VOLTAGE-GATED SODIUM CHANNEL MODULATION OF CALCIUM DYNAMICS AND NMDA RECEPTOR SIGNALING IN MURINE NEOCORTICAL NEURONS

by

SHASHANK MANOHAR DRAVID

(Under the Direction of Thomas F. Murray)

ABSTRACT

Physiological [Mg^{2+}] produces a voltage-dependent channel block of NMDA receptors (NMDAR). Removal of Mg^{2+} from the extracellular environment therefore induces spontaneous Ca^{2+} oscillations in neurons mediated by activation of NMDAR and L-type Ca^{2+} channels. In an effort to develop a physiologically relevant model of NMDAR-mediated Ca^{2+} signaling, we studied the mechanism of Ca^{2+} oscillations in murine neocortical neurons under physiological [Mg^{2+}]. AMPA receptors (AMPAR) regulated the frequency of Ca^{2+} oscillations, which were independent of L-type Ca^{2+} channels and partially mediated by NMDAR. Activation of metabotropic glutamate receptors and release of Ca^{2+} from endoplasmic reticulum were critical for the generation of Ca^{2+} oscillations.

We further studied whether Ca^{2+}-permeable AMPAR (Ca-AMPAR) contribute to the Ca^{2+} oscillations. Ca-AMPAR exhibit characteristic permeability to Co^{2+}, not seen among other divalent cation-permeable channels. Based on this conductance property, we developed a fluorometric method using dye calcein whose fluorescence is quenched by Co^{2+}, while unaffected by intracellular Ca^{2+} levels. AMPAR did not produce measurable Co^{2+} influx in the basal state. Activation of AMPAR by bath application of AMPA or kainate induced Co^{2+} influx in neocortical neurons, which was blocked by an AMPAR antagonist. These results suggest that Ca-AMPAR may not be activated during basal synaptic activity and only mediate Ca^{2+} influx when activated by an excitatory stimulus.

We further studied the effect of brevetoxin (PbTx), potent allosteric enhancers of voltage-gated sodium channel (VGSC) function associated with "Red Tide" blooms, on Ca^{2+} dynamics and downstream signaling in neocortical neurons. Depending on the extent of VGSC activation, PbTx-2 treatment produced distinct Ca^{2+} responses resulting in distinct temporal pattern of extracellular signal-regulated kinases 1/2 (ERK1/2) activation. Differential modulation of ERK1/2 by PbTx-2 may be explained by the activation of synaptic versus extrasynaptic NMDAR. An elevation of intracellular Na^{+} and Src kinase-mediated tyrosine phosphorylation of NMDAR is known to increase NMDAR function. Inhibition of Src kinase reduced PbTx-2 induced Ca^{2+} influx. PbTx-2

treatment also increased tyrosine phosphorylation of NR2B subunit. In addition, PbTx-2 augmented NMDAR-mediated Ca^{2+} influx and ERK2 activation. These results suggest that brevetoxin upregulates NMDAR signaling in neocortical neurons which may be mediated by coincidence of an elevation of intracellular [Na^{+}] and Src kinase activation.

INDEX WORDS: voltage- gated sodium channel, NMDA receptors, calcium oscillations, neocortical neurons, brevetoxin, ERK

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DEDICATION

To

My Parents

Mrs. Madhuri (Aai) and Dr. Manohar S. Dravid

For all I am today and will ever be.

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CHAPTER 1

INTRODUCTION AND SIGNIFICANCE

Neurons are excitable cells which exhibit fast changes in ion concentrations across the plasma membrane leading to the generation of an action potential. An action potential in one neuron is relayed to another neuron through synaptic transmission which involves release of neurotransmitter and hence the term chemical transmission. Glutamate is the most abundant neurotransmitter mediating excitatory signals in the central nervous system (CNS). Glutamate activates two types of glutamate receptors: ionotropic (iGluRs) and metabotropic glutamate receptors (mGluRs). Ionotropic glutamate receptors have been subdivided into N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors based on ligand specificity and primary structure. The AMPA and kainate receptors are together referred to as non-NMDA receptors. The iGluRs are permeable to cations and largely exclude anions from the pore. NMDA receptors are more permeable to Ca^{2+} than non-NMDA receptors. Intracellular Ca^{2+} levels are tightly regulated in any cell type by membrane proteins located on the plasma membrane, endoplasmic reticulum and mitochondria. Ca^{2+} is an important second messenger and slight changes in intracellular Ca^{2+} levels can transduce cellular responses mediated by Ca^{2+}-sensitive proteins in the cytosol. Disregulation of Ca^{2+} homeostasis in a cell leads to cellular injury and death. Oscillations in cytoplasmic Ca^{2+} levels are a common mode of signaling both in excitable and non-excitable cells and can increase the efficiency and specificity of gene expression (Dolmetsch et al., 1998). Ca^{2+} oscillations act as a biological Morse code, in the sense that alterations in the temporal features of Ca^{2+} oscillations produce differential activation of certain genes (Fields et al., 1997; Hu et al., 1999).

Neurons in culture exhibit the phenomenon of synchronous spontaneous Ca^{2+} oscillations that is represented by rhythmic activation of a group of neurons in characteristic temporal and spatial scales. Synchronized Ca^{2+} oscillations in culture systems were first identified in hippocampal neurons in Mg^{2+}-free media (Ogura et al., 1987). Similar oscillatory behavior has been reported in other neuronal cell types including neocortical neurons (Wang and Gruenstein, 1997) and cerebellar granule neurons (Lawrie et al., 1993), but these oscillations are only observed upon removal or lowering of extracellular Mg^{2+}. Ca^{2+} oscillations have also been reported in older cultures of cortical neurons, which do not require the removal of Mg^{2+} from the extracellular media (Murphy et al., 1992).

NMDA receptors are coincident receptors and their activation depends on agonist binding as well as on membrane depolarization. The NMDA receptor's voltage dependence is due to the channel block by submillimolar concentration of extracellular Mg^{2+} (Mayer et al., 1987) and membrane depolarization of –50mV or more is required for the release of this Mg^{2+} block. It has been seen that removal of Mg^{2+} from extracellular media induces epileptiform activity in hippocampal and neocortical cultures (Mody et al., 1987; Rose et al., 1990) and in immature cortical neurons Mg^{2+} -dependence of Ca^{2+} oscillations has also been demonstrated (Wang and Grunstein, 1997). Removal of Mg^{2+} from extracellular media creates non-physiologic conditions and biases the occurrence of Ca^{2+} oscillations in favor of NMDA receptors. We therefore studied the mechanism of Ca^{2+} oscillations in the presence of extracellular Mg^{2+}.

As mentioned earlier NMDA receptors are more permeable to Ca^{2+} than non-NMDA receptors. The Ca^{2+} permeability of the AMPA receptors is governed by the

GluR2 subunit, the presence of which renders the heteromeric receptor Ca^{2+}-impermeable. Ca^{2+} influx through the Ca^{2+}-permeable AMPA receptors has been shown to induce neuronal injury (Turetsky et al., 1994; Lu et al., 1996) and has been implicated in selective neurodegeneration observed in several neurological diseases such as global ischemia, Alzheimer's disease and amyotrophic lateral sclerosis. Ca^{2+}-permeable AMPA receptors therefore represent an important molecular target for prevention of neuronal injury after neurological insults. The Ca^{2+}-permeable AMPA receptors have characteristic permeability to Co^{2+} which is not observed in Ca^{2+}-impermeable AMPA receptors, NMDA receptors or voltage-gated Ca^{2+} channels. Using the selective permeability of the Ca^{2+}-permeable AMPA receptors we set forth to develop a fluorometric technique for the detection of activation of Ca^{2+}-permeable AMPA receptors in murine neocortical neurons.

Brevetoxins (PbTx-1 to PbTx-10) are potent allosteric enhancers of voltage-gated sodium channel (VGSC) function produced by the marine dinoflagellate *Karenia brevis* an organism linked to periodic red tide blooms (Baden, 1989). *K. brevis* blooms have been implicated in massive fish kills, bird deaths, and marine mammal mortalities, most notably involving the endangered West Indian manatee (O'Shea et al., 1991; Bossart et al., 1998). The two known forms of red tide associated clinical entities in humans first characterized in Florida are an acute gastroenteritis with neurologic symptoms after ingestion of contaminated shellfish (i.e. NSP) and an apparently reversible upper respiratory syndrome after inhalation of the aerosols of the dinoflagellate and their toxins (i.e., aerosolized red tide toxins respiratory irritation) (Asai, 1982, Baden, 1989). Brevetoxins are known to accumulate in the CNS when administered systemically in

animals at concentrations sufficient to affect CNS (Templeton et al., 1989). These findings underscore the importance of studying the cellular consequences of brevetoxin exposure in the CNS. Ca^{2+} oscillations exhibited by neurons regulate cellular responses (Fields et al., 1997). We explored whether an external stimuli such as activation of VGSC by brevetoxin can modulate these Ca^{2+} oscillations and associated signaling. Increase in intracellular Na^{+} by VGSC activator has been shown to increase NMDA receptor activity (Yu and Salter, 1998) which is observed when there is coincidence of Src tyrosine kinase activation. In addition, NMDA receptor activity is increased by tyrosine phosphorylation of the receptor. Since brevetoxin induces increase in intracellular [Na^{+}] by activation of VGSC we tested whether brevetoxin can induce augmentation of NMDA receptor-induced Ca^{2+} influx and associated downstream signaling. Furthermore, we studied whether Src kinase activation or tyrosine phosphorylation of the receptor had any role to play in brevetoxin-induced modulation of Ca^{2+} dynamics in neocortical neurons.

The following specific aims will be attempted to accomplish the objectives:

1. To elucidate the mechanism of synchronized spontaneous calcium oscillations in neocortical neurons in the presence of physiological Mg^{2+}.
2. To determine the effect of brevetoxin on calcium oscillations and downstream signaling in neocortical neurons.
3. To explore whether brevetoxin can augment the calcium influx and signaling mediated by NMDA receptors.

References:

Asai S., Krzanowski J. J., Anderson W. H., Martin D. F., Polson J. B., Lockey R. F., Bukantz S. C., and Szentivanyi A. (1982) Effects of toxin of red tide, Ptychodiscus brevis, on canine tracheal smooth muscle: a possible new asthma-triggering mechanism. *J Allergy Clin Immunol* **69,** 418-428.

Baden D. G. (1989) Brevetoxins: unique polyether dinoflagellate toxins. *FASEB J* **3,** 1807-1817.

Bossart G. D., Baden D. G., Ewing R. Y., Roberts B., and Wright S. D. (1998) Brevetoxicosis in manatees (Trichechus manatus latirostris) from the 1996 epizootic: gross, histologic, and immunohistochemical features. *Toxicol Pathol* **26,** 276-282.

Dolmetsch R. E., Xu K., and Lewis R. S. (1998) Ca^{2+} oscillations increase the efficiency and specificity of gene expression. *Nature* **392,** 933-936.

Fields R. D., Eshete F., Stevens B., and Itoh K. (1997) Action potential-dependent regulation of gene expression: temporal specificity in ca^{2+}, cAMP-responsive element binding proteins, and mitogen-activated protein kinase signaling. *J Neurosci* **17,** 7252-7266.

Hu Q., Deshpande S., Irani K., and Ziegelstein R. C. (1999) [$Ca(^{2+})$](i) oscillation frequency regulates agonist-stimulated NF-kappaB transcriptional activity. *J Biol Chem* **274,** 33995-33998.

Lawrie A. M., Graham M. E., Thorn P., Gallacher D. V., and Burgoyne R. D. (1993) Synchronous Ca^{2+} oscillations in cerebellar granule cells in culture mediated by NMDA receptors. *Neuroreport* **4,** 539-542.

Lu Y. M., Yin H. Z., Chiang J., and Weiss J. H. (1996) Ca($^{2+}$)-permeable AMPA/kainate and NMDA channels: high rate of Ca^{2+} influx underlies potent induction of injury. *J Neurosci* **16,** 5457-5465.

Mayer M. L., Westbrook G. L., and Guthrie P. B. (1984) Voltage-dependent block by Mg^{2+} of NMDA responses in spinal cord neurones. *Nature* **309,** 261-263.

Mody I., Lambert J. D., and Heinemann U. (1987) Low extracellular magnesium induces epileptiform activity and spreading depression in rat hippocampal slices. *J Neurophysiol* **57,** 869-888.

Murphy T. H., Blatter L. A., Wier W. G., and Baraban J. M. (1992) Spontaneous synchronous synaptic Ca^{2+} transients in cultured cortical neurons. *J Neurosci* **12,** 4834-4845.

Ogura A., Iijima T., Amano T., and Kudo Y. (1987) Optical monitoring of excitatory synaptic activity between cultured hippocampal neurons by a multi-site Ca^{2+} fluorometry. *Neurosci Lett* **78,** 69-74.

O'Shea T.J., Rathbun G.B., Buergelt C.D., Odell D.K. (1991) An epizootic of Florida manatees associated with a dinoflagellate bloom. *Marine Mammal Sci.* **7** 165-179.

Rose K., Christine C. W., and Choi D. W. (1990) Magnesium removal induces paroxysmal neuronal firing and NMDA receptor-mediated neuronal degeneration in cortical cultures. *Neurosci Lett* **115,** 313-317.

Templeton C. B., Poli M. A., and Solow R. (1989) Prophylactic and therapeutic use of an anti-brevetoxin (PbTx-2) antibody in conscious rats. *Toxicon* **27,** 1389-1395.

Turetsky D. M., Canzoniero L. M., Sensi S. L., Weiss J. H., Goldberg M. P., and Choi D. W. (1994) Cortical neurones exhibiting kainate-activated Co^{2+} uptake are selectively vulnerable to AMPA/kainate receptor-mediated toxicity. *Neurobiol Dis* **1,** 101-110.

Wang X. and Gruenstein E. I. (1997) Mechanism of synchronized Ca^{2+} oscillations in cortical neurons. *Brain Res* **767,** 239-249.

Yu X. M. and Salter M. W. (1998) Gain control of NMDA-receptor currents by intracellular sodium. *Nature* **396,** 469-474.

CHAPTER 2

LITERATURE REVIEW

Neurotransmitter glutamate

The excitatory action of glutamate in the mammalian brain and spinal cord has been known since the 1950s (Curtis and Watkins 1961, Hayashi 1952). It was not until the late 1970s, however that it became widely recognized that glutamate is the principal excitatory neurotransmitter in the vertebrate CNS. The receptors on which the neurotransmitter glutamate acts can be broadly divided into G-protein linked metabotropic glutamate receptors (mGluR) and ionotropic glutamate receptors (iGluR). The ionotropic glutamate receptors, named after their preferred agonists, include N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors. In the late 1980s it was proposed that glutamate also activates mGluR receptors that operate by releasing second messengers in the cytoplasm or by influencing ion channels through release of G protein subunits within the membrane (Conn and Pin 1997).

Ionotropic glutamate receptors

Functional iGluRs possess the stoichiometry of four or five highly sequence-related subunits. Mammals express four AMPA receptor subunits (GluR1-GluR4), five kainite receptor subunits (KA1, KA2, GluR5-7) and five NMDA receptor units (NR1, NR2A-D) (Nakanishi, 1992; Seeburg, 1993; Hollmann and Heinemann, 1994). All iGluR subunits contain three transmembrane domains (TM1, TM3 and TM4) and a re-entrant membrane loop (M2) on the cytoplasmic side that lines the inner channel pore and defines the distinct ion selectivities of the ion channel. The structure of the iGluR is quite conserved. The predicted secondary structure of Glu-receptor subunits includes the

following features: (1) a large extracellular N-terminus domain; (2) three transmembrane-spanning domains (TM1, TM3 and TM4); (3) a fourth hydrophobic segment (M2) that forms a channel-lining re-entrant hairpin loop similar to the pore-forming region of K^+ channels (Wo and Oswald, 1995); (4) a binding domain for agonists formed from the S1 and S2 extracellular regions (Stern-Bach et al., 1994); and (5) an intracellular C-terminus domain. NMDA receptors are either tetrameric or pentameric receptors with 2 NR1 subunits and 2 to 3 NR2 subunits. AMPA receptors are usually heteromeric, being comprised of at least two different subunits. These receptors can also form homomers (Martin et al., 1993; Wenthold et al., 1996). The large extracellular amino-terminal domain of each subunit includes a necessary component of the glutamate recognition site (S1) and customizes selective receptor modulation via several mechanisms including redox modulation of interdomain disulfide linkages or affinity to allosteric modulators such as extracellular protons, Zn^{2+} and polyamines. The TM3-TM4 loop includes a second required component of the glutamate recognition site (S2) and RNA splice variants that affect receptor desensitization. The intracellular carboxyl terminus is involved in signal transduction, receptor anchoring and contains phosphorylation sites that modulate receptor activity.

Ionotropic glutamate receptors are permeable to cations and largely exclude anions from the pore. Sodium and potassium are thought to be equally permeable and thus extensive comparisons between the two ions have not been made. NMDA receptors are more permeable to Ca^{2+} than non-NMDA receptors. The subunit composition determines the calcium conductance in the AMPA receptors. The presence of GluR2 subunit prevents the open channel from conducting Ca^{2+} ions. AMPA receptors have a

lower glutamate affinity than NMDA receptors, but have faster kinetics and are responsible for the fast initial component of the excitatory postsynaptic potential (EPSP). A distinctive feature of NMDA receptor is its voltage-sensitive block by Mg^{2+}. This is operative under normal circumstances, but is overcome by partial depolarization of the membrane. A further specific feature is the need of glycine as a coagonist. Each receptor unit appears to have two glycine and two glutamate binding sites (Laube et al. 1998).

Metabotropic glutamate receptors

The mGluR belong to the type 3 superfamily of G-protein-coupled receptors (GPCRs). The mGlu receptors are comprised of two main domains separated, in most cases, by a cysteine rich linker – a large extracellular domain (ECD) where the agonist binding site is located and a seven transmembrane domain (7TM) region which shares very low sequence similarity with other GPCRs. The ECD consist of two globular lobes separated by a cleft where the ligand binds. The second intracellular loop in mGluR is the longest and least conserved, and is crucial for G-protein coupling selectivity. The third loop is surprisingly short and highly conserved and plays a crucial role in G-protein activation and in cooperation with the first intracellular loop and the C-terminal tail, controls the coupling efficacy.

