GKAP-DLC2 interaction organizes postsynaptic scaffold complex to enhance synaptic NMDA receptor activity

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Enora Moutin\textsuperscript{1,2,3}, Fabrice Raynaud\textsuperscript{1,2,3}, Laurent Fagni\textsuperscript{1,2,3} and Julie Perroy\textsuperscript{1,2,3}.

1, CNRS, UMR-5203, Institut de Génomique Fonctionnelle, F-34000 Montpellier, France; 2, INSERM, U661, F-34000 Montpellier, France; 3, Universités de Montpellier 1 & 2, UMR-5203, F-34000 Montpellier, France.

Address correspondence to:
Julie Perroy, IGF, 141 rue de la Cardonille 34094 Montpellier Cedex 05.
Tel: +33 434359210; Fax: +33 467542432; E-mail: julie.perroy@igf.cnrs.fr
Summary

At glutamatergic brain synapses, scaffolding proteins regulate receptor location and function. The targeting and organization of scaffolding proteins in the postsynaptic density (PSD) is poorly understood. A core protein of the glutamatergic receptor postsynaptic scaffold complex, GKAP, interacts with DLC2, a protein associated with molecular motors. In the present study, we combined BRET imaging, immuno-staining and electrophysiological recording to assess the role of GKAP-DLC2 interaction in the functional organization of the glutamatergic synapse. We found that GKAP-DLC2 interaction in dendritic spine stabilizes scaffolding protein expression at the PSD and enhances synaptic NMDA receptor activity. Moreover, the GKAP-DLC2 functional interaction is favored by sustained synaptic activity. These data provide a novel regulatory pathway of synaptic transmission that depends on activity-induced remodeling of the postsynaptic scaffold protein complex.
Electron microscopic analysis of glutamatergic synapses revealed an electron-dense organelle, right beneath the postsynaptic membrane, which was named postsynaptic density (PSD) (Palay, 1958). The PSD consists of a network of proteins that anchor and link glutamate receptors and other postsynaptic proteins to the cytoskeleton and signaling pathways (Ehlers et al., 1996; Kennedy, 1997; Scannevin and Huganir, 2000; Sheng and Pak, 2000). Changes in PSD scaffolding protein integrity can influence excitatory synaptic transmission through glutamate receptor redistribution and signalisation (Ehrlich and Malinow, 2004; Stein et al., 2003). The guanylate kinase-associated protein (GKAP; also known as synapse-associated protein 90-postsynaptic density-95-associated protein/SAPAP and Discs-large-associated protein/DAP family proteins) are highly enriched and specifically localized in the PSD. There are at least four alternative splicings of the GKAP gene (Kim et al., 1997). GKAP proteins bind the guanylate kinase-like domain of PSD-95 (Kim et al., 1997; Naisbitt et al., 1997; Takeuchi et al., 1997), S-SCAM (synaptic scaffolding molecule) (Hirao et al., 1998) and PSD-95-Discs Large-zona occludens-1 (PDZ) domains of shank1-3 proteins (Boeckers et al., 1999; Naisbitt et al., 1999). GKAP binding to Shank proteins is mediated by a short C-terminal sequence common to all GKAP splice variants, whereas its binding to PSD-95 is mediated by the N-terminal region which contains five repeats of 14 amino acids highly conserved in all GKAP proteins (Kim et al., 1997). By interacting with PSD-95 and Shank, GKAP physically links the NMDA receptor/PSD-95 complex to the type I metabotropic glutamate receptor/Homer complex (Naisbitt et al., 1999; Tu et al., 1999). GKAP is therefore one of the major scaffold proteins organizing glutamate receptors in the PSD. The molecular mechanisms regulating the postsynaptic targeting and assembly of neurotransmitter receptors and associated scaffolding proteins in the PSD are still widely unknown. Indeed, PSD-95 seems to be involved in NMDA clusterization (Kornau et al., 1995; Niethammer et al., 1996) whereas GKAP may be involved in the modulation of the NMDA receptor-channel activity via PSD-95 (Yamada et al., 1999).

A few years ago, Naisbitt et al. (Naisbitt et al., 2000) identified a new GKAP interactor, Dynein Light Chain (DLC) (also known as DYNLL and LC8), a light chain that is shared by cytoplasmic dynein and myosin-V (Benashski et al., 1997). This adaptor protein functions as a molecular motor that drives the trafficking of cargoes along microtubules and actin filaments. DLC is an 8 kDa highly conserved protein, from rice to human (King et al., 1996; King and Patel-King, 1995) and it is ubiquitously expressed in organisms. In mammals, there
are two DLC isoforms, DLC1 and DLC2. Its crucial role has been demonstrated in several organisms, including *Drosophila*, where null mutations result in embryonic lethality (Dick et al., 1996). DLC associates with a wide variety of proteins, including neuronal nitric oxide synthase (nNOS) (Jaffrey and Snyder, 1996), gephyrin (Fuhrmann et al., 2002), bassoon (Fejtova et al., 2009), the proapoptotic factor Bim (Puthalakath et al., 1999), transcriptional factors Swallow (Schnorrer et al., 2000) and Trps1 (Kaiser et al., 2003), viral proteins (Raux et al., 2000), and p53 binding protein 1 (Lo et al., 2005). DLC being a light chain of motor proteins, its role in trafficking has been widely assessed. Thus DLC has been reported to link cargoes to the molecular motors (Lee et al., 2006; Navarro et al., 2004; Schnorrer et al., 2000). In 2008, Elisa Barbar proposed that DLC is an essential Hub protein in various protein networks (Barbar, 2008). A large number of studies indeed suggested involvement of DLC in many biological processes such as in viral and RNA transport (Raux et al., 2000), apoptosis (Puthalakath et al., 1999; Puthalakath et al., 2001), cell cycle (Lo et al., 2005; Vadlamudi et al., 2004), inhibition of nNOS (Jaffrey and Snyder, 1996), facilitation of Swallow folding (Schnorrer et al., 2000), macropinocytosis (Yang et al., 2005) and nuclear transport (Sodeik et al., 1997). Dimerization of DLC (Benashski et al., 1997) is required for its activity (Wang et al., 2003). Moreover, it has been observed in several cases that DLC interacts with partially disordered proteins and promotes their dimerization (Barbar, 2008).

