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Impact of second messenger modulation on activity-dependent and basal properties of excitatory synapses

By Chun Yun Chang

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Abstract of the dissertation

Impact of second messenger modulation on changes in activity-dependent and basal properties of excitatory synapses

by

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Cognitive processing in the central nervous system relies on accurate information propagation; neurotransmission is the fundamental mechanism underlying network information flow. Because network information is coded by the timing and the strength of neuronal activity, synaptic properties that translate neuronal activity into synaptic output profoundly determine the precision of information transfer. Synaptic properties are in turn shaped by changes in network activity to ensure appropriate synaptic output. Activity-dependent adjustment of synaptic properties is often initiated by second messenger signals. Understanding how second messengers sculpt synaptic properties and produce changes in synaptic output is key for elucidating the interplay between network activity and synaptic properties. We studied the effect of second messenger modification on activity-dependent and static properties of rat hippocampal excitatory synapses using electrophysiological and optical approaches. We focused on two second-messenger pathways that potentiate transmission: cAMP and diacyl glycerol (DAG) signals. In parallel, we also compared the effects of manipulating calcium influx, which is known to potentiate synaptic transmission through increasing release probability (P_r). During high frequency stimulation, we found that both cAMP and DAG signals potentiated phasic transmission, as previously characterized. In parallel with increasing phasic transmission, the modulators also enhanced high-frequency associated asynchronous transmission, which emerges late during stimulus trains

and is relatively long-lasting. However, such parallel potentiation of phasic and asynchronous transmission was not seen in elevated calcium; high calcium preferentially promoted asynchronous transmission. With low frequency stimulation, we found that cAMP and high calcium enhanced synaptic output by potentiating synapses with basally high P_r. Conversely, DAG signals recruited neurotransmission from both high P_r and low P_r terminals, which include presynaptically quiescent synapses. Taken together, these results suggest that second messenger modulation of synapses differentially shapes the static properties of the synapses; second messengers also fine-tune activity-dependent synaptic responses differently from manipulating calcium influx. These results likely have physiological relevance to second messenger-dependent sculpting of temporal and spatial synaptic properties.

Acknowledgment

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Chapter 1. Synaptic properties and transmitter release

1.1 Introduction

In a neuronal network, activity is propagated within individual neurons as action potentials (APs) and passed between neurons by synaptic transmission. The properties of synapses are important as they determine the signals that postsynaptic neurons will receive from presynaptic neurons. When the scale or the strength of neuronal activity within the network changes radically, the capacity of synapses needs to be reset to an appropriate range for optimized activity conduction.   Such activity-dependent setting of synapses is particularly important in the central nervous system (CNS), where neuronal activity from multiple external sensory inputs as well as internally generated responses needs to be propagated accurately for proper integration. After decades of extensive study, it is well established that such activity-dependent modification of neuronal connectivity is often initiated by second messenger signals (Ghosh and Greenberg, 1995).   While most studies have focused on changes in individual, isolated synaptic responses of preexisting synaptic connections, effects on activity-dependent properties of the synapses and changes in preexisting nonfunctional synapses by modulators are still unclear.

1.2 Hippocampal formation and synaptic plasticity

The concept of activity-dependent adjustment of neuronal connections was initially proposed by Cajal almost a century ago. In his pioneering idea, mental processing can be improved by reinforcing pre-established neuronal connections, or by establishing new connections through dendritic and axonal arborizations (Cajal, 1899; Cajal, 1911). This conceptual hypothesis, along with the idea put forward by Sherrington that neuronal contiguity offers opportunities for nervous impulse changes when passing across neurons (Foster, 1897), initiated the era of cognitive/functional-oriented modern neuroscience in the 20th century. The idea of synaptic connection based mental association and activity-driven strengthening of neuronal connections

were further elaborated almost a half century later by Hebb in his seminal hypothesis that synaptic plasticity can be driven by temporal contiguity of presynaptic and postsynaptic activity (Hebb, 1949). Experimental evidence for activity-driven changes of synaptic transmission was not observed until the early 70's in the rabbit hippocampus (Lømo, 1966; Bliss and Gardner-Medwin, 1973; Bliss and Lømo, 1973). After decades of studies, neuronal plasticity has been characterized in many other cortical areas, but the majority of research effort has focused on hippocampal synapses, as plasticity in hippocampal synapses is relatively easy to induce. Moreover, the physiological significance of the hippocampal formation in combination with the versatile plasticity of synaptic connectivity in hippocampal principal neurons make hippocampal synapses an attractive system to study activity-dependent changes in synaptic transmission.

The physiological significance of the hippocampal formation emerged in the late 1950's with growing reports showing deficits in the acquisition of new episodic memory in human patients with bilateral hippocampal lesions (Scoville and Milner, 1957). Behavioral experiments in animals with controlled lesions and in animals with molecular or pharmacological manipulations of hippocampal circuits confirmed the crucial role of the hippocampus in episodic memory formation (Neves et al., 2008). The observation of hippocampal synaptic plasticity ignited the fervent interest of neuroscientists in this topic, as the learning and memory-oriented significance of the hippocampus suggests that hippocampal synaptic plasticity may serve as a cellular mechanism underlying information storage.

In the hippocampus and other brain areas, the main neuronal signal is thought to propagate through excitatory neuronal activity. Hippocampal principal neurons utilize glutamate as the primary excitatory neurotransmitter. Anatomically, hippocampal neurons undergo elaborate wiring within the network. Characterizing neurotransmission is thus a challenging task, as multiple excitatory and inhibitory inputs merge onto the same neurons. In a more simplified

preparation, such as dissociated neurons cultured in isolation, neurons synapse onto themselves, resulting in autapses.   Autapses exhibit many advantages for electrophysiological and optical studies of neurotransmission. Firstly, the postsynaptic currents (PSC) sampled from the autapses are by definition derived from a single presynaptic neuron, leaving the PSC readout uncontaminated by heterosynaptic or neuromodulatory inputs from other sources.   Secondly, the discrete anatomical arrangements of autapses allow pharmacological reagents to be directly and rapidly delivered to the target neurons. In addition, synaptic properties of autapses, by electrophysiological measurements or ultrastructural assessments, are very similar to hippocampal synapses *in vivo*. These features make autapses an appealing neuronal preparation to study plastic properties of hippocampal neurotransmission.

1.3 The synapse and its properties

The word "synapse", derived from synapsis, comes from the Greek word συναπτω, meaning "to clasp". It was chosen to describe specialized compartments at the nerve terminals that allow conduction to occur in only one direction, as Sherrington and other scientists at the time observed at the end bulb of sensory terminals and at neuromuscular junctions (NMJs) (Bennett, 1999). The synapses they observed more than a century ago are the chemical synapses modern neuroscientists refer to.   In general, chemical synapses are composed of paired presynaptic and postsynaptic compartments. Both compartments acquire specialized features that enable versatile output and plasticity in response to fluctuating network activity.

1.3.1 Presynaptic compartment and vesicles in hippocampal excitatory synapses

The presynaptic compartment contains neurotransmitters as the output messenger. In the hippocampus as well as other cortical areas, neurotransmission from glutamatergic synapses is the major excitatory drive for signal propagation. Landmark studies from Katz and colleagues,

primarily at the cholinergic neuromuscular synapse, put forward an elegant hypothesis that

neurotransmitters are released as quantal units (del Castillo and Katz, 1954). This quantal theory

was validated later by ultrastructural and electrophysiological experiments in many other

synapses (Nagasawa J et al., 1970; Heuser et al., 1979). These results reveal that

neurotransmitters are packed in the small organelles, named presynaptic vesicles, at presynaptic

terminals, and transmitter release results from vesicle fusion to the plasma membrane. A typical

hippocampal glutamatergic presynaptic terminal contains up to hundreds of presynaptic vesicles

with diameters ranging 30~40 nm (Schikorski and Stevens, 1997). Vesicular glutamate loading is

mediated by vesicular glutamate transporters (vGluT) (Disbrow et al., 1982; Naito and Ueda,

1985; Floor et al., 1990; Bellocchio et al., 2000; Takamori et al., 2000; Chaudhry et al., 2008).

vGluT-mediated glutamate loading is powered by the action of the vacuolar H^+-ATPase that

generates an electrochemical proton gradient across the vesicle membrane. In mature

hippocampal synapses, vGluT1 is the predominant vGluT species present on excitatory terminals

(Fremeau et al., 2001; Fremeau et al., 2004). The fusion of the vesicles takes place at a

specialized, electron-dense active zone (Couteaux and Pécot-Dechavassine, 1970; Zhai and

Bellen, 2004) located at the presynaptic membrane precisely opposite the synaptic cleft and

opposite the postsynaptic specialization. Normally, vesicles undergo docking and priming,

processes that are mediated by serial protein-protein interactions to anchor (docking) and

immobilize (priming) vesicles onto the plasma membrane at the active zone prior to release

(Becherer and Rettig, 2006; Verhage and Sørensen, 2008).

Presynaptic vesicles, according to their relative readiness to participate in exocytic cycles, can be

further categorized into three classes: those that respond most immediately to the upcoming APs,

those that actively participate in exo-endo vesicle cycles, and those that hardly participate in the

vesicle cycle. Although this classification of presynaptic vesicles is widely accepted in the field,

the nomenclatures used to refer to these classes of vesicles sometimes vary from laboratory to

laboratory. In this manuscript, we refer to readily releasable vesicles as the vesicles that respond

to depolarization most immediately, recycling vesicles as the vesicles that participate in functional vesicle cycles, and reserve/resting vesicles as those that do not recycle under normal physiological conditions (Rizzoli and Betz, 2005; Becherer and Rettig, 2006; Kavalali, 2007).

Because vesicles within the readily releasable pool (RRP) respond to stimulation most rapidly, determining the size of the RRP is of interest as the size of RRP may be one of the important determinants of synaptic strength. Conventionally, the size of the RRP is estimated by measuring the fast PSC component from the release that is triggered by repetitive AP stimulation, rapid strong depolarization, or hypertonic challenge (Stevens and Tsujimoto, 1995; Mennerick and Matthews, 1996; Rosenmund and Stevens, 1996; Schneggenburger et al., 1999). Likewise, the sustained component from a strong, lasting stimulus is contributed from the recycling vesicles, which actively replenish the vacant vesicle slots at the active zone during ongoing activity (Neves and Lagnado, 1999).   Reserve vesicles, due to their lack of response to stimulation, are generally hard to identify by electrophysiological paradigms. By electromicroscopic techniques, the relative amount of reserve/resting pool (RP) vesicles can be estimated by 3D image reconstruction in combination with pre-imaging vesicle labeling. These analyses reveal that the RP accounts for ~80% of the total vesicle pool in hippocampal excitatory terminals (Schikorski and Stevens, 2001). Ultrastructural studies also suggest that the vesicles residing within the active zone quantitatively correlate with electrophysiologically defined RRP (Schikorski and Stevens, 2001). It is thus thought that the RRP corresponds to the population of docked and primed vesicles. In hippocampal CA1 and in cultured excitatory synapses, the average number of readily releasable vesicles is about 5~10 vesicles, or 5% of the total vesicles, per synapse. In addition, there seems to be a correlation between the relative number of recycling vesicles and active zone-docked RRP (Schikorski and Stevens, 1997).

Upon serial molecular docking and priming, the vesicles become responsive to the calcium that enters through presynaptic calcium channels triggered by AP-associated membrane depolarization. After sensing the calcium influx by vesicular calcium sensors (e.g. synaptotagmin. Geppert et al., 1994), vesicles fuse and collapse into the plasma membrane and release the transmitter content into the synaptic cleft (Chapman, 2002). Membrane materials from exocytic vesicles are normally retrieved back into the presynaptic compartment along with other vesicular proteins that temporally reside on the plasma membrane through clathrin-mediated endocytic processes (Dittman and Ryan, 2009). Such internalization of vesicular materials initiates another round of vesicular cycling. Recycled vesicles are normally deposited directly back to the recycling pool, reprocessed through endosomal pathways, or recycled back to the RRP (Südhof, 2004; Kavalali, 2007).

Instead of freely populating in the presynaptic compartment, the majority of presynaptic vesicles are tethered with actin filaments (Doussau and Augustine, 2000). In has been shown in many synapses that mobilization of actin-tethered vesicles requires phosphorylation of release-relevant molecules (e.g. synapsin I. Fdez and Hilfiker, 2006). During ongoing activity, mobilization of vesicles for vesicle replenishment has been observed in hippocampal synapses by activating myosin light chain kinases (Ryan, 1999) and calcium/calmodulin-dependent protein kinase (CaMK)(Chi et al., 2001), in fly NMJs by activating CaMK II (Shakiryanova et al., 2007), and in the calyx of Held by activating calmodulin (Sakaba and Neher, 2001). Therefore, during prolonged stimulation, the efficiency of vesicle replenishment might determine the upper limit rate of vesicle depletion. Our studies and others' work have previously described a heterogeneous release competence among readily releasable vesicles (Stevens and Tsujimoto, 1995; Wu and Borst, 1999; Hagler and Goda, 2001; Moulder and Mennerick, 2005). Release heterogeneity of the RRP results in incomplete RRP depletion during short, repetitive stimulation (Moulder and Mennerick, 2005). Because vesicle replenishment, either from recycling vesicles or from newly endocytosed vesicles, takes place constitutively during persistent synaptic activity, it would be

interesting to understand the extent to which vesicle replenishment contributes to transmitter release. In chapter 2, we will discuss results that suggest little contribution of newly endocytosed vesicles in transmission during short repetitive stimulation.

1.3.2 Postsynaptic compartment and glutamate receptors

Apposing the presynaptic active zone is the postsynaptic component of the recipient cells. The postsynaptic compartment contains a specialized postsynaptic density (PSD) consisting of receptors, second messenger proteins and scaffolding proteins (Kennedy, 1997). The complex yet organized postsynaptic structures ensure that presynaptically delivered transmitters are effectively received by postsynaptic receptors, and are faithfully translated into signals in the recipient cells.

Glutamate receptors can be classified into metabotropic and ionotropic receptors. When glutamate binds to the receptors, it activates the receptors that allow presynaptic activity to be delivered to post synaptic cells through either secondary signals (through metabotropic receptors) or electrical signals (through ionotropic receptors). Glutamate-activated ionotropic receptors can be further classified to N-methyl-D-aspartate (NMDA)-sensitive and non-NMDA-sensitive receptors. NMDA-insensitive receptors include kainate-sensitive receptors and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-sensitive receptors (AMPAR). Because kainate receptors play a relative small role in the excitatory PSCs (EPSCs) in our preparation, they are beyond the scope of this introduction.   AMPARs have characteristically fast activation and deactivation kinetics (Colquhoun et al., 1992). Although the rapid kinetics of the receptors make AMPAR-mediated EPSCs a better readout, compared with NMDAR EPSCs, for probing instantaneous changes in the presynaptic transmitter release. AMPARs also undergo fast receptor desensitization (Trussell et al., 1988; Trussell and Fischbach, 1989). Fast receptor

desensitization of AMPARs could compromise quantification accuracy of transmitter release when strong transmission saturates and/or desensitizes synaptic receptors (Trussell et al., 1993; Tang et al., 1994; Jones and Westbrook, 1996). Experimentally, desensitization-associated EPSC rundown can be minimized by rapidly dissociating antagonists (e.g. γ-DGG and kynurenate) or by allosteric receptor modulators (e.g. cyclothiazide).

Contrary to AMPARs, NMDA-sensitive receptors (NMDAR) have distinctively slow activation and deactivation kinetics. The different receptor kinetics allow NMDAR EPSCs to be easily distinguished from AMPAR EPSCs. Physiologically, NMDARs are sensitive to magnesium block in a voltage-dependent manner; the magnesium block is relived with depolarization (Mayer et al., 1984; Nowak et al., 1984). Therefore, physiological activation of NMDARs requires preceding membrane depolarization at the postsynaptic sites. Compared with AMPARs, NMDARs are more permeable to calcium (MacDermott et al., 1986; Ascher and Nowak, 1988). It is shown that NMDAR-mediated postsynaptic calcium inflow can trigger various signal cascades. NMDAR activation thus not only carries electrical signals but also converts postsynaptic electrical signals into molecular signals that orchestrate postsynaptic modifications (Bliss and Collingridge, 1993). Although slow activation and deactivation kinetics compromise the application of NMDAR EPSCs to characterize rapid transmission dynamics, certain pharmacological reagents (e.g. the used-dependent antagonist MK-801; Huettner and Bean, 1988) make NMDARs superior receptor targets for probing spatial-dependent changes in transmission. Because the binding of MK-801 to NMDARs is relatively irreversible (Huettner and Bean, 1988), MK-801 has been used to examine heterogeneity of synaptic release properties (Hessler et al., 1993; Rosenmund et al., 1993), spatial segregation of release sites (Atasoy et al., 2008), and changes in channel open probability (Jahr, 1992; Rosenmund et al., 1993). In chapter 3, we will describe using MK-801 to examine synaptic modulator-mediated changes in spatial synaptic connectivity.

In hippocampal excitatory synapses, AMPARs and NMDARs are in general colocalized (Forsythe and Westbrook, 1988; Bekkers and Stevens, 1989). The expression and localization of AMPARs and NMDARs, however, are not static. In the mature synapses, AMPARs and NMDARs undergo movement between synaptic and perisynaptic areas (Choquet and Triller, 2003). Such lateral movement of the receptors might serve as an alternative pathway to regulate the number of synaptic receptors (Cognet et al., 2006). Developmentally, the expression of NMDARs precedes the expression of AMPARs; NMDARs dominate postsynaptic receptor sites in many preparations early in development (Williams et al., 1993; Monyer et al., 1994; Hsia et al., 1998; Tovar and Westbrook, 1999). Strong synaptic activity through NMDARs later recruits AMPARs (Kerchner and Nicoll, 2008). In cultured embryonic hippocampal neurons, such NMDAR-domination dissipates at around DIV (days *in vitro*) 8 (Hsia et al., 1998). Like most receptors, NMDARs consist of multiple subunits. Receptor composition of NMDARs also undergoes drastic changes as neurons mature (Tovar and Westbrook, 1999). In particular, synapses in early developmental stages, or synapses with low presynaptic activity tend to be dominated by NR2B-containing NMDARs (Tovar and Westbrook, 1999; Ehlers, 2003). NR2B-containing NMDARs can be distinguished from other NMDARs by pharmacological reagents (e.g. ifenprodil), or by deactivation kinetics of NMDAR EPSCs (Cull-Candy and Leszkiewicz, 2004). Although NMDAR and/or NR2B predomination in developing neurons is relatively well accepted, these observations do not seem to apply to nascent synapses formed by mature neurons (Zito et al., 2009). In chapter 3, we will discuss results that show no receptor composition preference in synapses with low basal activity in mature hippocampal autapses.

1.3.3 Calcium-triggered transmitter release

The presynaptic neurotransmission cascade initiates from action potential-triggered calcium infux. Membrane depolarization activates voltage-gated calcium channels (VGCC), which allow rapid calcium influx. Calcium binds to vesicular calcium-sensors (e.g. synaptotagmin), which in turn

trigger sequential protein conformational changes in the vesicular SNARE protein complex that force vesicle fusion and collapse onto the plasma membrane, resulting in transmitter release (Wojcik and Brose, 2007).   Presynaptic voltage-gated calcium channels are rapidly activated by strong depolarization, which distingish them from the calcium channels in the other parts of the neuron (Catterall and Few, 2008). The majority of presynaptic calcium channels that mediate calcium influx for fast transmitter release are N type and/or P/Q type channels, depending on the synapse (Catterall and Few, 2008).   In hippocampal excitatory terminals, transmission is mediated by both N type and P/Q type channels, with minor contribution of R type channels (Wu and Saggau, 1994; Reid et al., 1997; Gasparini et al., 2001).

In fast synapses, ranging from the majority of fast central synapses to some peripheral synapses such as ribbon bipolar synapses, NMJs and squid giant synapses, the delay between presynaptic calcium influx and postsynaptically detected transmitter release is less than a half millisecond (Llinás et al., 1995; Sabatini and Regehr, 1996; von Gersdorff et al., 1998; Geiger and Jonas, 2000; Schneggenburger and Neher, 2000).   This time-locked transmission is defined as phasic transmission. The submillisecond response of transmitter release upon action potential-triggered calcium entry strongly suggests a geometrically tight coupling between a locally high concentration of calcium and the vesicular calcium sensors for transmitter release. Studies ranging from freeze-fracture electron microscopy to theoretical modeling have suggested that presynaptic calcium channels are enriched at the active zone in order to create a transient local calcium signal for rapid transmitter release (Pumplin et al., 1981; Chad and Eckert, 1984; Simon and Llinás, 1985; Zucker and Fogelson, 1986; Meinrenken et al., 2002). These studies also suggest that the depolarization-induced calcium concentration is highest near the calcium entry sites at the presynaptic terminals. As calcium diffuses, its concentration is further shaped by the presynaptic calcium buffering environment. Thus, near the calcium channels, calcium is not in equilibrium with presynaptic calcium buffers.

Experimentally, a radial calcium gradient around the calcium entry sites has been detected by optical assessments at squid giant synapses, frog sympathetic neurons and NMJs, ribbon bipolar neurons, inner hair cells as well as chromaffin cells (Lipscombe et al., 1988; Hernandez-Cruz et al., 1990; DiGregorio and Vergara, 1997; Becherer et al., 2003; Zenisek et al., 2003). These optical examinations yield an estimation of transient calcium signals with smaller than 1 µm in diameter and less than 1 ms in duration. The accuracy of optical measurements of transient calcium signals, however, also suffers from certain inadequacies, such as the limitation of spatial resolution in optical images, and the potential calcium signal distortion resulted from the buffering and saturation of fluorescent calcium indicator (Augustine et al., 2003).

Alternatively, the distance between presynaptic local calcium transient and vesicles can be probed by exogenous calcium chelators (Augustine et al., 1991). In studying synaptic physiology, BAPTA, BAPTA derivatives and EGTA are among the most common calcium chelators for examining local calcium signals. BAPTA has a very fast calcium binding rate, which enables rapid competition with vesicular calcium sensors for calcium binding. Direct presynaptic loading of BAPTA at squid giant synapses almost completely eliminates transmitter release (Adler et al., 1991). On the other hand, injecting EGTA, a chelator with similar calcium affinity but significantly slower binding rate compared with BAPTA, has little effect on transmitter release (Adams et al., 1985; Adler et al., 1991). These results are interpreted as that transmitter release from the vesicles within the calcium non-equilibrium zone is not affected by the presence of the slow chelator EGTA, thus supporting the idea of a transient calcium domain for rapid transmitter release. By incorporating the diffusion constant and the calcium binding constant of the chelators, studies estimate the range of local non-equilibrium zone relative to the calcium channel for a given presynaptic chelator concentration (Adler et al., 1991; Augustine et al., 1991). Such a calcium non-equilibrium zone is in a submicrometer range. Similar estimates for local calcium

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transient have also been applied in other synapses (von Gersdorff and Mathews, 1994; Borst and

Sakmann, 1996; Bucurenciu et al., 2008).   In the small synapses where directly presynaptic

loading of EGTA is not possible, a cell-permeable version of EGTA (EGTA-AM) is used. The

transient presynaptic calcium peak at different concentrations of EGTA-AM has been probed by

fluorescent calcium dye in granule to Purkinje synapses (Atluri and Regehr, 1996).   Incubation of

100 µM EGTA-AM reduces calcium peak by ~ 50% compared with a lower concentration of

EGTA-AM. In hippocampal excitatory synapses, incubation of 100 µM EGTA-AM has little effect

on isolated EPSCs, suggesting a tight coupling between vesicles and channels (Cummings et al.,

1996). Although EGTA-AM does not affect isolated EPSCs, application of EGTA-AM, however,

does change transmission dynamics during repetitive stimulation (Hagler and Goda, 2001; Otsu

et al., 2004). This may suggest that EGTA-AM not only determines the calcium non-equilibrium

domain, but also eliminates residual calcium buildup beyond the non-equilibrium domain during

repetitive depolarization. In chapter 2, we will discuss the results that show changes in release

during repetitive-stimulation, and EGTA-AM's effect on release dynamics during repetitive

stimulation.

It has been long known that the extracellular level of calcium is not linearly related to the strength

of transmitter release (Dodge and Rahamimoff, 1967). Rather, PSCs show a power-dependence

on the extracellular or presynaptic calcium level. This power relationship between PSC amplitude

and extracellular/presynaptic calcium suggests multiple calcium binding sites are present in the

calcium sensitive-release machinery, and/or multiple calcium channels with overlapping calcium

domains are required for vesicle release (Dunlap et al., 1995).   In hippocampal excitatory

synapses, such calcium-transmission relationship, termed cooperativity, is estimated to be

around 4 (Wu and Saggau, 1994; Reid et al., 1998). Some studies show specific channel-

dependent calcium cooperatively (Mintz et al., 1995). This channel-specific calcium cooperativity

may suggest channel type-dependent channel-vesicle coupling. There is evidence for channel

specific channel-vesicle coupling in different synapses. Hippocampal dentate gyrus parvalbumin

interneurons that predominately express P/Q type channels at presynaptic terminals show tight

channel-vesicle coupling, whereas CCK (cholecystokinin) interneurons that express N type

channels exhibit loose channel-vesicle coupling (Hefft and Jonas, 2005).   Because N type and

P/Q type presynaptic calcium channels are expressed unevenly across terminals in hippocampal

excitatory autapses (Reid et al., 1997). It is unclear if transmission dynamics are in part shaped

by specific channel types in hippocampal excitatory synapses. In chapter 2, we will offer

evidence that suggests low correlation between specific channel types and transmission

dynamics in hippocampal excitatory autapses.