Currently, eight mGluR subtypes (mGluR1-mGluR8) and splice variants have been cloned from rat brain. They are classified into three groups according to sequence homology, signal transduction mechanism and agonist selectivity. Group I includes mGluR1 and mGluR5, which are positively coupled to phosphoinositide hydrolysis/Ca2+ mobilization. Group II, containing mGluR2 and mGluR3 and group III including

mGluR4, mGluR6, mGluR7 and mGluR8, are both coupled in a inhibitory manner to adenylyl cyclase but have a completely different pharmacology. (Pellicciari and Costantino, 1999)

Calcium permeable AMPA receptors

AMPA receptors were originally purported to be permeable only to Na^+. But later it was determined that AMPA receptors can also allow Ca^{2+} to enter the pore. Electrophysiological studies on recombinant AMPA channels expressed in *Xenopus laevis* oocytes (Hollman et al., 1991) and mammalian cells (Verdoorn et al., 1991) demonstrated that the presence of the GluR2 subunit renders the heteromeric AMPA receptor Ca^{2+} impermeable. AMPA receptors assembled from GluR1, GluR3 or GluR4 alone or in combination are permeable to Ca^{2+} and have doubly rectifying current–voltage relationships. GluR2, expressed with other GluR subunits, forms channels that are Ca^{2+}-impermeable and electrically linear or outwardly rectifying. The property of Ca^{2+} impermeability of the GluR2 subunit is attributed to the presence of a positively charged arginine (R) in place of a glutamine (Q) residue within the M2 domain (Burnashev et al., 1992; Hume et al., 1991). Rectification of receptors lacking GluR2 subunits arises from fast voltage-dependent channel block by intracellular polyamines (Kamboj et al., 1995; Koh et al., 1995). Some positively charged polyamine spider toxins, such as argiotoxin and Joro spider toxin block Ca^{2+}-permeable AMPA receptors selectively (presumably because they are repelled by the positively charged arginine residue in the GluR2 subunit) and therefore serve as pharmacological probes to detect the presence or absence of GluR2 in heteromeric receptor assemblies.

Ca^{2+} influx through the Ca^{2+} permeable AMPA receptors has been shown to induce neuronal injury (Turetsky et al., 1994; Lu et al., 1996) and has been implicated in selective neurodegeneration observed in several neurological diseases such as global ischemia, Alzheimer's disease and amyotrophic lateral sclerosis. Neurotoxicity is not always observed in neurons possessing these Ca^{2+} permeable AMPA receptors. For example, hippocampal GABAergic interneurons lacking GluR2 are viable and relatively resistant to post-ischemic delayed neurodegeneration (Johansen et al., 1983). Other groups have shown that *gluR2* mRNA is downregulated in vulnerable neurons during ischemia and epilepsy and suggest that formation of Ca^{2+} permeable AMPA receptors leads to enhanced toxicity of endogenous glutamate following neurological insult (Pellegrini-Giampietro et al., 1997). In any event, Ca^{2+} permeable AMPA receptors represent an important molecular target for prevention of neuronal injury after neurological insults.

Oscillations in cytosolic calcium and their mechanisms

Calcium is an important second messenger that activates a number of downstream signalling elements. Oscillations in cytoplasmic calcium levels are a common mode of signalling both in excitable and non-excitable cells and can increase the efficiency and specificity of gene expression (Dolmetsch et al., 1998). Calcium oscillations act as a biological Morse code, in the sense that alteration of the temporal features of the oscillations, leads to differential activation of certain genes (Fields et al., 1997; Hu et al., 1999).

In the nervous system, spontaneous calcium transients have been implicated in regulating neuronal plasticity in developing neurons (Spitzer et al., 1994). Calcium spikes also promote normal neurotransmitter receptor expression and channel maturation in *Xenopus* embryonic neurons (Gu and Spitzer, 1995). Spontaneous calcium oscillations also play a critical role in regulating the advancement of the leading process of migrating cerebellar granule neurons (Komuro and Rakic, 1996), as well as, of chick retinal ganglion cell growth cones (Gomez et al., 1995; Gomez et al., 1999).

Individual cells in culture can exhibit transient, sustained or oscillatory calcium signaling. Neurons in culture exhibit the phenomenon of synchronous spontaneous calcium oscillations represented by rhythmic activation of a group of neurons in characteristic temporal and spatial pattern. Synchronized calcium oscillations in culture systems were first identified in hippocampal neurons in Mg^{2+} -free extracellular media (Ogura et al., 1987). Similar oscillatory behavior has been reported in other neuronal cell types including cerebral cortical neurons (Wang and Gruenstein, 1997) and cerebellar granule neurons (Lawrie et al., 1993), but these oscillations are only observed after removing or lowering extracellular Mg^{2+}. Calcium oscillations have also been reported in older cultures of cortical neurons, which do not require the removal of Mg^{2+} from the media (Murphy et al., 1992). Neurons in culture form functional synapses (Nakanishi and Kukita, 1998) and rhythmic neurotransmitter release may organize the synchronous activity in the neuronal cultures.

The mechanism involved in generation of calcium oscillations varies in different cell types. In general calcium oscillations can be initiated by influx of Ca^{2+} through the plasma membrane or mobilization from intracellular calcium stores. In cultured

cerebrocortical neurons, in the absence of extracellular Mg^{2+}, concomitant activation of NMDA and L-type voltage gated calcium channels is responsible for the generation of calcium oscillations (Wang and Gruenstein, 1997). The mGluRs have been implicated in the generation of calcium oscillations in other models. In HEK cells transfected with different mGluR subtypes, cyclic phosphorylation and dephosphorylation of mGluR5 by protein kinase C (PKC) is responsible for generation of calcium oscillations (Kawabata et al., 1996). In coronal slices from neocortex endogenous glutamate acting on group I mGluRs is responsible for generation of calcium oscillations (Flint et al., 1999).

It is important to identify the routes of calcium entry for the generation of calcium oscillations, inasmuch as different routes of calcium entry can stimulate distinct signaling pathways that can activate different sets of genes and result in alternative physiological outcomes (Gallin and Greenberg, 1995). For instance in hippocampal neurons, Ca^{2+} entry through NMDA receptors and L-type calcium channels are equally effective in activating serum responsive element mediated transcription, but only Ca^{2+}influx through L-type calcium channels potently stimulates the cAMP responsive element (CRE) (Bading et al., 1993). Calcium influx induced neurotoxicity is also dependent on distinct influx pathways and not on the overall calcium load (Sattler et al., 1998). Identification of the mechanism of calcium oscillations under physiologic conditions would, therefore, help understand the tonic influence of different routes of calcium entry, on the various downstream events.

Voltage-gated sodium channels (VGSC): molecular target for neurotoxins

VGSC are transmembrane proteins responsible for the voltage-dependent increase in sodium permeability that initiates action potentials. VGSC consist of a pore forming α subunit of 260 kDa associated with auxiliary subunits: β_1, β_2 and β_3 (Catterall, 1992; Isom et al., 1992; Isom et al., 1995; Morgan et al., 2000). The α subunit consists of four homologous domains (I–IV), each containing six transmembrane segments (S1–S6), and one reentrant segment (SS1/SS2) connected by internal and external polypeptide loops (Catterall, 1992 and Noda et al., 1986). The S4 segments are positively charged and serve as voltage sensors to initiate the voltage-dependent activation of VGSC by moving outward under the influence of the electric field (Armstrong, 1981; Stühmer et al., 1989; Yang et al., 1996). Inactivation is mediated by the short intracellular loop connecting domains III and IV (Stühmer et al., 1989; Vassilev et al., 1988; West et al., 1992). The membrane-reentrant loops between S5 and S6 (SS1-SS2) form the ion selectivity filter and the outer region of the pore (Noda et al., 1989; Terlau et al., 1991; Heinemann et al., 1992).

VGSC are molecular targets of several neurotoxins which bind to specific receptor sites and alter the function of the channel. Radiolabeled neurotoxin assays uncovered at least six distinct neurotoxin-binding sites associated with the sodium channel (Catterall, 1977 and 1980). Neurotoxin binding generally alters ion permeation and/or voltage-dependent gating and can be classified as the following: pore-blocking toxins, toxins that affect gating from membrane-embedded receptor sites and toxins that affect gating from extracellular receptor sites (Catterall, 1980 and 2000).

Receptor site 1 on the sodium channel is occupied by the water-soluble heterocyclic guanidines, tetrodotoxin (TTX) and saxitoxin (STX) and the peptidic μ-contoxins. TTX is isolated from the tissue of puffer fish and can also be found in mollusks, octopus, crabs, and Central American frogs (Catterall 2000). Binding of these toxins has been shown to block sodium conductance (Hille, 1966; Narahashi, 1974). Upon binding to receptor site 1, TTX enters the extracellular opening of the transmembrane pore, thus preventing access of transported monovalent cation to the cation pore.

Various toxins, including lipid-soluble grayanotoxins, veratridine, acotinine, and batrachotoxin, bind to neurotoxin intramembrane receptor site 2, which alters voltage-dependent gating. These toxins bind preferentially to the activated state of sodium channels, which causes a persistent activation at resting membrane potentials. It is suggested that the block of inactivation is due to their interaction with the IVS6 transmembrane segment that is required for fast inactivation.

Having effects similar to those of site 2 neurotoxins, the lipid-soluble brevetoxins and ciguatoxins bind to neurotoxin receptor site 5 and cause a shift in activation to a more negative membrane potential and a block of inactivation (Benoit et al., 1986; Huang et al., 1985). Transmembrane segments IS6 and IVS5 are both involved in the formation of receptor site 5.

The polypeptide toxins, α-scorpion toxins, sea-anemone toxins and some spider toxins act on extracellular neurotoxin receptor site 3. Upon binding, these toxins slow or block sodium channel inactivation. The binding affinity of this group of toxins is decreased by depolarization of rat brain sodium channels (Catterall, 1977; Couraud,

1978). Because the voltage dependence of neurotoxin binding correlates closely with the voltage dependence of channel activation, it can be implied that membrane potential affects the structure of receptor site 3 on rat brain sodium channels. This suggests that this region of the channel is important for coupling of activation and inactivation and toxin binding prevents the conformational change required for fast inactivation (Catterall, 1979).

Purification of the polypeptide toxin conotoxin-TxVIA from the venom of the cone snail *Conus textile* led to the discovery of neurotoxin site 6. Conotoxin-TxVIA causes a specific inhibition of sodium current inactivation by producing a marked prolongation of the action potential. The β-scorpion toxins act on neurotoxin receptor site 4. β-scorpion toxins induce both a shift in the voltage dependence of sodium channel activation in the hyperpolarizing direction and a reduction of the peak sodium current amplitude. The voltage dependence of activation of neuronal sodium channels is modified by β-scorpion toxin only after a strong depolarizing prepulse. This suggests that the interaction of the toxin with its receptor site must be dependent on the activated conformational state of the toxin receptor site (Cestele and Catterall, 2000).

Brevetoxins

Experiments utilizing neuroblastoma cells and rat brain synaptosomes have shown that brevetoxins interact with binding site 5 on the α-subunit of voltage-gated sodium channels (VGSCs) (Catterall and Gainer, 1985; Poli et al., 1986). Four distinct effects of brevetoxin on the VGSC have been elucidated and include a shift of the activation potential to more negative values, a prolongation of mean open time, an

inhibition of channel inactivation, and the induction of subconductance states (Jeglitsch et al., 1998). These alterations in sodium channel function promote the depolarization of neurons at resting membrane potentials and thus account for the acute central and peripheral toxicologic effects of brevetoxins observed in vivo.

Brevetoxins (PbTx-1 to PbTx-10) are potent lipid soluble polyether neurotoxins produced by the marine dinoflagellate *Karenia brevis* (formerly known as *Gymnodinium breve* and *Ptychodiscus brevis*). This organism has been linked to periodic red tide blooms in the Gulf of Mexico, along the western Florida coastline (Baden, 1989). As a consequence of their lipid solubility, these toxins are expected to easily pass through cell membranes including the blood brain barrier, as well as buccal mucosa and skin (Mehta et al., 1991; Apland et al., 1993). *K. brevis* blooms have been implicated in massive fish kills, bird deaths, and marine mammal mortalities, most notably involving the endangered West Indian manatee Trichechus manatus latirostris (Bossart et al., 1998).

Multiple die-offs of marine mammals have been reported in association with the Florida red tide and brevetoxins (Bossart et al., 1998). In 1996, a prolonged Florida red tide in the Gulf of Mexico resulted in the documented deaths of 149 endangered Florida manatees (Bossart et al., 1998). Exposure of brevetoxin to manatees was due to prolonged inhalation of toxin laden aerosol and ingestion of contaminated seawater over several weeks. The post-mortem investigation revealed severe pulmonary lesions, chronic hemolytic anemia with multiorgan hemosidreosis and evidence of neurotoxicity (particularly in cerebellum).

The two known forms of red tide associated clinical entities in humans first characterized in Florida are an acute gastroenteritis with neurologic symptoms after

ingestion of contaminated shellfish and an apparently reversible upper respiratory syndrome after inhalation of the aerosols of the dinoflagellate and their toxins (i.e., aerosolized red tide toxins respiratory irritation) (Asai et al., 1982; Baden et al., 1995).

Neurotoxic shellfish poisoning (NSP) can be viewed clinically as a milder form of paralytic shellfish poisoning (PSP) or ciguatera fish poisoning. NSP typically causes gastrointestinal symptoms of nausea, diarrhea and abdominal pain as well as neurologic symptoms primarily consisting of paresthesias similar to those seen with ciguatera fish poisoning. Cerebellar symptoms such as vertigo and incoordination also reportedly occur. In severe cases, bradycardia, headache, dilated pupils, convulsion and subsequent need for respiratory support have been reported (Morse et al., 1977; Baden et al., 1995).

Few reports have been published regarding human health effects associated with exposure to aerosolized red tide toxins. The exposure usually occurs on or near beaches with an active red tide bloom. Onshore winds and breaking surf results in the release of toxins into the water and into the onshore aerosol (Pierce and Kirkpatrick, 2001). Inhalation of aerosolized red tide toxins reportedly results in conjunctival irritation, copious catarrhal exudates, rhinorrhoea, nonproductive cough and bronchoconstriction (Music et al., 1973; Asai et al., 1982). Some people also report other symptoms such as dizziness, tunnel vision and skin rashes.

Our laboratory has demonstrated that brevetoxins produce acute neuronal injury and death in cerebellar granule neurons (CGNs) (Berman and Murray, 1999). The neurotoxicity was reversed by the coapplication of tetrodotoxin or antagonists of the NMDA receptor. The neuroprotective efficacy of NMDA antagonists is attributed to the ability of brevetoxins to evoke the release of glutamate from CGNs. Brevetoxins also

produce a rapid increase in cytoplasmic [Ca^{2+}] in CGNs with a potency consonant with that of neurotoxicity. In CGNs brevetoxins induced Ca^{2+} influx is through three pathways: NMDA receptors, L-type Ca^{2+} channels and the reverse mode of the Na^{+}/ Ca^{2+} exchanger (Berman and Murray, 2000). The downstream signaling events after brevetoxin exposure and other cellular effects on the nervous system remain unexplored.

Gain control of NMDA receptor

Gain control of NMDA receptor refers to an increase in NMDA receptor mediated excitatory transmission which usually involves phosphorylation of the NMDA receptor subunits leading to conformational changes of the receptor which affect channel gating properties.

Src kinase and potentiation of NMDA receptor activity

Protein tyrosine kinases (PTKs) fall into two main groups: receptor and non-receptor PTKs (Hunter and Cooper, 1985; Ullrich and Schlessinger, 1990). Src is the prototype of the group of non-receptor PTKs (Brown and Cooper, 1996). Src is a member of a family of tyrosine kinases having a total of nine members, five of which Src, Fyn, Lyn, Lck, and Yes are expressed in the CNS. Src is expressed widely in the CNS with high levels of expression in the cerebral cortex, hippocampus, pons, midbrain, cerebellum, and spinal cord (Sugrue et al., 1990; Ross et al., 1988). Within CNS neurons, Src is present both pre- and post-synaptically. The post-synaptic localization is particularly relevant with respect to the modulation of NMDA receptor function, as Src has been identified in the post-synaptic density (PSD) (Atsumi et al., 1993; Cudmore and

Gurd, 1991), the main structural component of excitatory synapses where glutamate receptors are concentrated.

Over the past several years, much evidence has accumulated to indicate that tyrosine phosphorylation upregulates the activity of NMDARs (Wang and Salter, 1994; Khor and Seeburg, 1996; Lancaster and Rogers, 1998; Liao et al., 2000; Zheng et al., 1998) and that the endogenous protein tyrosine kinase Src can mediate this upregulation (Yu et al., 1997; Salter, 1998). To summarize the main lines of evidence implicating Src: first, applying Src or a peptide that activates Src family members into cells increases both NMDA-evoked currents and the NMDAR component of excitatory postsynaptic currents (EPSCs); second, applying these reagents to the cytoplasmic face of inside-out membrane patches enhances NMDAR single-channel activity; third, the increases in NMDAR activity are prevented with Src-specific inhibitory reagents; fourth, Src co-immunoprecipitates as part of the NMDAR complex of proteins (Yu et al., 1997) and localises in the PSD (Suzuki and Okamura-Noji, 1995) and last, Src phosphorylates the NR2A and NR2B subunits (Suzuki and Okamura-Noji, 1995; Hisatsune, 1999).

Intracellular Na^+ regulation of NMDA receptor activity

The influx of Na^+ is fundamental to electrical signaling in the nervous system and is essential for such basic signals as action potentials and excitatory postsynaptic potentials. During periods of bursting or high levels of discharge activity, large increases in intracellular Na^+ concentration are produced in the soma and dendrites. Yu and Salter in 1998 reported that increase in intracellular Na^+ increases the NMDA-receptor-mediated whole-cell currents and NMDA-receptor single-channel activity. Raising the intracellular Na^+ by 24±2 mM significantly increased the NMDA single-channel overall

open probability and mean open time. Using veratridine, a plant-derived alkaloid that leads to the block of sodium channel inactivation, it was shown that influx of Na^+ through TTX-sensitive voltage-gated sodium channels is sufficient to potentiate NMDA-channel activity (Yu and Salter, 1998). The exact mechanism of intracellular Na^+ mediated upregulation of NMDA receptor activity is not known but the effect of intracellular Na^+ requires a concurrent of Src kinase activation (Yu and Salter, 1998).

Extracellular signal-regulated kinase (ERK): Regulation of ERK in the nervous system

Mitogen activated protein kinases constitute a family of serine/threonine kinases, among which the extracellular signal-regulated kinases (ERKs) are extensively studied. Regulation of the ERK cascade is complex (Grewal et al., 1999). The ERK cascade, like the other MAPK cascades, is distinguished by a characteristic core cascade of three kinases. The first kinase is the MAP kinase kinase kinase (Raf-1 and B-Raf) which activates the second, a MAP kinase kinase (MEK), by serine/threonine phosphorylation. MAPKKs (MEKs) are dual specificity kinases which in turn activate a MAP kinase (p44 MAPK = ERK1, p42MAPK = ERK2) by phosphorylating both a threonine and a tyrosine residue.

ERK1/2 have been studied extensively for their role in regulation of cell proliferation. These enzymes are activated in response to growth factors and tumor promoters and control cell cycle progression (Sweatt, 2001). The ubiquitous Raf-1 pathway is activated by Ras, which is stimulated by growth factor tyrosine kinase receptors acting through Grb2 and Sos; PKC also activates this pathway by interacting

with either Ras or Raf-1. Activation of Raf-1 leads to activation of MEK and consequently the ERKs. Ras/Rap guanine nucleotide exchange factors (GEFs) also activate ERK1/2 through the second messenger diacylglycerol (Ebinu et al. 1998; Kawasaki et al., 1998).