Because GKAP is a core protein of the glutamate receptor complex, its association with DLC2 might be of significant importance in the targeting and organization of the receptors and associated scaffolding proteins in dendritic spines. It is therefore essential to understand the spatio-temporal dynamics of GKAP-DLC2 interaction and its function in glutamate receptor activity. Here we identified the molecular determinants involved in GKAP-DLC2 interaction. We further investigated by single cell Bioluminescence Resonance Energy Transfer (BRET) imaging the spatio-temporal dynamics of this interaction in cultured hippocampal neurons, and its consequences on NMDA receptor function. We found that GKAP-DLC2 interaction was prominent in dendritic spines and could be further increased by sustained synaptic activity. This GKAP-DLC2 interaction enabled the accumulation of GKAP and PSD-95 in dendritic spines and potentiated synaptic NMDA currents.

**Results**

*GKAP interacts with DLC2 in living cells.*
To assess the occurrence of GKAP-DLC2 interaction in a physiological context by Bioluminescence Resonance Energy Transfer (BRET), we fused the amino terminus of GKAP to the energy donor *Renilla Luciferase* (*Rluc8*) and the amino terminus of DLC2 to the acceptor Yellow Fluorescent protein, Venus (*Rluc8-GKAP* and *Venus-DLC2*). Addition of the tag did not impair the known properties of these proteins: *Rluc8-GKAP* interacted with PSD-95 and Shank3 (Fig. S1A) and *Venus-DLC2* was able to form dimers (Fig. S1B). In hippocampal neurons, these two tagged-proteins were ubiquitously expressed and colocalized in dendritic spines of cultured hippocampal neurons (Fig. 1A).

Under condition of constant level of *Rluc8-GKAP* expression, BRET signal increased hyperbolically as a function of *Venus-DLC2* expression level (Fig. 1B). Saturation of the BRET signal when all the donor was linked to the acceptor indicated a specific interaction between GKAP and DLC2 proteins. This interaction was further confirmed by co-immunoprecipitation (Fig. 1C). We assessed by BRET whether DLC2 could also bind other PSD-associated scaffolding elements. None of the protein tested (PSD95, Shank3 and Homer3) produced specific BRET signal with DLC2, indicating a specificity of binding of DLC2 towards GKAP (Fig. S1C).

To characterize the molecular determinants involved in the interaction of GKAP with DLC2, we screened the amino acid sequences of other DLC2 interactors and found two conserved consensus binding sites: GIQVD and GVQVEE (Lajoix et al., 2004; Lo et al., 2001; Navarro-Lerida et al., 2004). The glycine residue as well as the -1 and +1 flanking amino acids might be crucial for the interaction (Lajoix et al., 2004). Accordingly, we engineered by molecular biology the cDNA coding for a GKAP mutant in which GIQVD and GVQVEE were respectively mutated in GNQND and in GVEVEE (*GKAPmut*, Fig. 1D). BRET and co-immunoprecipitation experiments evidenced a loss of interaction between *GKAPmut* and DLC2 (Fig. 1B and C). Taken together, these data showed that GKAP and DLC2 interacted in living cells and three punctual mutations on GKAP were sufficient to impair this interaction.

We further characterized the association between GKAP and DLC2 at the subcellular level by imaging protein-protein interactions on single hippocampal neuron using BRET imaging technology (Coulon et al., 2008; Perroy, 2010). The BRET signal between *RLuc8-GKAP* and *Venus-DLC2* was homogenous in the cell body and punctiform in dendrites (Fig. 2A). The mean BRET intensity was significantly higher in dendrites than in soma (124.5 ± 4.2 and 99.8 ± 1.8, respectively, Fig. 2B left) and importantly, the standard deviation was almost three times higher in dendrites versus soma (80.3 ± 2.4 and 22.4 ± 2.3, respectively, Fig. 2B right), which was consistent with a higher punctiform distribution in the dendrites than in soma.
Neuronal activity potentiates GKAP-DLC2 interaction.

We next analyzed the effect of neuronal activity on GKAP-DLC2 interaction. To enhance neuronal activity, we used a specific blocker of transient outward voltage-dependent potassium currents, 4-AP (1 mM) (Buckle and Haas, 1982). BRET signal between Rluc8-GKAP and Venus-DLC2 in hippocampal neurons was recorded before and during 4-AP exposure (Fig. 3A). After 4-AP exposure, the mean BRET signal increased in soma (109.33% ± 3.13 at 10 min; n=21) and dendrites (111.96% ± 3.39 at 10 min; n=21, Fig. 3B). The standard deviation increased in soma (155.06% ± 11.41; n=21, Fig. 3C), suggesting an important clusterization of GKAP-DLC2 complex induced by 4-AP application. In dendrites, the standard deviation also significantly increased after 4-AP application, but to a lower extent (104.11% ± 1.21; n=21, Fig. 3C), probably because of an already important basal clusterization (Fig. 2B). Indeed, despite the strong increase in soma, BRET signal standard deviation remained higher in dendrites than in soma after 4-AP stimulation (absolute mean BRET values: 32.4 ± 2.6 in soma and 86.5 ± 1.1 in dendrites). The observed activity-dependent increase in BRET intensity and standard deviation in dendrites may be due to an increase of BRET in dendritic spines. Consistent with this hypothesis, BRET intensity was measured in dendritic spines specifically, where we found a significant increase after 4-AP exposure (128.78% ± 7.63 increase, 10 min after 4-AP application compared to control; n=72 spines, Figure 3D). A similar modulation of DLC2-GKAP interaction could be obtained by stimulation of postsynaptic NMDA receptors with a specific agonist (Fig. S2). These experiments highlighted a neuronal activity-induced potentiation of GKAP-DLC2 interaction in postsynaptic elements.

