

## **Role of Calcium calmodulin-dependent protein kinase II (CaMKII) in synapse formation and synaptic Transmission between Lymnaea neurons.**

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### **Abstract**

Networks of synaptically connected neurons underlie all brain functions. Various cell-cell signaling and extrinsic molecules influence synapse assembly at the synaptic site. Calcium ions play a significant role in signal transduction pathways that control various neuronal functions. Multifunctional Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMK II) is an important mediator of calcium signaling in neurons, it plays an essential role in controlling synaptic strength and plasticity, and it is highly expressed in the cytosol of developing neurons, especially in presynaptic neurons. However, the precise role of CaMKII in synapse formation and synaptic transmission has not yet been determined. We hypothesized that CaMKII activity could be necessary for synapse formation and synaptic transmission. To test whether CaMKII activity is required for the synapse formation and synaptic transmission, the identified neurons visceral dorsal 4 (VD4 – presynaptic) and its postsynaptic partner left pedal dorsal 1 (LPeD1) from the freshwater snail *Lymnaea stagnalis* were paired in soma-soma configuration in cell culture. The soma-soma paired cells recapitulated their excitatory connections in vitro. To test the possible role of CaMKII in synapse formation and synaptic transmission, the in vitro paired neurons were exposed to a CaMKII-specific inhibitor KN-93 and its inactive analog KN-92. The incidence of synapse formation and efficacy of synaptic transmission was tested electrophysiologically.

In this study, we have demonstrated that paired neurons cultured in the presence of CaMKII inhibitor KN-93 do not affect the incidence of synapse formation; however, it reduces synaptic transmission. In contrast, paired neurons exposure to KN-92 did not affect the synapse formation and synaptic transmission at synapses between identified neurons. Taken together, CaMKII activity was not required for normal synapse formation; nevertheless, it plays an essential role in synaptic transmission between the soma-soma-paired *Lymnaea* neurons. *ASEAN Journal of Psychiatry, Vol. 23: October–November 2022: 01- 09.*

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### **Introduction**

The human brain comprises an estimated 100 billion inter-connected neurons through specialized structures

called synapses. "synapse" – a term coined by Sherrington- is a contact point between two communicating nerve cells [1, 2]. The receiving cell is

called the postsynaptic neuron, whereas the cell transmits the signal is the presynaptic cell. At the synaptic site, an electrical signal is conveyed from the presynaptic neuron to the postsynaptic cell - a process referred to as 'synaptic transmission.

Synaptic transmission is a process by which some chemical messengers are released from the presynaptic neurons and bind to their specific receptors on the postsynaptic membrane to change its permeability for selection. A presynaptic terminal may contain a few hundred synaptic vesicles, though the synaptic vesicles' numbers vary widely among synapses [3-5]. Presynaptic terminals release neurotransmitters from the docked synaptic vesicles via the process of calcium-regulated exocytosis [6-9]. An action potential invades the presynaptic membrane to induce the opening of voltage-gated calcium channels in the plasma membrane of the presynaptic neuron. These channels allow a rapid influx of calcium into the presynaptic cell, and the probability of vesicle fusion is increased dramatically by the elevations of cytosolic calcium [10]. Calcium is the main messenger involved in a variety of cellular processes; such as the activation of various kinases (protein kinase A and C, calcium-calmodulin dependent protein kinase II), and these, in turn, activate synaptic vesicles by phosphorylating the vesicle-associated protein-like, synapsin, synaptophysin, synaptophysin, synaptotagmin [8, 9, 11-16]. Phosphorylation of vesicle-associated proteins triggers synaptic vesicles' fusion at the presynaptic membrane specialized area known as the "Active Zone." Following vesicle fusion and neurotransmitter release, the lipid and protein constituents of the vesicle transiently become part of the presynaptic plasma membrane and are subsequently endocytosed for reuse [4, 9, 17].

