Differential involvement of \(\text{Ca}^{2+}/\text{calmodulin-dependent protein kinases and mitogen-activated protein kinases in the dopamine D1/D5 receptor-mediated potentiation in hippocampal CA1 pyramidal neurons}

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**Abstract**

Dopaminergic neurotransmission modulates and influences hippocampal CA1 synaptic plasticity, learning and long-term memory mechanisms. Investigating the mechanisms involved in the slow-onset potentiation induced by the dopamine D1/D5 receptor agonists in hippocampal CA1 region, we have reported recently that it could play a role in regulating synaptic cooperation and competition. We have also shown that a sustained activation of MEK/MAP kinase pathway was involved in the maintenance of this long-lasting potentiation (Shivarama Shetty, Gopinadhan, & Sajikumar, 2016). However, the molecular aspects of the induction of dopaminergic slow-onset potentiation are not known. Here, we investigated the involvement of MEK/MAPK pathway and \(\text{Ca}^{2+}/\text{calmodulin-dependent protein kinases (CaMKII and CaMKIV)}\) in the induction and maintenance phases of the D1/D5 receptor-mediated slow-onset potentiation. We report differential involvement of these kinases in a dose-dependent manner wherein at weaker levels of dopaminergic activation, both CaMKII and MEK1/2 activation is necessary for the establishment of potentiation and with sufficiently stronger dopaminergic activation, the role of CaMKII becomes dispensable whereas MEK activation remains crucial for the long-lasting potentiation. The results are interesting in view of the involvement of the hippocampal dopaminergic system in a variety of cognitive abilities including memory formation and also in neurological diseases such as Alzheimer’s disease and Parkinson’s disease.

**1. Introduction**

Induction mechanism of long-term potentiation (LTP) involves a transient rise in intracellular \(\text{Ca}^{2+}\) that in turn leads to the activation of many protein kinases (Lynch, 2004; Mayford, Siegelbaum, & Kandel, 2012). These active protein kinases, such as, \(\text{Ca}^{2+}/\text{calmodulin-dependent protein kinase II (CaMKII), mitogen-activated protein kinases (MAPKs), protein kinase-A (PKA), Protein kinase-C (PKC)}\) and others link the transient calcium signals to signal transduction mechanisms essential for the expression of LTP (Giese & Mizuno, 2013). However, the involvement of the kinases in the induction and maintenance mechanisms differs between different types of LTP depending on the nature of the LTP-inducing stimulus and the brain region (Baudry et al., 2015; Lynch, 2004; Mayford et al., 2012).

The NMDA receptor-dependent LTP in the hippocampal CA1 region is associated with activation of CaMKII as it is blocked by the inhibitors of CaMKII (Malenka et al., 1989; Malinow, Schuman, & Tsien, 1989). CaMKII remains persistently activated even after the induction of LTP as a result of autophosphorylation at Thr-286 (Fukunaga, Muller, & Miyamoto, 1995; Fukunaga, Stoppini, Miyamoto, & Muller, 1993; Ouyang, Kantor, Harris, Schuman, & Kennedy, 1997) and mutation of this site to prevent autophosphorylation leads to impairments in LTP and learning (Giese, Fedorov, Filipkowski, & Silva, 1998). Further, mice lacking the CaMKII gene also show deficient hippocampal LTP and impaired spatial learning (Silva, Paylor, Wehner, & Tonegawa, 1992; Silva, Stevens, Tonegawa, & Wang, 1992), and CaMKII inhibitors block the potentiation induced by activation of cAMP pathways (Makhinson, Chotiner, Watson, & O’Dell, 1999). On the other hand, CaMKII is not involved in the LTP induction during early development (Yasuda, Barth, Stellwagen, & Malenka, 2003) and in certain other hippocampal synapses (Cooke et al., 2006). Recent evidence shows that LTP can be induced in CA1 region in...
the absence of CaMKII Thr286 autophosphorylation (Villers, Giese, & Ris, 2014). Therefore, the extent of involvement of CaMKII in the LTP induction and expression could differ with the type of LTP-inducing stimulus.