The Ras/Raf-1 pathway is inhibited by cyclic AMP-dependent protein kinase (PKA). In neurons cAMP can be positively coupled to ERK activation via Rap-1 and B-Raf (Vossler et al., 1997). The B-Raf pathway is stimulated by PKA and signals through the Ras homolog, Rap1. In addition, more recently a cAMP-responsive GEF was discovered that can also lead to ERK activation independent of PKA (Kawasaki et al., 1998). Despite this complexity, one simplifying feature of the cascade is that ERK (both ERK1 and ERK2) activity is exclusively regulated by MEK, the upstream dual-specificity kinase that phosphorylates the ERKs. This dual phosphorylation is both necessary and sufficient for ERK activation. This attribute has been capitalized upon to develop several pharmacologic tools used to investigate the ERK MAPK cascade experimentally: the MEK inhibitors PD098059 (Alessi et al., 1996); U0126 (DeSilva et al., 1998); and SL327 (Atkins et al., 1998). The dual phosphorylation and activation of ERKs can be monitored using commercially available phospho-site antibodies, allowing for a relatively convenient method of directly assaying ERK activation in cell extracts.

Initial insights into the possible regulation of the ERK cascade in neurons came from studies using neuronal cell cultures. For example, several studies have demonstrated that pharmacological stimulation of the NMDA receptors leads to the activation of ERK2 in both cortical and hippocampal neurons in culture (Bading and Greenberg 1991; Fiore et al., 1993; Kurino et al., 1995; Xia et al., 1996). In addition,

stimulation of muscarinic receptors elicits p42 MAPK activation in cortical neurons (Stratton et al., 1989; Wang and Durkin 1995). Stimulation of protein kinase C also produces a robust activation of ERK2 in both hippocampal and cortical neurons (Stratton et al. 1989). Activation of NMDA receptors using long term potentiation (LTP)-inducing physiologic stimulation similarly results in ERK2 activation (English and Sweatt 1996). In recent studies it was also observed that hippocampal ERK activation occurs in response to activation of metabotropic glutamate receptors, muscarinic acetylcholine receptors and DA receptors (Roberson et al., 1999). Moreover, ERK activation by DA was blocked by the PKA inhibitor H89, and MAPK activation by metabotropic receptor and muscarinic receptor agonists was blocked by the PKC inhibitor chelerythrine (Roberson et al., 1999).

Thus ERK activation in the neurons is regulated by a wide variety of neurotransmitter receptors coupled to either PKA or PKC: NMDA receptors, adrenergic receptors, DA receptors, muscarinic acetylcholine receptors and metabotropic glutamate receptors. ERK activation in the neurons is not limited to neurotransmitter receptors. Brain derived neurotrophic factor (BDNF) receptors also couple to ERK activation in hippocampal neurons, and BDNF mediated ERK activation contributes to BDNF-induced synaptic plasticity in area CA1 (Gottschalk et al., 1998, 1999; Pozzo-Miller et al., 1999).

Roles of ERK in the nervous system

ERK activation in neurons has been shown to be important for the induction of LTP, a prerequisite for synaptic plasticity. Numerous studies have shown that hippocampal LTP is inhibited by MEK inhibitors (English and Sweatt, 1997; Atkins et al.,

1998; Watabe et al., 2000; Giovannini et al., 2001). Two different models have been proposed to account for the role of ERK signaling in the induction of LTP (Sweatt, 2001; Zhu et al., 2002). In one model (Sweatt, 2001), ERK activation contributes to LTP induction by facilitating activation of synaptic NMDA receptors through downregulation of dendritic A-type K^+ channels (Adams et al., 2000). In the second model (Zhu et al., 2002), ERK activation has a more central role in LTP induction and is required for activity-dependent membrane insertion of AMPA receptors.

In addition to modulating LTP induction, ERK activation is also thought to have an important role in transcriptional events involved in the later, mRNA synthesis and protein synthesis-dependent stages of LTP (Impey et al., 1998; Davis et al., 2000). One of the important transcription factors regulated by ERK is cAMP responsive element binding protein (CREB). CREB further regulates a number of genes, including brain derived neurotrophic factor (BDNF) which is important for cell survival. The ERK-CREB-BDNF cascade has been shown to be important for neuronal survival (Xia et al., 1995; Bonni et al., 1999). Duration of ERK1/2 activation can also modify cellular responses. In PC12 cells, transient ERK1/2 activation leads to cell proliferation while sustained activation leads to differentiation (Traverse et al., 1992). Though ERK is widely accepted as a pro-survival protein, a few reports suggest that sustained overstimulation of ERK may lead to cell death (Runden et al., 1998: Murray et al., 1998).

References:

Adams J. P., Roberson E. D., English J. D., Selcher J. C., and Sweatt J. D. (2000) MAPK regulation of gene expression in the central nervous system. *Acta Neurobiol Exp (Warsz)* **60,** 377-394.

Alessi D. R., Caudwell F. B., Andjelkovic M., Hemmings B. A., and Cohen P. (1996) Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase. *FEBS Lett* **399,** 333-338.

Apland J. P., Adler M., and Sheridan R. E. (1993) Brevetoxin depresses synaptic transmission in guinea pig hippocampal slices. *Brain Res Bull* **31,** 201-207.

Armstrong C. M. (1981) Sodium channels and gating currents. *Physiol Rev* **61,** 644-683.

Asai S., Krzanowski J. J., Anderson W. H., Martin D. F., Polson J. B., Lockey R. F., Bukantz S. C., and Szentivanyi A. (1982) Effects of toxin of red tide, Ptychodiscus brevis, on canine tracheal smooth muscle: a possible new asthma-triggering mechanism. *J Allergy Clin Immunol* **69,** 418-428.

Atkins C. M., Selcher J. C., Petraitis J. J., Trzaskos J. M., and Sweatt J. D. (1998) The MAPK cascade is required for mammalian associative learning. *Nat Neurosci* **1,** 602-609.

Atsumi S., Wakabayashi K., Titani K., Fujii Y., and Kawate T. (1993) Neuronal pp60c-src(+) in the developing chick spinal cord as revealed with anti-hexapeptide antibody. *J Neurocytol* **22,** 244-258.

Baden D. G. (1989) Brevetoxins: unique polyether dinoflagellate toxins. *FASEB J* **3,** 1807-1817.

Baden D. G., Melinek R., Sechet V., Trainer V. L., Schultz D. R., Rein K. S., Tomas C. R., Delgado J., and Hale L. (1995) Modified immunoassays for polyether toxins:

implications of biological matrixes, metabolic states, and epitope recognition. *J AOAC Int* **78,** 499-508.

Bading H. and Greenberg M. E. (1991) Stimulation of protein tyrosine phosphorylation by NMDA receptor activation. *Science* **253,** 912-914.

Bading H., Ginty D. D., and Greenberg M. E. (1993) Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. *Science* **260,** 181-186.

Benoit E., Legrand A. M., and Dubois J. M. (1986) Effects of ciguatoxin on current and voltage clamped frog myelinated nerve fibre. *Toxicon* **24,** 357-364.

Berman F. W. and Murray T. F. (1999) Brevetoxins cause acute excitotoxicity in primary cultures of rat cerebellar granule neurons. *J Pharmacol Exp Ther* **290,** 439-444.

Berman F. W. and Murray T. F. (2000) Brevetoxin-induced autocrine excitotoxicity is associated with manifold routes of Ca2+ influx. *J Neurochem* **74,** 1443-1451.

Bonni A., Brunet A., West A. E., Datta S. R., Takasu M. A., and Greenberg M. E. (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science* **286,** 1358-1362.

Bossart G. D., Baden D. G., Ewing R. Y., Roberts B., and Wright S. D. (1998) Brevetoxicosis in manatees (Trichechus manatus latirostris) from the 1996 epizootic: gross, histologic, and immunohistochemical features. *Toxicol Pathol* **26,** 276-282.

Bossart G. D., Baden D. G., Ewing R. Y., Roberts B., and Wright S. D. (1998) Brevetoxicosis in manatees (Trichechus manatus latirostris) from the 1996 epizootic: gross, histologic, and immunohistochemical features. *Toxicol Pathol* **26,** 276-282.

Brown M. T. and Cooper J. A. (1996) Regulation, substrates and functions of src. *Biochim Biophys Acta* **1287,** 121-149.

Burnashev N., Monyer H., Seeburg P. H., and Sakmann B. (1992) Divalent ion permeability of AMPA receptor channels is dominated by the edited form of a single subunit. *Neuron* **8,** 189-198.

Catterall W. A. (1977) Activation of the action potential Na+ ionophore by neurotoxins. An allosteric model. *J Biol Chem* **252,** 8669-8676.

Catterall W. A. (1979) Binding of scorpion toxin to receptor sites associated with sodium channels in frog muscle. Correlation of voltage-dependent binding with activation. *J Gen Physiol* **74,** 375-391.

Catterall W. A. (1980) Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. *Annu Rev Pharmacol Toxicol* **20,** 15-43.

Catterall W. A. and Gainer M. (1985) Interaction of brevetoxin A with a new receptor site on the sodium channel. *Toxicon* **23,** 497-504.

Catterall W. A. (1992) Cellular and molecular biology of voltage-gated sodium channels. *Physiol Rev* **72,** S15-S48.

Catterall W. A. (2000) From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels. *Neuron* **26,** 13-25.

Cestele S. and Catterall W. A. (2000) Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. *Biochimie* **82,** 883-892.

Conn P. J. and Pin J. P. (1997) Pharmacology and functions of metabotropic glutamate receptors. *Annu Rev Pharmacol Toxicol* **37,** 205-237.

Couraud F., Rochat H., and Lissitzky S. (1978) Binding of scorpion and sea anemone neurotoxins to a common site related to the action potential Na+ ionophore in neuroblastoma cells. *Biochem Biophys Res Commun* **83,** 1525-1530.

Cudmore S. B. and Gurd J. W. (1991) Postnatal age and protein tyrosine phosphorylation at synapses in the developing rat brain. *J Neurochem* **57,** 1240-1248.

Davis S., Vanhoutte P., Pages C., Caboche J., and Laroche S. (2000) The MAPK/ERK cascade targets both Elk-1 and cAMP response element-binding protein to control long-term potentiation-dependent gene expression in the dentate gyrus in vivo. *J Neurosci* **20,** 4563-4572.

DeSilva D. R., Jones E. A., Favata M. F., Jaffee B. D., Magolda R. L., Trzaskos J. M., and Scherle P. A. (1998) Inhibition of mitogen-activated protein kinase kinase blocks T cell proliferation but does not induce or prevent anergy. *J Immunol* **160,** 4175-4181.

Dolmetsch R. E., Xu K., and Lewis R. S. (1998) Calcium oscillations increase the efficiency and specificity of gene expression. *Nature* **392,** 933-936.

Ebinu J. O., Bottorff D. A., Chan E. Y., Stang S. L., Dunn R. J., and Stone J. C. (1998) RasGRP, a Ras guanyl nucleotide- releasing protein with calcium- and diacylglycerol-binding motifs. *Science* **280,** 1082-1086.

English J. D. and Sweatt J. D. (1997) A requirement for the mitogen-activated protein kinase cascade in hippocampal long term potentiation. *J Biol Chem* **272,** 19103-19106.

Fields R. D., Eshete F., Stevens B., and Itoh K. (1997) Action potential-dependent regulation of gene expression: temporal specificity in ca2+, cAMP-responsive element binding proteins, and mitogen-activated protein kinase signaling. *J Neurosci* **17,** 7252-7266.

Fiore R. S., Murphy T. H., Sanghera J. S., Pelech S. L., and Baraban J. M. (1993) Activation of p42 mitogen-activated protein kinase by glutamate receptor stimulation in rat primary cortical cultures. *J Neurochem* **61,** 1626-1633.

Flint A. C., Dammerman R. S., and Kriegstein A. R. (1999) Endogenous activation of metabotropic glutamate receptors in neocortical development causes neuronal calcium oscillations. *Proc Natl Acad Sci U S A* **96,** 12144-12149.

Gallin W. J. and Greenberg M. E. (1995) Calcium regulation of gene expression in neurons: the mode of entry matters. *Curr Opin Neurobiol* **5,** 367-374.

Giovannini M. G., Rakovska A., Benton R. S., Pazzagli M., Bianchi L., and Pepeu G. (2001) Effects of novelty and habituation on acetylcholine, GABA, and glutamate release from the frontal cortex and hippocampus of freely moving rats. *Neuroscience* **106,** 43-53.

Gomez T. M., Snow D. M., and Letourneau P. C. (1995) Characterization of spontaneous calcium transients in nerve growth cones and their effect on growth cone migration. *Neuron* **14,** 1233-1246.

Gomez T. M. and Spitzer N. C. (1999) In vivo regulation of axon extension and pathfinding by growth-cone calcium transients. *Nature* **397,** 350-355.

Gottschalk W., Pozzo-Miller L. D., Figurov A., and Lu B. (1998) Presynaptic modulation of synaptic transmission and plasticity by brain-derived neurotrophic factor in the developing hippocampus. *J Neurosci* **18,** 6830-6839.

Gottschalk W. A., Jiang H., Tartaglia N., Feng L., Figurov A., and Lu B. (1999) Signaling mechanisms mediating BDNF modulation of synaptic plasticity in the hippocampus. *Learn Mem* **6,** 243-256.

Grewal S. S., York R. D., and Stork P. J. (1999) Extracellular-signal-regulated kinase signalling in neurons. *Curr Opin Neurobiol* **9,** 544-553.

Gu X. and Spitzer N. C. (1995) Distinct aspects of neuronal differentiation encoded by frequency of spontaneous Ca2+ transients. *Nature* **375,** 784-787.

Heinemann S. H., Terlau H., Stuhmer W., Imoto K., and Numa S. (1992) Calcium channel characteristics conferred on the sodium channel by single mutations. *Nature* **356,** 441-443.

Hille B. (1966) Common mode of action of three agents that decrease the transient change in sodium permeability in nerves. *Nature* **210,** 1220-1222.

Hisatsune C., Umemori H., Mishina M., and Yamamoto T. (1999) Phosphorylation-dependent interaction of the N-methyl-D-aspartate receptor epsilon 2 subunit with phosphatidylinositol 3-kinase. *Genes Cells* **4,** 657-666.

Hollmann M., Hartley M., and Heinemann S. (1991) Ca2+ permeability of KA-AMPA--gated glutamate receptor channels depends on subunit composition. *Science* **252,** 851-853.

Hollmann M. and Heinemann S. (1994) Cloned glutamate receptors. *Annu Rev Neurosci* **17,** 31-108.

Hu Q., Deshpande S., Irani K., and Ziegelstein R. C. (1999) [Ca(2+)](i) oscillation frequency regulates agonist-stimulated NF-kappaB transcriptional activity. *J Biol Chem* **274,** 33995-33998.

Huang J. M., Wu C. H., and Baden D. G. (1984) Depolarizing action of a red-tide dinoflagellate brevetoxin on axonal membranes. *J Pharmacol Exp Ther* **229,** 615-621.

Hume R. I., Dingledine R., and Heinemann S. F. (1991) Identification of a site in glutamate receptor subunits that controls calcium permeability. *Science* **253,** 1028-1031.

Hunter T. and Cooper J. A. (1985) Protein-tyrosine kinases. *Annu Rev Biochem* **54,** 897-930.

Impey S., Obrietan K., Wong S. T., Poser S., Yano S., Wayman G., Deloulme J. C., Chan G., and Storm D. R. (1998) Cross talk between ERK and PKA is required for Ca2+ stimulation of CREB-dependent transcription and ERK nuclear translocation. *Neuron* **21,** 869-883.

Ishida H., Nozawa A., Totoribe K., Muramatsu N., Nudaya H., Tsuji K., Yamaguchi K., Yasumoto T., Kaspar H., Berkett N., Kosuge T. (1995) Brevetoxin B1, a new polyether marine toxin from the New Zealand shellfish, *Austrovenus stutchburyi. Tetrahedron Lett.* **36** 725-728.

Isom L. L., De Jongh K. S., Patton D. E., Reber B. F., Offord J., Charbonneau H., Walsh K., Goldin A. L., and Catterall W. A. (1992) Primary structure and functional expression of the beta 1 subunit of the rat brain sodium channel. *Science* **256,** 839-842.

Isom L. L., Ragsdale D. S., De Jongh K. S., Westenbroek R. E., Reber B. F., Scheuer T., and Catterall W. A. (1995) Structure and function of the beta 2 subunit of brain sodium channels, a transmembrane glycoprotein with a CAM motif. *Cell* **83,** 433-442.

Jeglitsch G., Rein K., Baden D. G., and Adams D. J. (1998) Brevetoxin-3 (PbTx-3) and its derivatives modulate single tetrodotoxin-sensitive sodium channels in rat sensory neurons. *J Pharmacol Exp Ther* **284,** 516-525.

Kamboj S. K., Swanson G. T., and Cull-Candy S. G. (1995) Intracellular spermine confers rectification on rat calcium-permeable AMPA and kainate receptors. *J Physiol* **486 (Pt 2),** 297-303.

Kawabata S., Tsutsumi R., Kohara A., Yamaguchi T., Nakanishi S., and Okada M. (1996) Control of calcium oscillations by phosphorylation of metabotropic glutamate receptors. *Nature* **383,** 89-92.

Kawasaki H., Springett G. M., Mochizuki N., Toki S., Nakaya M., Matsuda M., Housman D. E., and Graybiel A. M. (1998) A family of cAMP-binding proteins that directly activate Rap1. *Science* **282,** 2275-2279.

Kawasaki H., Springett G. M., Toki S., Canales J. J., Harlan P., Blumenstiel J. P., Chen E. J., Bany I. A., Mochizuki N., Ashbacher A., Matsuda M., Housman D. E., and Graybiel A. M. (1998) A Rap guanine nucleotide exchange factor enriched highly in the basal ganglia. *Proc Natl Acad Sci U S A* **95,** 13278-13283.

Koh D. S., Burnashev N., and Jonas P. (1995) Block of native Ca(2+)-permeable AMPA receptors in rat brain by intracellular polyamines generates double rectification. *J Physiol* **486 (Pt 2),** 305-312.

Kohr G. and Seeburg P. H. (1996) Subtype-specific regulation of recombinant NMDA receptor-channels by protein tyrosine kinases of the src family. *J Physiol* **492 (Pt 2),** 445-452.

Komuro H. and Rakic P. (1996) Intracellular Ca2+ fluctuations modulate the rate of neuronal migration. *Neuron* **17,** 275-285.

Kurino M., Fukunaga K., Ushio Y., and Miyamoto E. (1995) Activation of mitogen-activated protein kinase in cultured rat hippocampal neurons by stimulation of glutamate receptors. *J Neurochem* **65,** 1282-1289.

Lancaster B. and Rogers M. V. (1998) A peptide activator of endogenous tyrosine kinase enhances synaptic currents mediated by NMDA receptors. *Eur J Neurosci* **10,** 2302-2308.

Laube B., Kuhse J., and Betz H. (1998) Evidence for a tetrameric structure of recombinant NMDA receptors. *J Neurosci* **18,** 2954-2961.

Lawrie A. M., Graham M. E., Thorn P., Gallacher D. V., and Burgoyne R. D. (1993) Synchronous calcium oscillations in cerebellar granule cells in culture mediated by NMDA receptors. *Neuroreport* **4,** 539-542.

Liao G. Y., Kreitzer M. A., Sweetman B. J., and Leonard J. P. (2000) The postsynaptic density protein PSD-95 differentially regulates insulin- and Src-mediated current modulation of mouse NMDA receptors expressed in Xenopus oocytes. *J Neurochem* **75,** 282-287.