In addition, we noticed that the BRET signal was different between spines. These differences may reflect physiological disparities between spines such as different amount of proteins per spine. Since the BRET signal between GKAP and DLC2 depends on DLC2 and GKAP expression ratio, small differences in protein expression in the spine would influence the level of BRET. But more importantly, spines are particularly advantageous to neurons by compartmentalizing biochemical and electrical signals. This can help to encode changes in the state of an individual synapse without necessarily affecting the state of other synapses of the same neuron. The neuronal activity is not homogenous but varies from one spine to another. The observed differences in BRET signal between spines were therefore consistent with and
reinforced the idea that synaptic activity regulated the interaction between DLC2 and GKAP in spines.

**GKAP-DLC2 interaction enables a synaptic activity-dependent accumulation of GKAP in dendritic spines.**

As 4-AP increased GKAP-DLC2 interaction in spines, we studied the consequences of neuronal activity on CFP-GKAP accumulation in dendritic spines further (Fig. 4A). To this aim, we measured the ratio of fluorescence intensity in dendritic spines versus dendritic shaft, before and during 4-AP application. Before 4-AP application, the spine/shaft fluorescence ratio was 2.4, indicating a preferential expression of GKAP in spines, under basal conditions. Interestingly, after 5 min of 4-AP application, CFP-GKAP spine/shaft fluorescence intensity displayed a $32.9 \pm 9.2\%$ increase, thus highlighting an activity-dependent increase of GKAP protein in dendritic spines (Fig. 4B). To assess the role of GKAP-DLC2 interaction in the accumulation of GKAP in spines, we used the CFP-GKAPmut construct that did not interact with DLC2 (Fig. 1). The synaptic localization of CFP-GKAPmut was significantly reduced (~16.4%) as compared to CFP-GKAP, but its spine/shaft fluorescence ratio was still superior to one, suggesting that although interaction with DLC2 could modulate spine-preferential localization of GKAP, this interaction was not essential in basal conditions. In contrary to GKAP, the fluorescence intensity of GKAPmut was not significantly modulated by 4-AP application, suggesting that GKAP-DLC2 interaction was necessary for activity-induced GKAP accumulation in spines (Fig. 4B). In contrast to GKAP, DLC2 content in the postsynaptic compartment was not modulated by 4-AP treatment (Fig. S3), suggesting that the activity regulates only GKAP in a DLC2 interaction dependent manner. The 4-AP-induced GKAP accumulation in spines was blocked by co-application of CNQX (Fig. 4C), a competitive AMPA/kainate antagonist, indicating that 4-AP-induced increase in GKAP accumulation in spines was mediated by elevated synaptic activity rather than intrinsic neuronal activity *per se*. Interestingly, co-expression of DLC2 abolished the re-location of GKAP in spines by the activity (Fig. S4). The overexpression of light chains is known to prevent the correct association of molecular motor complex and can indeed be used as a dominant negative tool causing the molecular motor complex to dissociate and decoupling the motor from its cargo (see for example (Echeverri et al., 1996)). In this condition GKAP expression is no longer enhanced in spines upon neuronal activity. To summarize, GKAP-DLC2 interaction enabled trafficking and accumulation of GKAP in dendritic spines and this
effect was promoted by sustained synaptic activity.

**GKAP-DLC2 interaction modulates the synaptic localization of PSD-95.**

Because GKAP is a core protein of the postsynaptic glutamatergic receptor complex, we further studied the importance of GKAP-DLC2 interaction in the accumulation of GKAP scaffold partners in the dendritic spine. We thus assessed the spine/shaft fluorescence ratio of PSD-95-YFP, Venus-Shank3, Venus-Homer3 or Venus-GIT1 (for G protein-coupled receptor kinase interacting protein 1, a multifunctional adaptor protein expressed in dendrites) when co-expressed with GKAP or GKAPmut (Fig. 5A), a construct that still interacted with Shank3 and PSD-95 (Fig. S5), but not DLC2 (Fig. 1). We found that GKAP induced a significant increase in Shank3 (15.2% ± 6.6), Homer3 (18.4% ± 4.4) and PSD-95 (52.6 ± 8.0%) spine/shaft fluorescence ratio but not GIT1. By opposition, none of these partners expression was affected by the presence of GKAPmut. This differential effect of GKAP and GKAPmut highlighted the importance of GKAP-DLC2 complex in the preferential location of GKAP partners in spine. The observed PSD-95 accumulation in spine induced by GKAP-DLC2 interaction could be explained by GKAP targeting and trafficking of the PSD-95-GKAP-DLC2 complex to the spine, or stabilization of PSD-95 by GKAP-DLC2 complex at the spine. To further discriminate between the role of GKAP accumulation *per se* versus its localization ratio as a function of GKAP or GKAPmut expression level (Fig. 5B). The preferential localization of PSD-95 in spines increased as a function of GKAP, but not GKAPmut expression level (Fig. 5B). The absence of correlation between GKAPmut expression level and PSD-95 preferential localization in spines suggested that local interaction of GKAP with DLC2, rather than accumulation of GKAP *per se* stabilized PSD-95 in spines.

As neuronal activity increased GKAP-DLC2 interaction in spine, we examined whether this also applied to PSD-95 accumulation. Five minutes application of 4-AP indeed stably increased accumulation of PSD-95 in spine (Fig. 5C). Consistent with the hypothesis that neuronal activity-induced PSD-95 accumulation relied on GKAP accumulation in spines, transfection of GKAP increased per se the basal PSD-95 accumulation in spines (as previously shown, fig. 5A), but precluded additional effect of sustained neuronal activity on PSD-95 accumulation in dendritic spines (Fig. 5C). Furthermore, while PSD-95 spine/shaft ratio was not significantly different when co-transfected with CFP or CFP-GKAPmut in basal condition (Fig. 5A), 4-AP application failed to increase PSD-95 accumulation in spines in
presence of GKAPmut (Fig. 5C). Taken together, these experiments suggested that GKAP-DLC2 interaction was responsible for GKAP and PSD-95 accumulation in spine and this could be enhanced by neuronal activity. Note that the role of GKAP-DLC2 interaction in organizing the postsynaptic scaffold complex also applies to endogenous proteins, since endogenous PSD95 was re-located to spines upon neuronal activity in a GKAP-DLC2 interaction-dependent manner (Fig.S6).