Multifunctional, Ca<sup>2+</sup>/calmodulin-dependent protein kinase or (CaM kinase II) is a significant mediator of calcium signaling in neurons, where it provides a molecular switch whose activity is, although triggered by Ca<sup>2+</sup> but is not contingent upon its presence [18]. Specifically, once activated by Ca<sup>2+</sup>, it autophosphorylates itself at threonine 287 (T287) and can be active long after Ca<sup>2+</sup> returns to its basal levels [19-21]. CaMKII is one of the most abundant proteins in neurons, comprising 1-2% of the total protein concentration. The unique properties of CaMKII, including its abundance, multifunctional nature, key

location, and sophisticated regulation, may allow the enzyme to take part in important synaptic functions, including neurotransmitter synthesis and release, modulation of ion channels activity, synaptic plasticity, and gene expression (reviewed in [4, 20, 22, 23]). Even though CaMK II is one of the most abundantly expressed kinases in the brain and its role in the adult brain, CaMKII may serve developmental functions during the formation of synapses during development. However, the precise role of CaMKII in synapse formation and synaptic transmission remains to be determined. In this study, we have examined the role of CaMKII in synapse formation and synaptic transmission by using identified respiratory pre-and postsynaptic neurons from freshwater pond snail *Lymnaea stagnalis* paired in soma-soma configuration in cell culture. The main objectives of the present study were to determine: (1) whether CaMKII is involved in synapse formation and (2) whether CaMKII activity is required in normal synaptic transmission between VD4-LPeD1 synapses. We used *in vitro* cell culture and electrophysiological techniques to achieve these objectives. The identified neurons were paired in the presence of the *Lymnaea* brain conditioned medium (CM) alone or CM+ CaMKII specific inhibitor KN-93 and CM+ KN-92 (inactive analog KN-93). We have provided direct evidence that long-term (12-18 hrs) application of KN-93 (CaMK II specific to inhibitor) of soma-soma paired cells does not affect the incidence of synapse formation between VD4 and LPeD1, but it decreases the synaptic transmission at the VD4-LPeD1 synapse. In contrast, paired neurons exposure to KN-92 did not affect the synapse formation and synaptic transmission at synapses between identified neurons. The outcome of the present study demonstrates that the CaMKII activity is not required for synapse formation. However, it is required for proper normal synaptic transmission between the soma-soma paired *Lymnaea* neurons.

## **Materials and Methods**

### **Animals and Cell culture**

Laboratory-raised stocks of the freshwater snail, *Lymnaea stagnalis*, were maintained at room temperature (18-20 oC) in an aquarium containing

well-aerated, de-chlorinated tap water and kept at a 12/12 hours light/dark cycle. Animals were fed lettuce and fish food. 1 to 2 months old animals (10 – 15 mm shell length) were used for cell isolation, whereas the Lymnaea brain conditioned medium (CM) was prepared from 2 to 3 months old snails (15 – 25 mm shell length). The CM was prepared by incubating the isolated Lymnaea brains (central ring ganglia) in Define media (DM; L-15 Special Order; Life Technologies, Gaithersburg, MD, USA), 12 ganglia / 6ml DM for 4-6 days, and frozen until it was used.

Neurons were isolated individually and cultured as described previously [24-27](Syed. Briefly, snails were deshelled with forceps and anesthetized for 7-10 minutes in 10% Listerine (21.0% ethanol, 0.042% menthol; Pfizer Canada, Toronto, Ontario, Canada) in normal Lymnaea saline (51.3 mM NaCl, 1.7 mM KCl, 4.0 mM CaCl<sub>2</sub>, 1.5 mM MgCl<sub>2</sub>), buffered to pH 7.9 with 2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid (HEPES) (Syed et al., 1990; Woodin et al., 2002; Munno et al., 2003). The central ring ganglia were isolated and then sterilized with three fifteen-minute antibiotic washes (50 µg/ml gentamycin in normal saline). Ganglia were subsequently treated with trypsin (2 mg/ml, Sigma type III; Sigma Chemical Company, St. Louis, MO) for 23 minutes and then soybean trypsin inhibitor (2 mg/ml, Sigma type 1-S; Sigma Chemical Company) for 15 minutes, each dissolved in 50% L-15 (Gibco special order) defined medium (DM). DM was prepared with added inorganic salts (similar to saline) and 20 µg/ml gentamycin with the pH adjusted to 7.9 with 1N NaOH. Following enzymatic treatment, the ganglia were pinned to the bottom of a dissection dish containing high osmolarity DM (DM with added 20 mM D-glucose) and unsheathed with a pair of fine forceps [28]. The identified neurons were isolated by applying gentle suction pressure to fire-polished, Sigmacoate-treated pipettes (Sigma, T. Louis, MO). The isolated neurons were plated onto poly-L-lysine treated dishes in the presence of CM. Isolated identified neurons were juxtaposed in a soma-soma configuration in the culture dishes and left undisturbed overnight. The well-established and previously used soma-soma cholinergic synapse model between VD4 and LPeD1 neurons was used in this study [27, 29].