Lu Y. M., Yin H. Z., Chiang J., and Weiss J. H. (1996) Ca(2+)-permeable AMPA/kainate and NMDA channels: high rate of Ca2+ influx underlies potent induction of injury. *J Neurosci* **16,** 5457-5465.

Martin L. J., Blackstone C. D., Levey A. I., Huganir R. L., and Price D. L. (1993) AMPA glutamate receptor subunits are differentially distributed in rat brain. *Neuroscience* **53,** 327-358.

Mehta M., Kemppainen B. W., and Stafford R. G. (1991) In vitro penetration of tritium-labelled water (THO) and [3H]PbTx-3 (a red tide toxin) through monkey buccal mucosa and skin. *Toxicol Lett* **55,** 185-194.

Morgan K., Stevens E. B., Shah B., Cox P. J., Dixon A. K., Lee K., Pinnock R. D., Hughes J., Richardson P. J., Mizuguchi K., and Jackson A. P. (2000) beta 3: an additional auxiliary subunit of the voltage-sensitive sodium channel that modulates channel gating with distinct kinetics. *Proc Natl Acad Sci U S A* **97,** 2308-2313.

Morse E. V. (1977) Paralytic shellfish poisoning: a review. *J Am Vet Med Assoc* **171,** 1178-1180.

Murphy T. H., Blatter L. A., Wier W. G., and Baraban J. M. (1992) Spontaneous synchronous synaptic calcium transients in cultured cortical neurons. *J Neurosci* **12,** 4834-4845.

Murray B., Alessandrini A., Cole A. J., Yee A. G., and Furshpan E. J. (1998) Inhibition of the p44/42 MAP kinase pathway protects hippocampal neurons in a cell-culture model of seizure activity. *Proc Natl Acad Sci U S A* **95,** 11975-11980.

Music S. I., Howell J. T., and Brumback C. L. (1973) Red tide. Its public health implications. *JFMA* **60,** 27-29.

Nakanishi K. and Kukita F. (1998) Functional synapses in synchronized bursting of neocortical neurons in culture. *Brain Res* **795,** 137-146.

Nakanishi S. (1992) Molecular diversity of glutamate receptors and implications for brain function. *Science* **258,** 597-603.

Narahashi T. (1974) Chemicals as tools in the study of excitable membranes. *Physiol Rev* **54,** 813-889.

Noda M., Ikeda T., Kayano T., Suzuki H., Takeshima H., Kurasaki M., Takahashi H., and Numa S. (1986) Existence of distinct sodium channel messenger RNAs in rat brain. *Nature* **320,** 188-192.

Noda M., Suzuki H., Numa S., and Stuhmer W. (1989) A single point mutation confers tetrodotoxin and saxitoxin insensitivity on the sodium channel II. *FEBS Lett* **259,** 213-216.

Ogura A., Iijima T., Amano T., and Kudo Y. (1987) Optical monitoring of excitatory synaptic activity between cultured hippocampal neurons by a multi-site Ca2+ fluorometry. *Neurosci Lett* **78,** 69-74.

O'Shea T.J., Rathbun G.B., Buergelt C.D., Odell D.K. (1991) An epizootic of Florida manatees associated with a dinoflagellate bloom. *Marine Mammal Sci.* **7** 165-179.

Pellegrini-Giampietro D. E., Gorter J. A., Bennett M. V., and Zukin R. S. (1997) The GluR2 (GluR-B) hypothesis: Ca(2+)-permeable AMPA receptors in neurological disorders. *Trends Neurosci* **20,** 464-470.

Pellicciari R. and Costantino G. (1999) Metabotropic G-protein-coupled glutamate receptors as therapeutic targets. *Curr Opin Chem Biol* **3,** 433-440.

Pierce R. H. and Kirkpatrick G. J. (2001) Innovative techniques for harmful algal toxin analysis. *Environ Toxicol Chem* **20,** 107-114.

Poli M. A., Mende T. J., and Baden D. G. (1986) Brevetoxins, unique activators of voltage-sensitive sodium channels, bind to specific sites in rat brain synaptosomes. *Mol Pharmacol* **30,** 129-135.

Pozzo-Miller L. D., Gottschalk W., Zhang L., McDermott K., Du J., Gopalakrishnan R., Oho C., Sheng Z. H., and Lu B. (1999) Impairments in high-frequency transmission, synaptic vesicle docking, and synaptic protein distribution in the hippocampus of BDNF knockout mice. *J Neurosci* **19,** 4972-4983.

Roberson E. D., English J. D., Adams J. P., Selcher J. C., Kondratick C., and Sweatt J. D. (1999) The mitogen-activated protein kinase cascade couples PKA and PKC to cAMP response element binding protein phosphorylation in area CA1 of hippocampus. *J Neurosci* **19,** 4337-4348.

Ross C. A., Wright G. E., Resh M. D., Pearson R. C., and Snyder S. H. (1988) Brain-specific src oncogene mRNA mapped in rat brain by in situ hybridization. *Proc Natl Acad Sci U S A* **85,** 9831-9835.

Runden E., Seglen P. O., Haug F. M., Ottersen O. P., Wieloch T., Shamloo M., and Laake J. H. (1998) Regional selective neuronal degeneration after protein phosphatase inhibition in hippocampal slice cultures: evidence for a MAP kinase-dependent mechanism. *J Neurosci* **18,** 7296-7305.

Salter M. W. (1998) Src, N-methyl-D-aspartate (NMDA) receptors, and synaptic plasticity. *Biochem Pharmacol* **56,** 789-798.

Sattler R., Charlton M. P., Hafner M., and Tymianski M. (1998) Distinct influx pathways, not calcium load, determine neuronal vulnerability to calcium neurotoxicity. *J Neurochem* **71,** 2349-2364.

Seeburg P. H. (1993) The TINS/TiPS Lecture. The molecular biology of mammalian glutamate receptor channels. *Trends Neurosci* **16,** 359-365.

Spitzer N. C., Olson E., and Gu X. (1995) Spontaneous calcium transients regulate neuronal plasticity in developing neurons. *J Neurobiol* **26,** 316-324.

Stern-Bach Y., Bettler B., Hartley M., Sheppard P. O., O'Hara P. J., and Heinemann S. F. (1994) Agonist selectivity of glutamate receptors is specified by two domains structurally related to bacterial amino acid-binding proteins. *Neuron* **13,** 1345-1357.

Stratton K. R., Worley P. F., Huganir R. L., and Baraban J. M. (1989) Muscarinic agonists and phorbol esters increase tyrosine phosphorylation of a 40-kilodalton protein in hippocampal slices. *Proc Natl Acad Sci U S A* **86,** 2498-2501.

Stuhmer W., Conti F., Suzuki H., Wang X. D., Noda M., Yahagi N., Kubo H., and Numa S. (1989) Structural parts involved in activation and inactivation of the sodium channel. *Nature* **339,** 597-603.

Sugrue M. M., Brugge J. S., Marshak D. R., Greengard P., and Gustafson E. L. (1990) Immunocytochemical localization of the neuron-specific form of the c-src gene product, pp60c-src(+), in rat brain. *J Neurosci* **10,** 2513-2527.

Suzuki T., Matozaki T., Mizoguchi A., and Kasuga M. (1995) Localization and subcellular distribution of SH-PTP2, a protein-tyrosine phosphatase with Src homology-2 domains, in rat brain. *Biochem Biophys Res Commun* **211,** 950-959.

Sweatt J. D. (2001) The neuronal MAP kinase cascade: a biochemical signal integration system subserving synaptic plasticity and memory. *J Neurochem* **76,** 1-10.

Terlau H., Heinemann S. H., Stuhmer W., Pusch M., Conti F., Imoto K., and Numa S. (1991) Mapping the site of block by tetrodotoxin and saxitoxin of sodium channel II. *FEBS Lett* **293,** 93-96.

Traverse S., Gomez N., Paterson H., Marshall C., and Cohen P. (1992) Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor. *Biochem J* **288 (Pt 2),** 351-355.

Turetsky D. M., Canzoniero L. M., Sensi S. L., Weiss J. H., Goldberg M. P., and Choi D. W. (1994) Cortical neurones exhibiting kainate-activated Co2+ uptake are selectively vulnerable to AMPA/kainate receptor-mediated toxicity. *Neurobiol Dis* **1,** 101-110.

Ullrich A. and Schlessinger J. (1990) Signal transduction by receptors with tyrosine kinase activity. *Cell* **61,** 203-212.

Vassilev P. M., Scheuer T., and Catterall W. A. (1988) Identification of an intracellular peptide segment involved in sodium channel inactivation. *Science* **241,** 1658-1661.

Verdoorn T. A., Burnashev N., Monyer H., Seeburg P. H., and Sakmann B. (1991) Structural determinants of ion flow through recombinant glutamate receptor channels. *Science* **252,** 1715-1718.

Vossler M. R., Yao H., York R. D., Pan M. G., Rim C. S., and Stork P. J. (1997) cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. *Cell* **89,** 73-82.

Wang X. and Gruenstein E. I. (1997) Mechanism of synchronized Ca2+ oscillations in cortical neurons. *Brain Res* **767,** 239-249.

Wang Y. T. and Salter M. W. (1994) Regulation of NMDA receptors by tyrosine kinases and phosphatases. *Nature* **369,** 233-235.

Watabe A. M., Zaki P. A., and O'Dell T. J. (2000) Coactivation of beta-adrenergic and cholinergic receptors enhances the induction of long-term potentiation and synergistically activates mitogen-activated protein kinase in the hippocampal CA1 region. *J Neurosci* **20,** 5924-5931.

Wenthold R. J., Petralia R. S., Blahos J., II, and Niedzielski A. S. (1996) Evidence for multiple AMPA receptor complexes in hippocampal CA1/CA2 neurons. *J Neurosci* **16,** 1982-1989.

West J. W., Patton D. E., Scheuer T., Wang Y., Goldin A. L., and Catterall W. A. (1992) A cluster of hydrophobic amino acid residues required for fast Na(+)-channel inactivation. *Proc Natl Acad Sci U S A* **89,** 10910-10914.

Wo Z. G. and Oswald R. E. (1995) A topological analysis of goldfish kainate receptors predicts three transmembrane segments. *J Biol Chem* **270,** 2000-2009.

Xia Z., Dickens M., Raingeaud J., Davis R. J., and Greenberg M. E. (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* **270,** 1326-1331.

Xia Z., Dudek H., Miranti C. K., and Greenberg M. E. (1996) Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. *J Neurosci* **16,** 5425-5436.

Yang N., George A. L., Jr., and Horn R. (1996) Molecular basis of charge movement in voltage-gated sodium channels. *Neuron* **16,** 113-122.

Yu X. M., Askalan R., Keil G. J., and Salter M. W. (1997) NMDA channel regulation by channel-associated protein tyrosine kinase Src. *Science* **275,** 674-678.

Yu X. M. and Salter M. W. (1998) Gain control of NMDA-receptor currents by intracellular sodium. *Nature* **396,** 469-474.

Yu X. M. and Salter M. W. (1999) Src, a molecular switch governing gain control of synaptic transmission mediated by N-methyl-D-aspartate receptors. *Proc Natl Acad Sci U S A* **96,** 7697-7704.

Zheng F., Gingrich M. B., Traynelis S. F., and Conn P. J. (1998) Tyrosine kinase potentiates NMDA receptor currents by reducing tonic zinc inhibition. *Nat Neurosci* **1,** 185-191.

Zhu J. J., Qin Y., Zhao M., Van Aelst L., and Malinow R. (2002) Ras and Rap control AMPA receptor trafficking during synaptic plasticity. *Cell* **110,** 443-455.

CHAPTER 3

SPONTANEOUS SYNCHRONIZED CALCIUM OSCILLATIONS IN CEREBROCORTICAL NEURONS IN THE PRESENCE OF PHYSIOLOGICAL $[Mg^{2+}]$: INVOLVEMENT OF AMPA/KAINATE RECEPTORS AND METABOTROPIC GLUTAMATE RECEPTORS[1]

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Abstract

Primary cultures of neocortical neurons exhibit spontaneous Ca^{2+} oscillations under zero or low extracellular [Mg^{2+}] conditions. We find that mature murine neocortical neurons cultured for 9 days produce spontaneous Ca^{2+} oscillations in the presence of physiological [Mg^{2+}]. We have investigated the mechanism of these Mg^{2+}-independent Ca^{2+} oscillations. Ca^{2+} oscillations were action potential mediated inasmuch as tetrodotoxin eliminated their occurrence. AMPA receptors were found to regulate the frequency of Ca^{2+} oscillations. Ca^{2+} oscillations were independent of activation of L-type Ca^{2+} channels and NMDA receptors provide only a minor contribution. Release of intracellular Ca^{2+} stores was involved in the oscillatory activity since thapsigargin reduced the amplitude of the oscillations. S-4-carboxyphenylglycine, an antagonist of group I metabotropic glutamate receptor (mGluR), also reduced the amplitude of oscillations. In addition trans-ACPD, a group I mGluR agonist increased the oscillation frequency, suggesting critical role of mGluR for the generation of Ca^{2+} oscillations. mGluR mediated release of intracellular Ca^{2+} stores may be mediated by phospholipase C (PLC) since the PLC inhibitor U73122 eliminated the Ca^{2+} oscillations. These results indicate that Mg^{2+}-independent Ca^{2+} oscillations in neocortical cultures are primarily initiated by excitatory input from AMPA receptors and involve mobilization of intracellular Ca^{2+} stores following activation of mGluR.

Keywords: Neocortical neurons, calcium, magnesium, NMDA receptor, AMPA receptor, metabotropic glutamate receptors, oscillations.

1. Introduction

Ca^{2+} is an important second messenger that activates a number of downstream signaling elements. Oscillations in cytoplasmic Ca^{2+} levels are a common mode of signaling both in excitable and non-excitable cells and can increase the efficiency and specificity of gene expression (Dolmetsch et al., 1998). Ca^{2+} oscillations act as a biological Morse code, in the sense that alterations of the temporal features of Ca^{2+} oscillations produce differential activation of certain genes (Fields et al., 1997; Hu et al., 1999). Neurons in culture exhibit synchronous spontaneous Ca^{2+} oscillations that are represented by rhythmic activation of populations of neurons in characteristic temporal and spatial patterns. It has been shown that neurons in culture form functional synapses (Nakanishi and Kukita, 1998) and rhythmic neurotransmitter release in the neuronal network may lead to the synchronous oscillatory activity.

Ca^{2+} oscillations play an important physiologic role in the nervous system. Spontaneous Ca^{2+} transients have been implicated in regulating neuronal plasticity in developing neurons (Spitzer et al., 1995). Ca^{2+} spikes also promote neurotransmitter receptor expression and channel maturation in *Xenopus* embryonic neurons (Gu and Spitzer, 1995) and play a critical role in regulating the advancement of the leading process of migrating cerebellar granule neurons (Komuro and Rakic, 1996).

Synchronized Ca^{2+} oscillations in neuronal cultures were first reported in hippocampal neurons in Mg^{2+}-free extracellular media (Ogura et al., 1987). Similar oscillatory behavior have been reported in other neuronal cell types including neocortical neurons (Robinson et al., 1993; Wang and Gruenstein, 1997) and cerebellar granule neurons (Nunez et al.,1996; Lawrie et al., 1993), but these oscillations are only observed

upon removal or lowering of extracellular Mg^{2+}. Ca^{2+} oscillations have also been reported in mature cultures of neocortical neurons, which do not require the removal of Mg^{2+} from the extracellular media (Murphy et al., 1992). Mg^{2+} is a critical ion in the regulation of NMDA receptors. NMDA receptors are ligand gated channels and at resting membrane potentials NMDA receptors are blocked by submillimolar concentrations of extracellular Mg^{2+} (Mayer et al., 1984). Removal of Mg^{2+} from the extracellular environment will consequently favor Ca^{2+} influx through NMDA receptors. Accordingly it has been reported that NMDA receptors are indeed necessary for generation of Ca^{2+} oscillations in the absence of extracellular Mg^{2+} (Wang and Gruenstein, 1997; Nunez et al., 1996). We find that neocortical neuronal cultures (10-13 days in vitro) obtained from embryonic mice produce spontaneous Ca^{2+} oscillations in the presence of physiological [Mg^{2+}]. This study was done to understand the mechanisms underlying these spontaneous and Mg^{2+}-independent Ca^{2+} oscillations.

The mechanisms involved in generation of Ca^{2+} oscillations varies in different cell types. In general Ca^{2+} oscillations may be initiated by influx through the plasma membrane or mobilization from the intracellular Ca^{2+} stores. In non-excitable cells formation of inositol triphosphate (IP_3) and cyclical release of Ca^{2+} from IP_3-sensitive stores is believed to be responsible for the oscillatory behavior (Berridge, 1993). In sympathetic neurons caffeine induces oscillations through a mechanism that involves Ca^{2+} induced Ca^{2+} release (CICR) (Friel and Tsien, 1992). In HEK cells transfected with different metabotropic glutamate receptor (mGluR) subtypes it has been shown that cyclic phosphorylation and dephosphorylation of mGluR5 by protein kinase C (PKC) is responsible for generation of Ca^{2+} oscillations (Kawabata et al., 1996). In coronal slices

from neocortex endogenous glutamate acting on the group I mGluR is responsible for generation of Ca^{2+} oscillations (Flint et al., 1999). Intracellular Ca^{2+} waves have been observed in cortical slices and in neocortical cultures that are plated at a high cell density. In cultured neocortical neurons the Ca^{2+} waves propagate at a rate of 100-200 μm/sec (Charles et al., 1996). These Ca^{2+} waves involve communication through gap junctions rather than synaptic transmission (Kandler and Katz, 1998; Charles et al., 1996).

Approximately 13% of neurons in neocortical cultures possess Ca^{2+} permeable AMPA receptors (Turetsky et al., 1994; Jensen et al., 1998) and Ca^{2+} entry through these receptors may serve as the initial trigger for generation of calcium oscillations. Neocortical neurons also express mGluR receptors (Fotuhi et al., 1993) and activation of these mGluR receptors by endogenous glutamate can induce the release of Ca^{2+} from the intracellular Ca^{2+} stores. Thus it is necessary to evaluate the roles of both AMPA and mGluR in the generation of Ca^{2+} oscillations.

We investigated the role of glutamate receptors in the generation of Ca^{2+} oscillations in cerebral cortical neurons in the presence of physiological [Mg^{2+}]. AMPA receptors and mGluR were both found to play a critical role in the generation of oscillations, and a combination of Ca^{2+} influx from extracellular media and mobilization from intracellular stores contributed to the generation of Ca^{2+} oscillations. Furthermore we found that Ca^{2+} oscillations are tonically regulated by adenosine receptors.