GKAP-DLC2 interaction enhances NMDA synaptic currents.

As GKAP-DLC2 interaction promotes synaptic localization of PSD-95, we investigated the modulatory role of this protein assembly on endogenous NMDA current. Neurons transfected with CFP-GKAP or CFP-GKAPmut were recorded in the whole-cell configuration of the patch-clamp technique and transiently perfused with NMDA. GKAP induced a 28.8 ± 6.4 % (n=24) increase in NMDA currents while GKAPmut had no significant effect (11.8 ± 8.6 % decrease compared to control; n=17). Down-regulation of DLC2 by specific siRNA (Fig. S7) abolished the GKAP-induced increase of NMDA currents (1.4 ± 8.7 % decrease compared to control; n=9, fig. 6A and 6B). These results suggest that GKAP expression potentiated NMDA currents via interaction of the protein with DLC2.

We further studied the modulatory action of GKAP-DLC2 interaction on NMDA current at synaptic level by analyzing the slow component of miniature excitatory postsynaptic currents (mEPSCs) carried by NMDA currents (Lu et al., 2001). The NMDA component was measured 7 ms after the peak amplitude of averaged mEPSCs, in neurons transfected or not with GKAP or GKAPmut. Over expression of GKAP significantly increased the amplitude of the synaptic NMDA component (synaptic NMDA current in presence of GKAP: 130.9 ± 9.0 % of control condition; Fig. 6C). By opposition, GKAP mutant that could not interact with DLC2 had not significant effect on synaptic NMDA current (GKAPmut: 103.0 ± 6.8 % of synaptic NMDA current in control condition; Fig. 6C). These results highlight the potentiation of synaptic NMDA currents by GKAP, enhancement that requires the interaction of GKAP with DLC2.

Discussion

GKAP is a core protein of the scaffolding complex that governs glutamate receptor location and function in dendritic spines. Identification of the new GKAP interactor, DLC2, raised the
question of its functional interaction in the organization and activity of the glutamate
receptors. Thanks to recent development in single cell BRET imaging (Coulon et al., 2008;
Perroy, 2010), we have examined this issue by studying the spatio-temporal dynamics of
GKAP-DLC2 interaction in living hippocampal neurons. We found that GKAP-DLC2
interaction was prominent in dendritic spines and could be exacerbated by sustained synaptic
activity. We identified the molecular determinant of the interaction, and engineered a GKAP
mutant that lacked the ability to interact with DLC2. This allowed us to show that GKAP-
DLC2 interaction enabled accumulation of GKAP and PSD-95 in dendritic spines, and
potentiated postsynaptic NMDA currents. Thus, combination of BRET imaging with
immunofluorescence staining and electrophysiological recording allowed us to better
understand the physiological role of GKAP-DLC2 complex in the postsynaptic glutamate
receptor assembly and function.

Our results showed that GKAP-DLC2 interaction favored the spine preferential expression of
GKAP. DLC2 being a light chain of myosin V, this adaptor protein may function as a
molecular motor that would drive the specific trafficking of GKAP towards dendritic spines
along actin filaments, up to the PSD. This would explain the essential role of actin
cytoskeleton in both maintenance and reorganization of the PSD (Kuriu et al., 2006).
Accordingly, we further found that GKAP-DLC2 interaction also affected the spine targeting
of GKAP partners such as Shank3, Homer3 and PSD-95. This extended role of GKAP-DLC2
complex was consistent with previous findings documenting non-synaptic clusters of synaptic
proteins (Gerrow et al., 2006). However it is worth noting that PSD scaffolding proteins
contain multiple binding motifs, and their interactions with other scaffolds and cytoskeletal
proteins might also be important in their postsynaptic accumulation. For example, Shank
interacts with α-fodrin (Bockers et al., 2001), cortactin (Hering and Sheng, 2003), and Abp1
(Qualmann et al., 2004) proteins that bind to F-actin. Homer proteins were also reported to
bind F-actin (Shiraishi et al., 1999) and drebrin (Shiraishi-Yamaguchi et al., 2009).
Interestingly, scaffolding proteins with mutations in their binding motifs to other PSD
partners (for example disruption of GKAP-Shank interaction) can still be targeted to the
postsynapse but are less stable and more dependent on F-actin, highlighting once again the
importance of scaffold interactions with the cytoskeleton and further more putting emphases
on the need to interact with scaffolding partners to stabilize the protein complexes in the PSD
(Kuriu et al., 2006). In addition to its role in the targeting of synaptic proteins to dendritic
spine, GKAP-DLC2 interaction seems therefore to stabilize the postsynaptic complex at the
PSD. Indeed we showed that GKAP expression is no longer enhanced in spines upon
neuronal activity in conditions of DLC2 over-expression (Fig. S4). In these conditions, the activity-induced increase in GKAP-DLC2 interaction described with BRET experiments highlights the importance of a local enhancement of GKAP-DLC2 interactions in spines (rather than accumulation of GKAP per se) to stabilize GKAP and PSD-95 in spines. This hypothesis was further confirmed by the experiment performed in Fig. 5B, showing that despite increasing concentration in spines of a GKAP mutant that could not interact with DLC2 but correctly binds PSD-95 and Shank3, the absence of GKAP-DLC2 interaction within the spine impairs the stabilization of the scaffolding complex. This result highlights the role of DLC2 as a hub protein that would interact with partially disordered proteins to promote their adequate organization and stabilize the scaffolding complex at the PSD. Such a role of DLC2 has been previously described for other interactors, and this structuring feature was proposed to rely on the DLC2-induced promotion of protein dimerization (for review, see Barbar, 2008). Whether DLC2 enables GKAP dimerization to stabilize functional protein complex in the PSD is currently under investigation.