MAP-kinases ERK1 (p44 MAPK) and ERK2 (p42 MAPK) are one of the key signal integrators in various forms of LTP and its associativity (Shivarama Shetty, Gopinadhan, & Sajikumar, 2016; Sweatt, 2001) and are known to contribute to both local and nuclear mechanisms following a plasticity inducing event [(Reviewed in (Davis & Laroche, 2006)]. Dopaminergic neurotransmission through D1-like receptors influences protein synthesis-dependent late phase-LTP and long-term memory in the hippocampus (Frey, Matthews, & Reymann, 1991; Hansen & Manahan-Vaughan, 2014; Jay, 2003; Rossato, Bevilaqua, Izquierdo, Medina, & Cammarota, 2009). Notably, a synergistic interaction between dopamine D1 receptors and NMDA receptors involves ERK1/2 signaling mechanisms in rat hippocampus (Sarantis, Matsokis, & Angelatou, 2009) and application of dopamine to acute hippocampal slices leads to robust activation of ERK2 in CA1 region (Roberson et al., 1999). However, MAPKs could be differentially involved in the different phases of plasticity as reported in Aplysia (Sharma et al., 2003).

Interestingly, dopamine or D1/D5 receptor agonists induce a long-lasting, slow-onset potentiation (SOP) in hippocampal CA1 Schaffer collateral synapses (Huang & Kandel, 1995; Navakkode, Sajikumar, & Frey, 2007; Navakkode, Sajikumar, Korte, & Soong, 2012) suggesting that D1/D5 receptor activation may be involved in the mechanisms of tag-setting and expression of synaptic tagging and capture (STC), a widely studied model for associative plasticity (Redondo & Morris, 2011). We have recently reported that the D1/D5 receptor-mediated potentiation could lead to differential activation of intracellular mechanisms involving sustained activation of ERK2 in CA1 region (Roberson et al., 1999). However, MAPKs could be differentially involved in the different phases of plasticity as reported in Aplysia (Sharma et al., 2003).

2. Field-potential recordings

Monopolar, lacquer-coated, stainless steel electrodes (AM Systems) were used as stimulating (<1 MΩ) and recording electrodes (5 MΩ). Stimulating electrodes were positioned within the stratum radiatum of the CA1 region to stimulate Schaffer collaterals and the field-EPSP responses were recorded with an electrode placed in the CA1 distal apical dendritic region. After the pre-incubation period, an input–output curve (stimulus intensity vs. fEPSP slope) was plotted for each input and the test stimulus intensity was set to obtain a fEPSP slope 40% of the maximal response. The signals were amplified by a differential amplifier, digitized using a CED 1401 analog-to-digital converter (Cambridge Electronic Design, Cambridge, UK) and monitored online with custom-made software. In all experiments, a stable baseline was recorded for at least 30 min before starting the drug treatments. Four 0.2-Hz biphasic, constant current pulses (spaced at 5 s) given every five minutes were used for baseline and post-induction recording and the average slope value from the four sweeps was considered as one repeat while used for plotting. Initial slopes of fEPSPs were expressed as percentages of baseline averages.

2.3. Drugs

The D1/D5 receptor agonist SKF-38393 ([±)-1-Phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine-7,8-diol hydrochloride] (SKF; #D047, Sigma-Aldrich) was stored as 15–50 mM stock in deionised water at −20 °C protected from light. SKF was diluted to final concentration and bath applied to the slices at 1 ml/min flow rate for three 5-min durations with a 5-min interval between each application (Navakkode et al., 2007, 2012; Shivarama Shetty et al., 2016).

The CaM kinase inhibitor KN-93 and its inactive analog KN-92 were purchased from Calbiochem (#422708, #422712, #422709) and reconstituted in DMSO to 5–20 mM stock and stored as aliquots at −20 °C protected from light. KN-93, at low concentration (1 μM) specifically inhibits CaMKII whereas at a higher concentration (10 μM) inhibits many CaM kinases, including CaMKII and CaMKIV (Redondo et al., 2010). The MAPK/ERK kinase (MEK) inhibitor U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene) was purchased from Promega (#V1121), and stored as aliquots of 20 μM stock in DMSO at −20 °C.