Since vesicle release is tightly associated with presynaptic calcium concentration, the temporal

profile of presynaptic calcium has remarkable influence on transmitter release (Zucker and

Regehr, 2002).   In some synapses, short stimulation could result in short-lived calcium

accumulation, which leads to short-term facilitation or augmentation (Zucker and Regehr, 2002).

Persistent high intensity activity could result in buildup of calcium that triggers activation of

second messenger signals. This cascade in turn leads to facilitation of transmitter release lasting

for minutes as augmentation or hours as potentiation (Bliss and Collingridge, 1993; Korogod et al.,

2007).   In several central synapses, presynaptic calcium accumulation with repetitive activity also

encourages another form of release that emerges late and loosely couples temporally with the

presynaptic action potential, as opposed to phasic transmission. This form of release is termed

asynchronous release. Presynaptic calcium thus sculpts the strength and the dynamics of

transmitter output. The interplay between the presynaptic calcium fluctuation and the release

dynamics is therefore a crucial determinant of the neuronal signals delivered from the presynaptic

neurons to the postsynaptic cells.   In chapter 2, we will explore the influence of altering

presynaptic calcium dynamics in affecting phasic and asynchronous release.

1.3.4 The physiological advantages of chemical synapses

The features of chemical synapses offer versatile ways to fine tune the output strength (presynaptic strength) from presynaptic neurons to their target cells. The readout of transmitter release can be described by the following parameters: the postsynaptic response to release of a single vesicle's contents (quantal size, Q), probability for each quantal event to happen (P, also known as P_r, see below), and the number of release sites (N) (Fatt and Katz, 1952; del Castillo and Katz, 1954). Therefore, the multiplied products of Q, P and N describe synaptic strength. Changes in any of the parameters could result in rescaled connectivity between neurons. It is known that the preceding history of synaptic activity shapes the probability of quantal release (P) and/or the number of release sites (N) (Lisman and Harris, 1993), suggesting mutual influences among synaptic properties, neurotransmission, and neuronal activity.

1.4 Probability of vesicle release (P_r)

Release probability (P_r, ranging from 0 to 1) characterizes the likelihood of transmitter release in response to an isolated depolarization event. Because a release event is stochastic, the higher P_r is, the more likely vesicle fusion will occur.   Conventionally, the measurement of P_r can be achieved electrophysiologically. P_r changes in a population of synapses can be measured by paired-pulse ratio (PPR), in which initially low P_r leads to facilitation, and high P_r leads to depression (Zucker and Regehr, 2002). P_r from a population of synapses can also be estimated by the ratio of charge integral in an isolated synaptic EPSC relative to the charge sum of the entire RRP (Fernandez-Chacon et al., 2001), or by the kinetics of progressive receptor blockade with used-dependent receptor antagonist (Hessler et al., 1993; Rosenmund et al., 1993). In brain slices, P_r can be measured by the success versus failure rate of evoking a PSC when input from single or a few synapses is stimulated (Allen and Stevens, 1994). Alternatively, the P_r profile can be measured by optical quantal analysis through presynaptic activity-dependent vesicle labeling (Murthy et al., 1997).

P_r has drawn vast interest in studies of synaptic functionality. Because P_r represents the likelihood of transmitter release, P_r is affected by multiple factors and is the target of many forms of synaptic plasticity. P_r could be a predetermined factor set by the molecular composition of the vesicle release machinery, vesicle-channel distance, and other synaptic components. In hippocampal excitatory synapses, P_r is somewhat correlated with the size of the RRP and the size of the recycling vesicle pool (Dobrunz and Stevens, 1997; Murthy et al., 1997). In addition to presynaptic determination, synaptic P_r can be influenced by its postsynaptic targets, in which the properties or activity of postsynaptic compartments affect the presynaptic P_r retrogradely (Koester and Johnston, 2005; Branco et al., 2008). P_r is also shaped by the preceding history of synaptic activity (Bekkers and Stevens, 1990; Larkman et al., 1992). In this case, P_r is not a predetermined static factor, but rather a temporally adjusted readout influenced by ongoing synaptic activity. Below, a few major factors that affect P_r in baseline conditions and in an activity-dependent manner will be discussed.

1.4.1 Calcium and P_r

As discussed above, transmitter release is tightly coupled to calcium dynamics; altered presynaptic calcium profiles profoundly affect kinetics of vesicle release. Because release dynamics are dependent on the transient exposure of vesicles to the calcium concentration peak, vesicle-channel coupling influences the baseline P_r. The factors that alter vesicle-channel coupling will therefore affect P_r. These predisposed factors include background presynaptic calcium level, endogenous presynaptic calcium buffer and calcium handling machineries (e.g. calcium extrusion or internal uptake by transporters). It is worth noting that these factors are not always static, as some of them are susceptible for activity-dependent changes. For example, presynaptic calcium channels on their own are under various activity-dependent regulation, ranging from G-protein mediated channel inhibition to calcium-dependent facilitation or inhibition, depending on the synapse types and/or channel subunit composition. Activity-dependent

modulation can affect channel open probability, thus altering presynaptic calcium dynamics and

leading to temporal P_r changes (Catterall and Few, 2008).   Moreover, background level calcium

at the presynaptic terminals may change in an activity-dependent manner by calcium-induced

calcium release from intracelluar calcium stores (e.g. mitochondria or endoplasmic reticulum)

(Rose and Konnerth, 2001; Rusakov, 2006). Such elevation of background presynaptic calcium

level can contribute to P_r increase.

Experimentally, raising extracellular calcium promotes transmission by increasing P_r.   It is likely

that increasing driving force with elevated extracellular calcium acts by expanding the transient

high calcium domain at presynaptic terminals. The enlarged calcium domain enables more

vesicles to be exposed to the transient high calcium. Interestingly, elevating extracellular calcium

also strongly enhances asynchronous transmission during repetitive stimulation (Hagler and

Goda, 2001; Otsu et al., 2004).   Despite a long observed link between high calcium and

enhanced asynchronous transmission, it is still not clear whether increased asynchrony is always

a necessary result of increasing P_r. In Chapter 2, we will discuss the correlation between

asynchronous release and P_r changes by altering extracellular calcium as well as other

pharmacological reagents which are well documented to change P_r.

1.4.2 Second messenger modulation and P_r

Persistent activity within the network generates molecular signal cues that temporally or

persistently modify synaptic properties. The importance of second messenger components in

shaping synaptic properties has been demonstrated in genetically modified animals and by

pharmacological approaches. Persistent activity generates accumulated calcium in both

presynaptic and postsynaptic compartments. Elevated calcium is rapidly converted into second

messenger-mediated signals by binding to various calcium binding proteins (e.g. calmodulin) that

translate calcium signals into signaling cascades for long lasting modification of neuronal and/or synaptic properties (de Jong and Verhage, 2009).   One example of a calcium-initiated signal is calcium/calmodulin-dependent activation of adenlylate cyclases that produce cyclic AMP (cAMP) (Kamenetsky et al., 2006). Presynaptically, increased cAMP potentiates presynaptic output, possibly through epac- (exchange protein directly activated by cAMP) and protein kinase A (PKA) -mediated signals (Seino and Shibasaki, 2005). The increase of cAMP and PKA activity is important in activity- and learning-associated long-term potentiation (LTP) in many synapses across species (Pittenger and Kandel, 2003). Pharmacological-dependent increases of cAMP also rapidly potentiate synaptic outputs in different synaptic preparations (Seino and Shibasaki, 2005). Elevating cAMP by pharmacological reagents (e.g. adenylate cyclase activator forskolin or cAMP analogs) increases miniature PSC frequency, potentiates evoked PSCs in hippocampal (Huang et al., 1994; Trudeau et al., 1996), cortical (Chavis et al., 1998), and calyceal synapses (Kaneko and Takahashi, 2004), NMJs in flys (Cheung et al., 2006), and *Aplysia* (Brezina et al., 1994). Despite long-established importance of cAMP in presynaptic potentiation, the downstream targets of cAMP pathways that mediate P_r increases remain inconclusive.   Several molecular candidates have been proposed and experimentally tested (Seino and Shibasaki, 2005), yet final consensus is still lacking. Identifying downstream mediators for cAMP-mediated potentiation is certainly of future interest in studying molecular mechanisms underlying activity-dependent neuronal/synaptic modification.

Persistent presynaptic activity also results in phospholipase C (PLC) activation. Activated PLC hydrolyzes phosphoinositol lipid precursor and generates inositol 1,4,5-triphosphate (IP_3) and diacyl glycerol (DAG) as secondary messengers at presynaptic terminals (Micheva et al., 2001). IP_3 activates IP_3 receptors that release calcium from internal calcium stores to either increase intracellular level of background calcium, or generate other internal calcium-dependent signals (Rose and Konnerth, 2001).   DAG, on the other hand, activates its downstream C1-domain targets. At presynaptic terminals, the DAG targets include protein kinase C (PKC) and vesicular

priming protein Munc13.   Studies have shown that the DAG analogs phorbol esters profoundly

potentiate synaptic transmission in various types of synapses (Malenka et al., 1986a; Muller et al.,

1988; Searl and Silinsky, 1998; Hori et al., 1999; Millán et al., 2003) . Similar to increasing cAMP,

phorbol esters reproducibly increase mPSC frequency and potentiate evoked PSCs (Malenka et

al., 1986b; Honda et al., 2000; Waters and Smith, 2000). DAG and phorbol esters likely

potentiate synaptic transmission by activating both Munc13 and PKC, as neurons with deficit in

Munc13 or potential PKC targets are insensitive to phorbol esters potentiation (Rhee et al., 2002;

Wierda et al., 2007). The requirement of PKC for phorbol esters potentiation, however, is

somewhat controversial across studies, as inhibiting PKC activity abolishes phorbol potentiation

in some synapses (Stevens and Sullivan, 1998; Hori et al., 1999; Wierda et al., 2007), but not

others (Searl and Silinsky, 1998; Rhee et al., 2002; Lou et al., 2008). In chapter 2 and 3, we will

discuss results that show a relative minor PKC-dependent contribution to phorbol-mediated

synaptic potentiation assessed by isolated EPSCs and a more prominent role with repetitive

stimulation.

 Synaptic potentiation by increasing cAMP (by forskolin or cAMP analogs) and by phorbol esters

stimulation has been of research interest for decades.   In some synapses, the potentiation

effects of phorbol esters and forskolin are additive, suggesting that the downstream signal targets

is somewhat distinct (Kaneko and Takahashi, 2004). Other studies, however, suggest partially

overlapping mechanisms underlying potentiation from these two modulations (Gekel and Neher,

2008). Despite being long studied in parallel, most studies use isolated PSCs or mPSCs to

examine potentiating effects of forskolin and phorbol esters. These studies conclude that

forskolin and phorbol esters potentiate synaptic output by increasing P_r. Yet not much is known

about the differences underlying potentiation mediated by these two modulators. In chapter 3, we

will discuss the results that suggest differential mechanisms underlying forskolin and phorbol

esters potentiation at the level of quantitatively altering synaptic connection.

1.4.3 P_r and the functional connection

Both electrophysiological and imaging studies show that P_r is heterogeneous across synapses. Interestingly, these studies all suggest predominantly low P_r in the identified terminals. Since transmitter release is stochastic and dependent on P_r, low P_r would predict low reliability of synaptic output. Although this stochastic nature of transmitter release seems to contradict the high demand of transmission fidelity at the level of single synapse per se, predominantly low P_r among population of synapses is more efficient from an energy-saving point of view. Energy consumption during structural and functional recovery at the presynaptic and postsynaptic compartments after transmitter release is costly. A high proportion of low P_r synapses permits sufficient signal output at relatively low energy cost by generating transmission at some but not all terminals (Branco and Staras, 2009). From a plasticity point of view, the presence of low P_r synapses allows the sum of synaptic strength to be scaled efficiently by increasing P_r at the synapses that minimally contribute to transmission at baseline (Voronin and Cherubini, 2004). In some neuronal circuits, activity-dependent long-term potentiation (LTP) involves increasing transmission reliability at preexisting low P_r terminals (Voronin and Cherubini, 2004).

In addition to low P_r terminals, there is also a subset of synapses that appears to be silent. Silent synapses could originate from presynaptic or postsynaptic causes. Postsynaptically silent terminals are the synapses that devoid of AMPARs. The activation of such postsynaptically silent synapses is through the recruitment of AMPARs by activation of NMDARs (Kerchner and Nicoll, 2008). There is also evidence for presynaptically silent synapses. These presynaptically quiescent synapses do not undergo functional vesicle recycling despite the presence of presynaptic vesicles (Jack et al., 1981; Kullmann et al., 1996; Ma et al., 1999; Kim et al., 2003; Moulder et al., 2004; Slutsky et al., 2004). The presence of these presynaptically quiescent terminals might serve as a reservoir to quickly expand the plasticity of population of synapses

(Voronin and Cherubini, 2004).   Despite a potential role in plasticity, it is still not clear whether

these quiescent terminals are recruited for transmission by second messenger systems. In

chapter 3, we will discuss the results that show differential responses of presynaptically quiescent

terminals to forskolin and phorbol esters.

Recent work in dissociated hippocampal cultures shows that the restoration of release activity at

presynaptically quiescent terminals may involve protein synthesis (Ma et al., 1999) and/or

cytoskeleton reorganization (Slutsky et al., 2004; Shen et al., 2006; Yao et al., 2006), suggesting

an incomplete or immature release apparatus at basally quiescent terminals. The presence of

presynaptically quiescent terminals appears to be activity- dependent, as our previous work

shows that sustained depolarization increases the proportion of presynaptically silent terminals

(Moulder et al., 2004) . It is, however, not clear whether these two classes of quiescent synapses,

basally quiescent synapses and depolarization-silenced synapses, originate from common

molecular causes.   In chapter 3, we will discuss results that suggest potentially different

mechanisms underlying presynaptic silencing of these two classes of quiescent synapses.

1.5 Summary of objectives

In the human, there are about a hundred billion neurons in the CNS. The majority of these

neurons rely on chemical synapses to communicate with one another. Synapses thus translate,

convey, filter, and integrate neuronal activity that travel across the network.   The design of

chemical synapses enables instant modification of synaptic output, which stabilizes the network

by constantly optimizing synaptic signal-carrying capacity that is proper for the scale of network

activity. Because most synaptic resetting is initiated from second messenger modulation,

understanding how second messenger modulation shapes static and activity-dependent

properties of synapses is a key to understanding bidirectional adjustment between network

activity and synaptic transmission.

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Chapter 2. Dynamic modulation of phasic and asynchronous glutamate release in hippocampal synapses

2.1 Abstract

Although frequency-dependent short term presynaptic plasticity has been of long standing interest, most studies have emphasized modulation of the synchronous, phasic component of transmitter release, most evident with a single or a few presynaptic stimuli. Asynchronous transmitter release, vesicle fusion not closely time locked to presynaptic action potentials, can also be prominent under certain conditions, including repetitive stimulation. Asynchrony has often been attributed to residual Ca^{2+} buildup in the presynaptic terminal. We verified that a number of manipulations of Ca^{2+} handling and influx selectively alter asynchronous release relative to phasic transmitter release during action potential trains in cultured excitatory autaptic hippocampal neurons. To determine if other manipulations of vesicle release probability also selectively modulate asynchrony, we probed the actions of one thoroughly studied modulator class whose actions on phasic versus asynchronous release have not been investigated. We examined the effects of the phorbol ester PDBu, which has protein kinase C (PKC)-dependent and independent actions on presynaptic transmitter release.   PDBu increased phasic and asynchronous release in parallel. However, while PKC inhibition had relatively minor inhibitory effects on PDBu potentiation of phasic and total release during action potential trains, PKC inhibition strongly reduced phorbol-potentiated asynchrony, through actions most evident late during stimulus trains. These results lend new insight into PKC-dependent and independent effects on transmitter release and suggest the possibility of differential control of synchronous versus asynchronous vesicle release.

2.2 Introduction

Short-term, frequency-dependent modulation of transmitter release has been of interest for many decades. Primarily, studies have focused on modulation of phasic, synchronous synaptic transmitter release. However, asynchronous release can complement phasic release and contributes strongly to postsynaptic responses under certain conditions (Lu and Trussell, 2000; Hefft and Jonas, 2005; Taschenberger et al., 2005; Iremonger and Bains, 2007; Best and Regehr, 2009). Less is known about this form of release. Asynchrony is characterized by temporal dispersion of vesicle release following presynaptic action potential arrival and Ca^{2+} influx. The slow buildup and removal of Ca^{2+} in the presynaptic terminal by buffering and clearance mechanisms likely participate in asynchrony (Barrett and Stevens, 1972; Goda and Stevens, 1994; Cummings et al., 1996; Atluri and Regehr, 1998). In addition, separate Ca^{2+} sensors for phasic and asynchronous release are possible (Geppert et al., 1994; Goda and Stevens, 1994; Sun et al., 2007). Mutations, genetic deletions, and non-physiological divalent ion substitution alter the relative proportion of phasic to asynchronous release (Rahamimoff and Yaari, 1973; Geppert et al., 1994; Calakos et al., 2004; Tang et al., 2006; Pan et al., 2009), However, whether second messengers and synaptic modulators can alter the proportion of phasic to asynchronous release is less clear.

Hippocampal principal neurons *in vivo* experience wide range of firing frequencies partly dependent on the behavioral task (Czurkó et al., 1999; Hirase et al., 1999). During high frequency activity, phasic transmitter release gradually depresses, partly resulting from vesicle depletion (Zucker and Regehr, 2002). During the same repetitive stimulation, asynchronous vesicle release in hippocampal neurons becomes more prominent in the postysynaptic response and can carry most of the postsynaptic charge late in EPSC trains (Cummings et al., 1996; Hagler and Goda, 2001; Otsu et al., 2004). Modulation of the phasic to asynchrony ratio could have an important influence on the temporal relationship between the arrival of a presynaptic action potential and the corresponding postsynaptic spike (Wyart et al., 2005; Iremonger and

Bains, 2007; Jones et al., 2007). Strong phasic release will promote a postsynaptic spike soon after the presynaptic action potential; strong asynchronous release will introduce a delay and more temporal jitter between presynaptic and postsynaptic firing.

In this study, we examined the correlation between the depression of phasic release and the increase of asynchrony in evoked release during action potential trains delivered to autaptic synapses from dissociated hippocampal excitatory neurons. We tested the possibility of vesicle recycling in promoting asynchronous release, and compared the effect of elevating release probability (P_r) by different manipulations, including elevated Ca^{2+} and phorbol ester stimulation, on phasic versus asynchronous transmitter release. Although a phorbol ester strongly promoted increased phasic release and asynchrony in parallel, protein kinase C (PKC) inhibition more strongly compromised the phorbol-potentiated asynchronous component. However, we found no evidence that PKC activation is involved in the asynchrony generated by augmented Ca^{2+} influx during action potential trains, suggesting that PKC activity is not required for increases in asynchrony during strong Ca^{2+} influx.

2.3 Materials and Methods

Materials. Unless otherwise specified, reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cultures. Microisland cultures were prepared as previously described (Mennerick et al., 1995; Moulder et al., 2007). Briefly, hippocampal neurons from Sprague-Dawley rats at postnatal day 1-3 were dissociated with 1 mg/ml papain. The dissociated neurons were then seeded at ~ 100 cells/mm^2 in 35 mm culture dishes that were pre-coated with 0.15% agarose and type I collagen (0.5 mg/ml) as the substrate. Plating media was composed of Eagle's minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 5% heat-inactivated horse serum, 5% fetal bovine

serum, 17 mM glucose, 400 μM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Glial proliferation was inhibited by 6.7 μM cytosine arabinoside 3-4 days after plating. Half the culture media was removed and replaced with Neurobasal medium plus B27 supplement 4-5 days after plating. Cells were recorded 9 -15 days after plating.

Solutions. Whole-cell recordings were conducted in extracellular solution (bath) consisting of (in mM) 138 NaCl, 4 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 glucose and 10 HEPES (Invitrogen) (pH 7.25). α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor-mediated current was isolated with 25-50 μM D-amino-5-phosphonovaleric acid (D-APV, Tocris, Ellisville, MO) in bath solutions. 400 μM kynurenate was added to minimize AMPA receptor saturation and to reduce access resistance errors caused by the large autaptic currents. ESPCs were quantified by subtracting traces obtained in the presence of 1-2 μM 2,3 Dioxo-6-nitro-1,2,3,4 tetrahydrobenzo [f] quinoxaline-7-sulfonamide (NBQX, Tocris). Solution was perfused by a gravity-based multibarrel perfusion system at the rate of 0.2 ml/min. For application of hyperkalemic (45 mM KCl, equimolar substitution for NaCl) or hyperosmolaric (0.5-0.75 M sucrose) solution, the solution was perfused at 1.6 ml/min with < 50 ms complete solution switch. The internal pipette solution contained (in mM) 140 K-gluconate, 4 NaCl, 0.5 CaCl$_2$, 1 EGTA, and 10 HEPES (pH 7.25). Stock solutions of ω-Agatoxin IVA (1 mM) and ω-Conotoxin GVIA (1 mM, Tocris) were dissolved in distilled water and diluted to the indicated concentrations. Stock solutions of EGTA-AM (100 mM, Invitrogen), folimycin (67 μM, Calbiochem, Gibbstown, NJ), Gö6983 (2 or 20 mM) and phorbol 12,13-dibutyrate (PDBu, 1 or 5 mM), phorbol 12-myristate 13-acetate (PMA, 5 mM) were made in DMSO and diluted as indicated.

Electrophysiology and data analysis. Whole-cell recordings were performed with a MultiClamp 700B amplifier and Digidata 1440A acquisition system (Axon Instruments, Sunnyvale, CA); data were acquired in Clampex10 (Molecular Devices, Axon instruments). Electrode pipettes were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL) with pipette resistance 3-6 MΩ. After the whole-cell mode was established, only the cells with access resistance < 15

MΩ, membrane resistance > 150 MΩ, and leak current < 200 pA were accepted for analysis.

Series resistance was compensated at 80%. Cells were voltage clamped at -70 mV, EPSCs

were evoked by a brief (1 ms) depolarization to 0 mV. Signals were sampled at 5 or 10 kHz and

filtered at 2 kHz. All recordings were performed at room temperature. Leak current was

subtracted offline; data were analyzed by Clampfit10 (Molecular Devices, Axon Instruments) or by

customized Igor Pro procedures (WaveMetrics. Lake Oswego, OR). Data fitting was performed

by commercially available fitting routines (Clampfit and Igor Pro). Results are presented as mean

± SEM. Paired or unpaired *t*-test was used for statistic analysis; Pearson's test was used to

evaluate the significance of correlation.

2.4 Results

Asynchronous charge transfer in train-evoked release

We examined excitatory autaptic currents evoked by single or repetitive stimulation from island

cultures of hippocampal neurons (Bekkers and Stevens, 1991). To improve the accuracy of

quantifying transmitter release, we recorded in the presence of a rapidly dissociating glutamate

receptor antagonist (400 μM kynurenate) to reduce voltage-clamp errors associated with large

currents and to shield postsynaptic receptors from saturation and desensitization (Jones and

Westbrook, 1996; Neher and Sakaba, 2001). Under standard conditions, a single evoked AMPA

receptor-mediated EPSC, due to receptor deactivation kinetics and the brief transmitter

concentration profile (Clements et al., 1992), had an average decay time constant (τ of 2.83 ±

0.3 ms (mean amplitude = 3.5 ± 0.6 nA, n = 7). Repetitive high frequency stimulation at 20 Hz for

2 s generated an EPSC waveform that exhibited progressive alteration during stimulation. As

observed by others (Cummings et al., 1996; Hagler and Goda, 2001; Otsu et al., 2004), the

amplitude of each sequential EPSC early in the stimulus train showed strong depression (Figure

1A1), which has been previously attributed to a combination of vesicle depletion (Zucker and

Regehr, 2002) and to incompletely defined mechanisms not directly related to depletion (Brody

and Yue, 2000; He et al., 2002; Moulder and Mennerick, 2005). By contrast, during the same stimulus train a late component of EPSCs emerged (Figure 1A2), which has previously been attributed to asynchronous vesicle release at hippocampal and other synapses (Cummings et al., 1996; Lu and Trussell, 2000; Hagler and Goda, 2001; Otsu et al., 2004; Hefft and Jonas, 2005; Taschenberger et al., 2005; Hjelmstad, 2006; Iremonger and Bains, 2007; Best and Regehr, 2009). This delayed charge transfer resulted from a gradual EPSC waveform widening associated with increased synaptic noise (Figure 1A2), and a failure of the postsynaptic current to decay completely to baseline between stimuli (Figure 1A2, B). When fitted with a single exponential function, the final EPSC of trains exhibited an apparent decay $\square$ of 5.03 ± 0.68 ms (n = 7, *P* < 0.012 compared with the initial EPSC of the train).

