thus metaplasticity at CA1 synapses. Finally, we will discuss how downstream signaling from a RTK (itself regulated by a GPCR) regulates NMDAR activity indirectly by targeting proteins that regulate polymerization of the actin cytoskeleton.

4. GPCRs and PKA Regulation of NMDARs

In striatal neurons D_1 dopamine receptors ($G\alpha_s$-coupled) enhance adenylate cyclase, increase cAMP, and activate PKA which then phosphorylates and activates the protein phosphatase inhibitor, DARPP-32. Phosphorylated DARPP-32 is a potent inhibitor of the serine/threonine protein phosphatase 1 (PP1) [35]. In hippocampal neurons PP1 is inhibited by a different protein, Inhibitor 1, which is also a substrate of PKA [36,37]. Therefore PKA activation leads to inhibition of PP1 and decreased dephosphorylation (i.e. enhanced phosphorylation) of downstream substrates including NMDARs [38]. In CA1 neurons PKA phosphorylated inhibitor 1 likely interacts and inhibits PP1 causing an enhanced phosphorylation of NMDARs [36,37]. NMDAR activity in hippocampal neurons is down regulated by PP1/PP2A and is enhanced by PP1 inhibition [39]. Others have shown that PKA and NMDARs are closely linked via an A kinase anchoring protein (AKAP) [40]. In this model, constitutively active PP1 keeps NMDA channels in a dephosphorylated and low activity state. Both PKA and PP1 are bound to the AKAP scaffolding protein, yotiao. Upon activation PKA phosphorylates PP1 and decreases its activity, leading to a shift in the balance of the channel to a higher phosphorylation state and a higher activity state [33,41]. The scaffolding and localization of PKA and PP1 to the NMDAR enhances the efficiency and specificity of this signaling pathway [41]. Others have reported that β-adrenergic receptors acting via PKA regulate the desensitization of NMDAR mediated synaptic currents ($epsc_{NMDA}$) [42], possibly through a direct phosphorylation of NMDAR subunits.

A recent and elegant study by Skeberdis et al. [43] has demonstrated that the Ca^{2+} permeability of NMDARs is enhanced by PKA without any alteration in the amplitude of the $epscs_{NMDA}$. Blockers of PKA inhibited the induction phase of LTP at CA1 synapses presumably by decreasing the NMDAR-induced entry of Ca^{2+} and supporting a role for PKA as a "switch" (metaplasticity) mechanism of LTP control [37,44,45].

5. GPCR regulation of NMDARs via PKC and tyrosine kinases

We and others have established that a number of GPCRs (those that couple to $G_{\alpha q}$) upregulate NMDAR-mediated currents and/or $epscs_{NMDA}$ recorded in hippocampal neurons in culture or *in situ* in the hippocampal slice. These include muscarinic, LPA, and mGluR5 receptors [46–55]. They do so via a sequential signal pathway composed of PLC, PKC, Ca^{2+}, IP_3R, CAKβ or Pyk2 (cell adhesion kinase β also called proline rich tyrosine kinase 2) and Src (Fig. 1). Therefore, several $G_{\alpha q}$-coupled receptors act upon NMDARs by enhancing tyrosine kinase activity. The importance of this signaling cascade is highlighted by the finding that LTP at CA1 synapses requires activation of Pyk2 and Src [48]. One means by which $G_{\alpha q}$-coupled receptors may increase NMDAR signaling is by increasing their surface expression. Indeed, in a *Xenopus* expression system and in cultured hippocampal neurons