### **Soma-soma Pairing**

Isolated cell bodies of identified neurons visceral dorsal 4 (VD4) and left pedal dorsal 1 (LPeD1) located in the visceral and left pedal ganglia, respectively, were juxtaposed to create soma-soma pairs in the poly-L-lysine treated culture dish containing CM or CM + CaMKII inhibitor KN-93 or its inactive analog KN-92 maintain at room temperature (18-20 oC) and left undisturbed overnight [30, 31]. After 12-18 hours of pairing, synaptic transmission was investigated with intracellular recordings.

### **Electrophysiology**

Neuronal activity was monitored using conventional intracellular recording techniques (see [24, 25, 27, 32-34]). Specifically, glass microelectrodes (1.5 mm ID, with filament; World Precision Instruments, Sarasota, FL) were pulled on a vertical electrode puller (Kopf, 700C; David Kopf Instruments, Tujunga, CA) and filled with a saturated solution of K<sub>2</sub>SO<sub>4</sub> (electrode resistance; 20-60 MΩ). Neurons were viewed under an inverted microscope (Zeiss, Axiovert 200M) and impaled with micromanipulators (Narashige, MM204). Neuronal electrical signals were amplified (Cygnus Tech, Neuro Data IR-283), displayed on a digital storage oscilloscope (Philips, PM3394), and recorded with a digitizer (Axon Instruments, Digidata 1322A) and data acquisition software (Axon Instruments, Axoscope 10.0).

### **Specific Drugs used and their application.**

Pharmacological agents were used to perturb the functions of CaMKII. The chemicals were bath applied; KN-93 (Calbiochem) and KN-92 (Sigma) were first dissolved in dimethyl sulfoxide (DMSO; Sigma). The stock solution (2 mM) was diluted in Lymnaea saline to make a final concentration of 100 nM immediately before the application. Final DMSO concentration in the diluted working solution was less than 0.01 % (DMSO < 0.01%).

### **Statistical Analysis**

Statistical data analysis was conducted using SPSS (IBM SPSS statistics 25), and graphs were constructed with Microsoft Office Excel 2019. Parametric data are expressed as means ± standard errors (SE) and were analyzed for significance using a t-test. Nonparametric data are expressed as a percent and were analyzed for

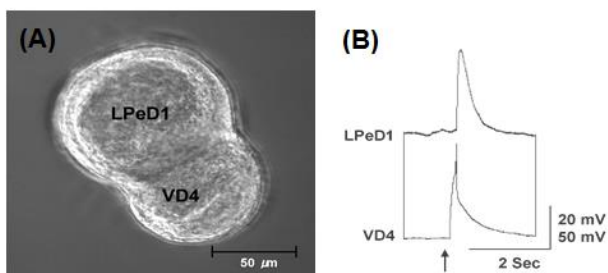
significance using the  $\chi^2$  test. Significance was assumed if the P-value was less than 0.05 ( $p < 0.05$ ).

## Results

### Specific Synapses between VD4 and LPeD1 reform in a Soma-soma configuration

The identified cholinergic Lymnaea neuron VD4 forms excitatory synapses with its postsynaptic partner's neuron LPeD1. To determine whether synapse between VD4 and LPeD1 neurons was also re-established in vitro, identified presynaptic neuron VD4 and its postsynaptic partner LPeD1 were extracted from visceral and left pedal ganglia, respectively. The isolated somata of neurons VD4 and LPeD1 were juxtaposed to create the soma-soma configuration (Figure 1A). The cells were incubated for 12-18 hrs in culture dishes containing CM [31]. The simultaneous intracellular recording revealed that depolarizing current injection in VD4 (at small arrow; Figure 1B) generates 1:1 excitatory postsynaptic potentials (EPSPs) in its postsynaptic neuron LPeD1. These results suggest that excitatory synapse reform between VD4 and LPeD1 is similar in vivo. These in vitro reconstructed soma-soma synapses are highly reliable. The synaptic transmission between VD4 and LPeD1 was previously shown to be cholinergic [25, 32].