We quantified the phasic and the late components of EPSCs by scaling and superimposing the initial EPSC to the peak of each subsequent EPSC in the train, after accounting for residual asynchrony contributed by preceding EPSCs (Figure 1A2). The area under the scaled EPSC was designated phasic charge. The charge contributed by the residual current following the phasic event (the shading immediately following the phasic EPSCs in Figure 1A2) plus the steady-state component remaining from the preceding EPSC (the horizontal shaded area in Figure 1A2) were denoted asynchronous charge.   The total charge transfer, therefore, was the cumulative integral of the entire EPSC waveform (the sum of phasic and asynchronous postsynaptic charge). Figure 1C shows a summary from 8 cells in which phasic and asynchronous charge were quantified in this way. The measured opposing effects of stimulation on phasic and asynchronous charge are similar to previously published work using alternate analysis methods (Hagler and Goda, 2001, Otsu et al, 2004). We found that there was considerable variability among cells in the contribution of asynchrony to total release during train stimulation, but this variation did not correlate with the initial EPSC size (Figure 1D1). Therefore, initial transmitter output did not predict the relative contribution of the late EPSC components during trains. However, we found that the synapses with the stronger depression of phasic EPSCs resulted in a greater contribution of asynchrony to total release (Figure 1D2). These

results are consistent with the idea that the two release processes work in opposition by competing for a common pool of vesicles (Otsu et al., 2004), but the results do not exclude the possibility of differential regulation of the two components.

Selective sensitivity of asynchrony to Ca^{2+} manipulations

Previous work has suggested that asynchronous release at hippocampal synapses is sensitive to presynaptic Ca^{2+} level (Cummings et al., 1996; Hagler and Goda, 2001; Otsu et al., 2004). We verified this by examining the sensitivity of phasic versus asynchronous EPSC components to the slow Ca^{2+} buffer EGTA-AM. In several studies, EGTA has been shown to block the late, presumed asynchronous phase of release selectively. However, under the conditions of most studies, EGTA-AM also changes the dynamics of phasic release during a train (Cummings et al., 1996; Hagler and Goda, 2001; Otsu et al., 2004).   We found that >10 min exposure to 100 µM EGTA-AM resulted in 47 ± 7% reduction in total charge transfer over the course of a 40 pulse train (n = 7). Phasic release was depressed by 22 ± 14%, even though early pulses in the train usually exhibited facilitation relative to baseline (pre-EGTA). In this condition, asynchrony was reduced by 73 ± 3%.

To determine whether putative asynchrony can be selectively depressed without affecting total release, we titrated EGTA-AM concentration and exposure time to 20 µM for 5 min, which produced minimal effects on initial release (Figure 2A, initial EPSC was reduced by 15 ± 5%, *P* = 0.07, n = 7) or total release over a 40 pulse, 20 Hz train (Figure 2C).   Under these conditions the asynchronous charge was reduced to 57 ± 6% of control, while total release was maintained at 94 ± 8% of control (n = 7 ; Figure 2C).   Phasic release during the 40 pulse train under this condition was slightly but not significantly increased by 20 ± 14% relative to baseline. The effect on asynchrony was strongest toward the middle of the pulse train (Figure 2B, left), with a gradual re-emergence of asynchrony late in the train (Figure 2B, right). This late re-emergence likely resulted from buffer saturation, because it was less prominent with the longer incubations in 100

μM EGTA-AM (data not shown). These results suggest that asynchrony can be selectively depressed by Ca^{2+} buffering and is very sensitive to presynaptic Ca^{2+} concentration, even when overall phasic release is nearly intact.

The sensitivity of asynchronous release to a Ca^{2+} chelator and the prerequisite of repetitive presynaptic activity for promoting asynchronous release suggest that changing Ca^{2+} influx may differentially influence phasic and asynchronous release. Work from others has suggested a positive correlation between asynchrony during stimulus trains and the concentration of extracellular Ca^{2+} (Hagler and Goda, 2001; Otsu et al., 2004; Hjelmstad, 2006). In agreement with these previous reports, we saw an increase in both phasic and asynchronous EPSC components as the extracellular calcium concentration ($[Ca^{2+}]_o$) was increased (Figure 3A-C). The preferential enhancement of the asynchronous component was revealed when the asynchronous charge was normalized to the total synaptic charge (Figure 3B1). Increasing $[Ca^{2+}]_o$ from 1 to 1.6 to 4 mM shifted the release mode from phasic-dominant release at low $[Ca^{2+}]_o$ to stronger asynchronous release in 1.6 and 4 mM $[Ca^{2+}]_o$ (25 ± 4% in 1 mM Ca^{2+}, 37 ± 5% in 1.6 mM Ca^{2+}, and 49 ± 3% in 4 mM Ca^{2+}, n = 6; Figure 3A, B1). Total charge transfer also increased modestly with increasing $[Ca^{2+}]_o$ (Figure 3B2). This could be consistent with incomplete depletion at lower $[Ca^{2+}]_o$ (Moulder and Mennerick, 2005) or with Ca^{2+}-dependent vesicle replenishment during stimulus trains (Stevens and Wesseling, 1998). These results emphasize that manipulating Ca^{2+} influx differentially modulates asynchrony versus phasic release during stimulus trains.

A closer examination of the release dynamics during the train revealed that phasic release was more strongly depressed in high $[Ca^{2+}]_o$ despite greater potentiation in the early part of the stimulus train. The coincidence of phasic depression and increased asynchrony late in the train is particularly prominent when the postsynaptic charge transfer during the last half of the train is quantified. When assessed only for the last 20 stimuli, asynchrony represented 55 ± 7% of total

release in 1.6 mM Ca^{2+} and 80 ± 5% of total release in 4 mM Ca^{2+}. These results, again, likely

reflect activity-dependent increases in the asynchronous component by gradual calcium

accumulation in the later part of the train. The increased depression of phasic release is usually

interpreted as a higher rate of vesicle depletion in higher calcium compared to low calcium

(Zucker and Regehr, 2002).

The sensitivity of asynchronous release to $[Ca^{2+}]_o$ and to the slow chelator EGTA suggests that

asynchrony may be driven by sensors relatively distant from the sites of Ca^{2+} influx, although we

cannot exclude the possibility of a separate Ca^{2+} sensor (Geppert et al., 1994; Goda and

Stevens, 1994; Sun et al., 2007). Ca^{2+} influx at hippocampal synapses is driven by two classes

of voltage-gated Ca^{2+} channels, N and P/Q classes of high-voltage activated channels

(Takahashi and Momiyama, 1993; Wu and Saggau, 1994; Reid et al., 1997), with the possibility

of some contribution of R type channels (Gasparini et al., 2001). At some synapses there may be

a looser association of N type channels with vesicles (Mintz et al., 1995). It is unknown whether

differential gating, kinetics or location of N and P/Q channels relative to sites of release might

favor one mode of release over the other. Therefore, we tested the possibility of channel-specific

influence on release mode using selective blockers of N or P/Q type Ca^{2+} channels. We used

ῶConotoxin GVIA (ConoTX) to block N type Ca^{2+} channels and □-Agatoxin IVA (AgaTX) to block

P/Q Ca^{2+} channels, and we compared asynchronous and total release under conditions of altered

extracellular $[Ca^{2+}]$ designed to match the initial output of phasic release.

Figure 4A shows the effect of ConoTX on initial phasic release under the various conditions used

to evaluate asynchrony. On average ConoTX (0.5-1 µM) reduced the initial EPSC to 50 ± 14% of

baseline (n = 5), similar to the effect of lowering $[Ca^{2+}]_o$ to 1 mM in the absence of blocker (Figure

4A; single EPSC in 1 mM Ca^{2+} was 63 ± 10% of baseline). In the presence of the Ca^{2+} channel

blocker, we found that the initial phasic release was restored to baseline levels (2 mM Ca^{2+}

without channel blocker) by increasing $[Ca^{2+}]_o$ to 5 mM (Figure 4A, right). The initial EPSC

43

amplitude in 5 mM $[Ca^{2+}]_o$ plus ConoTX was 124 ± 23% of baseline (n = 6). With train stimulation

during N type Ca^{2+} channel inhibition, asynchrony relative to total release decreased to a level

similar to that observed in the absence of blocker with low $[Ca^{2+}]_o$ (21 ± 5% in 1 mM Ca^{2+}, 18 ±

5% in ConoTX /2 mM Ca^{2+}, n = 5, Figure 4B, left bars). Likewise, when $[Ca^{2+}]_o$ was elevated in

the presence of ConoTX to match initial phasic release to the original levels, the degree of

asynchronous release during trains was indistinguishable from that under original baseline

conditions (n = 6. Figure 4B middle bars). These results were mirrored by experiments using the

selective P/Q channel blocker AgaTX (0.5-1 µM). P/Q channel blockade depressed single EPSC

amplitude by 58 ± 8%, and the EPSC recovered close to the original EPSC amplitude with 6 mM

$[Ca^{2+}]_o$ (100 ± 18% of baseline response, n = 5). Again, matching initial phasic transmitter output

in the presence of toxin produced a similar ratio of asynchrony to total release during 20 Hz trains

(Figure 4B, right bars).

These findings suggest that neither distinct functional properties of the Ca^{2+} channels nor relative

location of Ca^{2+} channels participate strongly in shaping the relative dynamics of asynchrony to

phasic release. Furthermore, it is interesting that manipulation of Ca^{2+} influx by either altering

driving force (manipulation of $[Ca^{2+}]_o$) or altering conductance (channel blockade) produces

similar effects on the asynchrony ratio. Taken together, it seems likely that bulk intraterminal

Ca^{2+} concentration dictates overall asynchrony. The results again highlight the ability to

differentially modulate asynchrony versus phasic release by manipulation of presynaptic Ca^{2+}

influx.

Asynchronous release and newly retrieved vesicles

To help explain the slow development of asynchrony during stimulus trains, it has been proposed

that asynchronous release may draw from the vesicle pool that has been replenished during

ongoing stimulation (Hagler and Goda, 2001; Otsu et al., 2004; Hjelmstad, 2006). Replenishment

can occur through endocytosis or through recruitment of a pool of reserved vesicles (Kavalali,

2007). Because endocytosis may support transmitter release during prolonged activity (Ertunc et al., 2007), we asked if vesicles endocytosed during stimulation participate in asynchronous release. We tested this idea using folimycin, an inhibitor of the vacuolar H^+-ATPase (Sara et al., 2005; Ertunc et al., 2007). With complete folimycin poisoning, neurotransmitter loading into vesicles is blocked, and subsequent vesicle fusion will be postsynaptically silent.

We first assessed the efficacy of folimycin by measuring the recovery of evoked EPSCs in the presence of folimycin after vesicle depletion (Figure 5A). Folimycin exposure (67 nM for 20 min) slightly reduced the evoked EPSC, although this effect was not statistically significant (Figure 5B1 left ; 27 ± 17% depression, n = 10, P = 0.31), suggesting that folimycin did not cause strong transmitter leak from vesicles within this time window.

Folimycin, however, significantly retarded the recovery of evoked EPSCs after extensive vesicle depletion by application of 45 mM K^+ for 90 s, designed to deplete the readily releasable and recycling vesicle pools (Harata et al., 2001). EPSC recovery 40 s after the K^+ stimulus was 68.7 ± 7.2% of the initial EPSC in the control condition (n = 4) and 25 ± 5.6% in folimycin (n = 4; Figure 5A). The strong attenuation of subsequent EPSC recovery after extensive vesicle depletion suggests that inhibition of transmitter refilling into vesicles was effective. Note that we did observe eventual recovery of EPSCs (data not shown), indicating that folimycin slowed but did not completely prevent vesicle refilling. Because this EPSC recovery was evaluated after washout of the folimycin, it could represent slow folimycin reversibility (Ertunc et al., 2007). Alternatively, it might suggest that there is slow mobilization of reserve pool vesicles following extensive vesicle depletion (Ikeda and Bekkers, 2009). Regardless of the reason for the slow recovery, the results show that over short intervals, such as during action potential trains, folimycin inhibition was effective.

During the brief, 40-pulse train, folimycin did not increase stimulus-dependent depression of phasic release (Figure 5B1 right). Previous work shows that folimycin aggravates depression during substantially longer stimulus trains by interfering with the contribution of recycled vesicles to sustained transmitter release (Ertunc et al., 2007). The lack of folimycin's effect on release in our results likely results from the short stimulation time period, in which release depends more heavily on internal supply of preexisting vesicles (Ryan et al., 1996; Sankaranarayanan and Ryan, 2000; Granseth et al., 2006).

Despite this confirmed inhibitory effect of folimycin on newly endocytosed vesicles, we observed no change in the degree of asynchronous release during action potential trains in folimycin-poisoned cultures (percentage of asynchronous release: 43 ± 4% in control, n = 10; 41 ± 4% in folimycin, n = 10. Figure 5B). We conclude that vesicle retrieval is not an important contributor to asynchronous release over short time periods.

Asynchronous release and phorbol ester modulation

The results thus far suggest that bulk $[Ca^{2+}]$ in the presynaptic terminal is prerequisite for asynchronous release. An obvious effect of increased $[Ca^{2+}]_o$ is an elevation of P_r. We tested the possibility that raising P_r by other methods may similarly increase relative asynchrony. We investigated whether second messenger activated P_r increases, like the Ca^{2+}-induced P_r increase, enhance phasic release depression while increasing asynchrony. Among modulators of transmission, phorbol esters have received intensive interest (Malenka et al., 1986; Brose and Rosenmund, 2002). Phorbol esters apparently activate PKC-dependent and independent mechanisms to potentiate transmitter release. We focused here on phorbol-mediated increases in P_r (Yawo, 1999; Oleskevich et al., 2000; Wu and Wu, 2001; Rhee et al., 2002). PDBu treatment (1 µM, 2-3 min) potentiated isolated EPSCs as expected, and the potentiation, although somewhat larger, was compatible with that caused by increasing $[Ca^{2+}]_o$ from 2 mM to 4 mM (Figure 6 A1and B1 inset, C).

Because the strong phorbol potentiation may involve presynaptic potentiation mechanisms in addition to increased vesicle release probability (Stevens and Sullivan, 1998; Waters and Smith, 2000), we examined whether Ca^{2+} increase and PDBu produce similar effects on P_r. A relatively straightforward estimate of P_r effects can be obtained from paired-pulse ratios. (Figure 6A1and B1 inset, D). PDBu's effect was consistent with the effect of increasing $[Ca^{2+}]_o$ to 4 mM. Both treatments significantly and similarly decreased the paired-pulse ratio (ratio of 2nd to 1st EPSC, 50 ms apart), suggesting an increase in P_r (Figure 6D). Moreover, during train stimulation, PDBu increased phasic depression similar to Ca^{2+} elevation (Figure 6A1 and B1). The decays of the phasic peak response were fitted to a double exponential function for each cell, and the weighted decay time constant was similar for both 4 Ca^{2+} and PDBu (4.0 ± 1.1 versus 4.7 ± 0.8 stimuli, respectively; P = 0.61). These results confirmed that PDBu and raising $[Ca^{2+}]_o$ to 4 mM similarly increased P_r and depressed phasic release.

Both PDBu and high $[Ca^{2+}]_o$ enhanced initial phasic release (Figure 6C), and enhanced asynchronous release (Figure 6A2, B2). However, when we compared the ratio of asynchronous to total release, elevated $[Ca^{2+}]_o$, but not PDBu treatment, changed the asynchrony ratio (Figure 6E). This result indicates that PDBu potentiated both release modes in parallel; unlike increased Ca^{2+}, P_r elevation by PDBu did not preferentially encourage either asynchrony or phasic release during repetitive stimulation. The differential effect of raising $[Ca^{2+}]_o$ and PDBu on release mode is particularly significant when only the charge transfer during the steady state of release was quantified. During the steady state release (the last 20 stimuli), asynchrony to total release increased from 56 ± 5% in baseline to 76 ± 2% in 4 mM Ca^{2+}, but was almost unchanged in PDBu (55 ± 7% in control; 59 ± 6% in PDBu). Note that although PDBu robustly potentiated single evoked EPSCs (280 ± 50% of control), a stronger depression during the train resulted in differential potentiation, in which the early EPSCs were more strongly potentiated then the steady

state EPSCs. Such differential potentiation led to a less then 2-fold (182 ± 30% of control) increase in overall phasic charge transfer during the train.

Because presynaptic phorbol ester effects have been attributed to a combination of PKC-dependent and PKC-independent mechanisms (Betz et al., 1998; Wierda et al., 2007), we wondered whether both pathways contributed to the overall parallel modulation of both phasic and asynchronous release, or whether dissection of the two pathways might reveal differential regulation. We used the broad-spectrum PKC inhibitor Gö6983 to block PKC activation by PDBu. To evaluate the efficacy of our Gö6983 treatment, we tested two previously documented presynaptic effects of PKC. First, we examined whether Gö6983 could depress the ability of phorbol ester to speed replenishment following Ca^{2+}-independent depletion of the readily releasable vesicle pool (Stevens and Sullivan, 1998) with a 3 s application of hypertonic sucrose (Rosenmund and Stevens, 1996; Stevens and Sullivan, 1998). Figure 7A shows that phorbol-stimulated speeding of EPSC recovery following sucrose challenge, as observed by Stevens and Sullivan (Stevens and Sullivan, 1998). Gö6983 completely prevented phorbol ester's ability to hasten EPSC recovery (Figure 7A).

As a second test of Gö6983 effectiveness, we examined the documented ability of phorbol esters to occlude effects of presynaptic inhibitory G-protein coupled receptor stimulation. This effect has been shown to be PKC-dependent (Zamponi et al., 1997). As previously reported, we found that A1 adenosine receptor stimulation with 2-chloroadenosine (1 µM) depressed isolated EPSCs by 64.5 ± 10.3% (n = 4) (Scholz and Miller, 1991; Swartz, 1993; Mennerick and Zorumski, 1995). Exposure to phorbol ester prior to 2-chloroadenosine application diminished the inhibition by 2-chloroadenosine (level of depression: 32 ± 9%, n = 4). Inhibiting PKC activity by Gö6983 reversed phorbol ester's occlusion (level of depression: 60 ± 8%, n = 4). Thus, by two measures, Gö6983 effectively prevented presynaptic PKC activation by PDBu under our experimental conditions.

In experiments designed to probe the effect of PDBu and Gö6983 on train evoked EPSCs, we again found that PDBu increased phasic and asynchronous release in parallel (Figure 7B, C first panel). When the effect of Gö6983 alone on synaptic transmission was examined, we found that Gö6983 did not affect EPSC amplitude (108 ± 10%, n = 10) or P_r estimated by paired-pulse ratio (2nd EPSC to 1st EPSC: 0.73 ± 0.07 in control; 0.71 ± 0.08 in Gö6983, n= 10). We did find that incubation in Gö6983 alone slightly reduced the overall asynchrony contribution to total release (42 ± 4% asynchrony to total release ratio before Gö6983, 38 ± 4% ratio after Gö6983, n = 10, *P* < 0.004, Figure 7D, top right), suggesting a possible small contribution of PKC-dependent mechanisms to basal asynchrony. Pre-exposure to Gö6983 (> 3 min) before PDBu application did not significantly inhibit PDBu potentiation of isolated EPSCs (ratio of EPSC potentiation: 2.4 ± 0.2 by PDBu alone, n = 8; 2.0 ± 0.3 in PDBu/Gö6983, n = 10, *P* = 0.49; Figure 7B1 right panels), suggesting the importance of PKC-independent pathways to the potentiation of phasic release (Rhee et al., 2002). PKC inhibition also slightly but non-significantly reduced PDBu-mediated potentiation of total charge transfer in train-evoked release (ratio of total charge transfer potentiation 1.6 ± 0.2 in PDBu, n = 8; 1.3 ± 0.1 in PDBu/Gö6983, n = 10, *P* = 0.08). In contrast with the trend-level effect on total release, PKC inhibition completely prevented the increase of asynchronous charge transfer in the presence of PDBu (Figure 7B1, C). PKC inhibition therefore reduced the ratio of asynchronous to total charge transfer (Figure 7D, bottom left). It should be noted that these effects were compared with the effect of Gö6983 alone; the results therefore represent the true effect of Gö6983 on PDBu rather than the small effect of Gö6983 on baseline asynchrony. The results suggest that PKC-dependent pathways selectively enhance asynchronous release, while PKC-independent pathways sustain the potentiation of phasic release, leading to the net parallel increase in both components when PKC-dependent and independent pathways are intact.

Although PKC inhibition only subtly decreased PDBu-induced potentiation of isolated EPSCs, a closer examination of phasic depression revealed that PKC inhibition also dampened potentiation during steady state phasic release. This effect led to an unchanged asynchronous to total release when only the steady state release (the last 20 stimuli) was quantified (47 ± 4% in Gö; 45 ± 3% in PDBu/Gö). Therefore, the PKC-dependent component of potentiation was most prominent during the steady-state phase of release in 20 Hz trains, where asynchrony was strongest. This led to the relatively selective inhibition of asynchrony over the course of the train.

To verify that the effects of PDBu on asynchrony were not specific to one particular phorbol ester, we also evaluated the effect of PMA (1 µM) on release during trains. Similar to PDBu, PMA increased individual EPSCs to 212 ± 20% of baseline (n = 9). Synchronous and asynchronous release increased largely in parallel over the course of the train, so that asynchrony contributed 39 ± 5% of total charge transfer at baseline prior to PMA treatment and 38 ± 4% after treatment. At steady state (stimuli 21-40), asynchrony contributed 57 ± 7% of postsynaptic current at baseline and 59 ± 6% after PMA treatment. Finally, Gö6983 selectively compromised the increased in asynchrony produced by PMA. After Gö6983 incubation, asynchrony's contribution to total release was reduced from 40 ± 4% to 33 ± 4% ($P < 0.03$ compared with Gö6983 alone, n = 9).

Presynaptic activity and Ca^{2+} influx could result in PKC activation directly or indirectly (Steinberg, 2008). We wondered if the Ca^{2+}-dependent increases in asynchronous release observed with the manipulations of $[Ca^{2+}]_o$ shown in Figures 2-4 could result from PKC activation. We therefore tested whether the preferential increase of asynchronous release in high $[Ca^{2+}]_o$ was sensitive to PKC inhibition. We assayed the asynchrony ratio in high Ca^{2+} in the presence of the PKC inhibitor. In 4 mM Ca^{2+}, asynchronous to total charge transfer was 47 ± 3% (n = 10). Inhibiting PKC activity during train stimulation did not significantly change the ratio of asynchronous to total charge transfer (44 ± 3%, n = 10; Figure 7B2 and 7D bottom right). The insensitivity of

asynchronous release to PKC inhibition in high $[Ca^{2+}]_o$ suggests that, unlike PDBu potentiation of asynchronous release, PKC activity likely does not contribute to increased asynchrony with Ca^{2+} elevation.

2.5 Discussion

In this study, we observed a disproportionate shift between phasic and asynchronous release with several manipulations of Ca^{2+} influx or buffering. Phasic depression was correlated with increased asynchrony. In contrast to the effects of manipulating P_r with $[Ca^{2+}]_o$ alterations, P_r modulation with phorbol esters increased phasic and asynchronous release in parallel. However, we found that PKC-dependent mechanisms explained most of the effect on asynchrony and a small component of the potentiation of phasic release. Our results are important because they are a proof of principle that modulators can influence asynchrony and phasic release through separate mechanisms. Furthermore, our results add to a list of PKC-dependent and PKC-independent presynaptic effects of DAG analogues.

An alternative interpretation of the late EPSC components that accumulates during repetitive stimulation is transmitter pooling. We have previously shown that transmitter pooling is evident in island cultures primarily with strong, synchronous synaptic output (Mennerick and Zorumski, 1995). Here, we failed to find any evidence of a positive correlation between initial EPSC amplitude and the degree of late (asynchronous) charge transfer (Figure 1D). Initial EPSCs decayed with time constants very similar to those of mEPSCs in this preparation (Diamond and Jahr, 1995; Zorumski et al., 1996). This confirms that asynchrony and spillover are both negligible following a single EPSC. Because phasic release strongly depresses during train stimulation, it is unlikely that pooling becomes prominent during the train. Finally, the sensitivity of late EPSC components to EGTA, even when phasic release was intact, strongly suggests that release asynchrony is the major contributor to late EPSC components. We cannot completely exclude a role for transmitter spillover/pooling in the present results; however, in the context of

previous work that has explored conditions promoting pooling, our results strongly support the idea that release asynchrony underlies the majority of late EPSC components during train stimulation in the present experiments (Cummings et al., 1996; Hagler and Goda, 2001; Otsu et al., 2004).

The mechanisms by which phorbol esters potentiate transmitter release have been debated (Rhee et al., 2002; Wierda et al., 2007; Lou et al., 2008). Some studies have indicated that potentiation of Ca^{2+} influx may be important to the effects of phorbol esters (Swartz et al., 1993; Bartschat and Rhodes, 1995; Honda et al., 2000) (Redman et al., 1997; but see Hori et al., 1999; Yawo, 1999; Waters and Smith, 2000). Our results and others demonstrate that alterations of Ca^{2+} buffering or influx (by manipulating either Ca^{2+} driving force or conductance) selectively alter the asynchrony contribution to total release. In addition, our results suggest that PKC-dependent mechanisms of phorbol ester stimulation selectively alter asynchrony. One parsimonious explanation for the effect of phorbol esters is that PKC-dependent mechanisms may alter presynaptic Ca^{2+} dynamics, such as Ca^{2+} influx or handling, which alter the temporal profile of bulk Ca^{2+} in the presynaptic terminal. In other words, changes in presynaptic intracellular $[Ca^{2+}]$ may be downstream of PKC activation. On the other hand, PKC-dependent increases in mEPSC frequency may be independent of Ca^{2+} influx (Capogna et al., 1995; but see Waters and Smith, 2000).