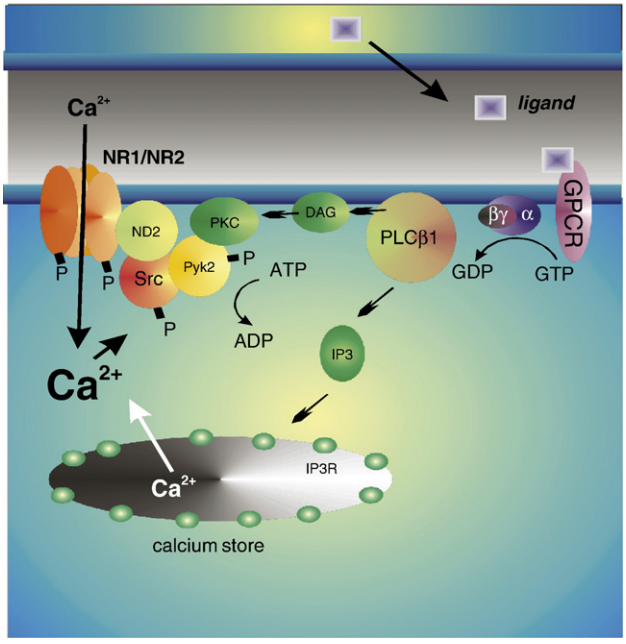

Fig. 1. A sequential $G\alpha_q$ signal cascade leads to a Src-dependent enhancement of NMDAR activity in CA1 pyramidal neurons. GPCR activation (e.g. M_1, mGluR5, LPA) leads to the stimulation of phospholipase β1 (PLCβ1) and the production of inositol triphosphate (IP3) leading the release of intracellular calcium. The conjoint production of diacylglycerol results in stimulation of protein kinase C (PKC). The increased calcium and PKC lead to the stimulation of the non-receptor tyrosine kinase, Pyk2; which then provides an SH2 docking site for Src. This interaction results in the enhancement of NMDAR activity. Src is anchored in the vicinity of the NMDAR via the binding of its unique domain to the scaffolding protein ND2.

stimulation of mGluR5 subtype of glutamate receptor enhances the insertion of new NMDARs in the plasma membrane and this requires activation of non-receptor tyrosine kinases [51]. Similarly the trafficking of NMDARs to the surface of cultured striatal neurons by D_1 dopamine receptors is dependent upon tyrosine kinase activity [56].

6. PACAP receptors in CA1 neurons

Pituitary adenylate cyclase activating peptide (PACAP) occurs as a 38-amino-acid or a truncated 27-amino-acid peptide [57]. Acting through PAC_1 receptors highly expressed in the hippocampus [58–61], PACAP has been reported to potentiate NMDAR responses in CA1 neurons [62,63]. The regulation of NMDARs by PACAP signaling through PAC_1Rs may underlie its reported ability to regulate hippocampal LTP as well as memory retention and consolidation [64–68]. PAC_1R signals either through $G_{q/11}$ to phospholipase C (PLC) [69,70] or alternatively to an adenylyl cyclase pathway via a $G_{\alpha s}$ [70,71]. Therefore, PACAP stimulates both PKA- and PKC-dependent signaling pathways [72] and raises the question of how NMDAR are regulated by PACAP receptors in CA1 hippocampal neurons (Fig. 2).

Our approach to answer this question was to examine the impact of cell signaling pathways on NMDARs in cultured, acutely isolated and slice CA1 hippocampal neurons using combined electrophysiological recordings and biochemical measurements. Parallel studies were done both on actual synaptic currents as well as currents evoked by exogenous applications of NMDA [73]. In cultured cells we used pharmacologically or kinetically isolated miniature $epscs_{NMDA}$ and in conventional slices we recorded evoked $epscs_{NMDA}$ using Schaffer Collateral stimulation. Each preparation has its advantages and disadvantages but isolated CA1 pyramidal neurons provide a method for studying rapid agonist applications with excellent accessibility of reagents. Rapid applications of NMDA (3 ms solution exchange time constant) evoke currents characterized by a rapid rise to a peak (I_{peak}) followed by desensitization to a quasi-steady state (see Fig. 4 for example). In actual experiments each neuron served as its own control. For example, we initially recorded stable I_{peak} before applying a GPCR agonist such as PACAP38. The agonist is applied for a short period of time and then it is rapidly washed away. Peak NMDAR currents are then monitored for periods of tens of minutes and observed changes are recorded long after exposure to the GPCR agonist. Responses of individual neurons are then averaged giving highly reliable measures of the degree of modulation. Isolated cells lose much of their dendritic processes during isolation. Nevertheless, peak NMDAR currents (I_{peak}) from isolated cells and $epscs_{NMDA}$ are modulated in parallel by D_4 dopamine [31], PDGFRs [74,75], muscarinic [49], mGluR5 [46] and PAC_1 (see below) receptors suggesting that synaptic receptors are still present in isolated cells. Similarly, PKC, Pyk2, and Src enhance both peak currents and $epscs_{NMDA}$ [48,49,76,77]. Indeed we suspect that I_{peak} is mediated in large part by receptors that only contain NR2A subunits (NR2AA, see below) (but not exclusively) whilst receptors containing only NR2B subunits (NR2BB) contribute more to the steady-state currents. This is based upon the much higher probability of opening of NR2AA versus NR2BB receptors [78,79] as well as by the sensitivity of I_{peak} to very low concentrations of the NR2AA selective antagonist, NVP-AAM077 [80].