**Figure 1**



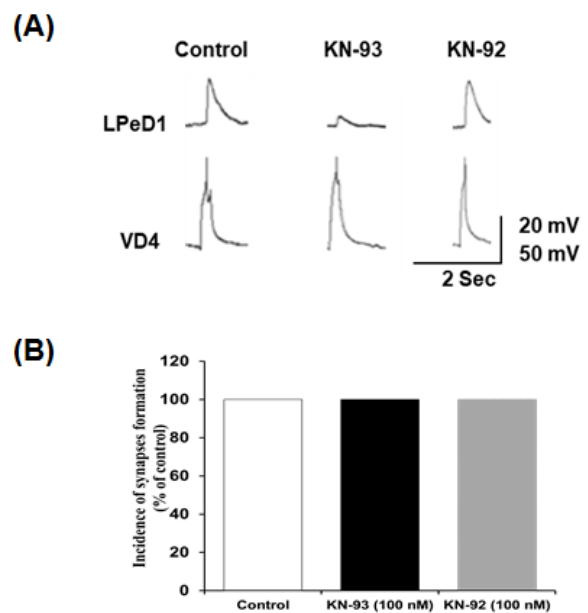
### Synapse formation between VD4 - LPeD1 does not require CaMKII activity

To test the possibility that various kinases, such as CaMKII mediating short and long-term plasticity, may also be involved in synaptogenesis, we pose the question of whether synapse formation between presynaptic neuron VD4 and postsynaptic neuron left LPeD1 also required CaMKII activity. To address this

possibility, neurons were paired in a soma-soma configuration (Figure 1A) either in the presence or absence of a selective CaMKII antagonist KN-93 (100 nM). The incidence of synapse formation was tested electrophysiologically after 18-24 hours of pairing. In all instances ( $n=10$ ), excitatory synapses similar to those observed under control conditions developed between the paired cells (Figure 2B). Specifically, induced action potentials in VD4 produced 1:1 excitatory postsynaptic potentials (EPSPs  $7.83 \pm 0.35$  mV) that were significantly smaller than their control counterparts (EPSPs  $15.72 \pm 0.36$  mV) (Figure 2A). These data demonstrate that chronic treatment of the pairs VD4 and LPeD1 neurons, CaMKII antagonist, or its carrier solution (DMSO) does not compromise neuronal viability. This data also indicates that CaMKII perturbation does not block synapse formation between VD4-LPeD1 synapses (Figure 2B). However, the amplitude of VD4-induced EPSPs in LPeD1 was significantly smaller than in the control (Figure 2A).

Because the amplitude of evoked EPSPs was smaller in pairs incubated in the CaMKII inhibitors, we reasoned that this kinase might instead regulate synaptic transmission between the VD4-LPeD1 synapses. To test this possibility, synaptic transmission between VD4-LPeD1 was tested either in the presence or absence of a CaMKII inhibitor.