An alternative, non-mutually exclusive explanation for the selective effect of PKC-dependent mechanisms on asynchrony is that asynchronous release draws from replenished vesicles (Otsu et al., 2004), and PKC-dependent effects may involve speeding vesicle replenishment following initial release (Minami et al., 1998; Waters and Smith, 2002; Wierda et al., 2007). Our results suggest that rapid retrieval of vesicles is not important for supporting asynchronous release. However, this result leaves open the possibility that recruitment from the reserve pool is important for fueling the development of asynchronous release during a train (Otsu et al., 2004; Hjelmstad,

2006). Past experiments (Stevens and Sullivan, 1998) and our present work (Figure 7A) suggest that replenishment of Ca^{2+}-independent sucrose-evoked release can be accelerated by PKC-dependent mechanisms, which could lead to increased asynchrony. Furthermore, PKC-dependent phorbol effects were more evident on phasic and asynchronous release late during 2 s, 20 Hz stimulus trains, possibly suggesting acceleration of replenishment. However, in previous studies of hippocampal neurons, phorbol stimulation did not speed replenishment during electrical stimulation; Ca^{2+}-dependent acceleration of vesicle replenishment occluded phorbol-induced replenishment (Stevens and Sullivan, 1998). Therefore, PKC-dependent mechanisms could selectively increase overall asynchrony by speeding replenishment. However, this explanation is difficult to test directly during stimulus trains. Other studies have assumed that most asynchrony during brief trains is part of the initial readily releasable vesicle pool (Moulder and Mennerick, 2005; Stevens and Williams, 2007). Accurate quantification of the RRP size accessible to action potentials will require further clarification of the contribution of asynchrony to this vesicle pool.

In contrast to PKC-dependent asynchrony increases, PKC-independent effects apparently underlie selective potentiation of phasic release. This is consistent with the idea that PKC-independent mechanisms (such as binding of Munc13 by phorbol esters) enhances fusion efficiency of vesicles (Rhee et al., 2002; Basu et al., 2007), rendering vesicles more easily released during the synchronous phase of transmission.

In summary our work demonstrates that second messenger pathways that potentiate vesicle release probability increase phasic and asynchronous release in parallel. However, in the case of phorbol ester modulation, this parallel increase can be subdivided into a component that is PKC-independent that mainly affects phasic release, and a PKC-dependent component that disproportionately affects asynchronous release. This PKC-dependent effect on asynchrony could be explained by Ca^{2+}-dependent increases in asynchronous vesicle release and/or by PKC-dependent effects on vesicle replenishment. The results suggest that asynchronous and phasic

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transmitter release may be controlled relatively independently. However, because physiological

PKC activation is usually associated with rises in presynaptic [Ca^{2+}] through IP3 receptor

activation and DAG increases, which can produce the PKC-independent effects studied here and

elsewhere, the precise physiological conditions under which differential regulation may occur

remain unclear. Nevertheless, this modulation has the potential to fine tune the temporal

relationship between incoming presynaptic firing and postsynaptic output.

2.6 References

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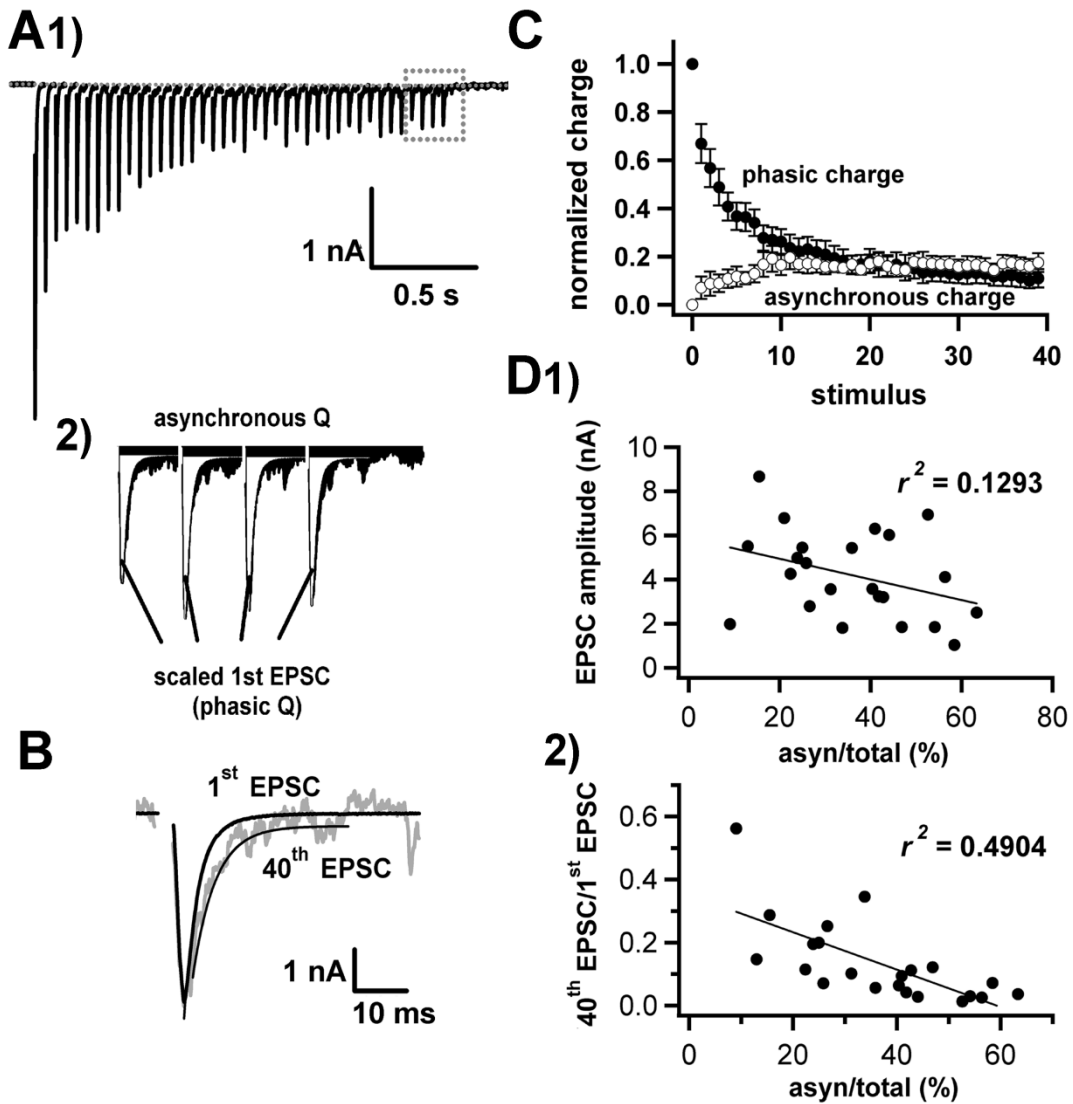
Figure 1. Repetitive stimulation at 20 Hz resulted in complementary alterations in phasic and asynchronous release. A1) and A2) An excitatory EPSC waveform evoked by a train of action potentials at 20 Hz for 2 s showed depression in the phasic component and increases in asynchronous component. The stimulus artifact in the sample trace (and hereafter) is blanked for clarity. A2) The magnified trace from the boxed region in A1. The first EPSC from the same

EPSC train in A1) was scaled to the amplitude of the 38th - 40th EPSCs. The charge transfer of

the scaled EPSC (open area) was defined as the phasic component (phasic Q), whereas the

residual area after subtracting the phasic component from the entire EPSCs was denoted as the

asynchronous component (asynchronous Q, shaded area).   Note that the block shading

indicates the residual, accumulated asynchronous current upon which the subsequent EPSC was

superimposed. B) Superimposed 1st (black thick) and the 40th EPSCs (gray) averaged from 3

independent recordings. The 40th EPSC was scaled to the peak amplitude of the 1st EPSC, and

the baseline current prior to the stimulation was aligned to the same level as the baseline of the

1st EPSC. The decay of both EPSCs was fitted to a single exponential function (thin black lines)

with τ_{1st} = 2.6 ms and τ_{40th} = 4.9 ms. C) Summary of the gradually depressed phasic component

and progressively increased asynchronous component in response to 20 Hz 2 s stimulation. The

charge transfer of phasic and asynchronous components from each EPSC was normalized to the

initial EPSC (n = 8). D1) No significant correlation between the size of the initial EPSC and the

proportion of asynchrony (asyn, asynchronous release is abbreviated as "asyn" in the figures

hereafter) in the same EPSC train. The amplitude of the initial EPSC was plotted against the

cumulative proportion of asynchrony to total release (asynchronous plus phasic). The correlation

of the initial EPSC amplitude with the proportion of asynchronous release was fitted by least

square linear regression with r^2 = 0.1293 (p > 0.05). D2) By contrast, the depression of the

phasic component (expressed as the 40th EPSC/1st EPSC) was correlated with the proportion of

asynchronous release. The response ratio of the phasic component from the same set of cells in

D1) was plotted against the proportion of asynchronous release. Linear regression of the plot

yields r^2 = 0.4904 (p < 0.05).
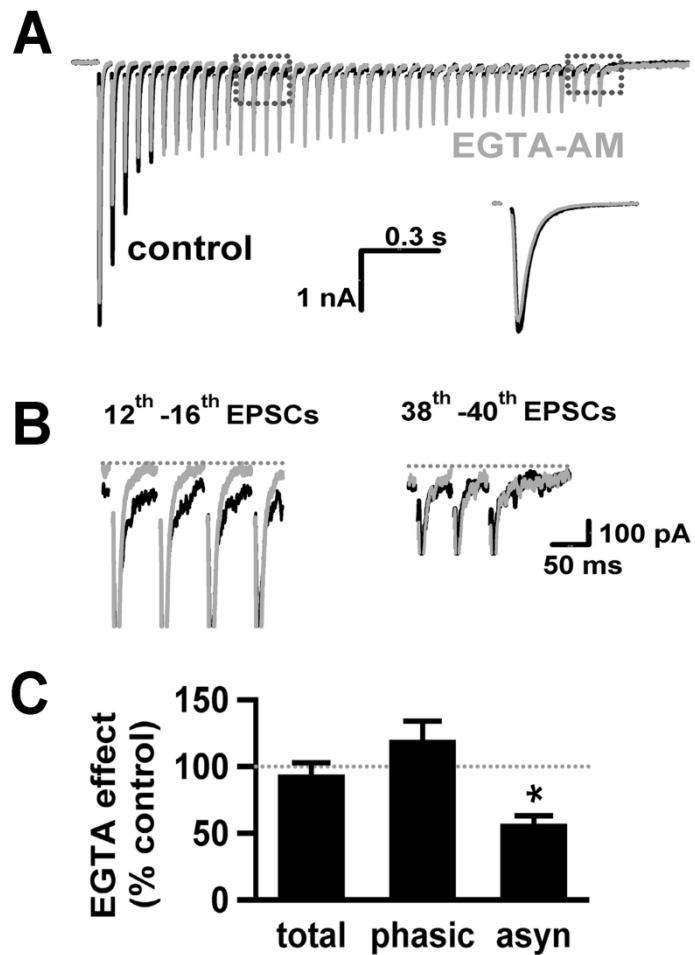
Figure 2. EGTA-AM preferentially eliminated the asynchronous component. A) and B) 20 µM EGTA-AM had little effect on the initial EPSC (A inset), but significantly reduced the asynchronous component. Superimposed EPSC waveforms were recorded before (black) and after (gray) EGTA-AM treatment from the same cell. The boxed areas are magnified in B). C) Summary of EGTA-AM's effect on total, phasic and asynchronous charge transfer. The charge transfer of each component after EGTA-AM treatment was compared to the charge transfer measured before EGTA-AM application (n = 7, *$P < 0.002$).
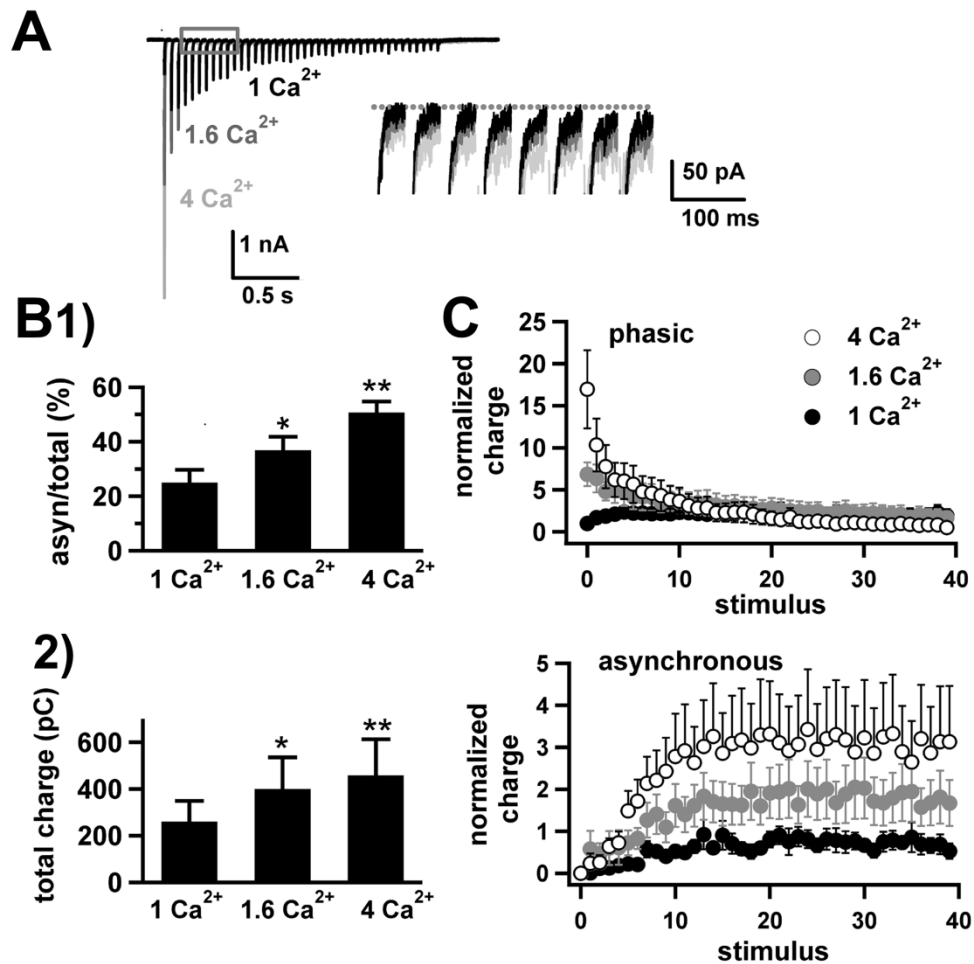
Figure 3. Asynchronous release was sensitive to $[Ca^{2+}]_o$. A) The proportion of asynchrony to total release over the train was increased as $[Ca^{2+}]_o$ was raised. EPSCs recorded from 1 (black), 1.6 (gray) and 4 mM Ca^{2+} (light gray) in the same cell were superimposed. Boxed areas are magnified in the inset. B) Summarized effect of altering $[Ca^{2+}]_o$ on the proportion of asynchronous (upper panel) and total release (lower panel)(upper panel, n = 6, *$P < 0.03$ compared to 1 Ca^{2+}, **$P < 0.003$ compared to 1.6 Ca^{2+}) (lower panel, n = 6, *$P < 0.04$ compared to 1 Ca^{2+}, **$P < 0.05$ compared to 1.6 Ca^{2+}). C) Higher $[Ca^{2+}]_o$ increased phasic depression (upper panel), and enhanced asynchronous release (lower panel). The phasic and asynchronous components of each EPSC were normalized to the 1st EPSC in 1 mM Ca^{2+} (n = 6).
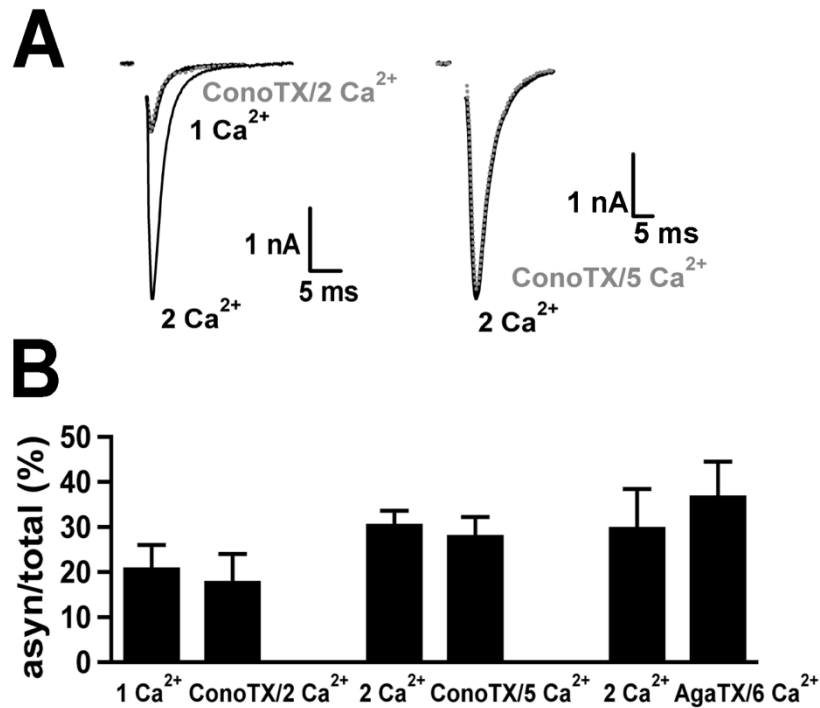
Figure 4. Asynchronous release was not dependent on specific presynaptic Ca^{2+} channels. A) By altering $[Ca^{2+}]_o$, the EPSC measured in the presence of N type channel blocker ω-Conotoxin GVII (0.5-1 μM; ConoTX) can be matched to the EPSC in the absence of channel blocker. The evoked EPSC in the presence of channel blocker in 2 mM Ca^{2+} (ConoTX/2 Ca^{2+}) was similar to the EPSC measured in 1 mM Ca^{2+} without the blocker (1 Ca^{2+}) (left panel). Similarly, the EPSC recorded in the presence of channel blocker can be matched to the control EPSC (2 mM Ca^{2+}) by increasing $[Ca^{2+}]_o$ to 5 mM Ca^{2+}(ConoTx/5 Ca^{2+}) (right panel). B) Blocking either N or P/Q type channels did not alter the proportion of asynchronous release compared with matched EPSCs. Summary of channel blockers' effects on proportion of asynchronous release (1 Ca^{2+}-ConoTX/2 Ca^{2+}, n = 5; 2 Ca^{2+}-ConoTX/5 Ca^{2+}, n = 6; 2 Ca^{2+}-AgaTX/6 Ca^{2+}, n = 5).
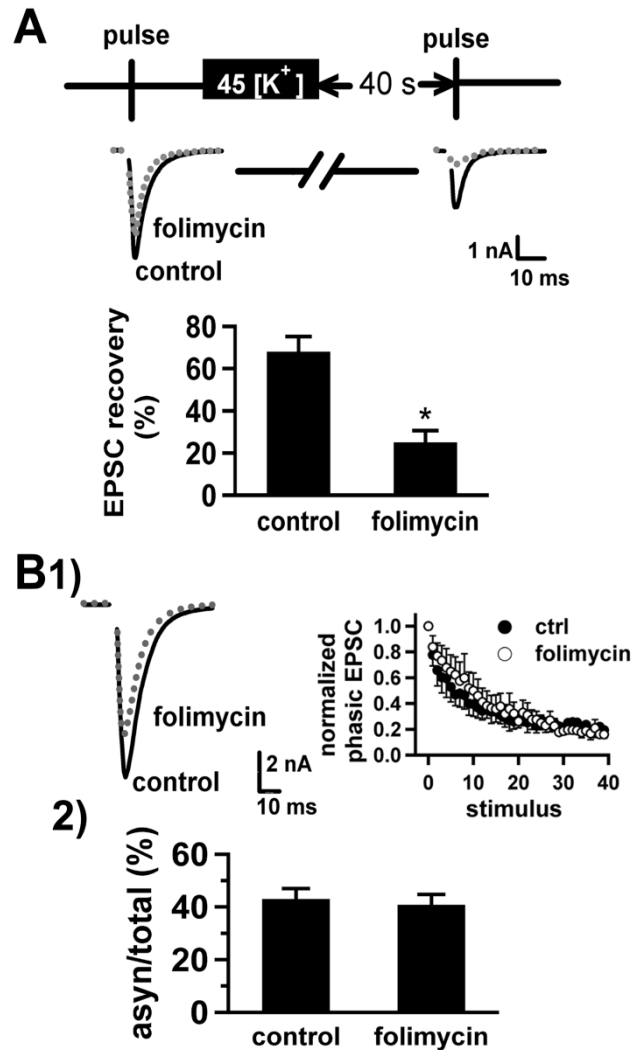
Figure 5. Asynchronous release was insensitive to blocking vesicle supply from newly recycled vesicles. A) The availability of newly endocytosed vesicles was functionally inhibited by folimycin (67 nM for 20 min). After incubation folimycin had little effect on the average amplitude of EPSCs (dotted line, n = 10, also see B1 left) compared to the control (solid line, n = 10). Folimycin effectively attenuated vesicle recovery after extensive vesicle depletion. Experimental protocol and the averaged EPSCs corresponding to the stimulation are illustrated in the upper panel. The ratio of EPSC recovery was assessed by comparing the recovered EPSC 40 s after vesicle depletion (45 mM K$^+$ for 90 s) to the initial EPSC before depletion. Lower panel, summary of folimycin's effect on EPSC recovery (n = 5, *P < 0.007). B) Despite the effectiveness at inhibiting

transmitter refilling, folimycin did not alter the level of phasic depression during 2 s, 20 Hz train (B1 right). B2) Summary of folimycin's effect on isolated evoked EPSCs (upper panel) and on the proportion of asynchronous release (lower panel) (n = 10).
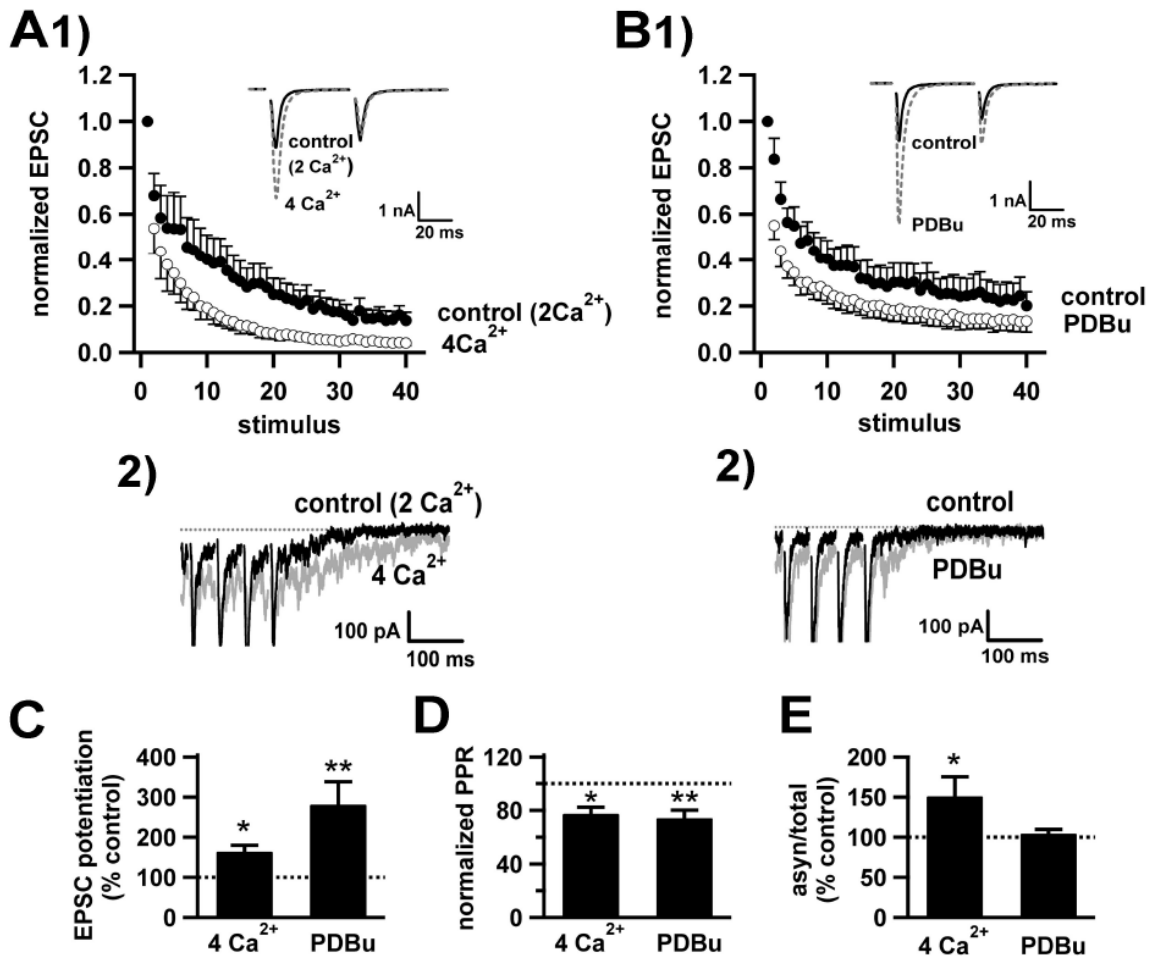
Figure 6. Pharmacological manipulation of P_r potentiated EPSCs but did not affect the proportion of asynchronous release. 4 mM Ca^{2+} (A1), and 1 μM PDBu (B1) had superficially similar effects on isolted EPSCs, paired-pulse modulation, and phasic depression during train-evoked EPSCs. A1) Comparison of release dynamics during stimulation stimulus train between control (2 mM Ca^{2+}) and 4 mM Ca^{2+}. The phasic component of each EPSC was normalized to the initial EPSC and plotted against the stimulus episode (n = 7). Inset, sample paired EPSCs (50 ms inter stimulus interval) recorded in control (2 mM Ca^{2+}, solid line) and in 4 mM Ca^{2+} (dotted line). A2) The late onset EPSCs (36th- 40th) from 20 Hz train stimulation in control (black) were superimposed on the EPSCs from the same stimulus episodes in 4 mM Ca^{2+} (gray). B1) and B2)