7. PACAP signals via PKC, Pyk2 and Src to NMDARs

Low concentrations (1 nM) of PACAP38 enhance pharmacologically isolated $epscs_{NMDA}$ at CA1 synapses (Fig. 3) and this effect is eliminated by the inclusion of the Src-specific antagonist Src(40–58) in the recording patch pipettes [73]. This is paralleled by an enhancement of I_{peak} in acutely isolated CA1 pyramidal neurons (taken from P14 to P20 rats) (Fig. 4). The effect of PACAP38 is seen at extremely low concentrations (10 pM to 1 nM, isolated cells) and it is suppressed by a selective antagonist of the PAC_1R. This response requires G-proteins as GTP-γ-S occludes and GDP-β-S blocks the enhancement induced by PACAP38 [73]. This might seem a self-evident result but there are reports of GPCR signaling via mGluRs in hippocampal neurons independent of G-proteins [81].

The G-protein subtype involved in this signaling pathway is $G_{\alpha q}$ as application of a specific RGS2 protein [82], which selectively prevents the binding of $G_{\alpha q}$ to GPCRs, eliminated

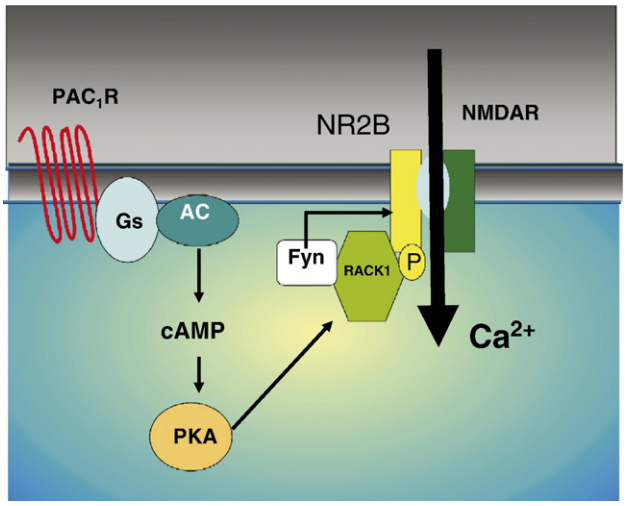

Fig. 2. An alternative $G\alpha_s$ signal cascade leads to a Fyn-dependent enhancement of NMDAR activity in CA1 pyramidal neurons. GPCR activation (e.g. PAC_1R) leads to the stimulation of adenylyl cyclase (AC), the production of cyclic adenosine monophosphate (cAMP) and the stimulation of protein kinase A (PKA). PKA leads to the dissociation of Fyn kinase from the receptor for activated C kinase 1 (RACK1) relieving the inhibition of Fyn and permitting it to increase the tyrosine phosphorylation of the NR2B subunit of the NMDAR and causing an enhancement in receptor activity. (Based on Yaka et al. [119]).

the PACAP38 induced enhancement (unpublished). One possibility is that PACAP38 binding induces a dynamic switching between $G_{\alpha s}$ and $G_{\alpha q}$ as described for β_2-adrenergic receptor [83]. However, the enhancement of NMDARs is substantially attenuated in mice lacking PLCβ1 whilst the PACAP38-induced increase in cAMP levels in the hippocampus is unaltered arguing against such a switch [73]. Inhibition of PKC, Pyk2, or Src each prevented the PACAP-38 induced potentiation of NMDAR peak currents confirming that the PAC_1R is linked via a $G_{\alpha q}$/PLCβ1/PKC/Pyk2/Src signal cascade.

The Ron group has proposed that the Src-family kinase, Fyn, and NR2B form a complex with RACK1 whereby the activity of Fyn is inhibited [62,63,84]. RACK1 is one member of a family of scaffolding proteins which interact only with activated PKC, in a potentially isoform-specific manner (e.g. βIIPKC to RACK1) [85,86]. RACK1 has sequence homology with the β subunit of G proteins and both are members of a family of regulatory proteins made up of highly conserved repeating units usually ending with Trp-Asp (WD). RACK1, with seven WD units, forms a "propeller-like" structure. Activated Src also binds via SH2 sites to phosphotyrosyl groups in the 6th propeller (WD) domain of RACK1 [87] and it is inhibited as a result of this interaction [85,87,88]. They have proposed that PACAP38 induced stimulation of PKA leads to a dissociation of Fyn and NR2B from the first WD propeller region of RACK1, freeing Fyn to phosphorylate NR2B receptors (Fig. 2). They also provided evidence that Fyn and the NR2B subunit may interact with the same binding region of RACK1. Recently, they have shown that PACAP38 enhances field $epsps_{NMDA}$ in the CA1 region of hippocampal slices. This likely involved NR2BB containing NMDARs as the effect of PACAP was sensitive to ifenprodil. Furthermore, in slices from fyn−/− knockout mice they reported that PACAP38 failed to potentiate field $epsps_{NMDA}$ [62]. They proposed that the activation of PKA by PACAP allows Fyn-dependent phosphorylation of NR2B receptors. Critical to this interpretation is the use of peptides designed to interfere with the binding of NR2B receptors and Fyn to RACK1. Salter points out a flaw in that one of the peptides targeted a region of Fyn that is shared with most other Src family kinases, including Src itself [24]. Therefore the peptide employed would modulate Src as well as Fyn's interactions with RACK1.