Just before application, the stocks were diluted to the final concentration in aCSF and bubbled with carbon. The drugs were protected from light during storage and the bath application was carried out under dark conditions. Whenever the drug stock was prepared in DMSO, the DMSO concentration in the final bath-application solution was kept below 0.1%, a concentration which has been shown to not affect basal synaptic responses (Navakkode, Sajikumar, & Frey, 2005).

2.4. Data presentation and statistics

The fEPSP slopes per time point were expressed as percentage of average baseline values in each experiment. The time-matched, normalized data were averaged across replicate experiments, plotted against time as ‘mean ± SEM’ and were then subjected to statistical analysis with GraphPad Prism 6.0. Nonparametric tests were used considering the normality violations at small sample numbers. Wilcoxon matched-pairs signed rank test was used when comparisons were made within a group (a post-induction value compared to its own baseline value). Mann-Whitney U test was used for comparisons between groups. Statistical significance was assumed at *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.
3. Results

3.1. Role of MEK/MAPK pathway in the induction phase of D1/D5 receptor-mediated potentiation

MEK/MAPK pathway can be activated within minutes following a plasticity-inducing stimulus (Davis & Laroche, 2006). Hence, we investigated whether MEK/MAPK activation during the induction phase is critically involved in the SKF-induced potentiation. We used the inhibitor U0126, which specifically targets the upstream kinases of ERK1/2 called MEKs (MAP2Ks) (Favata et al., 1998). U0126 potently inhibits both MEK1 and MEK2, and thus prevents the activation of ERK1/2 ([IC50 of 72 nM for MEK1 and 58 nM for MEK2); (Duncia et al., 1998)).

Following a stable baseline recording for 30 min, 5 μM U0126 was bath applied and 15 min later, SKF, at the respective concentration, was co-applied in three pulses similar to that of earlier reports (Navakkode et al., 2007, 2012; Shivarama Shetty et al., 2016). Application of U0126 was continued for another 15 min after the end of SKF application; thus, U0126 was applied for a total duration of 55 min. Co-application of 5 μM SKF with 5 μM U0126 failed to induce any potentiation (Fig. 1B; n = 7). The mean fEPSP slope values were not significantly different from the baseline values at any time-point during the entire period of recording (Wilcoxon test, P > 0.05). The mean potentiation at the end of the recording (100 ± 6%) was significantly less compared to that without the inhibitor treatment [inset in Fig. 1B, (Shivarama Shetty et al., 2016)]. The potentiation induced by 10 μM SKF was also affected by the blockade of MEK1/2 activity (Fig. 1C; n = 7). Though the induction was not affected and the potentiation became significantly different from the baseline by 20 min (113 ± 2% Wilcoxon test, P = 0.015), the magnitude of potentiation did not follow the time-course as that observed in the absence of U0126 [see inset in Fig. 1C, (Shivarama Shetty et al., 2016)]. Further, the potentiation was only transient and was not significantly different from the baseline values by 140 min (120 ± 7%, Wilcoxon test, P = 0.078) and decayed gradually to the baseline by the end of recording period (101 ± 6%, Wilcoxon test, P = 0.468).

Interestingly, co-application of U0126 blocked the persistence of the potentiation induced by 25 μM SKF [Fig. 1D, n = 8; See inset in Fig. 1D for control graph (Shivarama Shetty et al., 2016)]. The induction was not affected; the potentiation became significantly different from the baseline value 20 min after the start of SKF application (117 ± 6%, Wilcoxon test, P = 0.039) and remained significant until 160 min (124 ± 8%, Wilcoxon test, P = 0.039). From 165 min onwards the potentiation gradually decayed to the baseline values by the end of the recording period (270 min; 101 ± 6% Wilcoxon test, P = 0.843). The mean potentiation values 4 h following application of different concentration of SKF with MEK inhibition were compared to those observed in the absence of MEK inhibition [Fig. 1E; control data for comparison from (Shivarama Shetty et al., 2016), used with permission]. Collectively, these experiments indicated that MEK activation during the induction phase was crucial for the long-lasting potentiation induced by D1/D5 receptor activation.