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are similar to A1) and A2), using PDBu as modulator. C) Summarized effects of 4 mM Ca^{2+} and PDBu on synaptic potentiation. EPSCs measured in 4 mM Ca^{2+} or PDBu were normalized to the control (*P < 0.02, n = 7; **P < 0.004, n = 9). D) Summary of the effect of 4 mM Ca^{2+} and PDBu on paired-pulse ratio. Normalized paired-pulse ratio (PPR) was expressed as the ratio of the 2nd EPSC to the 1st EPSC, relative to the baseline paired-pulse ratio in 2 mM Ca^{2+}, which was taken as 100% (*P < 0.02, n = 7; **P < 0.03, n = 9). The paired-pulse interval was 50 ms. E) Summary of the effect of Ca^{2+} and PDBu on the proportion of asynchrony to total release during the train (*P < 0.05, n = 7).
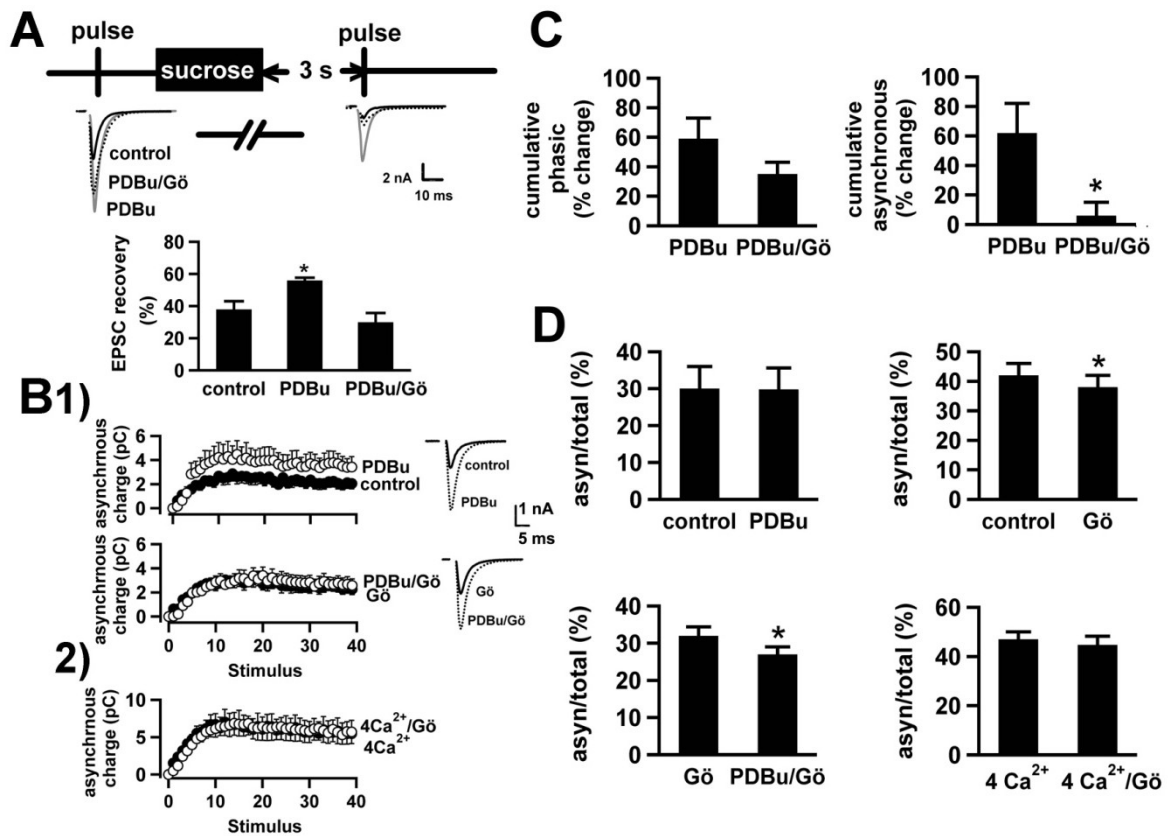
Figure 7. PKC inhibition prevented increases in asynchronous release in PDBu. A) PKC

inhibition by 2-3 μM Gö6983 (Gö) effectively attenuated PDBu (1 μM) stimulated recovery from

Ca^{2+}-independent vesicle depletion. EPSC recovery assay and the corresponding EPSC are

illustrated in the upper panel. The EPSC recovery was calculated by normalizing the EPSC

recovered 3 s after hyperosmotic-induced vesicle depletion to the initial EPSC before depletion.

0.5 - 0.75 M sucrose was applied for 3 s to induce depletion. The raw traces represent examples

from separate cells. Note especially the similar depression induced by depletion in the control

(black trace) and PDBu plus Gö6983 (PDBu/Gö dashed trace) conditions. By contrast, PDBu

alone (gray trace) promoted substantial recovery. The average ratios of EPSC recovery in

control, PDBu, or PDBu with Gö6983 (PDBu/Gö) are summarized in the lower panel (*P < 0.004,

n = 7). B1) The effect of PDBu (PDBu, upper panel) or PDBu plus Gö6383 (PDBu/Gö, lower

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panel) on a single EPSC (inset) and on asynchronous release during train stimulation. The

asynchronous component in each stimulus episode was plotted as a function of time (stimulus

number) (n = 8 in control-PDBu, n = 9 in Gö-PDBu/Gö. See C) for comparative statistics.) B2)

Effect of Gö6383 on increased asynchrony in 4 mM Ca^{2+} (n =10). C) Summarized effects (from

B1) of PDBu alone or PDBu/Gö on cumulative phasic (left) or asynchronous (right, *P < 0.04)

charge transfer. D) Summaries of asynchrony to total release for the conditions shown in B1 and

B2: PDBu (upper left, n = 8), Gö alone (upper right, n = 10; P < 0.05), PDBu/Gö (lower left, n =

10, *P < 0.007), and 4 mM Ca^{2+}/Gö (lower right, n = 10) on the proportion of asynchronous

release during the train.

Chapter 3. Rapid activation of dormant presynaptic terminals by phorbol esters

3.1 Abstract

Release probability (P_r) varies across synapses, and presynaptic stimulation stochastically recruits transmission from terminals according to their P_r. The majority of central synapses have relatively low P_r, which includes synapses that are completely quiescent presynaptically. The presence of presynaptically dormant versus active terminals presumably increases synaptic malleability when conditions demand synaptic strengthening or weakening, perhaps by triggering second messenger signals. However, whether modulators recruit transmission from dormant terminals remain unclear. Here, by combining electrophysiological and fluorescence imaging approaches, we uncovered rapid presynaptic awakening by select synaptic modulators. A phorbol ester (a diacylglycerol analog), but not forskolin (an adenylyl cyclase activator) orelevated extracellular calcium, recruited neurotransmission from presynaptically dormant synapses. This effect was not dependent on protein kinase C activation. After phorbol ester-induced awakening, these previously dormant terminals had a P_r spectrum similar to basally active synapses naive to phorbol treatment. Dormant terminals did not seem to have properties of nascent or immature synapses, judged by NR2B N-methyl-D-aspartate receptor (NMDAR) receptor subunit contribution after phorbol-stimulated awakening. Strikingly, synapses rendered inactive by prolonged depolarization, unlike basally dormant synapses, were not awakened by phorbol esters. These results suggest that the initial release competence of synapses can dictate the acute response to second messenger modulation, and the results suggest multiple pathways to presynaptic dormancy and awakening.

3.2 Introduction

Presynaptic modulators often act by altering the vesicle release probability (P_r), which is heterogeneous across the population of a neuron's presynaptic terminals (Hessler et al., 1993; Rosenmund et al., 1993; Murthy et al., 1997).   Conventionally, measuring P_r across synapses is accomplished by electrophysiological or optical assessments. Both approaches are biased toward sampling the responses of synapses with higher P_r. Populations of synapses with extremely low P_r or completely dormant presynaptic terminals are more difficult to detect, despite evidence for such populations (Tong et al., 1996; Murthy et al., 1997; Ma et al., 1999; Moulder et al., 2004; Slutsky et al., 2004). Whether these populations are subject to presynaptic modulation by second messenger systems has only begun to be investigated.

There is good evidence that prolonged activation of cyclic AMP (cAMP)/protein kinase A (PKA) pathways by forskolin (FSK) awakens presynaptically dormant synapses (Chavis et al., 1998; Ma et al., 1999; Moulder et al., 2008). At least in some systems, this presynaptic activation requires protein synthesis (Ma et al., 1999) and takes several hours. On the other hand, rapid activation of cAMP signaling does not seem to increase functional terminals (Trudeau et al., 1996), despite apparent enhancement of synaptic output by electrophysiological assessment (Weisskopf et al., 1994; Trudeau et al., 1996; Lonart et al., 1998). Whether rapid potentiation of transmission by other modulators involves awakening dormant synapses is less clear.

Here we focus on phorbol ester modulation of glutamate transmission. Classically, phorbol stimulation activates protein kinase C (PKC) pathways (Blumberg, 1991). However, phorbol esters also bind to proteins with diacylglycerol-binding domains, including of the vesicle priming protein Munc13; activation of Munc13 by phorbol esters promotes vesicle release through this PKC-independent mechanism (Betz et al., 1998b; Rhee et al., 2002). Both PKC-dependent and PKC-independent pathways may participate in acute synaptic modulation by phorbol esters (Wierda et al., 2007; Lou et al., 2008).   Although the phenomenon of phorbol potentiation and its

underlying molecular mechanisms have been intensively studied (Brose and Rosenmund, 2002; Silinsky and Searl, 2003), it remains unknown whether phorbol esters potentiate synaptic function in part by acting on dormant synapses.

Using both electrophysiological and imaging assessments, we show that phorbol esters activate preexisting presynaptically dormant synapses in addition to enhancing transmitter output at already active synapses. Interestingly, after activation by phorbol 12,13-dibutyrate (PDBu), presynaptic quiescent synapses appear to have heterogeneous P_r that is similar to basal, untreated active synapses. Despite its effects on basally dormant synapses, PDBu does not re-activate presynaptic terminals rendered inactive by sustained depolarization, suggesting multiple independent routes to presynaptic dormancy. In summary our work adds a further dimension of understanding to the effects of phorbol ester modulation of presynaptic function. Among its rapid effects is a restoration of presynaptic function at terminals that do not normally contribute to synaptic transmission.

3.3 Material and Methods

Cell cultures. Dissociated hippocampal cultures were prepared as previously described (Mennerick et al., 1995; Moulder et al., 2007). Briefly, hippocampi were harvested from Sprague-Dawley rats at postnatal day 1-3 and were dissociated enzymatically using 1 mg ml^{-1} papain, then mechanically with a glass pipette. Dissociated neurons were then plated at 100 cells mm^{-2} for low density microisland plating (for electrophysiological recording) or ~650 cells mm^{-2} for high density mass cultures (for imaging experiments). For island cultures, culture plates were pre-coated with 0.15% agarose and were stamped or sprayed with type I collagen (0.5 mg ml^{-1}) as the microdot substrate. For mass cultures, collagen was spread uniformly across a 25 mm diameter glass coverslip. Culture media consisted of Eagle's minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 5% heat-inactivated horse serum, 5% fetal bovine serum, 17 mM glucose, 400 μM glutamine, 50 U ml^{-1} penicillin, and 50 μg ml^{-1} streptomycin. To

suppress glial proliferation, 6.7 μM cytosine arabinoside was added to the cultures 3-4 days after

plating. Half the culture media was removed and replaced with Neurobasal medium plus B27

supplement one day after antimitotic addition. Cells were used for experiments 9 -14 days after

plating.

Electrophysiology. Whole-cell recordings were performed with a MultiClamp 700B amplifier and

Digidata 1440A acquisition system (Axon Instruments, Sunnyvale, CA); data were acquired in

Clampex10 (Molecular Devices, Axon instruments). Recordings were conducted in extracellular

solution (bath) consisting of (in mM) 138 NaCl, 4 KCl, 2 $CaCl_2$, 0.01 glycine, 10 glucose and 10

HEPES (Invitrogen) (pH 7.25). α-amino-3-hydroxy-5-methyl-4-isoxazole propionate -sensitive

receptor (AMPAR)-mediated current was isolated by 50-75 μM D-amino-5-phosphonovaleric acid

(D-APV, Tocris, Ellisville, MO); N-methyl-D-aspartate-sensitive receptor (NMDAR)-mediated

currents were isolated by 1-2 μM 2,3 Dioxo-6-nitro-1,2,3,4 tetrahydrobenzo [f] quinoxaline-7-

sulfonamide (NBQX, Tocris). 400 μM kynurenate (for AMPAR current) and 250 μM L- amino-5-

phosphonovaleric acid (L-APV, for NMDAR current, Tocris) were added to reduce receptor

desensitization and to minimize access resistance errors associated with the large autaptic

currents. During MK-801 blocking procedures, L-APV was omitted. Non-transmitter mediated

residual current was evaluated in the presence of 1-2 μM NBQX and 75 μM D-APV and was

subtracted from the evoked excitatory postsynaptic currents (EPSCs). Solution was perfused by

a gravity-based multibarrel perfusion system at the rate of 0.2 ml min^{-1} with < 100 ms complete

solution switch. Electrode pipettes with open tip resistance of 3-5 MΩ were filled with the internal

pipette solution containing (in mM) 140 K-gluconate, 4 NaCl, 0.5 $CaCl_2$, 10 EGTA, and 10 HEPES

(pH 7.25). After the whole-cell mode was established, cells were voltage clamped at -70 mV, and

EPSCs were evoked by a brief (1 ms) depolarization to 0 mV. Only cells with access resistance

< 11 MΩ, membrane resistance > 150 MΩ, and leak current < 200 pA were recorded. Series

resistance was compensated by 80%. Leak current was subtracted offline. Signals were sampled

at 5 or 10 kHz and filtered at 2 kHz. All recordings were performed at room temperature.

Pharmacological reagents. Unless otherwise specified, reagents were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of MK-801 (10 mM, Tocris), NMDA (100 mM), and ifenprodil (3 mM) were dissolved in distilled water and diluted to the indicated concentrations. Stock solutions of forskolin (50 mM), Gö6983 (2 or 20 mM), Ro31-8220 (2 mM) and phorbol 12,13-dibutyrate (PDBu, 1 or 5 mM) were made in DMSO and diluted as indicated.

Data analysis. Data were analyzed by Clampfit10 (Molecular Devices, Axon Instruments) or by customized Igor Pro procedures (WaveMetrics. Lake Oswego, OR). Residual stimulation-associated capacitive and presynaptic currents in the sample traces in all figures were blanked for clarity; sample traces in Figures 3 and 5 were lowpass filtered with a digital 8-pole Bessel filter at 500 Hz for clarity. Data fitting was performed by commercially available fitting routines (Clampfit and Igor Pro). All results are presented as mean ± SEM. Paired or unpaired *t*-test or one-way ANOVA were used for statistic analysis.

Imaging experiments, image acquisition and analysis. Mass cultures grown 11-14 days *in vitro* were used for imaging experiments. Prior to the experiment, the cultures were rinsed with extracellular solution plus 50 μM D-APV and 1 μM NBQX. Active synapses were labeled by FM1-43FX (10 μM, Invitrogen) in hyperkalemic extracellular solution (45 mM $[K^+]_o$, equimolar substitution for Na^+) with 25 μM D-APV and 1 μM NBQX for 2 min. After labeling, Advasep-7 (500 μM, CyDex, Overland Park, KS) was added to remove residual dye for another 10 seconds. Dye labeled cultures were rinsed and fixed in 4% paraformaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline (PBS) for 10 min. Fixed cultures were incubated in blocking solution containing 4% goat serum and 0.05% Triton X-100 for 15 min. For vesicular glutamate transporter 1 (vGluT-1) staining, the fixed cultures were exposed to primary vGluT-1 antisera (Chemicon, Temecula, CA) diluted 1 to 2000 in blocking solution for 3 h. Alexa Fluor 647 conjugated anti-guinea pig antisera (1:500, Invitrogen) were used to visualize vGluT-1 labeling. Cultures on glass were mounted on a microscope slide with Fluoromount-G (Southern Biotechnology, Birmingham, AL). Experimental conditions for each slide were coded before

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subsequent image acquisition and analysis, and the experimenter was kept naïve to experimental conditions through the data acquisition and analysis procedures.

All images were acquired under a 60x oil objective by a C1 scanning laser coupled to a Nikon TE300 inverted microscope (Nikon, Melville, NY).   Acquisition gain setting, scanning rate, and Z-stack setting were identical across the acquired fields within an experiment. Data analysis was carried out in MetaMorph (Molecular Devices). The regions of interest corresponding to vGluT-1-immunoreactive puncta were superimposed on the identical field imaged under wavelengths appropriate for FM1-43FX. Signal threshold area was set at 10 thresholded pixels. In general, 10 puncta per field, and 5-7 fields per experimental condition were analyzed.

3.4 Results

Phorbol ester recovers post-block NMDAR-mediated current

We first tested whether synaptic modulators could potentiate transmitter output from synapses with basally low release activity. To selectively eliminate neurotransmission from active synapses, we took advantage of an irreversible use-dependent NMDAR antagonist MK-801 (Huettner and Bean, 1988). At low frequency stimulation (0.1 Hz), MK-801 blocks NMDAR only at synapses that release glutamate in response to the stimulation (Hessler et al., 1993; Rosenmund et al., 1993). Consequently, the synaptic NMDAR remaining functional after MK-801 blocking trials should represent the receptors residing at synapses that are the least active during the stimulation. We examined if modulator application after synaptic block of NMDAR-current (I_{NMDAR}) causes recovery of synaptic I_{NMDAR}. The recovered I_{NMDAR} is presumably mediated by the receptors that remain functionally intact after MK-801 block, and therefore represent the transmitter output from synapses that release glutamate only after modulator treatment.

In the presence of MK-801 (5 µM), 25-35 action potential stimuli at 0.1 Hz depressed synaptic I_{NMDAR} to ~ 3% of the initial I_{NMDAR} (3 ± 0.4% of the initial I_{NMDAR}, n = 8, Figure 1A).   Isolated

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AMPAR current (I_{AMPAR}) and the paired-pulse ratio (PPR, 2nd EPSC/ 1st EPSC, 50 ms interpulse interval) measured by I_{AMPAR}, on the other hand, was minimally affected by MK-801 application (I_{AMPAR} amplitude after MK-801 blocking trial: 83 ± 7% of the initial I_{AMPAR}, P = 0.06; baseline PPR: 0.71 ± 12%, PPR after MK-801 blocking: 0.66 ± 6%, P = 0.09. n = 8). The insignificant trend toward reduced I_{AMPAR} likely resulted from weak, variable time-dependent rundown. These results indicate that MK-801 selectively antagonizes NMDARs; blocking synaptic NMDARs did not detectably change presynaptic release properties. There was very little recovery of I_{NMDAR} 2-3 min after MK-801 removal (Figure 1A, 1D2). The slight increase of I_{NMDAR} may reflect minor dissociation of MK-801 from the receptors due to the preceding trials of depolarization applied to sample the residual current after MK-801 block (Huettner and Bean, 1988). Alternatively, the weak recovery of residual current might represent a small contribution from receptor diffusion from perisynaptic to synaptic sites (Tovar and Westbrook, 2002). Regardless of the causes of the weak recovery, the small fraction of current increase under control conditions indicated that MK-801 block was relatively irreversible within the time interval of our experiments (Huettner and Bean, 1988). Applying similar blocking trials to neurons in 4 mM [Ca^{2+}] did not further reduce the residual current (blocking in 4 mM Ca^{2+}: 3.2 ± 1.0% to the initial I_{NMDAR}, n = 8; blocking in 2 mM Ca^{2+}: 4.6 ± 0.8%, P = 0.32, n = 9), suggesting application of MK-801 in 2 mM Ca^{2+} using our stimulation protocol was sufficient to block virtually all active synapses. Taken together, these results confirm that our protocol asymptotically blocked NMDARs at the population of presynaptically active terminals.

After similar blocking trials with MK-801, application of PDBu, as previously documented, strongly potentiated I_{AMPAR} (Figure 1E1 and 1E2). PDBu effects on AMPAR-mediated EPSCs have previously been shown to be primarily through presynaptic mechanisms (Malenka et al., 1986; Hori et al., 1999) (but see Carroll et al., 1998) Interestingly, PDBu also promoted a strong recovery of synaptic I_{NMDAR} after MK-801 block (Figure 1B and 1D). PDBu resulted in ~ 5-fold potentiation of the residual I_{NMDAR} after MK-801 block (Figure 1D1), and the net current recovery was about 20% of the initial current before receptor block (Figure 1D2).

cAMP increases awaken dormant presynaptic terminals, but the effect is slow to develop (Ma et al., 1999; Yao et al., 2006). We used acutely applied forskolin (FSK) to determine whether rapid cAMP increases, similar to rapid PDBu effects, recruit release from synapses that are not blocked by MK-801. As a presynaptic comparator, we also examined the effect of elevated extracellular Ca^{2+} on residual $I_{NMDAR.}$ As expected, FSK application robustly potentiated I_{AMPAR} (Figure 1E1, E2). However, in contrast to PDBu, acute FSK application after MK-801 block resulted in only a minimal recovery of post-block synaptic I_{NMDAR} (Figure 1C, 1D1 and 1D2). Evoked EPSCs in 4 mM Ca^{2+}, which is expected to increase P_r at active terminals but not to awaken dormant synapses, increased I_{AMPAR} to ~ 1.5 fold (Fig 1E1 and 1E2), but again this manipulation caused only a minimal increase of I_{NMDAR} after MK-801 block (Figure 1A1, 1D1 and 1D2). The residual current potentiation and the ratio of current recovery to the initial current before MK-801 block were similar in FSK and 4 mM Ca^{2+} (Figure 1D1 and 1D2), suggesting that acute FSK, similar to elevated Ca^{2+}, did not significantly recruit transmission from synapses protected from MK-801 block. The minimal recovery of I_{NMDAR} in 4 mM Ca^{2+} after MK-801 block, which potentiated I_{AMPAR}, also indicates that potentiation of residual I_{NMDAR} did not result from transmitter spillover to the receptor domains that were not previously exposed to transmitter.

To confirm further that the recovered I_{NMDAR} after PDBu potentiation was not mediated by residual receptors that were not completely blocked at the initially active terminals, we also examined PDBu's effect on I_{NMDAR} recovery after MK-801 block of synaptic current in elevated Ca^{2+} (4 mM). Blocking receptors with MK-801 in higher Ca^{2+}, which raises overall P_r while block is proceeding, ensures even more complete receptor block, including extrasynaptic receptors that might be recruited only under high P_r conditions. Following receptor block in elevated Ca^{2+}, PDBu still significantly promoted I_{NMDAR} recovery (PDBu potentiation: 515 ± 52% of residual current, n = 8). This result confirms that PDBu-dependent recovery of I_{NMDAR} was not caused by incomplete receptor block at the terminals in combination with transmitter spillover after potentiation. We

hypothesized that PDBu-induced I_{NMDAR} recovery after MK-801 block represents a unique

presynaptic effect of phorbol ester potentiation at initially dormant synapses.

Modulators equally enhance I_{AMPAR} and I_{NMDAR}.

To test our hypothesized explanation of phorbol induced current recovery after MK-801 block, we

first performed various tests of a postsynaptic explanation for the PDBu-induced I_{NMDAR} recovery.

Differential effects of PDBu and FSK on post-MK-801, residual I_{NMDAR} may result from preferential

potentiation of specific postsynaptic receptors (i.e. PDBu preferentially potentiated I_{NMDAR}, or FSK

preferentially potentiated I_{AMPAR}). In mature hippocampal neurons, AMPARs and NMDARs are

co-localized in excitatory terminals (Forsythe and Westbrook, 1988; Bekkers and Stevens, 1989);

pure presynaptic potentiation typically affects AMPAR- and NMDAR-mediated EPSCs in parallel

(Perkel and Nicoll, 1993; Tong and Jahr, 1994). If PDBu and FSK preferentially modulated

NMDARs or AMPARs, we would expect that PDBu and FSK should differentially affect EPSCs

mediated by NMDARs versus EPSCs mediated by AMPARs. We measured dual component

EPSCs before and after modulator application in the absence of MK-801 (Figure 2A). We defined

the amplitude of the AMPAR component of the EPSC as the current peak within 10 ms after

stimulation was initiated; whereas the amplitude of I_{NMDAR} was denoted as the averaged current

within a 5 ms bin 35 ms after stimulation initiation (Tsien et al., 1996; Myme et al., 2003).