The here described importance of GKAP-DLC2 interaction in the targeting and stabilization of synaptic proteins is physiologically relevant, as 4-AP-induced sustained synaptic activity enhanced GKAP-DLC2 interaction and synaptic proteins accumulation in the PSD. This result corroborates previous studies showing that treatment of neurons with bicuculline/4-AP resulted in accumulation of GKAP and suppression of its dynamics in synapses (Kuriu et al., 2006). We found that activity-induced GKAP accumulation in spine required GKAP interaction with DLC2. However the molecular mechanisms underlying the synaptic activity-dependent modulation of GKAP-DLC2 interaction and consequent scaffold stabilization are still unknown. Dimerization of DLC is required for its activity because the monomer lacks the groove that is necessary for binding (Liang et al., 1999; Wang et al., 2003). The DLC monomer/dimer equilibrium is controlled by electrostatic interactions at the dimer interface, such as by phosphorylation of residue Ser88, which is a regulatory mechanism for DLC in vivo (Benison et al., 2009). Enhanced synaptic activity may affect the balance between phosphatases and kinases activation, thus displacing DLC phosphorylation/dephosphorylation equilibrium and dimerization, which in turn would modulate its binding to molecular substrates such as GKAP. One interesting candidate is p-21-activated kinase (PAK) family. Phosphorylation of DLC is indeed an important regulatory mechanism in vivo, as phosphorylation at Ser88 by Pak1 inhibits apoptosis and promotes cancerous phenotypes (Puthalakath et al., 1999; Song et al., 2008; Vadlamudi et al., 2004). In cultured hippocampal neurons, the active form phospho-PAK accumulates in puncta that colocalize with PSD-95
Whether this depends on synaptic activity and affects DLC-GKAP interaction remains to be investigated.

Our results suggest that an important functional consequence of postsynaptic scaffold stabilization of the DLC-GKAP complex is the up-regulation of NMDA receptor-channel activity. Previous findings have described the clustering of NMDA receptors by PSD-95 at the surface of heterologous cells (Kornau et al., 1995; Lin et al., 2004; Niethammer et al., 1996), via inhibition of receptor internalization (Roche et al., 2001). In *Xenopus* oocytes, PSD-95 functionally increases NMDA currents, and GKAP markedly potentiates the channel activity of the receptor-PSD-95 complex (Yamada et al., 1999) suggesting that GKAP could make the signal transmission more efficient at postsynaptic sites. However to date, despite a couple of studies suggesting that functional localization of NMDA receptors in synapses might depend on PSD-95 (see (Kim and Sheng, 2004) for review), the impact of PSD-95 and associated proteins on the regulation of NMDA currents in neurons remained largely unknown. Here, we demonstrated that the over all consequence of GKAP-DLC2 interaction in spine and stabilization of GKAP and PSD-95 in the PSD is the potentiation of postsynaptic NMDA currents in hippocampal neurons. The role of GKAP-DLC2 interaction in organizing the postsynaptic scaffold complex may also affect AMPA receptors function. Indeed the transmembrane AMPA receptor regulatory proteins (TARPs) are important for the regulation of AMPA receptor activity at synapses. TARPS stabilize AMPA receptors at synapses via direct interactions with PSD-95 and other MAGUKs (Jackson and Nicoll). This interaction was shown to be necessary for synaptic AMPAR function by measuring AMPAR-mediated excitatory post-synaptic currents (EPSCs) following PDZ-domain mutation (Schnell et al., 2002), and by acute disruption of the interaction between TARPs and PSD-95 using biomimetic divalent ligands (Sainlos et al., 2011). By increasing PSD95 location in spine, GKAP-DLC2 interaction could therefore also controls AMPA receptor function.

Given that NMDA receptors are fundamental players of synaptic transmission, the herein identified mechanism of functional regulation of synaptic NMDA receptor activity by DLC2-GKAP interaction might be of physiological importance namely in synaptic plasticity. Impaired GKAP expression and abnormal NMDA-glutamatergic neurotransmission have been identified in psychiatric disorders, such as schizophrenia (Kajimoto et al., 2003), obsessive-compulsive disorder (Welch et al., 2007) and fragile X mental retardation (Schutt et al., 2009). Whether GKAP-DLC2 assembly is associated with these disorders is worth to be studied in order to validate the GKAP-DLC2 interaction as a new pharmacological target for the development of therapeutic compounds.
Material and Methods

Plasmids and siRNA -

The DLC2-pCMV-SPORT6 plasmid was purchased from Source BioScience Geneservice (Nottingham, UK). The Venus tag was added in frame with the 5' end coding sequence of DLC2 using Gateway Technology (Invitrogen, Paisley, UK) to obtain the p-Venus-DLC2 plasmid. The coding sequence of Venus in p-Venus-DLC2 and mCherry were exchanged by molecular subcloning to obtain pmCherry-DLC2. The siRNA raised against DLC2 (DYNLL2 siRNA mouse, Santa Cruz Biotechnologies, ref sc-143208, Heidelberg, Germany) was a pool of 3 target-specific 19-25 base paired siRNAs designed to knock down gene expression. The siRNA control was purchased from Invitrogen corporation (Paisley, UK). The pAmCyan1-N1 Vector was purchased from Clontech laboratories (ref 632442, Takara bio inc, Saint-Germain-en-Laye, France). The coding sequence of GKAPla was a generous gift from Carlo Sala (Institute of Neuroscience, CNR, Milan, Italy). The coding sequence of CFP and RLuc8 were added in the 5' end coding sequence of GKAPla using Gateway Technology to obtain the p-CFP-GKAPla and p-RLuc8-GKAPla expression plasmids, respectively. From these 2 expression plasmids, we constructed p-CFP-GKAPla-mutant and p-RLuc8-GKAPla-mutant by using 2 successive primers containing point mutations: tccagtctggctggtaagtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttgtagaagttg
glutamax (4mM, gibco), glutamic acid (25µM, gibco), antibiotics (Penicillin 100UI=ml and Streptomycine 100µg/ml) and 10% Fetal Bovine Serum (FBS). After 3 days in culture (DIV3), the culture medium was supplemented with Cytosine β-D-arabinofuranoside hydrochloride 5µM (Sigma-Aldrich, St Quentin Fallavier, France) for 12 hours. Then, 75% of the medium was replaced by neurobasal medium supplemented with B27, glutamax and antibiotics. Neurons were then transfected with expression plasmids or siRNAs at DIV11 using lipofectamine 2000 (Invitrogen, Cergy Pontoise, France) according to the manufacturer's standard protocol and studied between DIV12 and DIV14. The siRNAs were resuspended to reach a 10µM final solution, and 3µl of this solution were used for transfection in 35mm diameter culture dishes.