3.2. Role of CaMKII in the induction phase of D1/D5 receptor-mediated potentiation

To investigate the role of CaMKII in the induction phase of D1/D5 receptor-mediated potentiation, we made use of the specific inhibitor of the CaM kinases, KN-93. KN-93 is a cell-permeable, potent inhibitor with an IC50 of 0.37 μM for CaMKII (Sumi et al., 1991). It has been reported earlier that at a concentration of 1 μM, KN-93 selectively inhibits the activity of CaMKII while sparing other CaM kinases (Ishida, Kameshita, Okuno, Kitani, & Fujisawa, 1995; Redondo et al., 2010). Therefore, we co-applied 1 μM KN-93 along with the different concentrations of D1/D5 receptor agonist SKF. Following stable baseline recording for 30 min, 1 μM KN-93 was bath applied and 15 min later, SKF, at the respective concentration, was co-applied in three pulses. KN-93 application was continued for another 15 min after the end of SKF application i.e., KN-93 was applied for a total duration of 55 min.

Blocking the CaMKII activity with KN-93 during 5 μM SKF application completely blocked the SKF-induced potentiation (Fig. 2A; n = 7). No significant potentiation was observed at any time-point over the entire recording period (Wilcoxon test, P > 0.05). The mean potentiation at the end of recording was 106 ± 5% (Wilcoxon test, P = 0.218). KN-92 application, however, did not affect the induction or maintenance of the 5 μM SKF-induced potentiation (Fig. 2B; n = 6). The mean potentiation at the end of the recording period was 130 ± 4% (Wilcoxon test, P = 0.031). Interestingly, co-application of KN-93 with 10 μM SKF resulted only in a transient potentiation (Fig. 2C; n = 7). The potentiation became significantly different from the baseline by 30 min following SKF application (113 ± 3%, Wilcoxon test, P = 0.015). However, the potentiation started decaying gradually after 165 min (111 ± 6%, Wilcoxon test, P = 0.078) and was not significantly different from the baseline values afterwards. The mean potentiation at the end of the recording period was 104 ± 8% (Wilcoxon test, P = 0.468).

Application of KN-92 showed no effect on the maintenance of 10 μM SKF-induced potentiation (Fig. 2D; n = 6). The potentiation became statistically significant 15 min onwards and maintained for the entire recording period (Wilcoxon test, P = 0.031). Mean potentiation at the end of the recording period was 135 ± 6% (Wilcoxon test, P = 0.031).

When KN-93 was applied in the above fashion along with 25 μM SKF, the potentiation followed the usual time course and became significantly different from the baseline values 25 min after the start of SKF application (Fig. 2E; 113 ± 3%, Wilcoxon test, P = 0.023) and maintained over the entire recording period (133 ± 5%, Wilcoxon test, P = 0.007, n = 8). The magnitude of potentiation was less compared to that observed with KN-92 treatment, however, which was not statistically significant (Mann-Whitney U test, P > 0.05). The potentiation induced by 25 μM SKF was not affected by the application of KN-92 (Fig. 2F; n = 7). Mean potentiation at the end of the recording period was 153 ± 12% (Wilcoxon test, P = 0.015).

A comparison of the mean potentiation values at the end of the recording period between the different SKF concentration groups with KN-93 and KN-92 treatment is shown in (Fig. 2G). Together, these results indicate that at weaker D1/D5 receptor activation, CaMKII has a crucial role in the induction of long-lasting forms of plasticity. However, with stronger D1/D5 receptor activation its role appears to be dispensable, possibly due to coincidental activation of many other kinases, the concerted action of which can compensate for the blockade of CaMKII activity.