2. Materials and Methods

2.1 Mice Neocortical Primary Cell Cultures

Primary cultures of neocortical neurons were obtained from embryonic day 16-17 Swiss-Webster mice. Briefly, pregnant mice were euthanized by CO_2 asphyxiation and embryos were removed under sterile conditions. Neocortices were collected, stripped of meninges, minced by trituration with a Pasteur pipette and treated with trypsin for 25 min at 37˚ C. The cells were then dissociated by two successive trituration and sedimentation steps in soybean trypsin inhibitor and DNase containing isolation buffer, centrifuged and, resuspended in Eagle's minimal essential medium with Earle's salt (MEM) and supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 10% horse serum, 100 I.U./ml penicillin and 0.10 mg/ml streptomycin, pH7.4. Cells were plated onto poly-L-lysine coated 96-well (9mm) clear-bottomed black-well culture plates (Costar) at a density of 4.7 X 10^5 cells/cm^2, and incubated at 37 ºC in a 5% CO_2 and 95% humidity atmosphere. Cytosine arabinoside (10μM) was added to the culture medium on day 3 after plating to prevent proliferation of nonneuronal cells. The culture media was changed biweekly with MEM supplemented with 2 mM L-glutamine, 5% horse serum, 100 I.U./ml penicillin and 0.10 mg/ml streptomycin, pH 7.4. The cultures were used for experiments between 9 –13 days *in vitro*.

2.2 Intracellular Ca^{2+} monitoring

For intracellular Ca^{2+} monitoring the cells were first washed four times with Locke's buffer (154 mM NaCl, 5.6 mM KCl, 1.0 mM $MgCl_2$, 2.3 mM $CaCl_2$, 8.6 mM HEPES, 5.6 mM glucose, and 0.1 mM glycine, pH 7.4) using an automated cell washer

(Labsystems, Helsinki, Finland). Cells were then incubated for 1 h at 37˚C with dye loading buffer (100ul/well) containing 4 µM fluo-3 AM and 0.04% Pluronic acid in Locke's buffer. Fluo-3 AM is taken up by cells and entrapped intracellularly after hydrolysis to fluo-3 by cell esterases. Stock fluo-3 AM was prepared by dissolving 1mg of fluo-3 AM in 45 µl of DMSO to give a final DMSO concentration of 0.023% in the dye loading buffer. After 1h incubation in dye loading medium, cells were washed 5 times with Locke's buffer. The final volume of Locke's buffer in each well was 150 µl. All the drugs were made at 4-times the final concentration and 50 µl of the drugs were added to the culture wells.

FLIPR (Fluorometric Laser Imaging Plate Reader) operates by illuminating the bottom of a 96-well microplate with an argon laser and measuring the fluorescence emissions from cell-permeant dyes in all 96 wells simultaneously using a cooled CCD camera. Moreover, this instrument is equipped with an automated 96-well pipettor, which can be programmed to deliver precise quantities of solutions simultaneously to all 96-culture wells from two separate 96-well source plates. In all the experiments 50 µl of drug was added to each well yielding a final volume of 200 µl/culture well. Neurons were excited by the 488 nm line of the argon laser and Ca^{2+} bound fluo-3 emission in the 500 nm to 560 nm range was recorded with the CCD camera shutter speed set at 0.4 s. Prior to each experiment, average baseline fluorescence was set between 10,000 and 20,000 fluorescence units by adjusting the power output of the laser. Fluorescence readings were taken once every 6 s. Fluorescence recording was done for 30 min after addition of drug.

2.3 Quantification of results

Data were analyzed and graphs generated by using the GraphPad 3.0 analysis package (GraphPad Software, San Diego, CA, USA).

2.4 Materials

Trypsin, penicillin, streptomycin, heat-inactivated fetal bovine serum, horse serum and soybean trypsin inhibitor were obtained from Atlanta Biologicals (Norcross, GA.). Minimum essential medium (MEM), Deoxyribonuclease (DNAse), poly-L-lysine, cytosine arabinosoide, veratridine, MK-801, NBQX, concanavalin A, thapsigargin and tetrodotoxin were purchased from Sigma (St. Louis, MO). Dextrorphan, dextromethorphan, cyclothiazide, nifedipine, cyclopentyladenosine (CPA) and 8-(p-Sulfophenyl)theophylline (8-PSPT) were purchased from RBI (Natick, MA). 1-aminocyclopentane-trans-1,3-dicarboxylic acid (trans-ACPD), (S)-4-carboxyphenylglycine ((S)-4CPG) and U73122 were purchased from Tocris (Ballwin, MO). Tetanus toxin was obtained from List Biologicals (Campbell, CA). Pluronic acid and fluo-3 AM were purchased from Molecular Probes (Eugene, OR).

3. Results

3.1 Spontaneous synchronized Ca^{2+} oscillations at physiological Mg^{2+}

Neocortical neurons in culture develop extensive processes and form a synaptically connected network (Dichter, 1989; Nakanishi and Kukita, 1998). We found that murine neocortical cultures (10-13 days in vitro) reliably produce spontaneous Ca^{2+} oscillations in the presence of physiological Mg^{2+} (1 mM). The mechanisms underlying these Ca^{2+} oscillations were explored inasmuch as they are likely to differ from those

elicited by low or zero extracellular Mg^{2+} concentration. These Ca^{2+} oscillations were highly synchronized in populations of neurons in culture and their mean frequency was 2±1/min. The amplitude of the oscillations was variable among different cultures with average amplitudes ranging between 5,000 to 10,000 fluorescence units. The use of a sampling interval of 1 sec demonstrated that the amplitude of consecutive oscillations in a given culture was maintained at a constant level throughout the observation period (Fig. 3.1). To assess the time course of drug effects on Ca^{2+} oscillations a 30 min duration of observation was chosen. To accommodate this 30min experimental time period, a sampling interval of 6 sec was chosen inasmuch as the FLIPR software allows a total of 300 fluorescence recordings in a single experiment. Although the longer sampling interval permitted a longer duration of observation, it also gives the appearance of somewhat variable amplitude among consecutive Ca^{2+} spikes. This apparent variability in amplitude is, however, a consequence of the longer sampling interval.

We initiated the exploration of the mechanisms of the Ca^{2+} oscillations by evaluating the requirement for depolarization of neurons. In agreement with previous studies application of 1 μM tetrodotoxin (TTX) abolished the Ca^{2+} oscillations (Fig. 3.2B), indicating that the process is mediated by depolarization and activation of voltage-gated sodium channels (VGSC). Furthermore 1 μM veratridine, a VGSC activator, produced a sustained elevation in the Ca^{2+} response with attendant disruption of oscillations (Fig. 3.2C).

3.2 Contributions of NMDA and non-NMDA receptors

To determine the source of Ca^{2+} influx and the trigger for initiation of Ca^{2+} oscillations, we first evaluated ionotropic glutamate receptors. The noncompetitive

NMDA antagonists dizocilpine (MK-801), dextrorphan (DX) and dextromethorphan (DXM) were tested for their ability to suppress Ca^{2+} oscillations. Although all three NMDA antagonists produced a concentration dependent suppression of oscillations, their IC_{50} values for inhibiting Ca^{2+} oscillations were in the micromolar range (Fig. 3.3, 3.5 and Table 3.1). As shown in table 1 the IC_{50} values calculated from the concentration response curves (cumulative calcium spike area) are two orders of magnitude higher than their binding affinities (K_I) for the NMDA receptor (Franklin and Murray, 1992). Moreover the relative potencies of these compounds in blocking Ca^{2+} oscillations were not consonant with their relative affinities for the NMDA receptor. These data indicate that a molecular target other than NMDA receptors underlies the effects of these compounds on Ca^{2+} oscillations.

Non-NMDA glutamate receptors are capable of depolarization of neurons through glutamate-gated Na^+ and Ca^{2+} influx. Ca^{2+} permeable AMPA/kainate receptors have also been reported in cortical neurons (Turetsky et al., 1994; Lu et al., 1996). Thus we ascertained the involvement of AMPA/kainate receptors in the triggering of Ca^{2+} oscillations. 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX), an AMPA/kainate antagonist, potently suppressed the Ca^{2+} oscillations in a concentration dependent manner with an IC_{50} value of 5.32±1.61 nM (Fig. 3.4, 3.5). As shown in table 1, the IC_{50} value for NBQX is consonant with its affinity for AMPA/kainate receptors (Dev et al., 1996). NBQX selectively reduced the frequency of oscillations (Fig. 3.4), whereas NMDA antagonists primarily reduced the amplitude of oscillations (Fig. 3.3). To delineate the relative contributions of AMPA and kainate receptors in the generation of Ca^{2+} oscillations, we treated neurons with cyclothiazide (50

μM), an inhibitor of AMPA receptor desensitization and concanavalin A (50 μg/ml), an inhibitor of kainate receptor desensitization. Inhibition of AMPA receptor desensitization, led to an increase in the frequency of Ca^{2+} oscillations (Fig. 3.6B). In contrast, inhibition of kainate receptor desensitization did not affect the frequency of Ca^{2+} oscillations (Fig. 3.6C), implying that AMPA rather than kainate receptors are responsible for generation of Ca^{2+} oscillations.

Considered together these data indicate that AMPA receptors are responsible for generation of Ca^{2+} oscillations. As only a fraction of neurons in neocortical cultures express Ca^{2+}-permeable AMPA receptors, AMPA receptor-gated Ca^{2+} influx cannot account for the Ca^{2+} signal observed in the entire population of neurons. Rather, AMPA receptors more likely act as a trigger for depolarization which is required for the generation of Ca^{2+} oscillations.

3.3 Source of Ca^{2+} for generation of calcium oscillations

Since the NMDA antagonist MK-801 at a concentration of 100nM, which produces full occupancy of NMDA receptors, produced only 22±6% suppression of Ca^{2+} oscillations, we tested other potential sources of Ca^{2+} to support the oscillations. In order to delineate the source of Ca^{2+} for the oscillations, we first determined the effect of removal of Ca^{2+} from the extracellular medium. Removal of Ca^{2+} from the extracellular medium eliminated the Ca^{2+} oscillations (Fig. 3.7B). Complete elimination of Ca^{2+} oscillations upon removal of extracellular Ca^{2+} could be due to inhibition of Ca^{2+}-dependent neurotransmitter release and may not indicate that the Ca^{2+}-influx is entirely derived from extracellular media. We therefore assessed the role of intracellular Ca^{2+}

pool on the Ca^{2+} oscillations. Depletion of endoplasmic reticular (ER) Ca^{2+} stores with 1 μM thapsigargin reduced the amplitude but not the frequency of calcium oscillations (Fig. 3.7C). These results suggest that the main source or trigger for Ca^{2+} oscillations is influx of Ca^{2+} from the extracellular milieu while the mobilization of Ca^{2+} from intracellular stores contributes to the magnitude of cytosolic Ca^{2+} during a Ca^{2+} spike. The L-type voltage gated Ca^{2+} channels (VGCC) have been reported to be one of the major route of Ca^{2+} entry for generation of Ca^{2+} oscillations in neocortical neurons in the absence of extracellular Mg^{2+} (Wang and Gruenstein, 1997). Nifedipine (1 μM), however, did not affect Ca^{2+} oscillations indicating that L-type Ca^{2+} channels are not necessary for influx of calcium (Fig. 3.7D). Similarly, the Na^{+}-Ca^{2+} exchange inhibitor KBR7943 (1 μM) was ineffective in blocking oscillations (data not shown).

Since thapsigargin reduced the amplitude of Ca^{2+} oscillations, we tested whether mGluR's contribute to the rise in cytosolic Ca^{2+} by mobilization of Ca^{2+} from the endoplasmic reticulum (ER) stores. We used a Group I mGluR antagonist S-4-CPG, which in neocortical slices has been shown to inhibit Ca^{2+} oscillations (Flint et al., 1996), and found that S-4-CPG (500 μM) significantly inhibited the calcium oscillations (Fig. 3.8C). Furthermore, we ascertained the effect of stimulation of mGluR with a non-specific mGluR agonist, trans-ACPD. Application of 10 μM trans-ACPD caused an increase in the frequency of Ca^{2+} oscillations (Fig. 3.8B) indicating that mGluR are involved in the generation of Ca^{2+} oscillations. One of the key messengers involved in mGluR signaling is phospholipase C (PLC). Hence, we tested whether inhibition of PLC had any effect on Ca^{2+} oscillations. Application of PLC inhibitor, U73122 (10 μM), abolished the Ca^{2+} oscillations (Fig. 3.9). It may be inferred from these results that

mGluR are involved in the generation of Ca^{2+} oscillations which may occur through the mobilization of Ca^{2+} from ER Ca^{2+} stores after PLC activation.

3.4 Source of glutamate required for activation of glutamate receptors.

The results indicate that the Ca^{2+} oscillations are generated primarily by the activation of AMPA and mGluR receptors. We therefore tested whether the source of glutamate required for activation of these glutamate receptors is through vesicular release from the neurons. Neocortical cultures were therefore incubated with 50 nM tetanus toxin for 24 h to block vesicular release from neurons. This treatment abolished the Ca^{2+} oscillations indicating that the source of glutamate for activation of glutamate receptors is the vesicles in glutamatergic terminals (Fig. 3.10).

Adenosine receptors have been found to influence glutamatergic neurotransmission through both presynaptic and postsynaptic effects. The A1 adenosine receptor agonist cyclopentyladenosine (CPA) completely abolished the Ca^{2+} oscillations (Fig.3.11C). The adenosine receptor antagonist 8-p-sulfophenyltheophylline (8-PSPT) caused an increase in the frequency of Ca^{2+} oscillations (Fig. 3.11B). Thus spontaneous synchronized Ca^{2+} oscillations appear to be tonically regulated by A1 adenosine receptors.

4. Discussion

In the present study we have examined the routes of intracellular Ca^{2+} rise during generation of Ca^{2+} oscillations in neocortical neurons under physiological Mg^{2+} using FLIPR. We found that the mechanism of Ca^{2+} oscillations in the presence of physiological Mg^{2+} differed from the mechanisms described earlier with low or zero

extracellular Mg^{2+}. At low or zero extracellular Mg^{2+}, concomitant activation of NMDA receptors and L-type Ca^{2+} channels have been reported to be necessary for the generation of Ca^{2+} oscillations (Wang and Gruenstein, 1998). In contrast we found that Ca^{2+} oscillations which occurs in the presence of physiological Mg^{2+} are independent of activation of L-type Ca^{2+} channels and with a minor contribution of NMDA receptors. In agreement with the previous studies Ca^{2+} oscillations were primarily triggered by AMPA receptors (Murphy et al., 1992; Wang and Gruenstein, 1997). In addition we found that release of Ca^{2+} from intracellular Ca^{2+} stores was also involved in the generation of Ca^{2+} oscillations. Thus rather than being entirely driven by plasma membrane receptors, Ca^{2+} oscillations in neocortical neurons exhibit a more complex mechanism involving a combination of influx from the extracellular compartment and mobilization of intracellular Ca^{2+} stores.

NMDA receptors are coincident receptors and their activation depends on agonist binding as well as on membrane depolarization. The NMDA receptor's voltage dependence is due to the channel block by submillimolar concentration of extracellular Mg^{2+} (Mayer et al., 1987), and a membrane depolarization to –50 mV or more is required for the release of this Mg^{2+} block. Accordingly it has been seen that removal of Mg^{2+} from extracellular media induces epileptiform activity in hippocampal and neocortical cultures (Mody et al., 1987; Rose et al., 1990). In immature cortical neurons the Mg^{2+} - dependence of Ca^{2+} oscillations has also been demonstrated (Wang and Grunstein, 1997). Removal of Mg^{2+} is detrimental to neuronal survival and leads to excitotoxic cell death in cortical neurons due to excessive NMDA receptor activation (Rose et al., 1990). These studies indicate that removal of Mg^{2+} from extracellular media creates non-physiologic

conditions and biases the occurrence of Ca^{2+} oscillations in favor of NMDA receptors. Our results with the non-competitive antagonists of NMDA receptors indicate that only a small portion of the Ca^{2+} influx for the Ca^{2+} oscillations may be attributed to NMDA receptors. A concentration of MK-801 that results in full occupancy of NMDA receptors produced only a 22% reduction in the amplitude of Ca^{2+} oscillations.

AMPA receptors mediate fast excitatory neurotransmission in the nervous system. AMPA receptors usually gate Na^{+} but a fraction of neurons possess AMPA receptors that lack the GluR2 subunit and are therefore Ca^{2+}-permeable. AMPA receptors can also induce Ca^{2+} influx indirectly through NMDA receptors and VGCC by causing neuronal depolarization that is necessary for the activation of NMDA receptors and VGCC. AMPA receptors undergo rapid desensitization after stimulation with glutamate. Cyclothiazide, an AMPA receptor desensitization inhibitor, was found to augment the frequency of Ca^{2+} oscillations indicating that AMPA receptor desensitization is an important mechanism of frequency regulation of Ca^{2+} oscillations. These results indicate that AMPA receptors are key regulators of the frequency of Ca^{2+} oscillation.

Activation of mGluR, especially mGluR5, has been shown to induce oscillatory activity in a neocortical slice preparation when activated by endogenously released glutamate (Flint et al., 1999). mGluR were found to be essential for the Ca^{2+} oscillations in neocortical neurons in culture since inhibition of mGluR receptors decreased the amplitude of oscillations. PLC activation is coupled to the activation of G-protein mGluR and can induce Ca^{2+} release from intracellular stores through the IP_3 pathway. In neocortical neurons in the absence of Mg^{2+} the PLC inhibitor U73122 (20 μM) had no effect on calcium oscillations (Wang and Gruenstein, 1997). In contrast we observed that

10 μM U73122 completely eliminated calcium oscillations, indicating that mobilization of intracellular Ca^{2+} stores is essential for the generation of calcium oscillations under physiological Mg^{2+}. The mGluR mediated activation of PLC-β1 has been found to be necessary for activity-dependent differentiation in cerebral cortex (Hannan et al., 2001), thus underscoring the physiologic importance of the generation of Ca^{2+} oscillations by these receptors.

Synchronization of Ca^{2+} oscillations in neurons in culture is considered to be due to synaptic communication in the neuronal network. Ca^{2+} waves on the other hand are mediated by gap junctional communication and propagate at a much slower rate as compared to Ca^{2+} oscillations (Charles et al., 1996). The occurrence of these Ca^{2+} waves is independent of action potentials. It has been suggested that, the propagation of calcium waves is through the gap junctional diffusion of IP_3 and not Ca^{2+} (Kandler and Katz, 1998). In our culture system we did not observe Ca^{2+} waves among neurons. In addition the VGSC dependence and the complete elimination of Ca^{2+} oscillations in the tetanus toxin treated cultures further indicate that action potential generation and vesicular release of neurotransmitter are necessary for the synchronized activity.

Adenosine receptors have been shown to modulate glutamatergic neurotransmission in cortical neurons (Brand et al., 2001). Adenosine receptors may induce their modulatory effect by presynaptic as well as postsynaptic mechanisms. Complete elimination of Ca^{2+} oscillations by 10nM CPA and the increase in the frequency of oscillations by inhibition of adenosine receptors with the antagonist 8-PSPT establishes a role for A1 adenosine receptors in the oscillatory activity. Whether this

effect is due to pre- or postsynaptic receptors is unclear, but the results clearly indicate that adenosine receptors tonically regulate the Ca^{2+} oscillations.

It is important to identify the routes of Ca^{2+} entry for the generation of Ca^{2+} oscillations, inasmuch as different routes of Ca^{2+} entry can stimulate distinct signaling pathways that can activate different sets of genes resulting in alternative physiological outcomes (West et al., 2001). In addition the signaling mediated by synaptically mediated Ca^{2+} influx may be different than that mediated by extrasynaptic Ca^{2+} influx (Hardingham et al., 2002), highlighting the importance of studying the mechanism of synaptically mediated Ca^{2+} oscillations at physiological conditions. Knowledge of the mechanism of Ca^{2+} oscillations under physiologic conditions may therefore help understand the influence of different routes of Ca^{2+} entry, on the various downstream signaling pathways and gene expression.