Our results with the effects of PACAP38 on I_{peak} [73] conflict substantially with those of Yaka et al. [62,63,89]. The most critical point is our evidence that the Src selective inhibitor peptide Src(40–58) [24,26], which mimics the unique region of Src and not those of any of the other family tyrosine kinases, completely blocks the PACAP38 induced potentiation of I_{peak} as well as the pharmacologically isolated $epscs_{NMDA}$ in CA1 neurons *in situ*. Most importantly Src binds by its unique domain to an identified region of ND2 (residues 239–221), a protein which serves as the major scaffolding protein for Src at

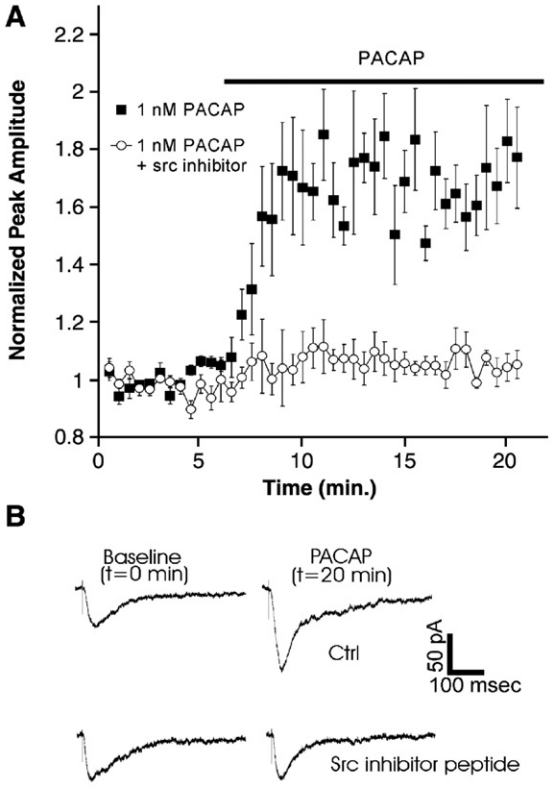

Fig. 3. PACAP38 enhances NMDA currents in hippocampal slice. (A) Application of 1 nM PACAP38 to hippocampal slices caused increased amplitude in NMDA currents. Pyramidal cells were recorded in a whole cell configuration. NMDA peak currents reached a maximal increase approximately 8 min following application. Normalized peak amplitude for PACAP treated cells was 167±10% compared to baseline ($n=6$). When Src (40–58) (25 μg/mL) was included in the patch pipette PACAP38 failed to elicit a response; normalized peak current 102±2% ($n=6$), $p<0.005$, unpaired *t*-test, data obtained at 20 min of recording. The *black bar* indicates time and duration of 1 nM PACAP38 application. (B) Sample traces from individual cells with and without Src(40–58) in the patch pipette at baseline ($t=0$ min) and following PACAP38 application ($t=20$ min). (From Macdonald et al. [66]).