3.3. Role of CaMKII activity in the maintenance phase

Having observed the differential role of CaMKII in the induction phase, we further investigated its involvement in the maintenance phase of the potentiation induced by different concentrations of SKF. Reports on the involvement of persistently active CaMKII in the maintenance phases of LTP have been confusing (Malinow et al., 1989; Feng, 1995; Chen, Otmakhov, Strick, Colbran, & Lisman, 2001; Lengyel et al., 2004). Here, we tested whether persistently active CaMKII is involved in the maintenance of D1/D5 receptor-mediated potentiation. As in the previous series of experiments, we made use of the strategy of specific inhibition of CaMKII...
with 1 μM KN-93. Following a stable baseline recording, SKF, at the respective concentration, was applied to the slices as explained in the initial experiments (Fig. 1). KN-93 (1 μM) was then bath applied from 90 min after the start of SKF application throughout the rest of the recording period. All these experiments were also repeated with KN-92, an inactive analog of KN-93, to rule out any non-specific treatment effects.

Blocking CaMKII activity with KN-93 during the maintenance phase had no effect on 5 μM SKF-induced potentiation (Fig. 3A; n = 7). The potentiation decayed to the baseline by the end of the recording period. (D) Inhibition of MEK1/2 activity also blocked the maintenance of the potentiation induced by 25 μM SKF (n = 8). (E) A histogram comparing the mean potentiation values 4 h after SKF application in the different SKF concentration groups with or without U0126 coapplication. The 'Control' group for each SKF concentration is shown as inserts in each case [Reproduced from (Shivarama Shetty et al., 2016) with permission of the publisher]. Asterisks indicate significant difference between two groups [(Mann-Whitney U test, *P < 0.05, **P < 0.01, ***P < 0.001); (For 5 μM SKF Group P = 0.046; for 10 μM SKF Group P = 0.006 and for 25 μM SKF Group P = 0.0002)]. Error bars in all graphs represent ±SEM. Representative fEPSP traces shown in each case were recorded at the respective time points as mentioned. Scale bars for all the traces, vertical: 3 mV, horizontal: 5 ms.

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Fig. 2. Role of CaMKII in the induction of D1/D5 receptor-mediated potentiation. Following a stable baseline recording for 30 min, CaMKII inhibitor KN-93 (1 µM) or its inactive analog KN-92 (1 µM) was bath applied and 15 min later, SKF, at the respective concentration, was co-applied in three pulses (three broken arrows). The duration of KN-93 application is represented with the grey bar in each case. (A) Inhibition of CaMKII completely blocked the potentiation induced by 5 µM SKF (n = 7). (C) Coapplication of 1 µM KN-93 with 10 µM SKF resulted in a transient potentiation (n = 7). The potentiation decayed to the baseline by the end of the recording period. (E) Inhibition of CaMKII activation did not affect the maintenance of the potentiation induced by 25 µM SKF (n = 8) but the magnitude of potentiation was reduced. Coapplication of KN-92 (1 µM) had no effect on the induction or maintenance of the potentiation induced by (B) 5 µM SKF (n = 6), (D) 10 µM SKF (n = 6) and (F) 25 µM SKF (n = 7). (G) A histogram comparing the mean potentiation values at the end of the recording period in the different SKF concentration groups with KN-93 or KN-92 coapplication. Asterisks indicate significant difference between two groups [Mann-Whitney U test, *P < 0.05, **P < 0.01]; (For 5 µM SKF group P = 0.008; for 10 µM SKF group P = 0.022 and for 25 µM SKF group P = 0.351)]. Error bars in all graphs represent ±SEM. Representative fEPSP traces shown in each case were recorded at the respective time points as mentioned. Symbols and traces as in Fig. 1.
Fig. 3. Role of CaMKII in the maintenance of D1/D5 receptor-mediated potentiation. Following a stable baseline recording for 30 min, SKF, at the respective concentration, was co-applied in three pulses (three broken arrows). The CaMKII inhibitor KN-93 (1 μM) or its inactive analog KN-92 (1 μM) was bath applied starting 90 min after the SKF application until the end of the recording. The duration of the inhibitor application is represented with the grey bar in each case. (A) and (B) Inhibition of CaMKII during maintenance phase did not affect the potentiation induced by 5 μM SKF (n = 7) as compared to that with the control analog (n = 7). (C) and (D) The maintenance of potentiation induced by 10 μM SKF was unaffected by the treatment with either the CaMKII inhibitor (n = 7) or the control drug KN-92 (n = 6). (E) and (F) Treatment with KN-93 did not affect the maintenance of potentiation induced by 25 μM SKF (n = 7), similar to the treatment with KN-92 (n = 6). (G) A histogram comparing the mean potentiation values at the end of the recording period in the respective SKF concentration groups with KN-93 or KN-92 treatment. No significant difference was observed between the treatments in any group (Mann-Whitney U test; P > 0.05). Error bars in all graphs represent ± SEM. Representative fEPSP traces shown in each case were recorded at the respective time points as mentioned. Symbols and traces as in Fig. 1.
specific effects (Fig. 3B; n = 7) and the potentiation observed was not significantly different from the KN-93 treatment group (Mann-Whitney U test, P > 0.05).