**BRET measurements** - BRET is a very sensitive technology that became in the past decade a technology of choice to study the dynamic of protein–protein interactions in living cells. The efficacy of the energy transfer depends on the close proximity (< 10 nm) and orientation of the donor and acceptor entities. The average radius of proteins being 5 nm, the occurrence of resonance energy transfer is interpreted as a strong indication that the proteins attached to the energy donors and acceptors respectively are indeed in direct contact (Boute et al., 2002; Pfleger and Eidne, 2006). BRET measurements in cell populations were performed as previously described (Perroy et al., 2004). Single cell BRET imaging in cultured hippocampal neurons to study the subcellular localization of protein-protein interactions were performed according to previous protocols (Coulon et al., 2008; Perroy, 2010). Briefly, images were obtained using a Plan-Apochromat 63X/1.40 Oil M27 objective, at room temperature. Hippocampal neurons were transfected at DIV11 and recorded at DIV14 in the external medium used for electrophysiological recordings (see following section of the methods). Transfected cells were first identified using a monochromatic light and appropriated filter to excite Venus (exciter HQ480/40 #44001 - emitter HQ600/50 #42017, Chroma). Light source was then switched off until the end of the experiment. Coelenterazine H (CoelH, 20µM) was applied for 5 min before acquisition with Metamorph software (Molecular Devices). Images were then collected using a cascade 512B camera from Photometrics. Sequential acquisitions of 30 sec each were performed at 5 MHZ (Gain 3950, binning 1) with emission filters D480/60nm (#61274, Chroma) and HQ535/50nm (#63944, Chroma) to select em480 and em535 wavelengths respectively. The pixel-by-pixel 535nm/480nm ratios were calculated by
dividing the absolute blue or yellow intensities per pixel of images obtained at 535 nm over 480 nm. These numerical ratios (comprised between 0 and 1.5) were translated and visualized with a continuous 256 pseudo-color look-up table (LUT) as displayed in the figures. To determine the average intensity and distribution of the 535 nm/480 nm fluorescence ratios, the mean intensity and standard deviation of pixels was calculated within a square region drawn on the cell of interest using Image J software (NIH). CoelH 20µM was applied 5 min before the first BRET image acquisition, and 4-AP (or NMDA) was added immediately after the first acquisition. The sequential acquisitions were then performed from the 535 and 480 nm channels, 1, 5 and 10 minutes after the beginning of 4-AP (or NMDA) application.

Electrophysiological recordings and data analysis - Hippocampal neurons were recorded in the whole-cell patch-clamp configuration, using an Axopatch 200B amplifier. Currents were filtered at 1 kHz, digitized at 3 kHz and analyzed using the pClamp 10.0 software of Axon Instrument (Molecular Devices, Sunnyvale, CA). Currents were recorded in DIV12-13 hippocampal neurons at room temperature, at a holding potential of -60mV. The recording pipettes had resistance of 3-7MΩ.

For mEPSC recordings, pipettes were filled with the following medium (in mM): 140 KCl, 10 HEPES, 10 D-glucose, pH 7.2 and osmolarity of 300 mOsm. Neurons were perfused continuously with the following external medium (in mM): 140 NaCl, 2 CaCl₂, 3 KCl, 10 HEPES, 10 D-glucose, 0.01 glycine, 0.01 bicuculline, 0.0003 tetrodotoxin, pH 7.4 and osmolarity of 330 mOsm. Once a minimal sample of at least 20 mEPSCs had been collected from a neuron, the average amplitude of these events was measured on the total duration of the sample. The average trace was normalized and pooled with other average traces recorded in the same condition to obtain a single representative trace, thus allowing us to study the slow component (NMDA dependent) of the event. For NMDA current recordings, pipettes were filled with the following medium (in mM): 5 EGTA, 0.5 CaCl₂, 140 CsCl, 10 HEPES, 10 D-glucose, pH 7.2 and osmolarity of 300 mOsm. Neurons were perfused continuously with the following external medium (in mM): 140 NaCl, 2 CaCl₂, 3 KCl, 10 HEPES, 10 D-glucose, 0.01 glycine, 0.0003 tetrodotoxin, pH 7.4 and osmolarity of 330 mOsm. Whole-cell NMDA currents were evoked in neurons by 10s applications of 100 µM NMDA (Sigma-Aldrich, St Quentin Fallavier, France). The agonist was applied 3 times at 60s intervals, and the averaged peak current amplitude was then calculated from the 3 pharmacological
stimulations. All electrophysiological data were analyzed using the Clampfit 10 software
from Axon Instrument (Molecular Devices).