Application of PDBu potentiated I_{AMPAR} and I_{NMDAR} in parallel, with no distinguishable difference in

the degree of potentiation (Figure 2A left and 2B left panel). This result is consistent with the idea

that acute PDBu treatment has primarily a presynaptic action. After acute incubation with FSK,

potentiation in I_{AMPAR} was also indistinguishable from I_{NMDAR} potentiation (Figure 2A right and 2B

right panel). Thus, FSK treatment also resulted in an equal enhancement of I_{AMPAR} and I_{NMDAR}.

These results showed that with brief incubation, phorbol esters and FSK did not preferentially

augment current mediated by specific postsynaptic receptors, and therefore suggest a

presynaptic effect of EPSC potentiation.

Increases in open probability (P_o) of NMDARs have been reported with phorbol ester stimulation and/or PKC activation (Durand et al., 1992; Xiong et al., 1998; Lan et al., 2001). An increase in P_o might be expected to result in non-parallel modulation of AMPAR and NMDAR EPSC components. Therefore, the results in Figure 2 argue against a PDBu effect on P_o under our conditions. In addition, if phorbol-stimulated increases in P_o of NMDARs, which contributes to the I_{NMDAR} recovery after MK-801 block, we would also expect that phorbol esters should retard the decay of NMDAR-mediated EPSCs (Jahr, 1992). However, we found that, in the presence of PDBu, the weighted time constant of NMDAR-mediated EPSCs was 178 ± 10 ms, which was not significantly different from the weighted decay constant in the baseline (164 ± 28 ms, n = 7, P = 0.86). Therefore, PDBu-mediated recovery of I_{NMDAR} does not result from increased P_o of NMDARs under our conditions.

Phorbol ester does not increase surface NMDARs.

Work from others has shown that prolonged treatment with phorbol esters promotes surface NMDAR insertion and receptor assembly in hippocampal neurons (Lan et al., 2001; Scott et al., 2001). We next verified if acute treatment with PDBu in our system leads to detectable increases in surface NMDARs that might cause I_{NMDAR} recovery following MK-801 block. To test if PDBu increased surface NMDARs, we blocked whole-cell NMDARs by applying MK-801 (20 μM) in the presence of the exogenous agonist NMDA (1 mM) and examined the increases of the whole-cell I_{NMDAR} after PDBu treatment. Whole-cell blockade of NMDARs depressed NMDA-evoked current to ~ 4% of the initial current (4.3 ± 1.8%, n = 6, Figure 3A1 upper panel). Whole-cell blockade of NMDARs affected neither evoked synaptic I_{AMPAR} (104 ± 10% of baseline, n = 6) nor PPR measured by I_{AMPAR} (baseline PPR: 0.89 ± 15%; PPR after whole-cell block: 0.78 ± 17%, P = 0.32), suggesting that blocking whole-cell NMDARs did not affect presynaptic transmitter output. Two min after MK-801 wash-off, residual whole-cell responses to NMDA were slightly increased (Figure 3A1 upper panel, 3A2). The slight increase of whole-cell I_{NMDAR} may result from weak

unblocking of antagonist from the receptors during the preceding trial of NMDA application, which measured the residual current after whole-cell MK-801 block (Huettner and Bean, 1988). Acute PDBu application after whole-cell receptor blockade resulted in minimal increases in the NMDA-evoked whole-cell current (Figure 3A1 lower panel). The mild increases in NMDA-evoked current in PDBu was indistinguishable from that in control (Figure 3A2), suggesting that acute application of PDBu did not significantly increase total NMDARs at the cell surface.

To test whether PDBu selectively increases synaptic NMDARs, we examined PDBu's effect on the synaptic I_{NMDAR} after whole-cell blockade of NMDARs. Consistent with the results from sampling whole-cell NMDA responses, we found that after whole-cell blockade of NMDARs, PDBu application did not significantly increase synaptic I_{NMDAR} compared to control cells (Figure 3B1 and 3B2), despite strong potentiation measured in I_{AMPAR} (225 ± 32% of baseline, n = 6. $P <$ 0.04). These results therefore suggest that acute treatment of PDBu did not lead to significant increases of NMDARs in postsynaptic sites. Taken together, our results suggest that acute application of PDBu does not trigger detectable increases of surface NMDARs.

Phorbol-mediated recovery of I_{NMDAR} is PKC-independent

Under certain conditions, phorbol esters enhance the clustering, movement, and channel gating of NMDARs (Durand et al., 1992; Kutsuwada et al., 1992; Lu et al., 2000; Lan et al., 2001; Scott et al., 2001; Groc et al., 2004), and these effects are PKC-dependent (Durand et al., 1992; Xiong et al., 1998; Lu et al., 2000; Lan et al., 2001; Scott et al., 2001; Fong et al., 2002), Presynaptically, phorbol esters act at least partly through PKC-independent mechanisms to potentiate transmission (Searl and Silinsky, 1998; Rhee et al., 2002; Lou et al., 2008). To distinguish whether postsynaptic PKC activation by PDBu results in NMDAR modification, which in turn leads to the current recovery after MK-801 block, we evaluated PDBu's action after pharmacological inhibition of PKC activity. Because PKC activity is present in both presynaptic and postsynaptic compartments, we first tested PKC-independent presynaptic potentiation of

PDBu. We measured PDBu potentiation of I_{AMPAR} in the presence a PKC inhibitor. We used the broad-spectrum PKC inhibitor Gö6983 to block PKC activation by phorbol esters. The pharmacological activity of Gö6983 was previously verified by two independent assays (Chang and Mennerick, 2009). Application of Gö6983 alone did not alter evoked AMPAR-mediated EPSCs (control: 5.06 ± 0.98 nA, n = 9; Gö6983: 4.82 ± 0.78 nA, n = 10; P = 0.85). Preincubation of Gö6983 did not prevent subsequent PDBu potentiation of I_{AMPAR} (Figure 4A1, 4A2, and 4A3), consistent with an important role of PKC-independent mechanisms in phorbol's potentiation (Rhee et al., 2002).

Having confirmed that PDBu effectively potentiated presynaptic neurotransmission in the absence of PKC activation, we next asked if PKC inhibition, which should also prevent postsynaptic modification of NMDARs, compromises current recovery by PDBu. After MK-801 block of synaptic NMDARs, PKC inhibition did not prevent PDBu-mediated recovery of synaptic I_{NMDAR} (Figure 4B2 and 4B3) compared to PDBu alone. Inhibiting PKC activity by another class of PKC inhibitor, Ro31-8220, yielded a similar result (I_{AMPAR} potentiation: 172.1 ± 12.8% in PDBu/Ro31-8220, n = 11; 246.1 ± 36.1% in PDBu, n = 10; P = 0.07. I_{NMDAR} recovery: 20.5 ± 4.7% in PDBu/Ro31-8220, n = 11; 32.5 ± 7.1% in PDBu, n = 10; P = 0.17). These results suggest that PKC-dependent modulation of postsynaptic NMDARs does not contribute strongly to the recovery of I_{NMDAR}. Taken together, these results suggest that PDBu's enhancement of synaptic strength at low P_r or dormant terminals is mainly PKC-independent.

Synaptic release properties and phorbol-mediated potentiation

Our results thus far rule out postsynaptic explanations for PDBu-mediated revival of I_{NMDAR} after synaptic MK-801 block, and support the idea that PKC-independent presynaptic actions of PDBu recruit release from dormant terminals.   In the literature, phorbol esters increase P_r of synapses and hence enhance synaptic strength (Malenka et al., 1986; Shapira et al., 1987). In most studies, the P_r effect of phorbol esters is evaluated by paired-pulse modulation (Shapira et al.,

1987; Muller et al., 1988; Yawo, 1999), or by the ratio of charge integral from an isolated evoked EPSC relative to the release from the entire release-ready vesicle pool (Basu et al., 2007; Wierda et al., 2007). These estimates, however, evaluate the conglomerate of changes of P_r from the entire population of synapses (Branco and Staras, 2009). Therefore, nothing is known about the P_r profile of the initially dormant synapses once their release activity is restored.

The P_r profiles of a population of synapses can be revealed by the kinetics of MK-801 progressive block of I_{NMDAR} (Hessler et al., 1993; Rosenmund et al., 1993). We used the kinetic changes in the progressive block after PDBu potentiation to probe changes of P_r distribution in the activated synapses after potentiation. We also examined the P_r profile from those basally quiescent terminals, once they were activated by PDBu, with similar MK-801 progressive block.

In control cells, the decay of the first 25 peak NMDAR EPSCs during MK-801 progressive block was best described by a sum of two exponentials (Figure 5 A2, open circle), suggesting that synaptic transmission was composed of fast and slow components, which represent at least two populations of synapses with high and low P_r (Hessler et al., 1993). Note that this observation does not exclude the idea of a continuous distribution of P_r (Rosenmund et al., 1993; Murthy et al., 1997).

PDBu treatment prior to any MK-801 application accelerated the subsequent MK-801 progressive block of the current (Figure 5A1, Figure 5A2, solid circle). Because we have previously ruled out the effect of PDBu on P_o of NMDARs (Figure 2), the most plausible explanation for the faster EPSC block by MK-801 is that PDBu elevated P_r across synapses. The accelerated decay during progressive current block also yielded a greater proportion of the fast component compared to the control (23.8 ± 9.8% vs. 64 ± 3%; see Figure 5A2 legend for full fit parameters), suggesting that PDBu increased the average P_r across synapses, as well as increasing the proportion of high P_r terminals (Rosenmund et al., 1993).

Blocking synaptic NMDARs at untreated synapses eliminated I_{NMDAR} from synapses that respond to our stimulus protocol. After receptor blockade, PDBu-recovered I_{NMDAR} as described previously. The MK-801 progressive block of PDBu-recovered I_{NMDAR} also consisted of bi-exponential components with significantly slower decay (Figure 5A2, gray circle) compared to the basal, unblocked population of terminals that were potentiated by PDBu prior to progressive block. The slower decay of the EPSC block in the recovered current suggests that the recovered current arises from a subset of terminals that exhibited significantly lower P_r than the unblocked PDBu-potentiated population.

Interestingly, upon PDBu activation, the progressive block profile of the quiescent synapses was very similar to that of the initially active terminals in the basal condition (Figure 5A2). The contrast in P_r profiles between PDBu-recovered current and the current that was derived from the active synapses in the presence of PDBu suggests that PDBu did not potentiate synaptic output uniformly across terminals.   The release activity of initially dormant synapses was restored by PDBu. However, such restoration did not translate to the P_r potentiation profile of the initially active terminals.   Instead, the P_r profile from the awakened terminals was closer to the baseline P_r profile of active synapses at baseline condition. The different P_r distribution from the initially active terminals and quiescent terminals after PDBu potentiation may suggest that initial release competence of the terminal affects the nature of PDBu potentiation.

To test further whether the initial release properties of synapses affects their response to PDBu, we examined if PDBu differentially potentiates synaptic output from terminals with different P_r. We reasoned that a single episode of brief depolarization should evoke transmitter release from the terminals with the highest P_r, the synaptic output from the high P_r terminals should dominate the isolated EPSC at baseline.   Likewise, after partially (achieved with MK-801 during a limited number of stimulus trials) or fully blocking the synaptic I_{NMDAR}, which is designed to eliminate the

current contribution from the terminals with higher P_r, the residual current should represent the synaptic output from the remaining low P_r terminals. If the initial P_r state affects synaptic response to PDBu potentiation, we would expect differential effects of PDBu potentiation on the residual current from partially or fully blocked cells compared to that in the control. We compared the level of PDBu potentiation in the control current, the residual current from partially blocked cells and the residual current from fully blocked cells.

In the control cells without any preceding receptor block, PDBu, as previously shown, increased baseline I_{NMDAR} about 2-fold (Figure 5B1 left). Partial elimination of I_{NMDAR} to ~ 15% of the initial NMDAR-mediated current did not affect I_{AMPAR} (102 ± 10% of the initial I_{AMPAR} before MK-801 block, n = 10). Interestingly, PDBu treatment in partially blocked cells resulted in ~ 3-fold potentiation from the residual I_{NMDAR} (Figure 5B1 middle). When compared to the cells in which the synaptic I_{NMDAR} was fully eliminated, PDBu potentiation of the residual current was, as previously shown, close to 5-fold (Figure 5B1 right).

Because there is considerable variability in the degree of PDBu potentiation across cells, we used PDBu potentiation of AMPAR-mediated EPSCs as the internal potentiation reference, and normalized the level of PDBu potentiation in I_{NMDAR} to I_{AMPAR} potentiation from the same cell. This normalization should represent the true PDBu potentiation of I_{NMDAR} from synapses with different P_r. After normalizing to the level of I_{AMPAR} potentiation, PDBu treatment in control, unblocked cells yielded the expected relative potentiation value near 100% (resulting from parallel NMDAR and AMPAR EPSC potentiation) (Figure 5B2). When similar normalization was applied to the current potentiation from partially blocked and from fully blocked cells, the ratio of I_{NMDAR} versus I_{AMPAR} potentiation increased from ~100% in control to ~150% in the partially blocked cells and to ~200% in fully blocked cells (Figure 5B2). The graded increases of PDBu potentiation in I_{NMDAR} suggest that PDBu more strongly potentiates low P_r terminals. Thus, the initial synaptic P_r may influence synaptic response to PDBu potentiation. This result, combined with the

progressive MK-801 block experiment, is consistent with the idea that level of potentiation might

be negatively correlated to the initial release activity.

NR2B contribution of the recovered current does not differ from baseline

The results thus far suggest that PDBu potentiated post-block I_{NMDAR} by presynaptic mechanisms;

the recovered currents arise from a subset of dormant synapses, or synapses with very low P_r,

that are not recruited by Ca^{2+} or by FSK. Studies have suggested that synaptic NMDAR

composition correlates with synaptic activity as well as with developmental stage.   Synapses with

lower functionality or immature (nascent) synapses possess greater NR2B subunit

postsynaptically (Williams et al., 1993; Monyer et al., 1994; Kirson and Yaari, 1996; Tovar and

Westbrook, 1999; Ehlers, 2003). Therefore, PDBu-recovered synapses may be dominated by

NR2B-containing receptors. We used the NR2B-specific antagonist ifenprodil to probe NR2B

contribution to the recovered I_{NMDAR}. We compared the ifenprodil sensitivity of the initial I_{NMDAR}

and of PDBu-recovered I_{NMDAR}. In the control condition, the peak of the initial (untreated,

unblocked) I_{NMDAR} was reduced by ~ 60% in the presence of ifenprodil (3 μM) (left panel of Figure

6A1 and A2). After MK-801 block, PDBu-recovered I_{NMDAR} showed similar ifenprodil sensitivity

(right panel of Figure 6A1 and A2), suggesting that PDBu-recovered I_{NMDAR} consisted of a similar

NR2B contribution compared with the initial EPSC.

In addition, we also examined the deactivation kinetics of the initial and the recovered I_{NMDAR}.

EPSCs mediated by NR2B-contaning receptors feature slower deactivation kinetics compared to

EPSCs mediated by NR2A containing receptors (Cull-Candy and Leszkiewicz, 2004). The

deactivation current of NMDAR-mediated EPSC can be fitted by a sum of two exponentials,

which yields a weighted τ =132 ± 13 ms (n = 6) in the initial current (Figure 6B1 and 6B2). In the

recovered current, we found a slightly but not significantly slower decay with weighted τ =181 ±

24 ms (Figure 6B1 and 6B2). Taken together, the pharmacological and kinetic results suggest

that there is no significant difference in the level of NR2B component in the initial current and the

recovered current. Thus, the synapses that give rise to the recovered current do not preferentially harbor NR2B-containing receptors.

Phorbol ester promotes transmitter release from presynaptically silent synapses

Our results suggest that phorbol esters promote synaptic output from synapses that contribute very little to baseline transmission. These synapses could represent synapses with very low P_r and/or synapses that are completely dormant. Work from our group and others has shown that a fraction of glutamate terminals appear to be completely dormant presynaptically (Rosenmund et al., 2002; Altrock et al., 2003; Moulder et al., 2004; Ting et al., 2007). These latent synapses are considered presynaptically inactive because they contain presynaptic vesicles, identified by immunoreactivity for vesicular glutamate transporter 1 (vGluT-1) or other vesicle markers, but fail to recycle vesicles, defined by FM1-43FX uptake upon strong depolarization designed to trigger recycling of all release-competent vesicles. We next asked if PDBu-recovered EPSCs represent, in part, restoration of synaptic activity at these presynaptically inactive terminals. We examined the percentage of inactive synapses from the cultures that were acutely exposed to FSK or to phorbol ester for 2 min. The synaptic activity of individual terminals was assessed by FM1-43FX labeling with hyperkalemic depolarization (45 mM $[K^+]_o$ for 2 min) (Pyle et al., 2000; Harata et al., 2001); excitatory terminals were then identified by positive immunoreactivity for vGluT-1. Inactive excitatory synapses were those that were immunoreactive for vGluT-1 but lacking FM1-43FX labeling. In the control condition, the percentage of inactive synapses from the identified excitatory terminals was about 30% (Figure 7A left and 7B). Acute application of PDBu prominently reduced the percentage of inactive synapses (Figure 7A middle and 7B), indicating restored vesicle cycling at initially dormant terminals. In contrast to PDBu, acute application of FSK did not change the percentage of inactive synapses (Figure 7A right and 7B).

Our previous work has shown that prolonged treatment (4 hr) with FSK reduces the percentage of inactive synapses (Moulder et al., 2008). In sibling cultures of those represented in Figure 7, we

repeated the experiments with 4 h FSK treatment and independently verified the reduction in dormant terminals with prolonged FSK treatment (13.6 ± 2.3 % presynaptically inactive glutamatergic terminals compared with 27.2 ± 2.9 % in control, n = 5; P < 0.0008). Therefore, the effects of prolonged activation of cAMP-dependent pathways are apparently distinguishable from acute potentiation by FSK. In summary, acute treatment of PDBu, but not FSK, effectively activated transmitter release from the preexisting presynaptically silent synapses.

Phorbol ester potentiation in depolarization-silenced terminals

We previously reported that increasing network activity by prolonged (4 h) depolarization with 35 mM extra cellular potassium ($[K^+]_o$) depresses synaptic output (Moulder et al., 2004). The synaptic output is depressed as a result of binary reduction of functional presynaptic terminals (Moulder et al., 2004). Therefore, prolonged depolarization presynaptically inactivates a subset of terminals. We next asked if depolarization-inactivated presynaptic terminals, like the dormant synapses in baseline conditions, can be reactivated by phorbol esters.   If PDBu restores release from depolarization-inactivated terminals, we would expect a greater I_{AMPAR} potentiation and a greater I_{NMDAR} recovery in depolarized cultures, relative to the depressed baseline in these cultures. We depolarized neurons with 35 mM $[K^+]_o$ for 4 h and examined if PDBu treatment more strongly potentiates I_{AMPAR}, and more strongly recovers post-block I_{NMDAR}. Depolarization reduced the average I_{AMPAR} by 70% (Figure 8A1 and A2) as previously reported (Moulder et al., 2004).   In the depolarized neurons, PPR did not change while the evoked EPSC was depressed (Figure 8A3), suggesting that P_r from the remaining active terminals did not change. These results are consistent with the idea of binary inactivation of terminals (Moulder et al., 2004). In the depolarized neurons, PDBu treatment did not cause more potentiation of I_{AMPAR} compared to the control (non-depolarized; Figure 8B). Similarly, PDBu resulted in a similar level of recovery in post-block I_{NMDAR} (Figure 8C). These results suggest that phorbol esters do not potentiate synaptic output from the synapses that are inactivated by prolonged depolarization.

3.5 Discussion

In this study, we used the irreversible open-channel blocker MK-801 and activity-dependent presynaptic labeling to probe the properties of quiescent synapses after synaptic potentiator treatment.   By electrophysiological and imaging approaches, presynaptically quiescent synapses are rapidly activated by PDBu, but not by FSK or by elevated extracellular Ca^{2+}; both basally release-active and dormant, refractory terminals are potentiated by PDBu. After PDBu activation, initially quiescent terminals display a heterogeneous array of P_r, reminiscent of the P_r of normally active synapses.   These quiescent synapses contain NMDA receptors pharmacologically and kinetically indistinguishable from those of active synapses. Lastly, PDBu restores only the release at basally quiescent synapses, but not at depolarization-inactivated synapses.   These observations highlight different mechanisms underlying seemingly similar second messenger-mediated presynaptic potentiation; while acute FSK-mediated potentiation mainly enhances synaptic output from the preexisting active terminals, phorbol esters increase P_r at the active terminals and restore release competence at basally quiescent synapses. Our work offers unique insight into the correlation between the basal functionality of the synapses and their response to second messenger modulation. These observations may help to elucidate the roles of different signaling pathways in temporal or spatial changes of neuronal connectivity.

Presynaptic causes of PDBu-mediated I_{NMDAR} recovery.

In addition to increasing the number of presynaptically functional synapses, several mechanisms could potentially cause the potentiation-associated revival of the synaptic I_{NMDAR} after MK-801 block.   One possibility is that transmitters spill out from synapses as a result of transmission enhanced by conventional increases in P_r. Such enhanced transmitter overlap from active terminals may taint receptors that are not previously exposed to transmitter during MK-801 application and could cause phorbol-induced recovery of I_{NMDAR}. Multiple studies have shown that enhanced presynaptic release increases the likelihood of transmitter spillover, especially when glutamate transporter are compromised (Mennerick and Zorumski, 1995; Asztely et al.,

1997; Diamond, 2001; Arnth-Jensen et al., 2002; Christie and Jahr, 2006). In the present study, however, neither of two additional presynaptic potentiators produced significant I_{NMDAR} recovery, suggesting that increasing transmission alone, with any accompanying spillover, is not sufficient to cause the level of current recovery by PDBu after MK-801 block.

Another alternative explanation for phorbol effects after MK-801 block is postsynaptic modification of NMDARs. Several studies show that phorbol esters increase surface expression of NMDARs (Lan et al., 2001; Scott et al., 2001), alter gating properties of the receptors (Durand et al., 1992; Lan et al., 2001; Lin et al., 2006), and enhance lateral movement of the receptors (Fong et al., 2002; Groc et al., 2004); inhibiting PKC activity abolishes phorbol's modification of the receptors (Lan et al., 2001; Fong et al., 2002; Lin et al., 2006). Multiple lines of evidence from our studies distinguish phorbol-dependent revival of I_{NMDAR} after MK-801 block from postsynaptic receptor modification. Firstly, with short treatment of phorbol esters, we failed to detect significant whole-cell or synaptic current recovery resulting from additional receptor insertion after blocking the preexisting surface receptors, despite robust presynaptic potentiation assessed by I_{AMPAR} . Secondly, although not all presynaptic effects of phorbol esters are PKC-dependent, the postsynaptic effects on NMDARs are PKC-dependent (Durand et al., 1992; Lan et al., 2001; Scott et al., 2001; Fong et al., 2002; Lin et al., 2006).   Preventing PKC activation in the presence of PDBu, which did not compromise presynaptic potentiation, did not hamper I_{NMDAR} recovery. Thirdly, the difference in progressive NMDAR block of PDBu-recovered I_{NMDAR} compared with progressive block of PDBu-potentiated naïve synapses excludes the possibility of simple lateral movement of NMDARs into active terminals. Finally, results from presynaptic, activity-dependent FM dye labeling (Figure 7) directly support a presynaptic explanation for PDBu-induced I_{NMDAR} recovery

Presynaptically dormant synapses

Synapses with low functional connectivity, caused either presynaptically or postsynaptically, serve as a reservoir that increases the potential malleability of neurons (Voronin and Cherubini, 2004; Kerchner and Nicoll, 2008). Presynaptically silent synapses have been shown in both developing and mature hippocampal cultures (Tong et al., 1996; Ma et al., 1999) and slices (Kullmann et al., 1996; Gasparini et al., 2000), spinal cord (Jack et al., 1981), and cultured sensory to motor neurons in *Aplysia* (Kim et al., 2003). In hippocampal cultures, release at these quiescent synapses can be recruited by repetitive stimulation (Shen et al., 2006; Yao et al., 2006), application of the neurotrophic factor BDNF (Collin et al., 2001; Slutsky et al., 2004), or by prolonged activation of cAMP signaling (Ma et al., 1999; Moulder et al., 2008). The neurotrophic or activity-dependent activation of these dormant synapses may involve cytoskeletal reorganization at the presynaptic terminals, which facilitates the maturation of release apparatus (Slutsky et al., 2004; Shen et al., 2006; Yao et al., 2006).

Our results highlight temporally distinct actions of FSK modulation. Consistent with previous studies, we show that FSK stimulates rapid presynaptic potentiation at basally active terminals (Trudeau et al., 1996). FSK also elicits longer term changes in static release properties by unsilencing dormant terminals (Chavis et al., 1998; Ma et al., 1999; Moulder et al., 2008). Unsilencing by chronic FSK treatment most likely depends on protein synthesis or other slowly developing effects that replenish the molecular components essential for functional vesicle release (Ma et al., 1999; Yao et al., 2006).