Application of 1 μM KN-93 had no effect on the maintenance of the potentiation induced by 10 μM SKF (Fig. 3C; n = 7) and 25 μM SKF (Fig. 3E; n = 7). The mean potentiation at the end of the recording period in both the cases was significantly different from the respective baseline values (Wilcoxon test, P < 0.05). Similar potentiation profile was also observed with 1 μM KN-92 treatment (Fig. 3D; n = 6 and Fig. 3F; n = 6) and there was no statistically significant difference between the respective KN-93 and KN-92 treatment groups (Mann-Whitney U test, P > 0.05).

A histogram comparing the mean potentiation at the end of recording period between the different SKF concentration groups with KN-93 and KN-92 treatment is shown in Fig. 3G. Together, these series of experiments showed that a sustained CaMKII activity is not involved in the maintenance phase of the D1/D5 receptor-mediated potentiation irrespective of the strength of stimulation.

3.4. Role of CaM kinase-IV (CaMKIV) in the maintenance phase of D1/D5 receptor-mediated potentiation

CaMKIV has been reported to take part in the induction of activity-dependent gene transcription and thus contribute to certain forms of transcription and translation dependent plasticity (Bito, Deisseroth, & Tsien, 1996; Kang et al., 2001; Kasahara, Fukunaga, & Miyamoto, 2001; Redondo et al., 2010). CaMKIV is a nuclear kinase and becomes activated after phosphorylation by CaM kinase kinase (CaMKK) which, in turn, is activated by nuclear calcium entry in response to strong stimulation. The active CaMKIV then phosphorylates and activates CREB, thus leading to transcription of plasticity-related genes (Bito et al., 1996; Kang et al., 2001; Kasahara et al., 2001). We were interested to know whether CaMKIV activation was involved in the maintenance phase of the potentiation induced by SKF. To investigate this, we made use of the CaM kinase inhibitor KN-93 but now at a higher concentration (10 μM), as it has been reported earlier that at this concentration KN-93 effectively inhibits many CaM kinases, including CaMKIV (Redondo et al., 2010).

The experimental strategy was similar to that followed in Section 3.3 but 10 μM KN-93 was applied starting 90 min after the SKF application and for the rest of the recording period. These experiments were also repeated with the same concentration of the inactive analog KN-92. The slow-onset, long-lasting potentiation observed following 5 μM SKF application was not affected by the 10 μM KN-93 treatment (Fig. 4A; n = 7). The potentiation became statistically significant by 75 min after the SKF application (109 ± 4%; Wilcoxon test, P = 0.046) and then lasted over 4 h. The mean potentiation at the end of the recording (130 ± 7% Wilcoxon test, P = 0.015) was similar to that in the KN-92 group (Fig. 4B; 131 ± 6%, Wilcoxon test, P = 0.031; n = 6) and when compared between two groups, there was no significant difference in the magnitude of potentiation (Mann-Whitney U test, P > 0.05). Treatment with 10 μM KN-93 had no significant effect on the potentiation induced by 10 μM SKF (Fig. 4C; n = 7). The potentiation followed the usual time course becoming significantly different from the baseline by 20 min after SKF application (114 ± 2%, Wilcoxon test, P = 0.031) and maintained at significant levels throughout the rest of the recording period (Wilcoxon test, P > 0.05). The mean EPSP slope at the end of the recording (134 ± 7% Wilcoxon test, P = 0.015) was similar to that in the KN-92 treatment group (Fig. 4D; 135 ± 8%, Wilcoxon test, P = 0.031; n = 6) and there was no significant difference in the magnitude of potentiation when compared between two groups (Mann-Whitney U test, P > 0.05).