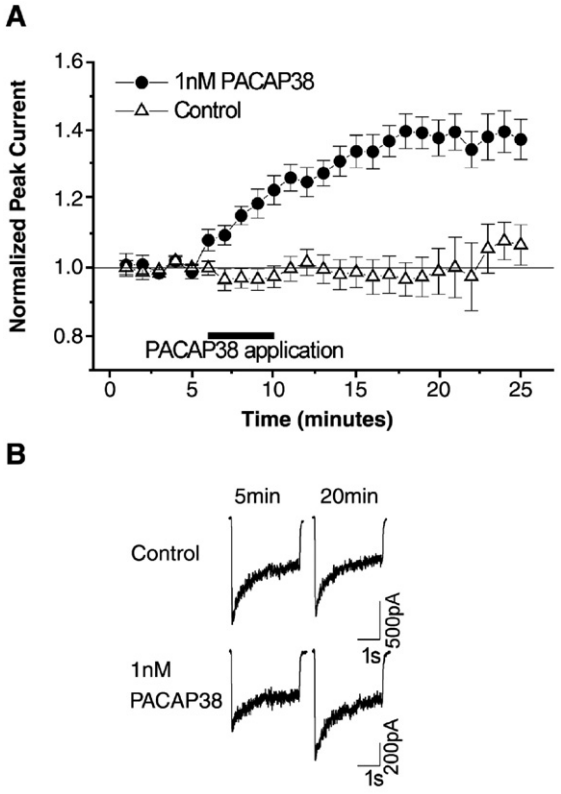

Fig. 4. PACAP38 enhances peak currents in isolated CA1 pyramidal neurons. (A) Application of PACAP38 (1 nM) to acutely isolated CA1 pyramidal neurons resulted in an increase of NMDA-evoked peak currents that outlasted the period of application, did not reverse during washout and persisted throughout the recording period. NMDA-evoked peak currents in control cells were unchanged throughout the time course of the experiment. Cells treated with 1 nM PACAP38 had significantly larger NMDA-evoked peak currents (control: 98±7%, $n=9$; 1 nM PACAP38: 140±5%, $n=9$, $p<0.001$, unpaired t-test, data obtained at 20 min of recording). The black bar indicates time and duration of 1 nM PACAP38 application. (B) Sample traces of NMDA-evoked currents for control and PACAP38 treated cells. Traces represent points immediately prior to PACAP38 application ($t=5$ min) and 10 min following PACAP38 application ($t=20$ min). (From Macdonald et al. [66]).

hippocampal synapses. Other Src kinases including Fyn do not. Indeed, Src(40–58) acts by displacing Src from ND2 and this inhibitor does not alter the association of ND2 with NMDARs nor does it alter the catalytic activity of Src [90]. In addition, we observed that the PACAP38 induced potentiation of I_{peak} is blocked by NV-AAM077 but not by ifenprodil or Ro 25-6981 (unpublished), suggesting that PACAP38 is regulating NR2A-containing receptors.

To address some of these discrepancies between our work and that of the Ron group we designed and synthesized two peptides which mimic either the Fyn binding site (peptide R1) or alternatively the Src binding site (peptide R6) of RACK1. We introduced these peptides into isolated CA1 neurons via the patch pipettes. As would be anticipated from Yaka et al. [62,63,89], R1 enhanced NMDAR currents on its own presumably by competing Fyn away from WD1. However, a similar, if not more robust enhancement, was observed when the R6 peptide was included in the patch pipette. Perhaps of some importance was our observation that the kinetics of the enhancement by R6 was substantially faster than that for R1. Unexpectedly, both the R6 and R1 induced enhancements of NMDAR currents were blocked by the co-application of the Src selective peptide, Src(40–58) suggesting that Src is the mediator of both R1 and R6 induced potentiation. These results imply that Fyn's modulation of NMDARs requires Src activation (thus Fyn maybe upstream of Src at least within the RACK1 complex). This conclusion conflicts with results from fyn−/− mice where PACAP38 induced potentiation was reportedly absent [62], but suggests that there is some kind of permissive relationship between Fyn and Src at CA1 synapses. One plausible explanation for our differing results is that we have primarily examined the Src-dependent modulation of NR2AA receptors in contrast to the Ron's groups focus on NR2BB containing receptors (see below). Indeed, PKA selectively enhances currents mediated by recombinant NR2BB but not NR2AA receptors when expressed in HEK293 cells [43]. Although this result is somewhat in contradiction to an earlier report demonstrating PKA-induced potentiation of NR2AA receptors [33]. These conflicting results are likely attributable to the specific substrates targeted by PKA. In the case of altered NR2AA Ca^{2+} permeability, a direct phosphorylation of the receptor protein would appear to be the mechanism of action. However, enhanced currents could result from PKA interactions with yotiao and the AKAP complex [33].