References:

Berridge M. J. (1993) Inositol trisphosphate and calcium signalling. *Nature* **361,** 315-325.

Brand A., Vissiennon Z., Eschke D., and Nieber K. (2001) Adenosine A(1) and A(3) receptors mediate inhibition of synaptic transmission in rat cortical neurons. *Neuropharmacology* **40,** 85-95.

Charles A. C., Kodali S. K., and Tyndale R. F. (1996) Intercellular calcium waves in neurons. *Mol Cell Neurosci* **7,** 337-353.

Dev K. K., Petersen V., Honore T., and Henley J. M. (1996) Pharmacology and regional distribution of the binding of 6-[3H]nitro-7-sulphamoylbenzo[f]-quinoxaline-2,3-dione to rat brain. *J Neurochem* **67,** 2609-2612.

Dichter M. A. and Zona C. (1989) Calcium currents in cultured rat cortical neurons. *Brain Res* **492,** 219-229.

Dolmetsch R. E., Xu K., and Lewis R. S. (1998) Calcium oscillations increase the efficiency and specificity of gene expression. *Nature* **392,** 933-936.

Fields R. D., Eshete F., Stevens B., and Itoh K. (1997) Action potential-dependent regulation of gene expression: temporal specificity in ca2+, cAMP-responsive element binding proteins, and mitogen-activated protein kinase signaling. *J Neurosci* **17,** 7252-7266.

Flint A. C., Dammerman R. S., and Kriegstein A. R. (1999) Endogenous activation of metabotropic glutamate receptors in neocortical development causes neuronal calcium oscillations. *Proc Natl Acad Sci U S A* **96,** 12144-12149.

Fotuhi M., Sharp A. H., Glatt C. E., Hwang P. M., von Krosigk M., Snyder S. H., and Dawson T. M. (1993) Differential localization of phosphoinositide-linked metabotropic glutamate receptor (mGluR1) and the inositol 1,4,5-trisphosphate receptor in rat brain. *J Neurosci* **13,** 2001-2012.

Franklin P. H. and Murray T. F. (1992) High affinity [3H]dextrorphan binding in rat brain is localized to a noncompetitive antagonist site of the activated N-methyl-D-aspartate receptor-cation channel. *Mol Pharmacol* **41,** 134-146.

Friel D. D. and Tsien R. W. (1992) Phase-dependent contributions from Ca2+ entry and Ca2+ release to caffeine-induced [Ca2+]i oscillations in bullfrog sympathetic neurons. *Neuron* **8,** 1109-1125.

Gu X. and Spitzer N. C. (1995) Distinct aspects of neuronal differentiation encoded by frequency of spontaneous Ca2+ transients. *Nature* **375,** 784-787.

Hannan A. J., Blakemore C., Katsnelson A., Vitalis T., Huber K. M., Bear M., Roder J., Kim D., Shin H. S., and Kind P. C. (2001) PLC-beta1, activated via mGluRs, mediates activity-dependent differentiation in cerebral cortex. *Nat Neurosci* **4,** 282-288.

Hardingham G. E., Fukunaga Y., and Bading H. (2002) Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. *Nat Neurosci* **5,** 405-414.

Hu Q., Deshpande S., Irani K., and Ziegelstein R. C. (1999) [Ca(2+)](i) oscillation frequency regulates agonist-stimulated NF-kappaB transcriptional activity. *J Biol Chem* **274,** 33995-33998.

Jensen J. B., Schousboe A., and Pickering D. S. (1998) Development of calcium-permeable alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors in cultured neocortical neurons visualized by cobalt staining. *J Neurosci Res* **54,** 273-281.

Kandler K. and Katz L. C. (1998) Coordination of neuronal activity in developing visual cortex by gap junction-mediated biochemical communication. *J Neurosci* **18,** 1419-1427.

Kawabata S., Tsutsumi R., Kohara A., Yamaguchi T., Nakanishi S., and Okada M. (1996) Control of calcium oscillations by phosphorylation of metabotropic glutamate receptors. *Nature* **383,** 89-92.

Komuro H. and Rakic P. (1996) Intracellular Ca2+ fluctuations modulate the rate of neuronal migration. *Neuron* **17,** 275-285.

Lawrie A. M., Graham M. E., Thorn P., Gallacher D. V., and Burgoyne R. D. (1993) Synchronous calcium oscillations in cerebellar granule cells in culture mediated by NMDA receptors. *Neuroreport* **4,** 539-542.

Lu Y. M., Yin H. Z., Chiang J., and Weiss J. H. (1996) Ca(2+)-permeable AMPA/kainate and NMDA channels: high rate of Ca2+ influx underlies potent induction of injury. *J Neurosci* **16,** 5457-5465.

Mayer M. L., Westbrook G. L., and Guthrie P. B. (1984) Voltage-dependent block by Mg2+ of NMDA responses in spinal cord neurones. *Nature* **309,** 261-263.

Mody I., Lambert J. D., and Heinemann U. (1987) Low extracellular magnesium induces epileptiform activity and spreading depression in rat hippocampal slices. *J Neurophysiol* **57,** 869-888.

Murphy T. H., Blatter L. A., Wier W. G., and Baraban J. M. (1992) Spontaneous synchronous synaptic calcium transients in cultured cortical neurons. *J Neurosci* **12,** 4834-4845.

Nakanishi K. and Kukita F. (1998) Functional synapses in synchronized bursting of neocortical neurons in culture. *Brain Res* **795,** 137-146.

Nunez L., Sanchez A., Fonteriz R. I., and Garcia-Sancho J. (1996) Mechanisms for synchronous calcium oscillations in cultured rat cerebellar neurons. *Eur J Neurosci* **8,** 192-201.

Ogura A., Iijima T., Amano T., and Kudo Y. (1987) Optical monitoring of excitatory synaptic activity between cultured hippocampal neurons by a multi-site Ca2+ fluorometry. *Neurosci Lett* **78,** 69-74.

Robinson H. P., Kawahara M., Jimbo Y., Torimitsu K., Kuroda Y., and Kawana A. (1993) Periodic synchronized bursting and intracellular calcium transients elicited by low magnesium in cultured cortical neurons. *J Neurophysiol* **70,** 1606-1616.

Rose K., Christine C. W., and Choi D. W. (1990) Magnesium removal induces paroxysmal neuronal firing and NMDA receptor-mediated neuronal degeneration in cortical cultures. *Neurosci Lett* **115,** 313-317.

Spitzer N. C., Olson E., and Gu X. (1995) Spontaneous calcium transients regulate neuronal plasticity in developing neurons. *J Neurobiol* **26,** 316-324.

Turetsky D. M., Canzoniero L. M., Sensi S. L., Weiss J. H., Goldberg M. P., and Choi D. W. (1994) Cortical neurones exhibiting kainate-activated Co2+ uptake are selectively vulnerable to AMPA/kainate receptor-mediated toxicity. *Neurobiol Dis* **1,** 101-110.

Wang X. and Gruenstein E. I. (1997) Mechanism of synchronized Ca2+ oscillations in cortical neurons. *Brain Res* **767,** 239-249.

West A. E., Chen W. G., Dalva M. B., Dolmetsch R. E., Kornhauser J. M., Shaywitz A. J., Takasu M. A., Tao X., and Greenberg M. E. (2001) Calcium regulation of neuronal gene expression. *Proc Natl Acad Sci U S A* **98,** 11024-11031.

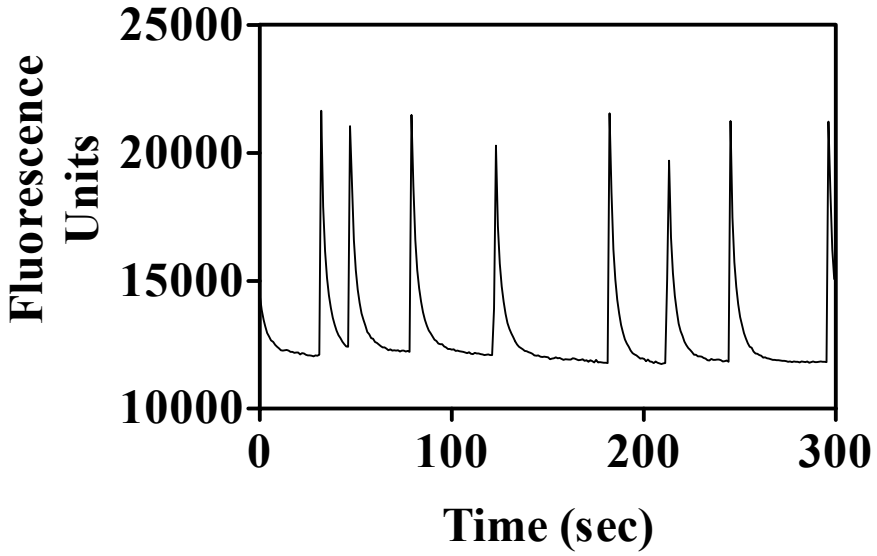


Figure 3.1. Cytosolic Ca^{2+} oscillations in neocortical neurons at physiologic [Mg^{2+}]. Intracellular Ca^{2+} levels were monitored every second in fluo-3 loaded neocortical neurons.

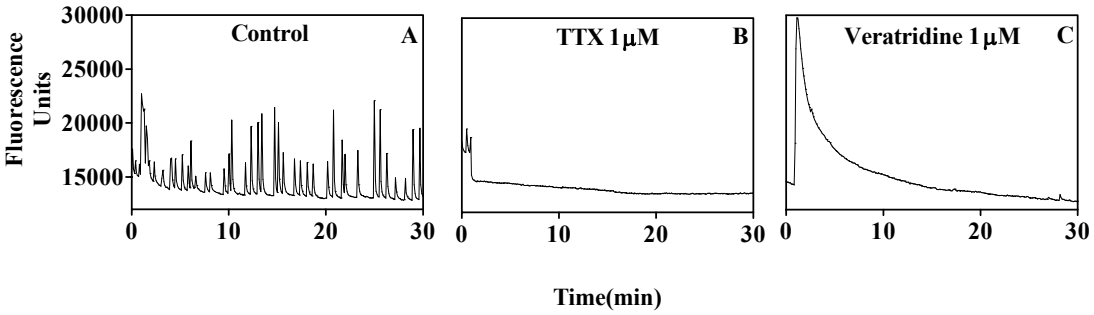


Figure 3.2. Depolarization mediated generation of Ca^{2+} oscillations.

Data are from a representative experiment performed in six replicates. The cerebral cortical neurons were exposed to either 1 μM tetrodotoxin (TTX) (B), 1 μM veratridine (C) or vehicle (A).

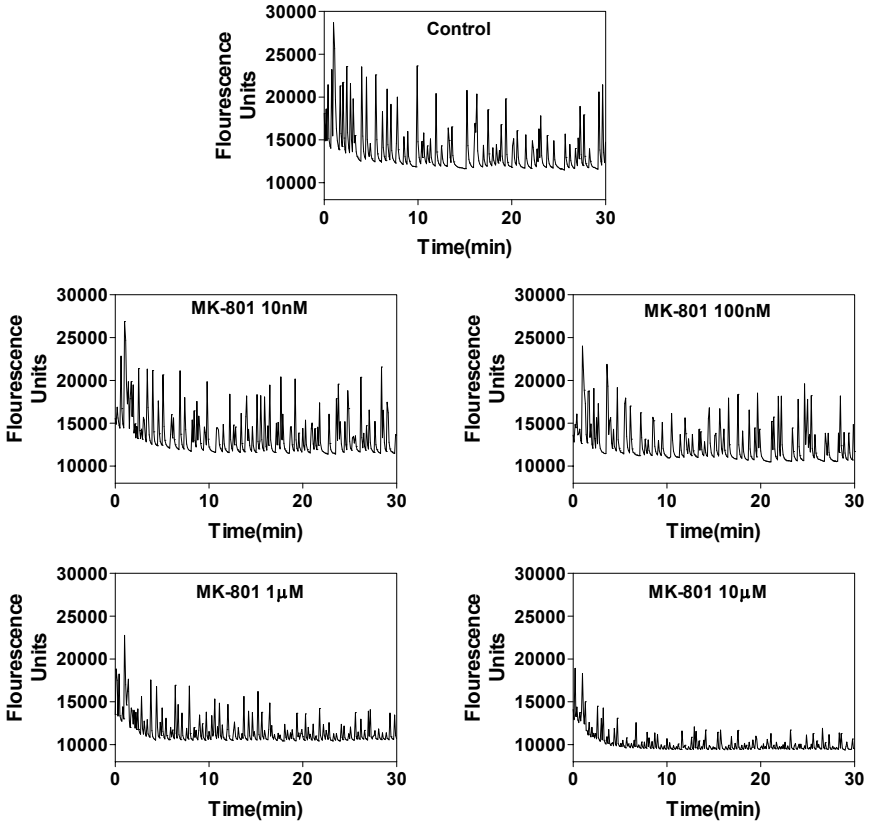


Figure 3.3. Concentration-dependent suppression of Ca^{2+} oscillation amplitude by MK-801. Data are from representative experiment performed in six replicates and repeated 3 times.

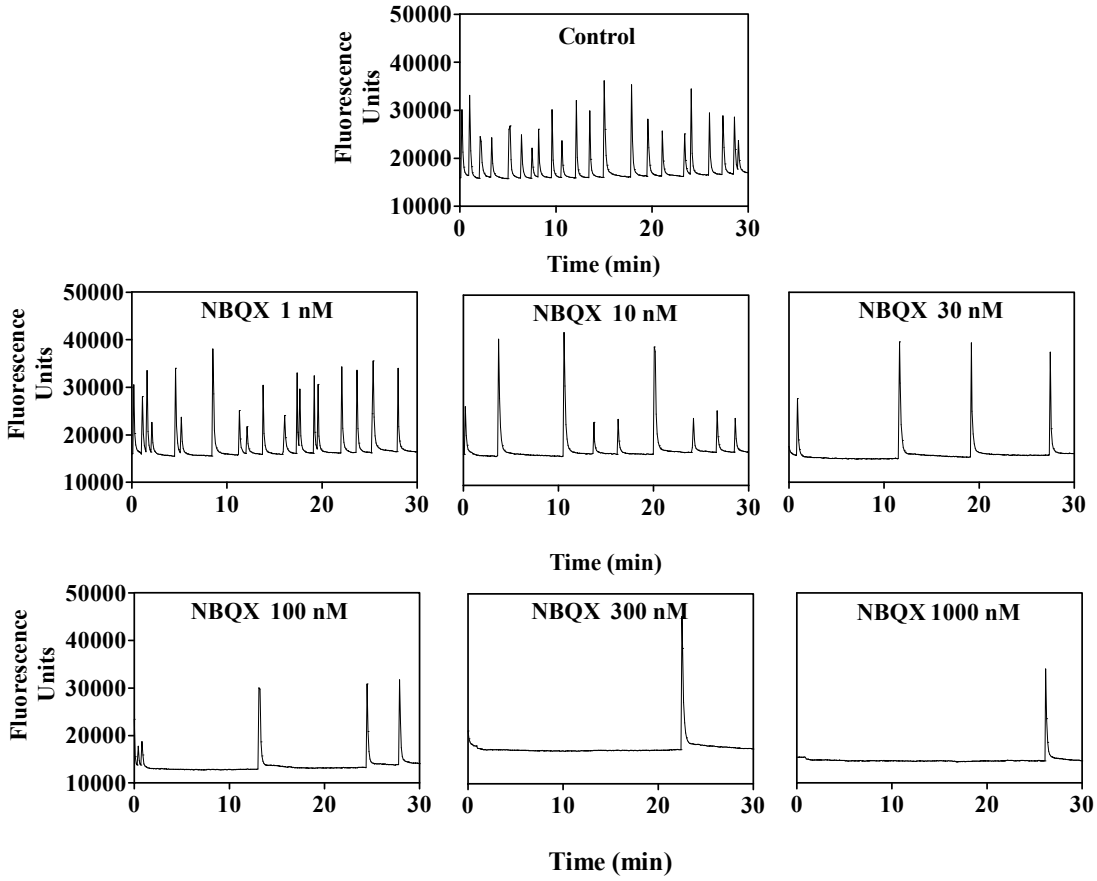


Figure 3.4. Concentration-dependent reduction in the frequency of Ca^{2+} oscillations by NBQX. Data are from representative experiment performed in six replicates and repeated 3 times.

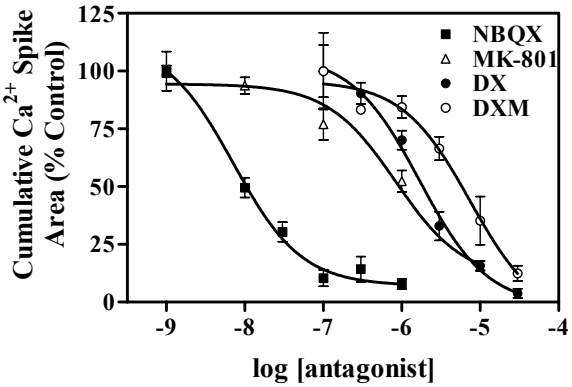


Figure 3.5. Concentration-dependent suppression of Ca^{2+} oscillations by non-NMDA and NMDA antagonists.

Each point in the concentration-response curve represents mean cumulative Ca^{2+} spike area from three independent experiments. The IC_{50} values for suppression of Ca^{2+} oscillations were derived by nonlinear regression analysis.

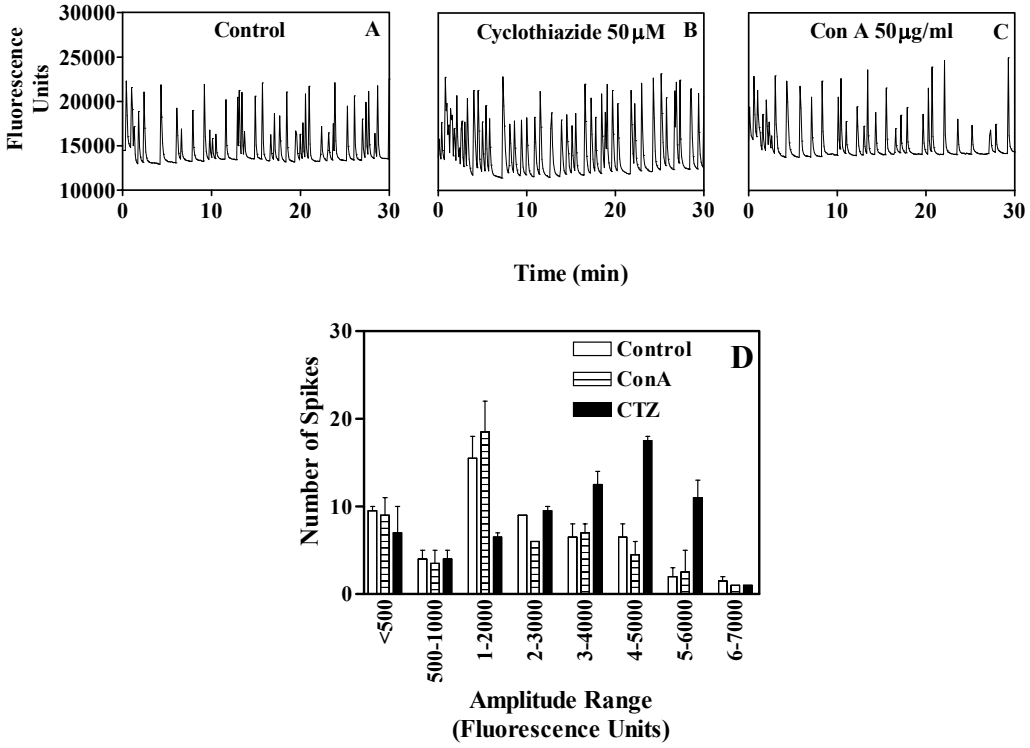


Figure 3.6. Relative contributions of AMPA and kainate receptors in the generation of Ca^{2+} oscillations.