Application of 25 μM SKF resulted in a slow-onset long-lasting potentiation that became significantly different from the baseline values 30 min onwards (Fig. 4E; 107 ± 2%, Wilcoxon test, P = 0.023; n = 8) and lasted for the entire recording period (270 min; Wilcoxon test, P = 0.007). Though the potentiation in the late phase was not completely blocked by the treatment with KN-93, the magnitude of potentiation was significantly less compared to that observed in the KN-92 treatment group (Fig. 4F; n = 6). When compared to the KN-92 treatment group, the potentiation in KN-93 treatment group was significantly reduced 135 min onwards (Mann-Whitney U test, P < 0.05). The mean potentiation at the end of the recording was 131 ± 7% in KN-93 group whereas it was 148 ± 7% in the KN-92 group. There was also a significant difference in the mean potentiation at the end of recording period, between the 25 μM SKF group [see insert in Fig. 1D; from (Shivarama Shetty et al., 2016)] and the 10 μM KN-93 treatment group (Mann-Whitney U test, P = 0.043). A histogram comparing the mean potentiation at the end of recording between the different SKF concentration groups with KN-93 and KN-92 treatment is shown in Fig. 4G. Taken together, these results showed that the active CaMKIV-mediated mechanisms contribute synergistically to the maintenance phase of potentiation only with stronger D1/D5R activation and were not activated by weaker D1/D5R stimulation.

A summary of the effects of inhibition of MEKs and CaMKIs during the induction and maintenance phase of the D1/D5 receptor-mediated potentiation are presented in Table 1A and B respectively.

4. Discussion

The experiments investigating the role of CaM kinases and MEKs in the induction phase of D1/D5 receptor-mediated potentiation revealed that at weaker levels of dopaminergic activation with its agonist (5 μM SKF), inhibiting either of the kinases, CaMKII or MEKs, completely blocked the induction of potentiation. At moderate levels of dopaminergic activation (10 μM SKF), inhibition of either of the kinases blocked the persistence of the potentiation. However, when the dopaminergic activation was sufficiently strong (25 μM SKF), inhibiting CaMKII during induction had no effect on the induced potentiation whereas inhibiting MEKs blocked the late phase. The late-phase mechanisms in this case were gated through MEK activation. When the dopaminergic activation was moderate, potentiation could still be maintained transiently by other active kinases but the expression of late-phase would require active MEK. With weaker D1/D5 receptor activation, a concerted action of these two kinases was necessary for the induction of the potentiation and thus blocking any of them would interfere with the induction.

Differential involvement of kinases in different forms of plasticity has been well appreciated (Baudry et al., 2015; Lynch, 2004; Mayford et al., 2012). Though CaMKII is known to be involved in many forms of LTP in the hippocampus, its role is not ubiquitous to all forms of NMDAR-dependent LTP: CaMKII inhibitors have no effect on the LTP induction in CA1 during early development (Yasuda et al., 2003) and its involvement is minimal in medial perforant path synapses of DG (Cooke et al., 2006). On the other hand, inhibitors of MEK block theta-induced LTP while sparing tetanus-induced or pairing-induced LTP (Opazo, Watabe, Grant, & O’Dell, 2003). Further, in some cases, CaMKII might play a role as a part of a parallel cascade involving MAPK and PKA (Cooke et al., 2006). Such parallel kinase cascades are known to operate in mice at an early age (Wikstrom, Matthews, Roberts, Collingridge, & Bortolotto, 2003), in the DG of adult mice (Cooke et al., 2006) and in rats (Wu, Rowan, & Anwyll, 2006). From these studies, two main limbs of such parallel cascades have been identified: one involving CaMKII and the other involving PKA/PKC/MAPK.