**Immunoprecipitation and Western Blot analyses** - Cells were lysed in 0.1% Triton X-100, 150
mM NaCl, 2 mM EGTA, anti-protease mixture (Roche Applied Science), and 20 mM Tris-
HCL, pH 7.4 (lysis buffer), and the mixture was centrifuged. The lysate obtained from 10^7
cells transfected with Cherry-DLC2 and CFP-GKAP or CFP-GKAPmut was co-
immunoprecipitated using RFP-Trap from ChromoTek according to the manufacturer
Trap, a small RFP binding protein coupled to agarose beads, enables purification of any
protein of interest fused to RFP (monomeric derivates of DsRed, including mRFP1, mCherry,
mPlum, mOrange). After extensive washing, the solid phase was incubated in Laemmli
Buffer at 90°C. A control assay was performed under the same conditions but with cells
transfected with Cherry instead of Cherry-DLC2 and CFP-GKAP or CFP-GKAPmut. Protein
samples were resolved by 7.5% PAGE, transferred to nitrocellulose, and subjected to
immunoblotting using rabbit anti-GFP antibody (1:1000, Invitrogen) or anti-RFP (1:1000,
MBL) for 1 hour. The blots were then washed three times with PBS containing 0.1% Tween
20 (PBST). The nitrocellulose was then incubated with goat anti-rabbit IgG (H+L) DyLight™
800 conjugated (PIERCE) for 1 hour. The blots were then washed three times with PBST.
Proteins were visualized by scanning on an Odyssey Infrared Imaging System (LI-COR
Biosciences) with 800 nm channel. The total level of the three proteins DLC2, GKAP and
GKAPmutant was revealed to evaluate equal transfection efficiency. We quantified the ratio
of immunoprecipitated proteins : CFP-GKAP / Cherry-DLC2 and CFP-GKAPmutant /
Cherry-DLC2. To quantify the loss of interaction due to the mutation of GKAP, the ratio of
CFP-GKAPmutant / Cherry-DLC2 was expressed as a percentage of the ratio CFP-GKAP /
Cherry-DLC2.

**Immunocytochemistry** - Hippocampal neurons were fixed at DIV12 in PBS containing 4%
paraformaldehyde for 15 min at room temperature. Cells were then washed and mounted on
coverslips with Moviol. To enhance synaptic activity, 4-AP 1 mM was added for 1 to 5
minutes and removed immediately before fixation, excepted for experiments in Fig. 5C where
neurons were washed 25 minutes before fixation in order to follow long lasting neuronal
remodeling. Images were acquired using a Apotome microscope (axioImagerZ1). For
quantification of the spine/shaft fluorescence ratio, the fluorescent intensity of spine and shaft
was measured using the Image J software. More than 100 spines were measured for each
construct and each experiment was repeated at least three times in separated neuronal
cultures.

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the monomeric 8-kDa dynein light chain and mechanism of the domain-swapped dimer

Welch, J. M., Lu, J., Rodriguz, R. M., Trotta, N. C., Peca, J., Ding, J. D.,


**Figure Legends**

**Fig. 1** - GKAP interacts with DLC2 in living cells. (A) Localization of CFP-GKAP and Venus-DLC2 in DIV14 hippocampal neurons. (B) HEK293 cells were cotransfected with constant concentrations of RLuc8-GKAP or RLuc8-GKAPmut and increasing concentrations of Venus-DLC2 expression plasmids. BRET was expressed as a function of the Venus-DLC2 acceptor expression level. Individual readings obtained from 3 independent experiments were pooled on the same graph. Curves were fitted using a nonlinear regression equation, assuming a single binding site (GraphPadPrism). (C) HEK293 cells were cotransfected with Cherry-DLC2 and CFP-GKAP or CFP-GKAPmut expression plasmids. Cherry-DLC2 was immunoprecipitated with the RFP-Trap and the amount of CFP-GKAP or CFP-GKAP mutant co-immunoprecipitated was quantified by Western Blot analysis using the anti-GFP antibody. The control assay was performed under the same conditions but with Cherry instead of Cherry-DLC2 (last column). In the IPs, the ratio of CFP-GKAPmut / Cherry-DLC2 was expressed as a percentage of the ratio of CFP-GKAP / Cherry-DLC2. Histograms are mean ± SEM of 3 individual experiments, Mann & Whitney test, *** significantly different p < 0.001. (D) Schematic representations of GKAP and GKAPmut. The putative GKAP amino acid sequences for binding to DLC2 are indicated in white, whereas mutations engineered to obtain GKAPmut are indicated in blue.

**Fig. 2** - GKAP interacts with DLC2 in neurons. (A) Neurons were co-transfected with RLuc8-GKAP and Venus-DLC2. BRET was imaged and zoomed in a 20 X 20 µm area in dendrites. The pictures show expression of Venus-DLC2 (GFP), RLuc8-GKAP (Em480), Venus-DLC2 excited by energy transfer (Em535) and BRET signal generated by the two tagged proteins (535/480). (B) On the left, histogram represents the BRET intensity in dendrites and soma. Note that highest BRET signals were found in the dendrites as compared to cell body. On the right, histogram represents the standard deviation of the BRET intensity in dendrites and soma. Please note that a high standard deviation indicates a clusterization in dendrites (n = 6 neurons, Wilcoxon test, * significantly different p < 0.01).

**Fig. 3** - Neuronal activity potentiates GKAP-DLC2 interaction. (A) Neurons were co-transfected with RLuc8-GKAP and Venus-DLC2. The pictures show expression of Venus-DLC2 (GFP) and BRET signal generated by the two tagged proteins (535/480) following 4-
AP (1mM) exposure. (B) Histograms of BRET intensity at different times after 4-AP treatment (% of basal signal), in soma and dendrites. (C) Histograms of Standard deviation of BRET intensity after 4-AP treatment (% of the basal signal), in soma and dendrites (B and C, Mean ± SEM, n=21 neurons, 4 independent experiments). (D) Left: Example of BRET signals in 4 dendritic spines during 4-AP stimulation. Note that the strong dynamic of protein-protein interaction in this area makes the BRET signal varying from one spine to another. Right: Histogram of BRET intensity at different times during 4-AP treatment (% of the basal signal), in dendritic spines. (Mean ± SEM, n=72 spines, from 17 neurons in four independent experiments). Student test, * significantly different p < 0.01.

Fig. 4 - GKAP-DLC2 interaction enables a synaptic activity-dependent accumulation of GKAP in dendritic spines. (A) Expression and location of CFP-GKAP or CFP-GKAPmut in neurons stimulated with 4-AP (1 mM). (B) Histogram of the spine/shaft fluorescence ratio of neurons transfected with CFP-GKAP (black) or CFP-GKAPmut (grey) after 4-AP (1mM) treatment. Ratio = mean ± SEM of 3 independent experiments, 10 neurons per experiment, 10 spines per neuron. (C) Histogram of the spine/shaft fluorescence ratio of neurons transfected with CFP-GKAP following 4-AP (1mM) + CNQX (10 µM) treatment. Same analysis than (B). Student test, * significantly different p < 0.01 and *** p < 0.001. NS = Not Significant.