**Figure 2**

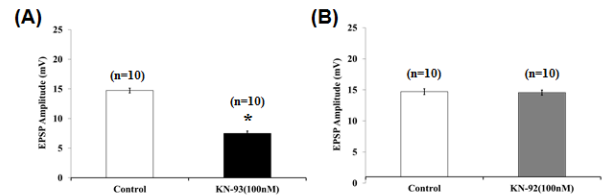


### **CaMKII activity required for normal synaptic transmission between VD4-LPeD1 synapse**

Synaptic transmission is initiated by exocytosis of docked vesicles at the active zone, resulting in the secretion of neurotransmitters into the synaptic cleft and subsequent activation of postsynaptic receptors [35]. Synaptic vesicles have proteins in their membrane, for example, synapsin 1, synaptophysin, synaptotagmin, and the vesicle-associated membrane protein (VAMP)/synaptobrevin (SYB), which anchor, dock, and probably fuse the synaptic vesicles with the plasma membrane [4, 11, 35]. Presynaptically, CaMKII may modulate transmitter release by phosphorylating some synaptic vesicle-associated proteins, such as synapsin and actin filaments. Synaptophysin is also a substrate for CaMKII, which is phosphorylated at its serine residues [4, 36]. Genetic knockdown of these proteins results in disruption of presynaptic transmitter release [37]. Therefore, to test the hypothesis that CaMKII may be involved in synaptic transmission, specific excitatory synapses between VD4-LPeD1 were reconstructed in a soma-soma configuration ((Figure 1A). VD4 and LPeD1 were extracted from the visceral and left pedal ganglia, respectively, and paired overnight. The synaptic transmission was tested electrophysiologically after 18-24 hours of pairing. Induced action potential in VD4 generated 1:1 excitatory postsynaptic potential (EPSPs) in LPeD1 (Figure 3A). To investigate the involvement of CaMKII in synaptic transmission, we bath-applied CaMKII inhibitor (final concentration of DMSO < 0.01%), KN-93 (100 nM in DMSO), Within 20 minutes of its bath application, the synaptic transmission between VD4 and LPeD1 was significantly reduced ( $P < 0.001$ ) (Figure 3A). Specifically, the control EPSPs amplitude was  $14.72 \pm 0.25$  mV ( $n=10$ ), which was reduced significantly in the bath applied KN-93 to  $7.50 \pm 0.34$  mV ( $n=10$ ; Figure 3A). To test for the specificity of KN-93 effects and to control for the carrier solution (DMSO), we used the inactive analog KN-92 (also 100 nM). KN-92 or DMSO alone did not affect the synaptic transmission between VD4 and LPeD1, [control EPSPs amplitude  $14.72 \pm 0.25$  mV ( $n=10$ ); KN-92  $14.56 \pm 0.47$  mV ( $n=7$ ; Figure 3B)]. These results indicate that: a) CaMKII-specific drug perturb its function in the Lymnaea model and b) that CaMKII is

involved in synaptic transmission between VD4 and LPeD1. These data also confirm my earlier results that KN-93-induced suppression of synaptic response (Figure 2A) may have involved acute effects of this drug on synaptic transmission and not synapse formation.

**Figure 3**



### **Discussions**

CaMK II is one of the most abundantly expressed kinases in the brain. CaMKII is involved in numerous forms of synaptic plasticity such as LTP [38-46], LTD [46-49] and PTP [50] short-term plasticity [51]. the present study could not deduce its precise role in synapse formation. Specifically, we demonstrated that blocking CaMKII activity during early synapse formation did not render the pairs incapable of synapse formation. It is, however, important to note that although synapses between VD4 and LPeD1 did form in the presence of CaMKII inhibitor, the amplitude of VD4-induced EPSPs was significantly reduced. This raises two possibilities: 1) It is feasible that KN-93 may have only suppressed normal synaptic transmission between VD4 and LPeD1, or 2) that it reduced the total number of synapses that had formed between the pair - thus indirectly reducing the efficacy of synaptic transmission between the cells. Because acutely applied CaMKII inhibitor to soma-soma synapsed pairs also reduced the efficacy of synaptic transmission between VD4-LPeD1, we would like to conclude that the overnight treatment of pairs with CaMKII inhibitor most likely reduced the efficacy of synaptic transmission and not the total number of synapses. Further work involving electron microscopic studies would be required to demonstrate this unequivocally.

The above data do not underscore the importance of Ca<sup>2+</sup>-mediated signaling in synapse formation, which may still be pivotal for both activity-dependent and independent mechanisms, albeit at a much lower concentration. For instance, previous studies in both vertebrates and invertebrates have shown that Ca<sup>2+</sup> is

essential not only for neuronal adhesion but also outgrowth and synapse formation [52-54]. Similarly, Feng et al. have shown in Lymnaea that an optimal Ca<sup>2+</sup> concentration is required for synapse formation and that perturbation of these levels renders the cellular machinery incapable of synapse formation between the soma-soma paired cells. These data thus suggest that low-frequency firing of VD4 may suffice to invoke sufficient Ca<sup>2+</sup> influx that may, in turn, regulate the cellular processes required for the synaptogenic program [54]. This argument does not, however, reconcile with the fact that VD4 is a bursting neuron and its spontaneous activity, which results in a high-frequency burst – analogs to that triggered in this study (6- 10 spikes) may invoke sufficient influx to activate CaMKII function. Interestingly, during early synapse formation, we found VD4 to be either

quiescent or firing single-action potentials. These data thus show that CaMKII function may be restricted to fully matured synapses when VD4 has acquired a full complement of its bursting behavior. The data presented in this study show that although CaMKII function may be required for "base-line" synaptic transmission, it does not appear to regulate synapse formation between the paired cells.

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#### **Conflict of interest**

The authors declare that they do not have any conflicts of interest.

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