Still greater complexity can occur with GPCR signaling as kinase substrates may include a variety of signaling proteins linked to the eventual alteration in NMDAR activity. For example, in previous experiments we demonstrated that cPKA does not alter NMDAR single channel activity in outside-out patches (see also [91]) taken from cultured hippocampal neurons unless the channel activity has been previously upregulated by applications of the Src selective activator phosphopeptide EPQ(pY)-EEIPIA [75,92]. In other words, Src's effects on NMDARs are permissive to the inhibitory

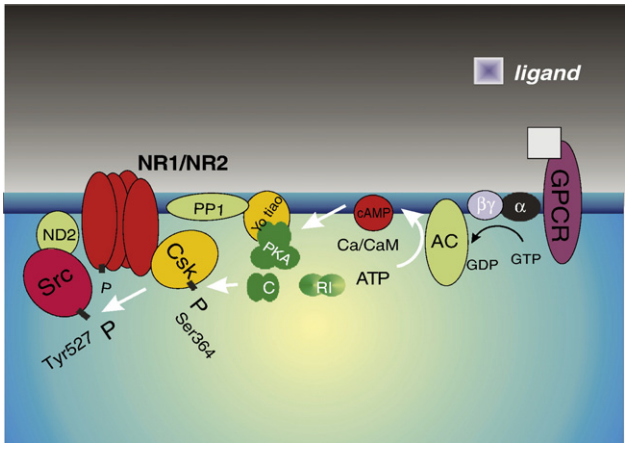

Fig. 5. A potential for cross talk between the $G_{\alpha s}$ and $G_{\alpha q}$ signal cascades regulating NMDAR activity in CA1 pyramidal neurons. In this scenario $G_{\alpha q}$ coupled receptors enhance Src-dependent NMDAR activity as previously described. However, stimulation of PKA activation via $G_{\alpha s}$ and cyclic AMP signaling targets the C-terminal Src kinase (Csk) by phosphorylating serine 364. Csk then phosphorylates tyrosine 527 of Src strongly inhibiting Src activity and down regulating its ability to enhance NMDAR activity.

effects of PKA in outside-out patches. The cross-talk between Src and PKA with regards to the regulation of NMDAR activity may depend upon the C-terminal Src kinase (Csk) [93,94]. Csk phosphorylates the c-terminus of Src and inhibits Src activity. We have extensive preliminary data that Csk regulates NMDARs and LTP via this mechanism. In turn, Csk is activated by phosphorylation by PKA [95,96] suggesting that PKA could inhibit the Src induced potentiation of NMDAR currents by stimulating Csk and inhibiting Src (Fig. 5). The extent of activation of $G\alpha_s$ vs. $G\alpha_q$ signaling pathways, especially by GPCRs such as the PAC_1 receptor that can couple to both G-proteins, may dictate the extent of Src potentiation of NMDARs, and subsequently the extent of LTP potentiation.

8. Could GPCRs act via the actin cytoskeleton to regulate NMDAR activity?

The amplitude of NMDAR mediated currents indirectly depends upon the balance between globular and filamentous actin (F-actin). Depolymerization is associated with a rundown of NMDAR currents [97]. The dependence of NMDARs upon the cytoskeleton arises because the receptors are tethered to F-actin via α-actinin [98–105]. Therefore, alterations in actin polymerization can potentially regulate $epscs_{NMDA}$.

Enhanced protein phosphatase activity is specifically associated with LTD of $epscs_{NMDA}$. For example, Morishita et al. [106] have shown that low frequency stimulation of CA1 hippocampal synapses induces LTD with a long-lasting depression of both isolated $epscs_{NMDA}$ and $epscs_{AMPA}$. The LTD of $epsc_{AMPA}$ was dependent upon activation of PP2B (calcineurin). In contrast, LTD of $epsc_{NMDA}$ was not. Instead the depression of NMDARs was blocked by serine–threonine phosphatase inhibitors (okadaic acid and microcystin; block protein phosphatases 1 and 2A, PP1/2A). Furthermore, the induction of LTD of $epscs_{NMDA}$ was associated with a stimulus induced depolymerization of the actin cytoskeleton. Microtubules were apparently not involved in this response [106]. This provides the first evidence that Schaffer Collateral stimulation can modulate actin polymerization and in turn the magnitude of NMDARs. These results are relevant to our own work showing that myosin light chain kinase (MLCK) also regulates NMDAR in cultured hippocampal neurons via enhanced phosphorylation and sustained polymerization of F-actin [34]. Furthermore, as PP1 and PP2A are key regulators of the actin cytoskeleton it is consistent with our earlier observations that NMDR activity is regulated by PP1/PP2A [39].