Fig. 5 - GKAP-DLC2 interaction enhances the synaptic accumulation of PSD-95. (A) Histograms of the spine/shaft fluorescence ratio of neurons transfected with Venus-Shank3 (left), Venus-Homer3 (middle left), Venus-GIT (middle right) or PSD-95-YFP (right). For each condition, neurons were co-transfected with either CFP (black), CFP-GKAP (waves) or CFP-GKAPmut (points). Each condition was compared to the CFP control. Same statistical analysis than in Fig. 4B. (B) Neurons were co-transfected with PSD-95-YFP and either CFP or CFP-GKAP or CFP-GKAPmut. The PSD-95 spine/shaft fluorescence ratio was expressed as a function of CFP or CFP-GKAP or CFP-GKAPmut expression level in spines. Each point of the scatter plots corresponds to one spine. The histogram represents the slopes of the linear regression curves of the scatter plots. (C) Fluorescence images of PSD-95-YFP and histograms of the spine/shaft fluorescence ratio of PSD-95-YFP measured in neurons transfected with PSD-95-YFP and CFP (left) or CFP-GKAP (middle) or CFP-GKAPmut (right), before (control) and after 4-AP (1mM) treatment. Note that the 4-AP treatment differs from previous figures: neurons are stimulated 5 min with 4-AP and then washed for 25 min before fixation to follow long lasting remodeling. Same statistical analysis than in Fig 4B.
Fig. 6 - GKAP-DLC2 interaction enhances NMDA synaptic currents. Neurons were transfected with Venus (control), and GKAP or GKAPmut, as indicated in the figures. (A) Representative traces of NMDA-induced current. Horizontal bars represent NMDA (100 µM) applications. (B) Each plot represents the mean ± SEM of NMDA current density measured from at least 10 neurons. Note that siRNAs were co-transfected with Venus as a transfection reporter. Same statistical analysis as in Fig. 4B, each condition was compared to the control. (C) Normalized averaged mEPSCs (> 20) recorded from neurons transfected or not with GKAP or GKAPmut (n = 11 in each condition). The slow NMDA channel-mediated component of the mEPSCs was analyzed 7 ms after the peak. Mann & Whitney test, * significantly different p < 0.05.
Figure 1

A

CFP-GKAP   Venus-DLC2   Merge
20µM   8µM

B

Venus-DLC2

Net BRET

0.10

0.05

0.00

0.00 0.01 0.02 0.03 0.04
Fluo/lumi net

RLuc8-GKAP   RLuc8-GKAPmut

C

Input   IP   Input   IP   IP

CFP          -   -   -   -   +
Cherry       -   -   -   -   +
Cherry-DLC2   +   +   +   +   -
CFP-GKAP     +   +   -   -   +
CFP-GKAPmut  -   -   +   +   -

% of CFP-GKAP or CFP-GKAPmut co-immunoprecipitated with Cherry-DLC2

D

5 x 14 aa repeats

GIQVD   GVQVEE

5 x 14 aa repeats

GNQND   GVEVEE

GAP

GAPmut
Figure 2

A

![Images of neuron - GFP, Em 480, Em 535, Em 535/480](images)

B

![Bar graphs](images)
Figure 3

A

GFP

0 min

1 min

5 min

10 min

B

soma

BRET intensity (% of basal)

4-AP time exposure (min)

dendrites

BRET intensity (% of basal)

4-AP time exposure (min)

C

soma

Std deviation (% of basal)

4-AP time exposure (min)

dendrites

Std deviation (% of basal)

4-AP time exposure (min)

D

dendritic spines

BRET intensity (% of basal)

4-AP time exposure (min)
Figure 4

A  

CFP-GKAP  CFP-GKAPmut

0 min  1 min  5 min

B

spine/shaft ratio intensity

4-AP time exposure (min)

GKAP  GKAPmut

0  1  5

***  *  

C

spine/shaft ratio intensity

CNOX + 4-AP time exposure (min)

NS  GS

5 µm
Figure 5

A

B

C

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**Figure 6**

A

![Graph showing current density over time with NMDA stimulation](image)

6 sec

control

NMDA

GKAP

GKAPmut

400 pA

B

![Bar graph showing current density](image)

Current density (pA/pF)

control

GKAP

GKAPmut

DLC2-siRNA

DLC2-siRNA-GKAP

control-siRNA-GKAP

C

![Graph showing amplitude over time](image)

Amplitude (normalized)

control

GKAP

GKAPmut

7 ms
A

RLuc8-GKAP / Venus-Shank3

RLuc8-GKAP / PSD95-YFP

B

Venus-DLC2

Cherry-DLC2

Input Control IP

C

DLC2-RLuc / PSD95-YFP

DLC2-RLuc / Venus-Homer3

Rluc-Shank3 / Venus-DLC2

RLuc8-GKAP / Venus-DLC2
A) GFP

B) BRET intensity (% of basal) vs. NMDA time exposure (min)
- Soma
  - 0 min: 90%
  - 1 min: 100%
  - 5 min: 110%
  - 10 min: 120%
- Dendrites
  - 0 min: 90%
  - 1 min: 100%
  - 5 min: 110%
  - 10 min: 120%

C) Std deviation (% of basal) vs. NMDA time exposure (min)
- Soma
  - 0 min: 90%
  - 1 min: 100%
  - 5 min: 110%
  - 10 min: 120%
- Dendrites
  - 0 min: 90%
  - 1 min: 100%
  - 5 min: 110%
  - 10 min: 120%

D) BRET intensity (% of basal) vs. NMDA time exposure (min)
- Dendritic spines
  - 0 min: 90%
  - 1 min: 100%
  - 5 min: 110%
  - 10 min: 120%