Data are from a representative experiment performed in six replicates. The neocortical neurons were exposed to either 50 µM cyclothiazide (B), 50 µg/ml concanavalin A (C), or vehicle (A) and changes in $[Ca]_i$ were monitored. Amplitude distribution analysis of the raw data (D). Data are mean ± SE of triplicates.

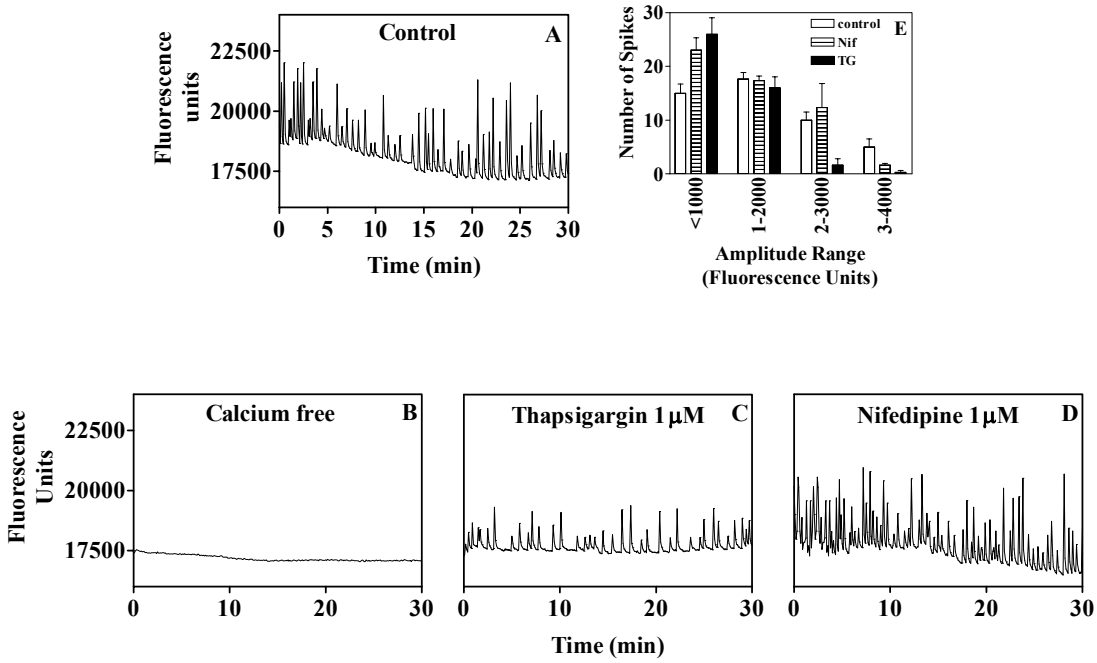


Figure 3.7. Routes of Ca^{2+} entry for the generation of Ca^{2+} oscillations.

Data are from a representative experiment performed in six replicates. Contributions of extracellular Ca^{2+} pool and intracellular calcium stores were evaluated by removal of Ca^{2+} from extracellular medium (B) and pretreatment of neocortical neurons with 1 µM thapsigargin for 1 h (C) respectively. Neocortical neurons were treated with 1 µM nifedipine (D) to assess role of L-type Ca^{2+} channel in generation of Ca^{2+} oscillations. Amplitude distribution analysis of the raw data (E). Data are mean ± SE of triplicates.

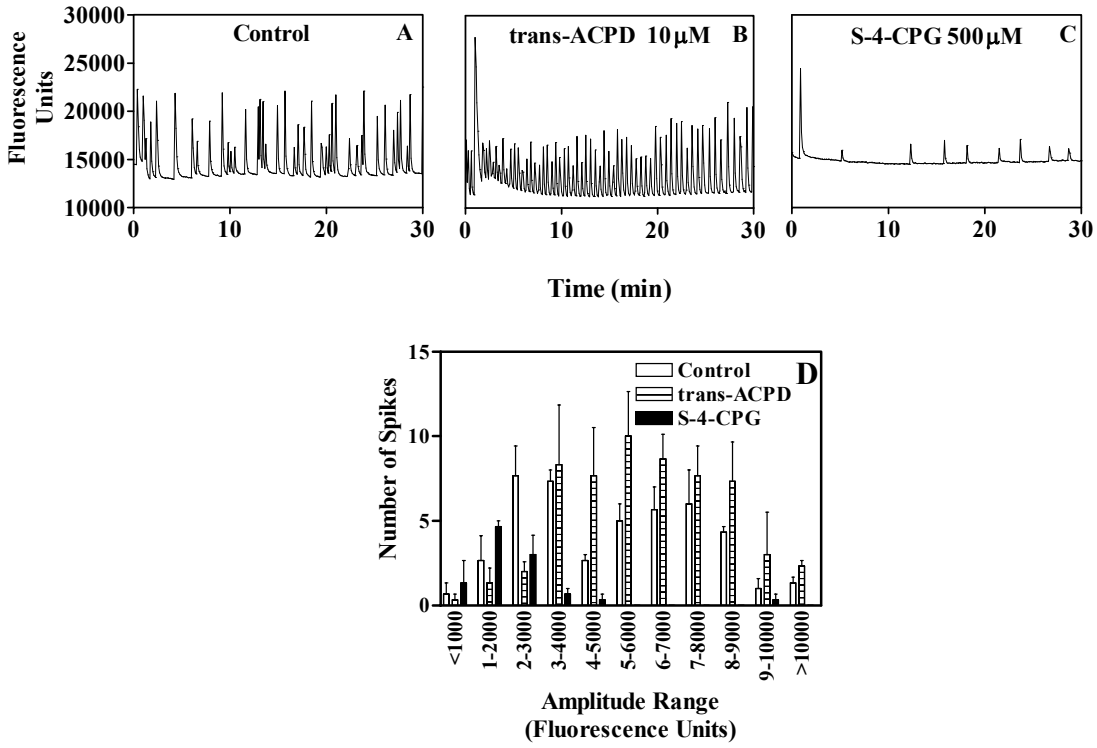


Figure 3.8. Involvement of mGlu receptors in the generation of Ca^{2+} oscillations. Data are from a representative experiment performed in six replicates. The neocortical neurons were exposed either to 50 µM trans-ACPD (B), 500 µM S-4-CPG (C) or vehicle (A). Amplitude distribution analysis of the raw data (D). Data are mean ± SE of triplicates.

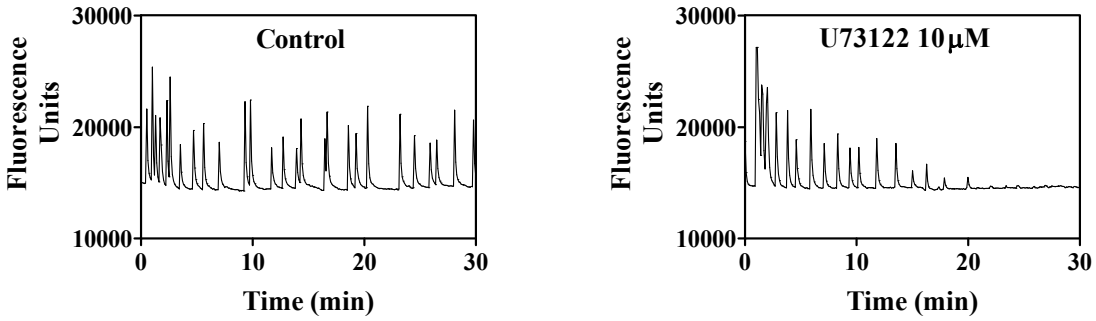


Figure 3.9. Role of PLC in the generation of Ca^{2+} oscillations.

10 µM of the PLC inhibitor U73122 completely eliminated the Ca^{2+} oscillations exhibited by neocortical neurons. Data are from a representative experiment performed in six replicates.

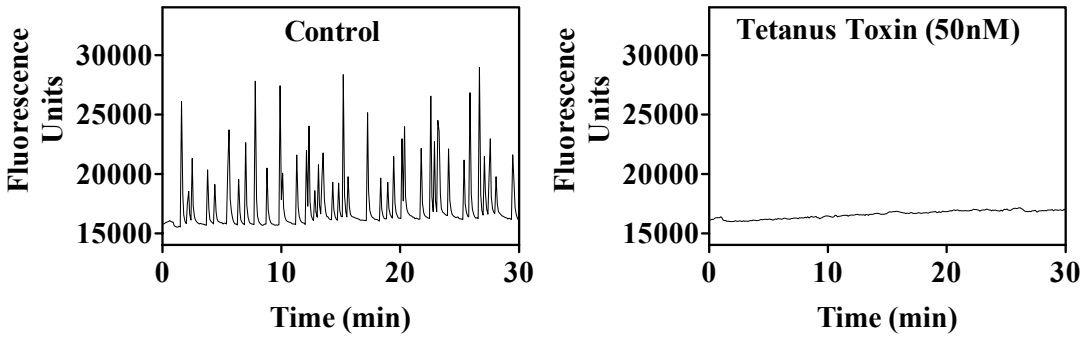


Figure 3.10. Tetanus toxin abolishes Ca^{2+} oscillations indicating that synaptic transmission is necessary for the synchronized activity in the neurons in culture.

Data are from a representative experiment performed in six replicates. The neocortical neurons were pretreated with either 50 nM tetanus toxin (B) or vehicle (A) for a period of 24 h.

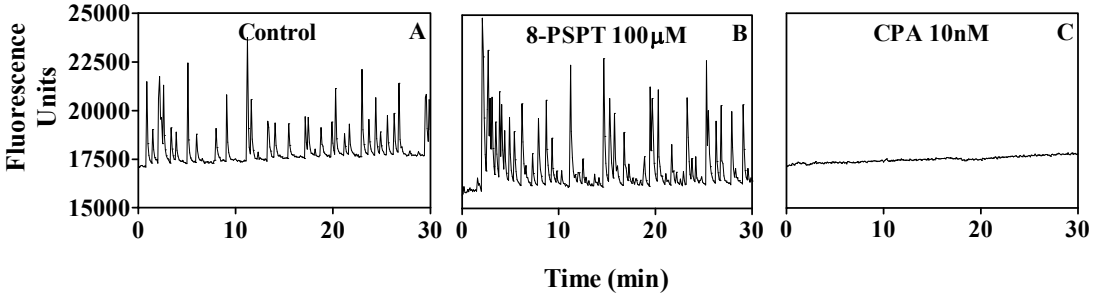


Figure 3.11. Tonic regulation of Ca^{2+} oscillations by adenosine receptors.

The neocortical neurons were exposed to either 100 µM 8-PSPT (B) or 10 nM CPA (C) or vehicle (A). Data are from a representative experiment performed in six replicates.

Table 3.1: Relative IC_{50} and Kd for non-NMDA and NMDA antagonists.

Compound	IC_{50}	Kd
NBQX	5.32±1.61 nM	18±1 nM
MK-801	0.76±0.17 μM	0.62±0.14 μM
DX	3.05±1.32 μM	39.5±5.1 μM
DXM	5.41±0.01 μM	321±17.9 μM

CHAPTER 4

FLUORESCENT DETECTION OF Ca^{2+}-PERMEABLE AMPA/KAINATE RECEPTOR ACTIVATION IN MURINE NEOCORTICAL NEURONS[1]

[1]Dravid S.M. and Murray T.F. submitted to Neuroscience Letters

Abstract

Agonist stimulated Co^{2+} uptake is used to identify neurons expressing Ca^{2+}-permeable AMPA/kaniate receptors (Ca-A/K receptors). Based on the selective permeability of Co^{2+} through these receptors we have developed a fluorometric method that utilizes the fluorescence laser imaging plate reader (FLIPR). We used the dye calcein whose fluorescence is stoichiometrically quenched by Co^{2+}, while being only minimally affected by variations in intracellular Ca^{2+}. Application of AMPA in the presence of cyclothiazide led to a concentration-dependent increase in Co^{2+} uptake in the neocortical neurons. Similar concentration-dependent increments in Co^{2+} uptake were observed with kainate treatment. 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX), an AMPA/kainate receptor antagonist, blocked the AMPA-induced Co^{2+} influx. The fluorometric method described affords a rapid, high throughput and quantitative procedure for investigation of Ca-A/K receptors in intact neurons.

Keywords: Ca^{2+}-permeable AMPA/kainate receptors, neocortical neurons, FLIPR, Calcein AM, cobalt, fluorometric.

AMPA receptors mediate fast excitatory synaptic transmission in the CNS and are involved in physiological events such as synaptogenesis, growth cone movement, and synaptic plasticity [16]. AMPA receptors are encoded by four genes designated as GluR1 to GluR4. The GluR2 subunit dictates the Ca^{2+} permeability of AMPA receptors, inasmuch as its presence renders the heteromeric AMPA receptor assemblies Ca^{2+}-impermeable [4, 5, 8]. Ca^{2+} influx through AMPA receptors lacking the GluR2 subunit is involved in modulation of synaptic transmission and communication between neurons and glia [6, 9]. Rapid Ca^{2+} influx through the Ca-A/K receptors has also been shown to contribute to excitotoxicity [3, 10, 14]. Therefore the Ca-A/K receptors may serve as important therapeutic targets.

Our results indicate that AMPA receptor activation is accompanied by both activation of NMDA receptors and voltage-gated calcium channels. Therefore, measurement of AMPA/kainate agonist-induced rise in intracellular Ca^{2+} does not selectively reflect Ca^{2+} influx through Ca-A/K receptors. Ca-A/K receptors are routinely detected using a histochemical method based on the selective permeability of Co^{2+} in Ca-A/K receptors [11]. Based on the selective permeability of Co^{2+}, Co^{2+} influx-induced quenching of a fluorescent dye represents a more selective means of monitoring the activation of these receptors. Agonist stimulated Co^{2+} influx and quenching of fura-2 fluorescence has been demonstrated in cerebellar granule neurons [11]. This strategy was modified in the present study to permit detection of Co^{2+} influx through Ca-A/K receptors in a population of neocortical neurons without the requirement of UV excitation. We have used the dye calcein whose fluorescence is rapidly and

stochiometrically quenched by divalent transition metals including Co^{2+}, while not being quenched by variations in intracellular Ca^{2+} [2] The use of calcein therefore offers the opportunity to delineate Co^{2+} influx selectively and without interference of Ca^{2+} influx.

Neocortical neuron culture: Primary cultures of neocortical neurons were obtained from embryonic day 17 Swiss-Webster mice. Briefly, neocortices were collected, minced by trituration and treated with trypsin. The cells were then dissociated using soybean trypsin inhibitor and DNase containing isolation buffer, centrifuged and resuspended in Eagle's minimal essential medium with Earle's salt supplemented with 2 mM L-glutamine, 10% fetal bovine serum and 10% horse serum. Cells were plated onto poly-L-lysine coated 96-well plates (Costar) at a density of $4.7X10^5$ cells/cm^2. Cytosine arabinoside (10 μM) was added on day 2 after plating to prevent proliferation of non-neuronal cells. The cultures were used for experiments between 9–12 days *in vitro*.

Intracellular Ca^{2+} Monitoring: Measurement of intracellular Ca^{2+} ($[Ca^{2+}]_i$) was performed using FLIPR (Molecular Devices) by the method described previously [1]. Briefly, the neocortical neurons cultured in 96 well plates were incubated for 1h with 4μM fluo-3 AM (Molecular Probes) and 0.04% Pluronic acid in physiological Locke's buffer. After the incubation period the neurons were washed with Locke's buffer, using an automated cell washer, to remove extracellular dye. Neurons were excited at 488 nm and Ca^{2+} bound fluo-3 emission was recorded at 500-560 nm.

Co^{2+} Influx Assay: Co^{2+} influx was detected by measuring the fluorescence quenching of calcein dye using FLIPR. The neocortical neurons were incubated for 8 min with 1 μM of calcein AM (Molecular Probes) in physiological Locke's buffer. After this incubation period neurons were washed to remove extracellular dye. Following washing, the neurons were allowed to equilibriate for an additional 40 min at 37°C. Agonist alone in the absence of Co^{2+} did not affect calcein fluorescence. Agonist-induced Co^{2+} influx was measured in the presence of 25 μM of extracellular $CoCl_2$. This optimum concentration of $CoCl_2$ was determined empirically to minimize basal quenching. The drug treatment occured 2 min after $CoCl_2$ addition.

Data analysis: Non-linear regression analysis curves and ANOVA results were derived using Prism software (Graphpad). Relaxation time (tau) was calculated using the following equation:

Y= (maximum fluorescence –baseline fluorescence)*exp(-t/tau) + baseline fluorescence

To determine that activation of AMPA/kainate receptors leads to Ca^{2+} influx, $[Ca^{2+}]_i$ monitoring was done in neocortical neurons treated with AMPA in the presence of the AMPA receptor desensitization inhibitor, cyclothiazide (CTZ). AMPA+50μM CTZ treatment produced a concentration-dependent increase in the Ca^{2+} influx as evident from the increase in the fluo-3 fluorescence in neocortical neurons (Fig. 4.1). Area under the curve analysis of the time-response was quantified and concentration-response curve was generated (EC_{50} = 6.91±2.99 μM). A pharmacological evaluation of AMPA stimulated Ca^{2+} influx was performed in an effort to define the pathways mediating the

increase in cytoplasmic Ca^{2+}. As depicted in Fig. 4.2, treatment with MK-801 and ω-Agatoxin IVA significantly reduced the Ca^{2+} influx after AMPA+CTZ exposure. These data indicate that AMPA/kainate receptor activation is accompanied by activation of both NMDA receptors and P-type calcium channels. The L-type calcium channel blockade with nifedipine did not alter the Ca^{2+} influx by AMPA+CTZ treatment.