9. Some GPCRs indirectly regulate NMDARs via growth factor transactivation

GPCRs can stimulate uncontrolled growth through an indirect activation or "transactivation" of tyrosine kinase growth factor receptors (e.g. PDGFR) [107]. Transactivation occurs when the agonist-induced engagement of a GPCR causes tyrosine autophosphorylation of the growth factor receptor and downstream activation of diverse signaling pathways *even though no growth factor ligand is present* (Fig. 6). Work by us has shown that D_4 dopamine receptors transactivate PDGFRs in CA1 hippocampal neurons leading to a long-lasting inhibition of NMDARs [31]. A similar transactivation of PDGFRs and inhibition of NMDAR is observed in prefrontal cortical neurons although it is mediated in these cells by D_2 or D_3 dopamine receptors [108]. Our work established transactivation as a unique physiological mechanism of inter-communication between transmitter systems [7,31,107,109] (Fig. 7).

As with transactivation, activation of PDGFRβ with PDGFBB (ligand) causes a long lasting inhibition of peak NMDAR currents (I_{peak}) [74]. The inhibition by PDGFBB is

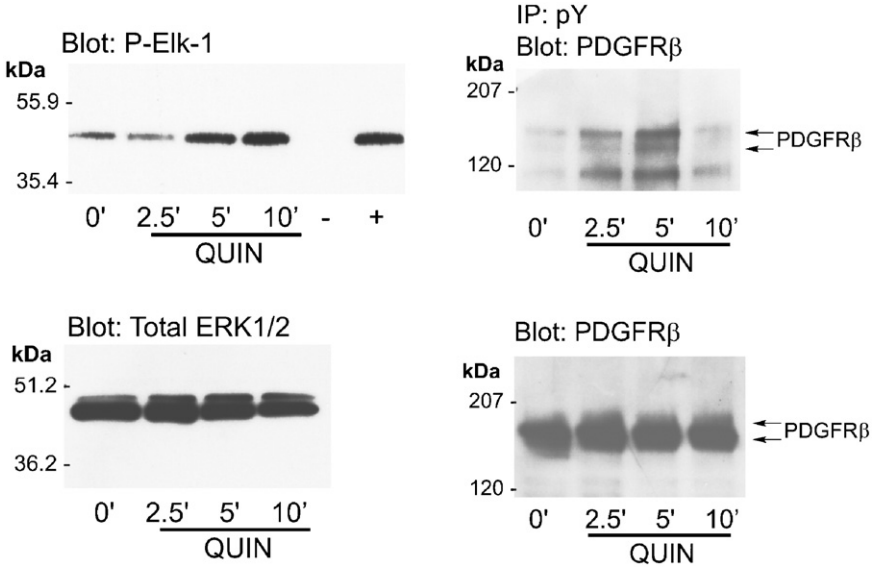

Fig. 6. Blots show that a D_2-class dopamine agonist, quinpirole, transiently induces autophosphorylation of PDGFRβ proving that D_2-class dopamine receptors transactivate PDGFRs in CA1 hippocampus. In addition, down stream Elk and ERK1/2 phosphorylation occurs as a result of transactivation. (From Kotecha et al. [50]).