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Fig. 4. Role of CaMKIV and other CaM-kinases in the maintenance of D1/D5 receptor-mediated potentiation. Following a stable baseline recording for 30 min, SKF, at the respective concentration, was co-applied in three pulses as in earlier experiments. The CaM-Kinase inhibitor KN-93 (10 μM) or its inactive analog KN-92 (10 μM) was bath applied starting 90 min after the SKF application until the end of the recording. The duration of the inhibitor application is represented with the grey bar in each case. (A) and (B) Inhibition of CaMKIV and other CaMKs during maintenance phase did not affect the potentiation induced by 5 μM SKF (n = 7) as compared to that with the control analog (n = 6). (C) and (D) The maintenance of potentiation induced by 10 μM SKF was unaffected by the treatment with either the CaMK inhibitor (n = 7) or the control drug KN-92 (n = 6). (E) and (F) Treatment with 10 μM KN-93 reduced the magnitude of the potentiation induced by 25 μM SKF (n = 8), compared to the treatment with 10 μM KN-92 (n = 6). (G) A histogram comparing the mean potentiation values at the end of the recording period in the respective SKF concentration groups with KN-93 or KN-92 treatment. No significant difference was observed between the treatments in 5 μM SKF and 10 μM SKF groups (Mann-Whitney U test; P > 0.05). However, treatment with 10 μM KN-93 significantly reduced the magnitude of potentiation in 25 μM SKF group (Mann-Whitney U test; P = 0.030). Error bars in all graphs represent ±SEM. Representative fEPSP traces shown in each case were recorded at the respective time points as mentioned. Symbols and traces as in Fig. 1.
In our study, we found no significant role for CaMKII activation or for CaMKIV activation in the maintenance phase of the D1/D5 receptor-mediated potentiation. Application of CaMKII inhibitors following LTP induction has been reported to have an effect on LTP (Chen et al., 2001; Ito, Hidaka, & Sugiyma, 1991; Malinow et al., 1989). Whether the CaMKII enzyme activity is transient or persistent has met with conflicting results (Fukunaga et al., 1995; Lee, Escobedo-Lozoya, Szatmari, & Yasuda, 2009; Lengyel et al., 2004). The inhibitor KN-93 acts by allosterically binding to CaMKII holoenzyme (in complex with Ca2+/CaM) and blocking CaMKII activation. Hence, it only prevented the Ca2+/calmodulin-dependent activation but not the autophosphorylated form of the enzyme (Sumi et al., 1991; Vest, O’Leary, Coulttrap, Kindy, & Bayer, 2010); but no direct inhibitors of the autophosphorylated form are available currently. Therefore, from these results we can only conclude that no persistent reactivation of the enzyme was involved during the maintenance phase. However, on another note, the autophosphorylated CaMKII is suggested to act as a molecular switch (Lisman, 1994) at the transition of early and late phases of LTP and in itself, this mechanism may not be able to sustain the potentiation for long durations [Reviewed by (Giese & Mizuno, 2013)]. A mutant deficient in threonine–286 autophosphorylation (T286A) (Giese et al., 1998) can still form memory upon stronger stimulation. Here, CaMKIV pathway might act synergistically with MEK/MAFK pathways to induce CREB activation (Wu, Deisseroth, & Tsien, 2001). Besides, studies using mutant mice lines have suggested that CaMKII may be necessary for only certain forms of memory, for instance, fear memory but not for spatial memory (Ho et al., 2000; Kang et al., 2001; Wei et al., 2002). The CaMKK-CaMKIV cascade is more sensitive to nuclear calcium entry necessary for the induction of the slow-onset potentiation. At moderate levels of stimulation, however, either one of these kinases alone could maintain the potentiation transiently but a concerted action of both appears to be necessary for the maintenance of late phase. Interestingly, when the dopaminergic activation was sufficiently stronger, CaMKII mediated mechanisms became dispensable while MEK activation remained necessary, which supports our earlier finding that MEK activation is crucial for D1/D5 receptor-mediated synaptic plasticity in hippocampal CA1 region (Shivarama Shetty et al., 2016). These results are important in understanding the dopaminergic modulation of hippocampal synaptic plasticity which is involved in long-term memory storage and also implicated in certain cognitive disorders.

**Conflict of interest**

The authors declare no conflict of interest.

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