The contrast between immediate actions of FSK and phorbol esters may reflect distinct downstream targets of the modulators. The need for slow, cAMP-dependent unsilencing pathways, nonetheless, makes the rapidity of PDBu unsilencing quite surprising, and may suggest multiple routes to synaptic unsilencing. Our results show that the rapid unsilencing by phorbol is PKC-independent. Because PKC-independent phorbol ester effects involve activation

of vesicle priming protein Munc13 (Betz et al., 1998a; Rhee et al., 2002), PDBu-induced synaptic awakening likely involves Munc13-induced vesicle priming at dormant terminals.

Our previous work has demonstrated a form of activity-dependent presynaptic adaptation of synaptic output; chronic depolarization presynaptically silences glutamate transmission (Moulder et al., 2004; Moulder et al., 2006). Depolarization-silenced terminals are apparently distinct from basally quiescent terminals, as PDBu restores the release activity at basally quiescent synapses but not at depolarization-silenced synapses. We have demonstrated this both with imaging (Moulder et al., 2008) and with electrophysiological assessments (present work). Although reduced levels of the priming protein Munc13-1 appears to be a commonality between basally silent and depolarization-silenced synapses (Jiang et al., 2009), other components contributing to release-competence must differ to explain the selective PDBu sensitivity of basally dormant synapses. Therefore, there may be at least two partially independent molecular pathways that lead to presynaptic silencing. Further study is required to delineate the molecular distinction between the two classes of presynaptically silent synapses.

Phorbol-mediated potentiation and P_r.

An increased average P_r from a population of synapses can be achieved by increasing P_r at each terminal, and/or by increasing the proportion of high P_r terminals. According to the accelerated fast decay and the increased fast component in MK-801progressively block after PDBu potentiation, phorbol esters seem to increase the proportion of high P_r terminals. This interpretation is consistent with the increases of vesicle turnover by PDBu observed by others (Waters and Smith, 2000), and echoes the idea that phorbol esters act on Munc13 to enhance fusion efficiency of vesicles (Basu et al., 2007). A similar P_r shift is observed with FSK treatment at the calyx of Held (Kaneko and Takahashi, 2004), suggesting that both FSK and phorbol esters increase P_r at functional terminals.

Interestingly, after PDBu potentiation, the P_r profile of initially quiescent synapses does not match the P_r profile of the entire population of PDBu-potentiated synapses (Figure 5A2). This could represent a deficiency in the quiescent synapses that renders these synapses unable to exhibit phorbol-induced P_r potentiation. By this view, phorbol esters have two entirely independent effects. One effect increases P_r at already active synapses, and the other effect restores dormant synapses to an active state with a baseline P_r distribution. Alternatively, the dormant synapses represent synapses at the extreme low end of the P_r spectrum. By this view, the phorbol effect on the dormant synapses is an extension of the observation that the lowest P_r terminals are most sensitive to phorbol stimulation. By this view, the similar rate of progressive block between the naïve synapses and PDBu-recovered synapses is coincidental. This latter view is somewhat more attractive on parsimony grounds since it does not postulate different molecular mechanisms for the P_r increases and unsilencing effects.   However, this interpretation is difficult to reconcile with the observations that elevated Ca^{2+} acute FSK, and FM1-43 labeling protocols are all ineffective in recruiting the PDBu-sensitive low P_r terminals.

Taken together, our observations provide evidence for differential potentiation by synaptic modulators and suggest that presynaptically dormant synapses can be rapidly recovered. Our results suggest multiple molecular and physiological pathways, including those that target vesicle priming, can trigger presynaptic unsilencing. Our studies also provide new insight into the multiple presynaptic actions of phorbol esters. Rapid awakening of dormant terminals is a form of plasticity that may be particularly well suited for altering the functional connectivity of neural networks.

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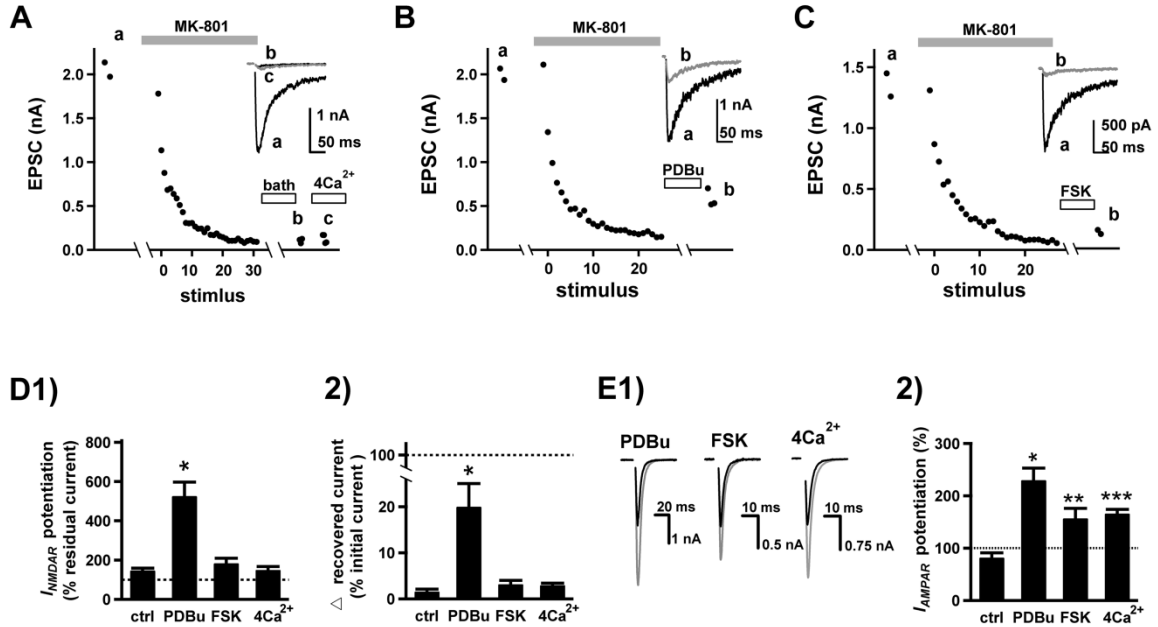
Figure 1. Modulators differentially potentiate NMDAR-mediated EPSCs after MK-801 block of synaptic NMDARs. A) Example of progressive MK-801block during evoked stimulation and subsequent recovery of I_{NMDAR}. Synaptic receptor blockade was accomplished by delivering low frequency presynaptic stimulation (0.1 Hz) in the presence of MK-801 (5 µM). Gray bar indicates the time period when MK-801 was applied; open bars application of regular extracellular saline solution (bath) or of elevated Ca^{2+} ($4Ca^{2+}$). The recovered I_{NMDAR} in regular external bath or 4 mM Ca^{2+} was sampled 2-3 min after MK-801 wash-off. The amplitude of I_{NMDAR} is plotted against the stimulus episode. Inset, superimposed initial (a), the basal recovered I_{NMDAR} (b) and the recovered current in 4 mM Ca^{2+} (c) shown from the corresponding time point in the amplitude-stimulus plot. B) and C) Similar to A), with PDBu (1 µM) (B) or FSK (50 µM) (C) applied during the 2 min interval after MK-801 wash-off. D1) Summary of synaptic I_{NMDAR} potentiation by modulators after synaptic receptor blockade. The residual I_{NMDAR} after MK-801 block is set as 100% (PDBu: 526 ± 71%, n = 10, *P < 0.00007; FSK: 184 ± 25%, n = 7; 4 mM Ca^{2+}: 150 ± 17%, n = 8. Statistical comparison is to the control: 147 ± 12%, n = 8). D2) Comparison of the

percentage I_{NMDAR} recovery (Δ recovered current = recovered current – residual current) after

modulator treatment. The Δ recovered I_{NMDAR} is normalized to the initial I_{NMDAR}. The initial I_{NMDAR}

is set to 100% (PDBu: 20 ± 5%, n = 10, *P < 0.005; FSK: 3.2 ± 0.8%, n = 7; 4 mM Ca^{2+}: 3.0 ±

0.4%, n = 8. Statistical comparison is to the control: 1.6 ± 0.5%, n = 8). E) Synaptic NMDAR

blockade did not affect I_{AMPAR} potentiation by modulators. E1) Sample traces of I_{AMPAR} in 4 mM

Ca^{2+}, PDBu and FSK (gray); traces are superimposed on their corresponding initial EPSC (black).

The percentage potentiation of EPSCs by modulators is summarized in E2) (PDBu: 230 ± 23% of

initial I_{AMPAR}, n = 10, *P < 0.0002; FSK: 157 ± 19% of the initial I_{AMPAR}, n = 7, **P < 0.02; 4 mM

Ca^{2+}: 163 ± 8% of the initial I_{AMPAR}, n = 7, ***P < 0.002. All statistics are compared to the control:

83 ± 8%, n = 8).
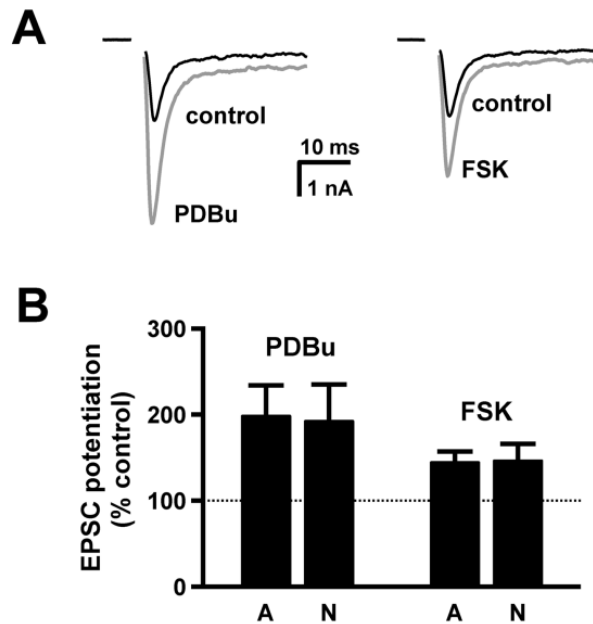
Figure 2. Modulators equally potentiate AMPAR- and NMDAR-mediated EPSCs.   A) The effect

of modulators on AMPAR- and NMDAR EPSCs was examined by comparing AMPAR- and

NMDAR components of EPSCs before and after 2 min of modulator application. Examples of

EPSC in PDBu (left panel, gray), or FSK (right panel, gray) are superimposed on the respective

baseline EPSCs (black). B) Summary of potentiation of AMPAR (A) or NMDAR (N) components

by PDBu (left bars. I_{AMPAR}: 202 ± 34% of baseline; I_{NMDAR}: 194 ± 42% of baseline, n = 6, P = 0.55)

and FSK (right bars. I_{AMPAR}: 146 ± 11% of baseline; I_{NMDAR}: 148.8 ± 18% of baseline, n = 5, P =

0.86).
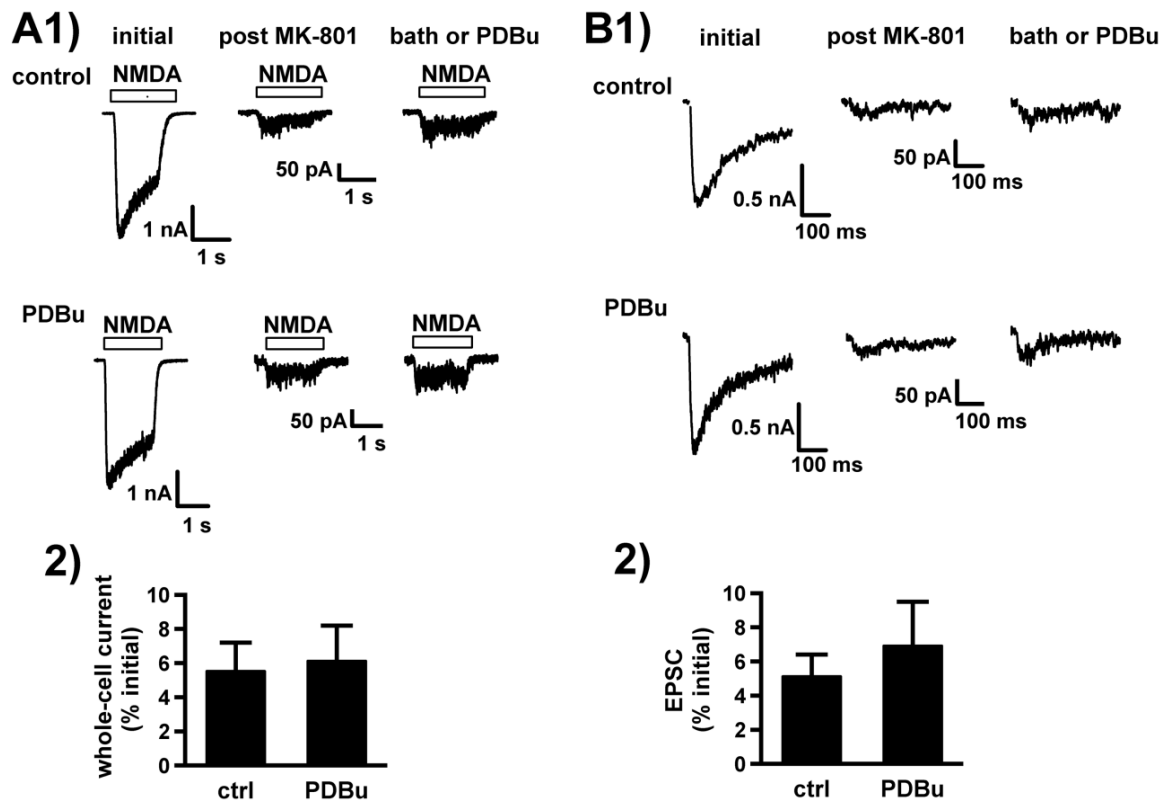
Figure 3. Acute PDBu application does not increase surface NMDARs. A1) After whole-cell blockade of NMDARs, PDBu caused minimal recovery in whole-cell I_{NMDAR}. Sample traces of time-dependent (3A1 upper) or PDBu-dependent (3A1 lower) effect on whole-cell I_{NMDAR} after blocking surface NMDARs. A2) Quantification of residual whole-cell I_{NMDAR} after control or PDBu treatment following surface receptor blockade. The peak currents are normalized to the initial current before MK-801 block (control: 5.8 ± 2.5%, n = 6; PDBu: 6.2 ± 2.2%, n = 6. P = 0.58). B1) and 2) Similar to A1) and 2), The comparison of time-dependent effect and PDBu's effect on synaptic I_{NMDAR} after whole-cell NMDAR blockade (control: 5.2 ± .1.2%, n = 6; PDBu: 7 ± 2.5%, n = 6. P = 0.59)
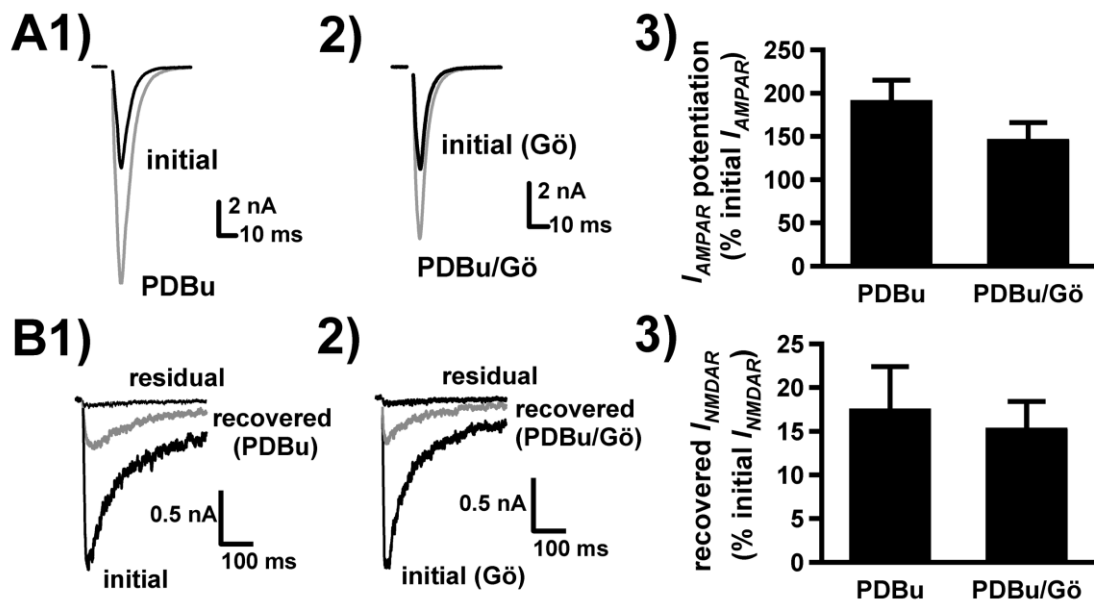
Figure 4. PKC inhibition does not prevent I_{NMDAR} recovery by PDBu. A) PDBu effectively potentiated I_{AMPAR} (A1) and robustly recovered I_{NMDAR} (B1) after MK-801 block. A1) Sample trace of I_{AMPAR} potentiation after PDBu treatment from the same cell is superimposed on the initial current. B1) PDBu-recovered I_{NMDAR} is superimposed on the initial (before blocking) and the post-block residual current. A2) and B2) Similar to A1) and B1), PKC inhibitor Gö6983 was present prior to and during the experiments. A3) Summary of PDBu's effect on I_{AMPAR} potentiation in the absence (PDBu) or presence (PDBu/Gö) of PKC inhibitor (192.3 ± 23.5% in PDBu, n = 9; 147.2 ± 19.1% in PDBu/Gö6983, n = 11. P = 0.15). Summarized effects on I_{NMDAR} recovery (% of initial current prior to MK-801 block) are presented in B3) (17.6 ± 4.7% in PDBu, n = 9; 15.4 ± 2.9% in PDBu/Gö6983, n = 11. P = 0.7).
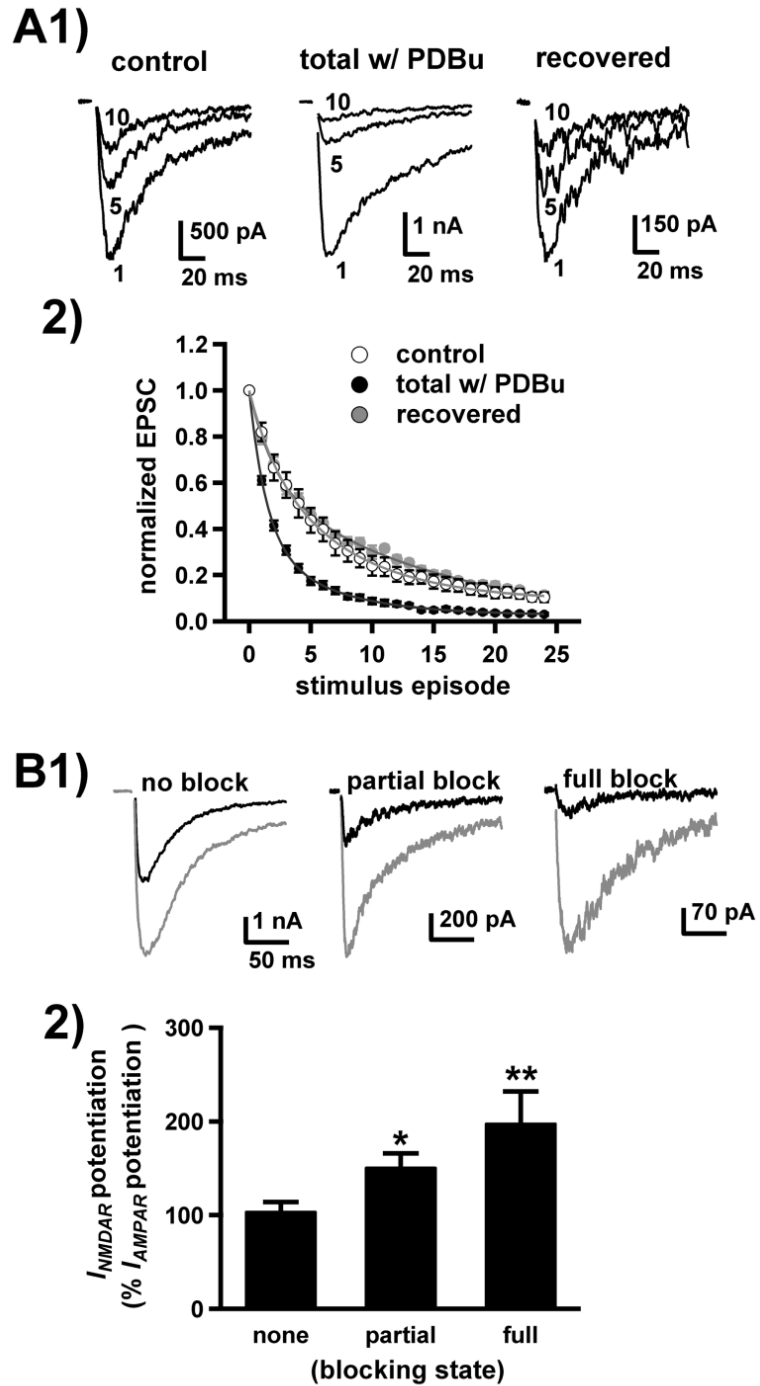
Figure 5. P_r differs at active versus dormant synapses after PDBu potentiation. A) PDBu-

activated quiescent synapses show lower overall P_r. A1) Examples of graded depression of

I_{NMDAR} during MK-801 progressive block in control synapses (left), synapses that were potentiated by PDBu without prior MK-801 block (middle) and PDBu-recovered synapses (right). EPSCs from the indicated stimulus episodes in the presence of MK-801 are superimposed. A2) Peak I_{NMDAR} during MK-801 is normalized to the initial I_{NMDAR} and plotted against stimulus episode. The plot, averaged from 8-9 cells, is best fitted by a sum of two exponentials in all three conditions. The kinetics of progressive block (25 stimuli) have τ_{fast} = 2.0 ± 0.6 (23.8 ± 9.8%) and τ_{slow} = 7.6 ± 0.9 (67.3 ± 9.2%) in control (open, n = 8), τ_{fast} = 1.38 ± 0.04 (64 ± 3%) and τ_{slow} = 5.99 ± 0.59 (32 ± 3%) in PDBu-potentiated current (solid, n = 9), and τ_{fast} = 1.98 ± 0.35 (38 ± 6%) and τ_{slow} = 14.58 ± 3.8 (62.6 ± 2%) in PDBu-recovered current (gray, n = 8). B) PDBu more strongly potentiated I_{NMDAR} at synapse with low P_r. B1) Examples of PDBu-dependent potentiation from a neuron without receptor blockade (left), a neuron with synaptic I_{NMDAR} that was partially blocked (~15% of initial current, middle), and a neuron with synaptic I_{NMDAR} that was fully blocked (right). The EPSCs after PDBu potentiation (gray) are superimposed on the EPSC prior to PDBu treatment (black). B2) Summary of PDBu-mediated potentiation in all three conditions. Level of I_{NMDAR} potentiation by PDBu is normalized to I_{AMPAR} potentiation from the same cells (no block: n = 12; partial block: n = 10; full block: n = 11. $P < 0.00003$ by one-way ANOVA. *$P < 0.003$ compared with no block and **$P < 0.05$ compared with partial block by paired t-test).
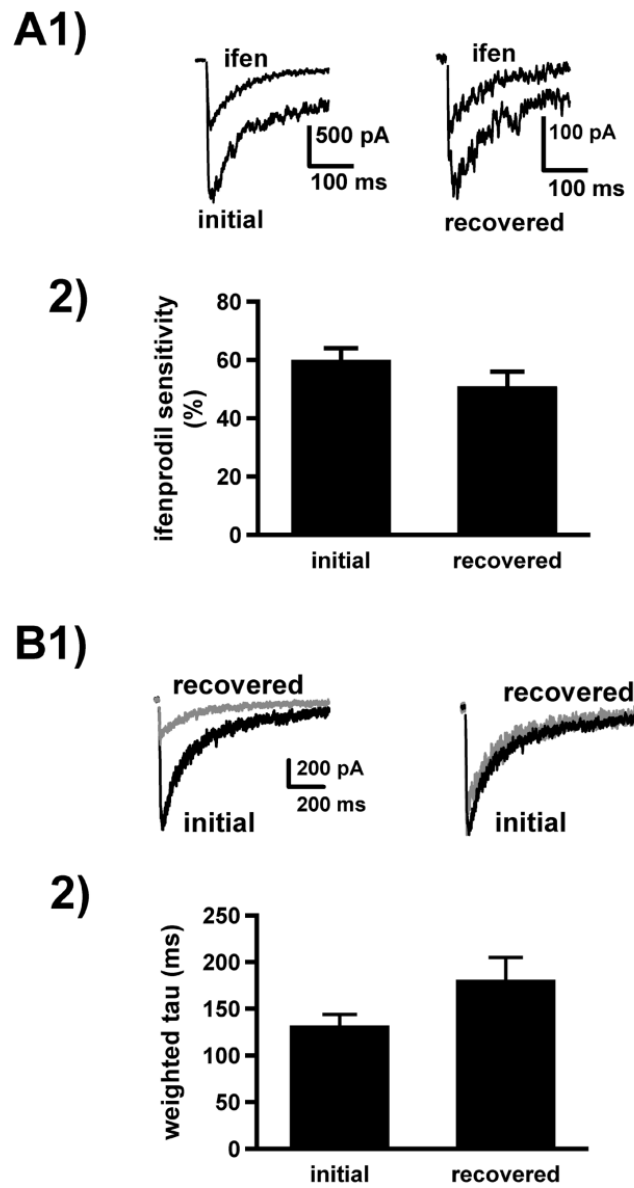
Figure 6. The initial I_{NMDAR} and PDBu-recovered I_{NMDAR} have similar NR2B contribution.   A) The initial (left) and the recovered I_{NMDAR} (right) exhibited similar sensitivity to ifenprodil (3 µM). A1) The sample traces of the initial (left) and the recovered (right) I_{NMDAR} in the presence of ifenprodil are superimposed on the current in the absence of Ifenprodil. A2) Quantitative comparison of ifenprodil-sensitivity in the initial current and in PDBu-recovered current (ifenprodil sensitivity:

59.3 ± 4.1% at baseline, n = 9; 51.8 ± 4.9% in PDBu-recovered current, n = 9. *P* = 0.06). B) The initial and the recovered I_{NMDAR} have similar deactivation kinetics. B1) The decay of the recovered I_{NMDAR} is indistinguishable from that of the initial current. Example of recovered I_{NMDAR} superimposed on the initial current (left). Right, the recovered I_{NMDAR} is scaled and superimposed on the peak of the initial current. B2) The average weighted tau of the baseline NMDAR-mediated EPSCs and the recovered EPSCs from 6 cells (weighted τ = 132 ± 13 ms in baseline; 181 ± 24 ms in PDBu-recovered current. *P* = 0.07).
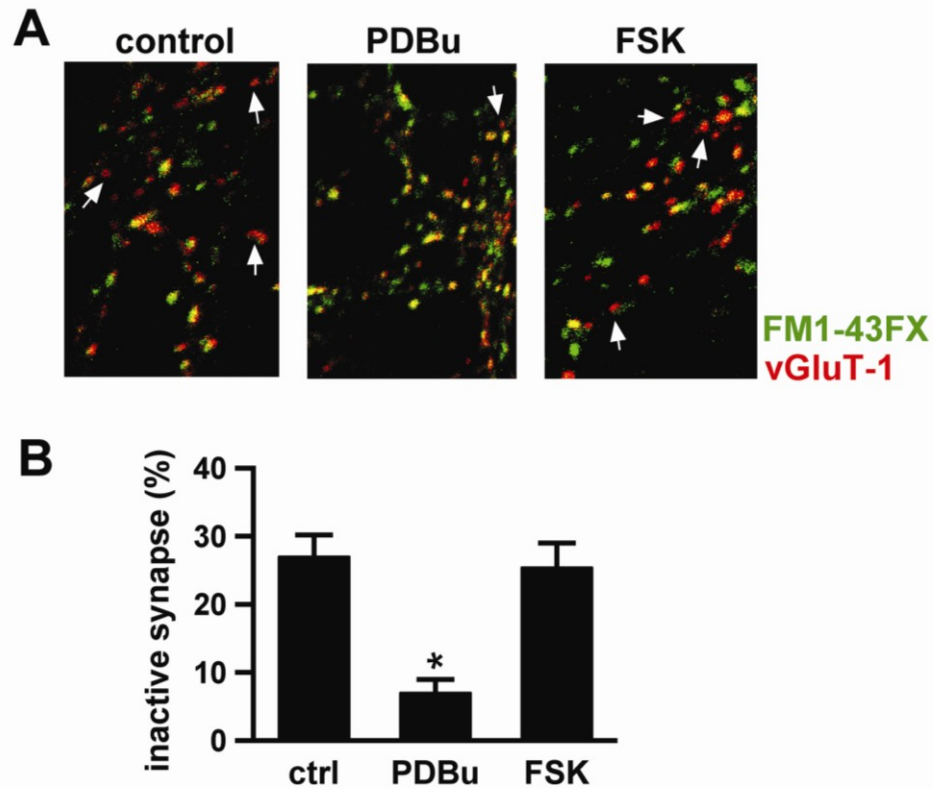
Figure 7. PDBu reduces the percentage of inactive excitatory synapses. A) Example images

from control (left), PDBu (middle) and FSK (right)-treated synapses. Active synapses were

labeled with FM1-43FX (green); glutamatergic terminals were identified by immunoreactivity to

vGluT-1 antisera (red). Puncta that are positive for vGluT-1 antisera but devoid of FM1-43FX

labeling are inactive excitatory synapses (arrows). B) Quantification of the percentage of inactive

glutamatergic terminals in control (27.2 ± 2.9%), or PDBu (7.2 ± 1.8%), and FSK (25.6 ± 3.4%)-

treated synapses (*P < 0.000002 compared to control, n = 5).
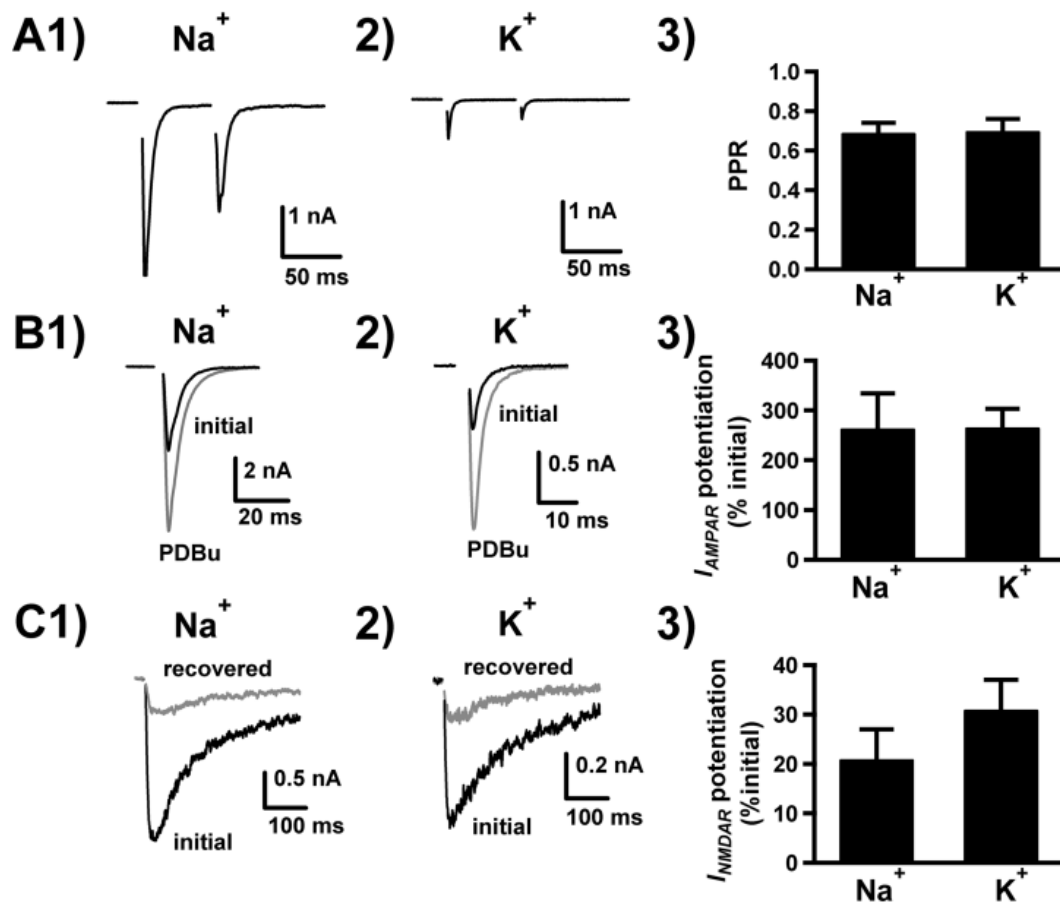
Figure 8. PDBu does not reactivate depolarization-inactivated synapses. A) Prolonged

depolarization (35 mM $[K^+]_o$ for 4 h) reduced synaptic current but did not affect paired-pulse ratio

(PPR). Sample paired AMPAR-mediated EPSCs (50 ms interpulse interval) in control (A1) and in

previously depolarized neurons (A2). EPSC measurements here and subsequently were all

performed in physiological $[K^+]_o$ (4 mM, see Material and Methods) as the rest of the

experiments . The average amplitude of I_{AMPAR} was 3.80 ± 0.94 pA in control (Na^+, n = 9), and

1.07 ± 0.34 pA in depolarized neurons (K^+, n = 10. $P < 0.03$). A3) Summary of PPR

(EPSC[2nd]/EPSC[1st]) in control (Na^+, PPR = 0.69 ± 0.05, n = 9) and in depolarized neurons (K^+,

PPR = 0.70 ± 0.06, n =10. $P = 0.84$). B) Level of I_{AMPAR} potentiation by PDBu was similar in

control and in depolarized neurons. Examples of PDBu potentiated I_{AMPAR} in control (B1) and in

depolarized neurons (B2). B3) Summary of PDBu potentiation in I_{AMPAR} in control (Na^+: 264.3 ±

70% to baseline, n = 9) and in depolarized cultures (K$^+$: 266 ± 37.7% to baseline, n = 10. P = 0.98). C1) Sample traces of PDBu-mediated recovered I_{NMDAR} in control (C1) and in depolarized neurons (C2). Recovered currents (gray) are superimposed on the initial currents (black).   C3) Summarized effect of PDBu on the recovered synaptic I_{NMDAR} in control (Na$^+$: 21.6 ± 6.1% to the initial current, n = 9) and in depolarized neurons (K$^+$: 31.3 ± 6.1% to the initial current, n = 10, P = 0.27).

Chapter 4. Summary and future directions

Synaptic potentiation has been of research interest for decades, as it likely serves as a cellular mechanism underlying memory storage. Presynaptic potentiation could result from transient accumulation of presynaptic calcium, potentiation of presynaptic calcium channels, increases in the number of readily releasable vesicles, or enhancement of vesicular fusogenic efficiency (Zucker and Regehr, 2002; Wojcik and Brose, 2007). While most studies focus on the readout of static properties from presynaptically functional terminals, not much is known about the impact of synaptic potentiation on the dynamics of activity-dependent transmission, or the impact on preexisting non-functional terminals.   In this thesis work, we used second messenger modulators that potentiate presynaptic output to study the changes in transmission dynamics and the changes in functional connectivity after presynaptic potentiation.

4.1 Asynchronous transmission and beyond

In the first part of the work, we studied the effect of calcium dynamics and P_r increases on stimulus-dependent changes in synaptic transmission.   In particular, we focused on activity-dependent asynchronous transmission. We showed that asynchronous transmission is strongly calcium-dependent, as increasing extracellular calcium exaggerates release asynchrony, whereas chelating presynaptic calcium with the slow calcium chelator EGTA-AM reduces release asynchrony. Activity-dependent asynchronous transmission is not dependent upon quick vesicle recycling, and is not selective to specific types of presynaptic calcium channels. The preferential increase of asynchronous transmission in high calcium might not be directly related to calcium-dependent P_r increases, because raising P_r by phorbol esters does not result in a similar preferential increase of asynchronous transmission. Phorbol esters, instead, potentiate phasic transmission in parallel with asynchronous transmission. PKC inhibition compromises phorbol ester-induced asynchronous increase, leading to preferential PKC-independent potentiation of

phasic transmission. PKC-independent increases of phasic transmission and PKC-dependent

increases of asynchrony therefore suggest a role of physiological DAG signals in altering

transmitter release modes through different downstream targets.   PKC activation may modulate

asynchronous transmission by altering presynaptic calcium profiles or by affecting vesicle

mobilization/replenishment.

Despite being observed almost four decades ago, asynchronous transmission was not re-

scrutinized until recent years (Rahamimoff and Yaari, 1973; Goda and Stevens, 1994; Atluri and

Regehr, 1998). While most studies focused on using non-physiological calcium concentrations or

non-physiological cations (e.g. strontium. Mellow et al., 1978; Zengel and Magleby, 1981) to

probe the action of asynchronous transmission, our work is the first evidence showing that

different components of second messenger signaling may distinctively regulate phasic and

asynchronous transmission modes. This raises the possibility that changes in transmission

dynamics by second messengers may serve as a plasticity mechanism, allowing neurons to

quickly adjust computing efficiency for information processing in the network.

There is a surging increase in identified synapses showing activity-associated asynchronous

transmission, including deep cerebellar nuclei inhibitory fibers to the inferior olive, cochlear

nucleus interneurons, hippocampal CCK (cholecystokinin) interneurons, glutamate synapses in

nucleus accumbens, hypothalamic glutamate synapses onto magnocellular neurosecretory cells,

calyceal synapses, as well as hippocampal excitatory autapses (Goda and Stevens, 1994; Lu and

Trussell, 2000; Neher and Sakaba, 2001; Hefft and Jonas, 2005; Hjelmstad, 2006; Iremonger and

Bains, 2007; Best and Regehr, 2009).   Functionally, inhibitory asynchrony is likely to prolong

inhibitory tone by transforming presynaptic firing patterns of interneurons into sustained inhibition

in the target neurons (Lu and Trussell, 2000; Hefft and Jonas, 2005; Best and Regehr, 2009).

The physiological role of excitatory asynchrony, however, remains enigmatic.

In our study, prominent activity-dependent asynchronous release was measured by AMPAR EPSCs. During the experiments, fast receptor desensitization was minimized by a rapid dissociating antagonist (kynurenate).   The physiological role of excitatory asynchronous transmission, nonetheless, would have to be considered in the absence of any exogenous antagonist.   It has been shown that, in addition to transmission depression, AMPAR EPSCs are strongly dampened due to receptor desensitization in the absence of rapid dissociating antagonists during the late phase of high frequency stimulation (Zhang and Trussell, 1994; Carter and Regehr, 2000; Chen et al., 2002). Because asynchronous transmission emerges late during high frequency stimulation when phasic transmission undergoes strong depression, it is possible that in physiological conditions (in the absence of antagonists), electrical signals triggered by asynchronous release are not mainly through AMPARs, but primarily through other ionotropic receptors, such as NMDARs. In the late phase of high frequency stimulation, the high affinity for glutamate allows NMDARs to be easily activated by transmitters when voltage-dependent inhibition of magnesium is relieved by preceding depolarization mediated by AMPAR activation. Thus, a short burst of presynaptic activity will be translated into two components in the postsynaptic neurons: the initial AMPAR-driven, fast and large transient conductance, which gradually transits to a smooth, voltage-dependent NMDAR conductance (Bal and Destexhe, 2009). Transition of postsynaptic responses from AMPAR dominant to NMDAR dominant during high frequency stimulation has been observed in retinogeniculate synapses (Chen et al., 2002). Such a postsynaptic conductance shift is accentuated by the depression of transmitter release. Since greater asynchrony is accompanied by greater phasic depression, it is possible that postsynaptic response to asynchronous transmission is dominated by the late-emerging NMDAR conductance. The time-dependent emergence of a stronger NMDAR conductance could have multiple physiological consequences on the computational power of neurons.

Neuronal information is likely coded by timing or strength (e.g., firing rate) of neuronal activity (Softky, 1995). Electrophysiological recording and theoretical simulation of cortical pyramidal neurons have shown that high-frequency stimulation, due to its association with transmission depression, results in postsynaptic responses that are more temporally coherent, and less sensitive to stimulation frequency (Tsodyks and Markram, 1997). In other words, frequency-dependent depression may help to translate presynaptic firing activity into "time-coded" information in postsynaptic firing patterns. In our study and others, phasic transmission depression associates with greater asynchronous transmission, which likely results in an extended late NMDAR-dominated conductance in postsynaptic neurons. It is possible that high frequency associated asynchronous transmission can generate a temporal "on" state through NMDAR conductance, causing tonic depolarization. This late depolarization in the dendrites may improve detection of weak heterosynaptic inputs by facilitating temporal or spatial summation of excitatory postsynaptic potentials (EPSPs), as well as enhancing input signal propagation along dendrites. EPSP summation has been observed in hippocampal CA1 pyramidal neurons in which distal entorhinal input propagation is enhanced by coincident CA3 excitatory inputs (Jarsky et al., 2005). Alternatively, transmission asynchrony may act as activity-dependent synaptic noise to increase dendritic signal detection (Hô and Destexhe, 2000; Stacey and Durand, 2001). In this case, the effect of asynchronous transmission may not necessarily depend on NMDARs. Thus, asynchronous transmission may offer temporal control over heterosynaptic signal amplification. This temporal enhancement ensures that weak synaptic inputs are detected only when appropriate reference activity from other synaptic inputs is present, thereby generating heterosynaptic activity cues for signal detection at the recipient neurons. This heterosynaptic enhancement of signal detection may be particularly important for the neurons whose synaptic inputs are segregated anatomically according to different input sources, and/or for the neurons that receive converging inputs from distinct network domains. Such neuron candidates include cortical and neocortical pyramidal neurons (e.g. hippocampal CA1 and cortical layer 5 pyramidal cells) (Gutnick and Mody, 1995; Petreanu et al., 2009; van Strien et al., 2009). In addition to increasing neuronal sensitivity to weak signal inputs, asynchrony-associated conductances could

also reduce the membrane time constant. A low membrane time constant may enhance temporal

resolution of suprathreshold neuronal responses to different signals arriving within a short time

interval by reducing refractory period (Halliday, 2000; Rudolph and Destexhe, 2001).   Increasing

neuronal responsiveness to synaptic inputs can be viewed as gain modulation (Salinas and Thier,

2000; Shu et al., 2003).   Thus, asynchronous transmission may temporally increase neuronal

input-output efficiency, either by prolonging the synaptic conductance through NMDARs, or by

increasing synaptic noise.

In the case that NMDARs are the predominant receptors mediating asynchronous transmission,

transmission asynchrony-associated NMDAR conductance could also modulate network activity

at the level of neuronal assemblies.   In cortical networks, high frequency stimulation generates

an initial phase of firing, in which the latency between presynaptic inputs and postsynaptic firing,

termed postsynaptic spike time, shows high precision among neurons, leading to a synchronous

firing pattern across neurons (Harsch and Robinson, 2000; Robinson and Harsch, 2002). High

precision spike time gradually degrades with the introduction of NMDAR conductance (Harsch

and Robinson, 2000), which causes an apparent break down of firing synchrony among neurons.

The breakdown of spike-time precision by NMDAR conductances may help to eliminate the

likelihood of synchronized runaway excitation in a network by temporally randomizing firing

activity among neurons. Therefore, asynchronous transmission may help pace network activity

by disrupting firing synchrony.

Because asynchronous transmission is activity-dependent, it is difficult to separate the

postsynaptic conductance generated by asynchronous transmission from the conductance

generated by phasic transmission using conventional electrophysiological methods. It is thus

difficult to evaluate the effect of asynchronous transmission on postsynaptic signal integration,

and on network activity patterning. Dynamic clamp is a powerful electrophysiological alternative

offering versatile applications that compensate for the limitations of conventional

electrophysiological methods (Bal and Destexhe, 2009). Dynamic clamp enables introducing to

neurons the simulated conductance components that are difficult to generate by conventional

electrophysiological methods. Using this technique, one may be able to obtain the conductance

of asynchronous transmission by subtracting the phasic synaptic conductance from the overall

postsynaptic conductance during high frequency stimulation. By combining dynamic clamp with

conventional electrophysiological approaches, it is possible to examine postsynaptic responses to

heterosynaptic inputs in the presence or absence of asynchronous transmission. This makes

possible deciphering the influences of temporal changes in transmission dynamics in

postsynaptic signal integration.

4.2 Presynaptically silent synapses - why they are silent, and why they break the silence

In the second part of the study, we offer evidence for distinct mechanisms underlying seemingly

similar synaptic potentiation in high calcium, FSK, and phorbol esters. We show that FSK and

high calcium mainly potentiate synaptic output from preexisting active terminals; phorbol ester,

however, is able to recruit transmission from presynaptically silent terminals in addition to basally

active terminals. We also show that presynaptic silencing may be a common outcome of different

molecular causes, as phorbol ester is able to activate basally silent synapses but not

depolarization-silenced synapses. These results highlight an interesting contrast to the results

from our previous work, in which prolonged FSK treatment, but not phorbol ester treatment,

restores depolarization-induced presynaptic silencing (Moulder et al., 2008). Mechanisms of

prolonged FSK-dependent unsilencing are perhaps dependent on protein synthesis (Ma et al.,

1999; Jiang et al., 2009), or cytoskeleton rearrangement (Yao et al., 2006). The differential

effects of modulators on functional connections thus reflect specific roles of distinct second

messenger pathways under different physiological circumstances. Our results raise the

possibility that presynaptically quiescent synapses could be rapidly recruited to expand neuronal connectivity when appropriate signals are delivered.

Although it is widely accepted that the presence of weak connecting synapses might serve as a reservoir to increase the malleability of neurons in response to constantly changing network activity (Voronin and Cherubini, 2004), it is still unclear how the low strength of synapses is maintained. In particular, presynaptically silent connections might represent an immature state of nascent synapses, or low connectivity might result from random competition or specific selection (e.g. postsynaptic-dependent selection) for inhibition/depression among a population of synapses.

The presence of presynaptically quiescent terminals seems to correlate with network activity (Moulder et al., 2006), it is possible that network activity determines the strength of individual synapses through synaptic plasticity. Without proper controls, synaptic plasticity, which allows strengthening or weakening of certain connections according to network activity patterns, may somehow risk destabilizing synaptic weight in a network, as the high activity connections might be further strengthened, or the low activity connections might be further weakened. In the case of excitatory strengthening, such adaptation could cause runaway excitation. To prevent network destabilization, compensatory mechanisms are required to constantly calibrate total synaptic weight in the network. This synaptic weight recalibration has been described in amygdala intercalated interneurons (Royer and Paré, 2003), and in hippocampal CA3 pyramidal cells (Kim and Tsien, 2008). In intercalated neurons, which receive multiple glutamatergic inputs from basal lateral amygdala, the potentiation of one excitatory input triggers the depression of another, leading to a relatively unchanged net synaptic weight.   Similar observations have been made in hippocampal CA3 pyramidal neurons, in which network inactivation strengthens dentate granule excitatory inputs to CA3 pyramidal neurons but weakens CA3 recurrent inputs to CA3 pyramidal neurons.   Synaptic weight calibration could result from heterosynaptic depression. Activity-

dependent heterosynaptic depression has been shown in the hippocampal CA1 region (Serrano et al., 2006; Andersson et al., 2007). Heterosynaptic depression can be mediated by calcium-induced calcium release from internal stores at the input recipient neurons (Royer and Paré, 2003), or through the intervention of astrocytes or other glial cells (Serrano et al., 2006; Andersson et al., 2007) .   It is therefore possible that presynaptic quiescent terminals observed in hippocampal dissociated cultures are the result of such persistent heterosynaptic depression for the purposes of maintaining network stability. From an excitatory feed forward regulation point of view, presynaptic silencing of terminals could set the upper limit of overall excitability, thus preventing over excitation of local network by limiting the amount of functional excitatory connections. In this case, a simplified network with minimized circuit components is fitting to decipher the potential causes of presynaptic silencing under basal conditions.

Alternatively, presynaptically quiescent terminals could result from target-specific postsynaptic effects.   It has been shown in hippocampal dissociated mass cultures that synaptic P_r is correlated with local dendritic activity; synapses at the same dendritic branch tend to exhibit similar P_r (Branco et al., 2008). In brain slices, target-specific heterogeneity of synapses that originate from the same axon are also observed in cortical layer 2/3 pyramidal neurons and in hippocampal dentate granule cells (Koester and Johnston, 2005; Pelkey et al., 2006).   In this scenario, presynaptically quiescent terminals should be geometrically segregated according to specific target cells or specific dendritic branches to which synapses innervate.   Such design may enable quick recruitment of components into existing networks, thus expanding the computational power of the circuit. The maintenance of target-specific presynaptically quiescent terminals may rely on local retrograde signals (Regehr et al., 2009).

Advances in optical techniques such as multiphoton microscopy make optical observation of synaptogenesis possible (Niell and Smith, 2004).   With appropriate cellular markers, time-lapse

imaging could reveal the patterns and processes of synapse formation in a temporal order, enabling synapses of different developmental stages to be identified (Zito et al., 2009).   The presynaptic properties of maturing synapses can be further examined by activity-dependent presynaptic labeling or by presynaptic calcium imaging, whereas postsynaptic properties can be assessed by whole cell recording in combination with transmitter uncaging. The combination of these approaches with neurons carrying appropriate genetic manipulations can help address synapse-autonomous and non-autonomous influences on P_r in situ with single-synapse resolution. The autonomous factors include the influence of synaptic maturation on presynaptic P_r and on postsynaptic functionality. Non-autonomous factors might include the requirement of presynaptic or postsynaptic activity in setting P_r, the correlation of P_r between neighboring synapses, and target-specific influences on P_r.

4.3 Conclusion

Neuronal networks cannot function without properly tuned synaptic properties. Changes in transmitter release not only reflect plastic properties of terminals, but also profoundly influence postsynaptic activity at both the neuronal and network levels. The influences of transmission on neuronal activity signify the importance of activity-dependent changes in synaptic properties. Thus, studying network dynamics will be incomplete without considering ongoing synaptic changes. Likewise, it is equally important to extend the current understanding of changes in synaptic properties to their impact on information processing in neuronal assemblies. This knowledge will shed light on the importance of codependence between macroscopic (network) and microscopic (synaptic) activity fluctuation in various forms of cognitive processing.

4.4 Reference

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