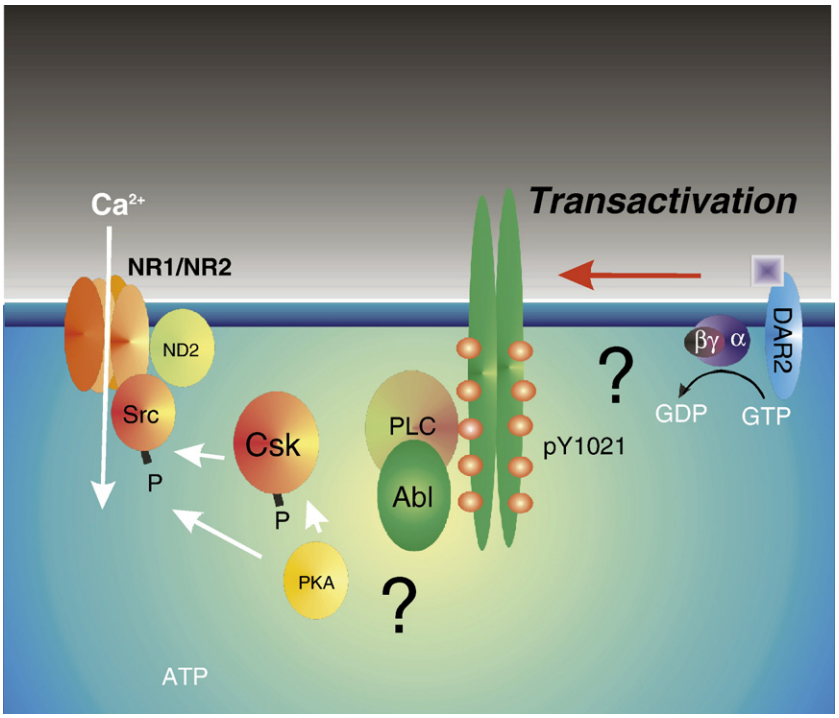

Fig. 7. Schematic shows that transactivation of PDGFR inhibits NMDARs by a Src-dependent mechanism. Dopamine D_2 class receptors transactivate PDGFR in CA1 hippocampal neurons in the absence of any ligand for the PDGFR. The mechanism of the transactivation is poorly understood. Autophosphorylated PDGFR then leads to the inhibition of NMDARs by a Src-dependent mechanism. Evidence suggests that phosphorylation of Y1021 and stimulation PLCγ [74,108] as well as a non-receptor tyrosine kinase, Abelson's kinase (Abl) (unpublished) is required for this signaling pathway. We speculate that PKA phosphorylation of Csk could be an intermediate component of the pathway.

blocked by inhibitors of PKA as well as by the intracellular applications of the selective Src inhibitor peptide Src(40–58) [75]. This peptide mimics the unique domain of Src and interferes with its binding to the mitochondrial subunit protein ND2 [90]. The depression was also blocked by inhibitors of the serine–threonine phosphatases, PP1 and PP2A, but the mechanisms by which PDGFRs inhibit NMDARs are poorly understood. We reported that PDGFR-induced inhibition was blocked by the actin stabilizing agent phalloidin suggesting that PDGFR signaling is actin-dependent and may require an intact cytoskeleton [74]. It remains unclear how the interplay between PKA and Src, phosphatase activity, and regulation of the actin cytoskeleton, lead to the long-lasting inhibition of peak NMDAR currents in CA1 neurons.

10. Could subtypes of NMDARs be differentially regulated by GPCRs?

NMDARs are tetrameric channels composed of dimers of NR1 and NR2 subunits (NR1, NR1, NR2*x*, NR2*x*) [110] (and in some cases NR3x). CA1 pyramidal neurons predominantly express NR1a, NR2A and NR2B [111,112]. These receptors are composed of either dimers of NR1 and NR2AA (NR1, NR1, NR2A, NR2A) or NR2BB (NR1, NR1, NR2B, NR2B), but *triheteromeric* channels NR2AB (NR1, NR1, NR2A, NR2B) are also present [113]. Triheteromeric channels have pharmacological properties in between those of NR2AA and NR2BB receptors. For example, recombinant NR2AA and triheteromeric receptors are sensitive to a potent block by Zn^{2+}(about 15 nM) whereas NR2BB and triheteromeric receptors are selectively blocked by ifenprodil [114]. The N-terminal domains of NR2A and NR2B subunits impart this differential sensitivity to Zn^{2+} and ifenprodil [114].

In cultured hippocampal neurons NR2AA and NR2BB receptors are preferentially sequestered in synaptic versus extrasynaptic regions [115], respectively. This is supported by evidence that extrasynaptic NR2BB receptors target a signal cascade which modulates CREB transcription [116] whilst synaptic NR2AA receptors do not [117–119]. Indeed, NR2BB receptors are activated in excitotoxicity and lead to ischemic cell death whilst NR2AA receptors may be neuroprotective [117–122]. These results are consistent with demonstrations that activation of extrasynaptic receptors induces "chemically-induced" LTD (the LTD which occurs following bath applications of NMDA) whereas activation of synaptic receptors induces LTP [123,124]. It should be recognized that synaptically-induced LTD is not necessarily mediated by the same mechanisms as "chemically-induced" LTD [123]. In spite of this evidence, there is dynamic exchange of NMDARs between extrasynaptic and synaptic regions [125] and NR2BB and NR2AA receptors are likely both found in synapses. We have preliminary evidence that NR2AA receptors are selectively regulated by PAC_1Rs. This is an important finding as different signaling mechanisms may regulate NR2AA versus NR2BB receptors and therefore GPCRs may be able to regulate different compartments of NMDARs as well as different downstream

events following NMDR activation. The induction of synaptic plasticity at CA1 synapses has also been reported to be dependent upon NR2AA versus NR2BB receptors. In some cases LTP could be dependent upon activation of NR2AA and LTD upon NR2BB containing receptors [126]. However, these results are controversial with evidence both for and against this hypothesis. It remains to be determined whether or not GPCRs target specific subtypes of NMDARs and thereby potentially serve to differentially regulate the thresholds for induction of LTP and LTD.

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