In order to detect Ca^{2+} influx selectively through Ca-A/K receptors we studied agonist induced Co^{2+} influx in neocortical neurons. A basal level of Co^{2+} influx is observed even in the absence of agonist treatment (Fig. 4.3). This basal level of quenching was not blocked by the AMPA/kainate receptor antagonist, NBQX, the NMDA antagonist, MK-801, or by inhibition of synaptic activity with tetrodotoxin (data not shown). Thus the exact mechanism of this basal Co^{2+} influx in neocortical neurons is not known. Treatment of neurons with AMPA+50 μM CTZ produced a concentration-dependent increase in quenching of calcein fluorescence representing Co^{2+} influx (Fig. 4.4A). Furthermore, the Co^{2+} influx induced by 30 μM AMPA+CTZ was completely blocked NBQX (Fig. 4.3) suggesting that the Co^{2+} influx occurred through the Ca-A/K receptors. Nonlinear regression analysis was performed on the concentration-response curve obtained using the peak fluorescence quenching produced by each concentration of AMPA (Fig. 4.4B) (EC_{50} = 7.54±1.6 μM). Nonlinear regression analysis of the reciprocal relaxation time (1/tau) derived from a fit of the raw data versus log [AMPA] (Fig. 4.4B) yielded a similar concentration-response relationship (EC_{50} = 2.60±0.76 μM). The EC_{50} values for AMPA+CTZ induced Ca^{2+} influx utilizing fluo-3 and Co^{2+} influx utilizing calcein were therefore not significantly different. We also tested kainate,

another AMPA/kainate receptor agonist, to determine whether or not it mimicked the response of AMPA in neocortical neurons. As shown in Fig. 4.5, kainate produced a concentration-dependent increase in quenching of calcein fluorescence suggesting that it also produced an activation of Ca-A/K receptors.

Finally we confirmed the selectivity of Co^{2+} influx through Ca-A/K receptors by using agents that activate other Ca^{2+} influx pathways. As shown in Fig. 4.6, only AMPA+CTZ treatment lead to Co^{2+}-induced quenching of calcein fluorescence. Activation of NMDA receptors by 30 μM NMDA or activation of voltage-gated Ca^{2+} channels by 55 mM KCl did not lead to calcein quenching thereby demonstrating this method as selective as the previously reported histochemical procedure in monitoring Ca-A/K receptors.

We have described a fluorometric method that can be applied for rapid, real-time detection of Ca-A/K receptors in intact neurons. Prevention of AMPA receptor desensitization amplified Co^{2+} influx through Ca^{2+}-permeable AMPA receptors (Fig 6). In agreement with previous reports we found that Co^{2+} influx occurs selectively through Ca-A/K receptors. Calcein dye has been used to detect changes in cell volume [7]. The time course of cell swelling mediated calcein fluorescence quenching is however much slower than what we have observed in the present study. In addition, prevention of cell swelling with 200 mM sucrose did not alter AMPA+CTZ induced calcein fluorescence quenching (data not shown).

Previous studies have shown that about 13% of cultured cortical neurons show Co^{2+} positive staining and are vulnerable to cytotoxicity due to rapid Ca^{2+} influx through the Ca-A/K receptors [14]. The majority of these Co^{2+} positive neurons are GABAergic [15]. Selective cytotoxicity of these neurons due to activation of AMPA/kainate receptors would therefore affect cortical inhibition and may lead to specific disease states such as epilepsy, Alzheimer's disease and amyotrophic lateral sclerosis. During cerebral ischemia, GluR2 mRNA and subunit expression is downregulated, inducing excessive Ca^{2+} influx through AMPA receptors contributing to neurotoxicity [13]. Ca-A/K receptors therefore represent important therapeutic targets for neurological diseases. The method described here can be used to explore the regulation of Ca-A/K receptors in populations of cultured neurons.

Acknowledgement: This work was supported by NIH grant GM63554 to TFM.

References:

[1] Berman,F.W., Murray,T.F., Brevetoxin-induced autocrine excitotoxicity is associated with manifold routes of Ca^{2+} influx, J. Neurochem., 74 (2000) 1443-1451.

[2] Breuer,W., Epsztejn,S., Millgram,P., Cabantchik,I.Z., Transport of iron and other transition metals into cells as revealed by a fluorescent probe, Am. J. Physiol, 268 (1995) C1354-C1361.

[3] Brorson,J.R., Manzolillo,P.A., Miller,R.J., Ca^{2+} entry via AMPA/KA receptors and excitotoxicity in cultured cerebellar Purkinje cells, J. Neurosci., 14 (1994) 187-197.

[4] Geiger,J.R., Melcher,T., Koh,D.S., Sakmann,B., Seeburg,P.H., Jonas,P., Monyer,H., Relative abundance of subunit mRNAs determines gating and Ca^{2+} permeability of AMPA receptors in principal neurons and interneurons in rat CNS, Neuron, 15 (1995) 193-204.

[5] Hume,R.I., Dingledine,R., Heinemann,S.F., Identification of a site in glutamate receptor subunits that controls calcium permeability, Science, 253 (1991) 1028-1031.

[6] Iino,M., Goto,K., Kakegawa,W., Okado,H., Sudo,M., Ishiuchi,S., Miwa,A., Takayasu,Y., Saito,I., Tsuzuki,K., Ozawa,S., Glia-synapse interaction through Ca^{2+}-permeable AMPA receptors in Bergmann glia, Science, 292 (2001) 926-929.

[7] Inglefield,J.R., Schwartz-Bloom,R.D., Activation of excitatory amino acid receptors in the rat hippocampal slice increases intracellular Cl^- and cell volume, J. Neurochem., 71 (1998) 1396-1404.

[8] Jonas,P., Racca,C., Sakmann,B., Seeburg,P.H., Monyer,H., Differences in Ca^{2+} permeability of AMPA-type glutamate receptor channels in neocortical neurons caused by differential GluR-B subunit expression, Neuron, 12 (1994) 1281-1289.

[9]Liu,S.Q., Cull-Candy,S.G., Synaptic activity at calcium-permeable AMPA receptors induces a switch in receptor subtype, Nature, 405 (2000) 454-458.

[10] Lu,Y.M., Yin,H.Z., Chiang,J., Weiss,J.H., Ca^{2+}-permeable AMPA/kainate and NMDA channels: high rate of Ca^{2+} influx underlies potent induction of injury, J. Neurosci., 16 (1996) 5457-5465.

[11] Pruss,R.M., Akeson,R.L., Racke,M.M., Wilburn,J.L., Agonist-activated cobalt uptake identifies divalent cation-permeable kainate receptors on neurons and glial cells, Neuron, 7 (1991) 509-518.

[12] Song,I., Huganir,R.L., Regulation of AMPA receptors during synaptic plasticity, Trends Neurosci., 25 (2002) 578-588.

[13] Tanaka,H., Grooms,S.Y., Bennett,M.V., Zukin,R.S., The AMPAR subunit GluR2: still front and center-stage, Brain Res., 886 (2000) 190-207.

[14] Turetsky,D.M., Canzoniero,L.M., Sensi,S.L., Weiss,J.H., Goldberg,M.P., Choi,D.W., Cortical neurones exhibiting kainate-activated Co^{2+} uptake are selectively vulnerable to AMPA/kainate receptor-mediated toxicity, Neurobiol. Dis., 1 (1994) 101-110.

[15] Yin,H., Turetsky,D., Choi,D.W., Weiss,J.H., Cortical neurones with Ca^{2+} permeable AMPA/kainate channels display distinct receptor immunoreactivity and are GABAergic, Neurobiol. Dis., 1 (1994) 43-49.

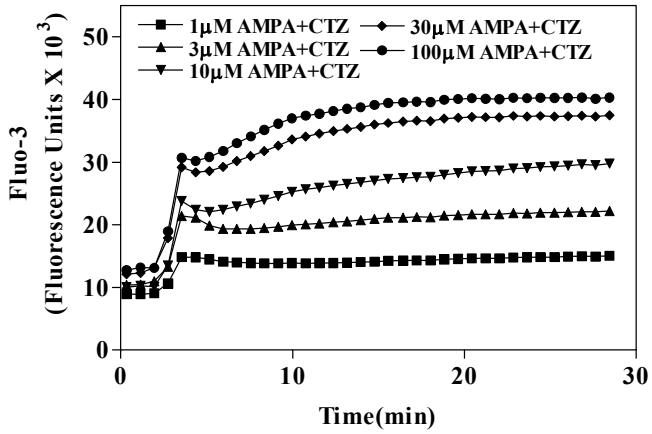


Figure 4.1. Changes in fluo-3 fluorescence of neocortical neurons after exposure to increasing concentrations of AMPA+50 μM cyclothiazide (CTZ). Each point represents mean ± SEM of triplicate determinations.

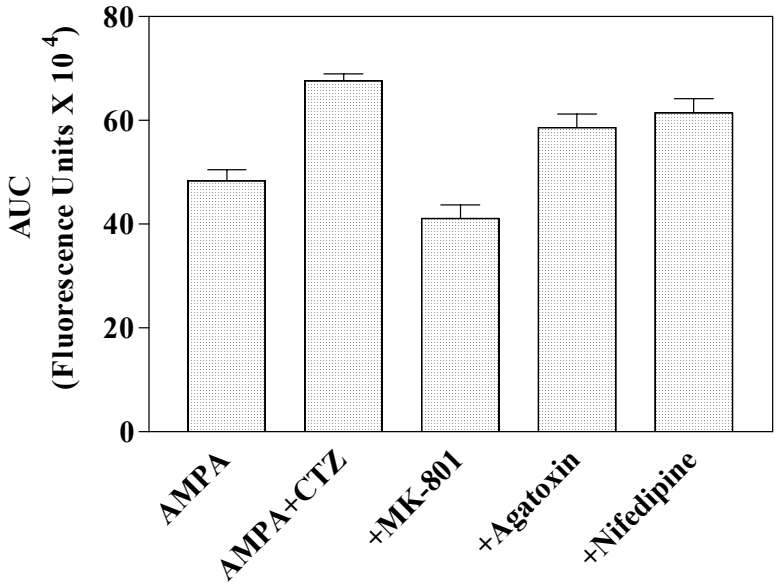


Figure 4.2. Pharmacological evaluation of routes of Ca^{2+} entry after AMPA receptor activation.

Neocortical neurons were exposed to 30 µM AMPA, 30 µM AMPA+50 µM CTZ, or 30 µM AMPA+50 µM CTZ and the antagonists 100 nM MK-801, 100 nM ω-Agatoxin IVA or 1 µM nifedipine. All antagonists were added 5 min prior to AMPA+CTZ. Integrated fluo-3 fluorescence response [area under the curve (AUC)] were obtained from the analysis of raw data. The values are mean±SEM of duplicate determinations.

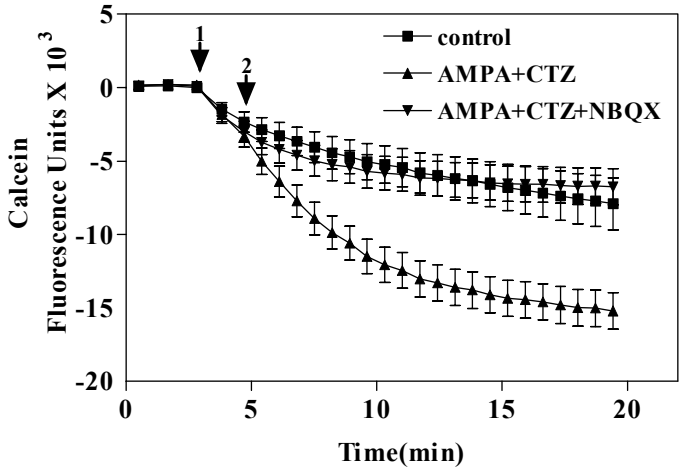


Figure 4.3. AMPA receptor activation induces Co^{2+} influx in neocortical neurons. Co^{2+} influx is enhanced by 30 µM AMPA+50 µM CTZ treatment. NBQX, an AMPA/kainate antagonist, blocks this response to AMPA+CTZ. The arrowhead (1) represents point of $CoCl_2$ addition while arrowhead (2) represents point of drug addition. Each point represents mean ± SEM of six replicates.

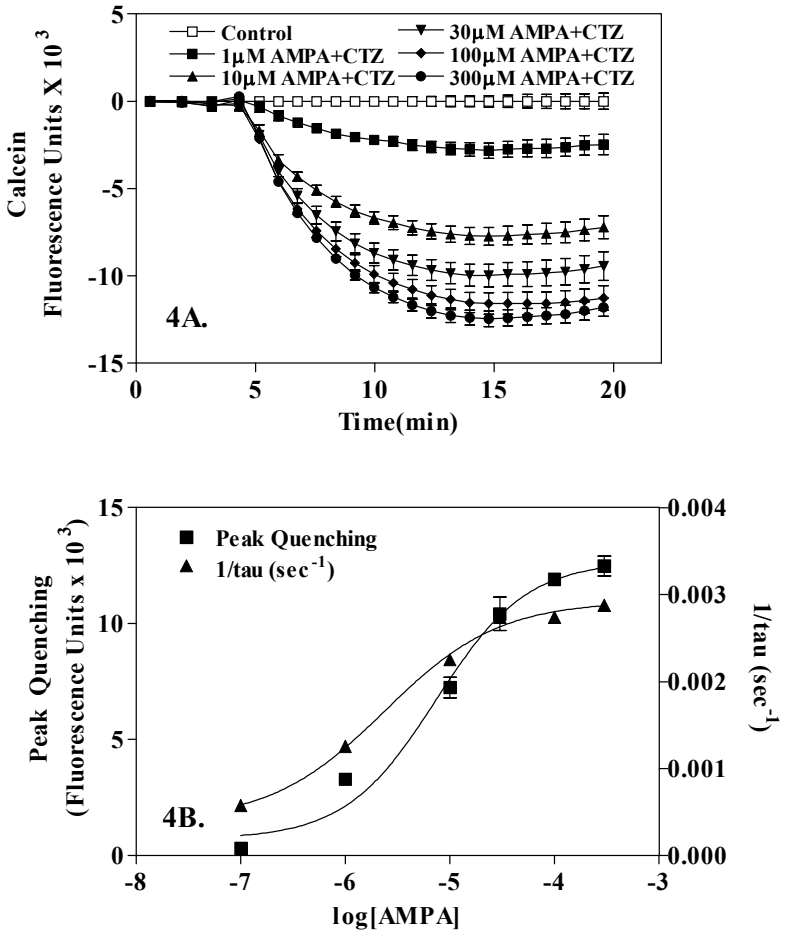


Figure 4.4. A: Concentration-dependent increase in quenching of calcein fluorescence by AMPA+50 μMCTZ.

Each point represents mean ± SEM of quadruplicates. Correction for basal quenching was done for each time point. B: Nonlinear regression analysis of concentration-response relationships for peak quenching (■) and reciprocal relaxation time (1/tau) (▲).

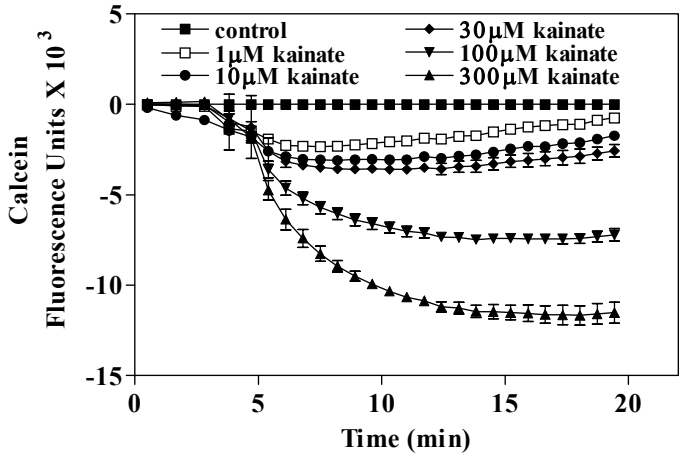


Figure 4.5. Concentration-dependent increase in quenching of calcein fluorescence by kainate. Each point represents mean ± SEM of triplicate determinations. Correction for basal quenching was done for each time point.

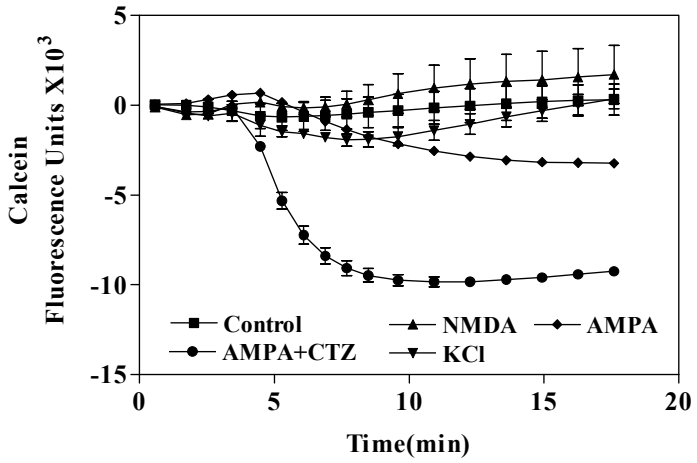


Figure 4.6. Specificity of AMPA-induced Co^{2+} influx through Ca^{2+} permeable AMPA receptors.

Co^{2+} influx was assayed with 30 µM AMPA, 30 µM AMPA+50µM CTZ, 30 µM NMDA or 55 mM KCl. Each point represents mean ± SEM of triplicates determinations.

CHAPTER 5

VOLTAGE-GATED SODIUM CHANNEL REGULATION OF Ca^{2+} DYNAMICS AND ERK1/2 ACTIVATION IN MURINE NEOCORTICAL NEURONS[1]

[1]Dravid S.M. and Murray T.F., to be submitted to Journal of Neurochemistry

Abstract

Voltage-gated sodium channels (VGSC) are involved in the generation of action potentials in neurons. Brevetoxins (PbTx) are potent allosteric enhancers of VGSC function and are associated with the periodic "Red Tide" blooms. These neurotoxins produce neuronal injury and death in cerebellar granule cells following acute exposure. Using PbTx-2, we have characterized the effects of activation of VGSC on Ca^{2+} dynamics and extracellular signal-regulated kinases 1/2 (ERK1/2) signaling in neocortical neurons. Neocortical neurons exhibit synchronized spontaneous Ca^{2+} oscillations which are mediated by glutamatergic signaling. PbTx-2 (100nM) increased the amplitude and reduced the frequency of basal Ca^{2+} oscillations. This modulatory effect on Ca^{2+} oscillations produced a sustained rise in ERK1/2 activation. At 300nM PbTx-2 disrupted the oscillatory activity leading to a sustained increase in intracellular Ca^{2+} and induced a biphasic response with activation followed by dephosphorylation of ERK1/2. PbTx-2 induced ERK1/2 activation was Ca^{2+} dependent and was mediated by Ca^{2+} entry through manifold routes. PbTx-2 treatment also increased CREB phosphorylation and increased gene expression of BDNF. These findings indicate that brevetoxins, by influencing the activation of key signaling proteins, can alter physiologic events involved in learning, growth and survival in neocortical neurons.

Keywords: Ca^{2+}, oscillations, brevetoxin, neocortical neurons, ERK, CREB, BDNF, NMDA, glutamate, voltage-gated sodium channel.

Introduction

Voltage-gated sodium channels (VGSC) are involved in the generation of action potential in the neurons. VGSC represent the molecular target for several groups of neurotoxins which alter channel function by binding to specific sites on the alpha subunit of the channel (Cestele and Catterall, 2000). Brevetoxins (PbTx-1 to PbTx-10) are potent lipid soluble polyether neurotoxins produced by the marine dinoflagellate *Karenia brevis* (formerly known as *Gymnodinium breve* and *Ptychodiscus brevis*) an organism linked to periodic red tide blooms in Gulf of Mexico, along the western Florida coastline (Baden, 1989) and New Zealand (Ishida et al., 1995). Brevetoxins interact with site 5 of the α-subunit of the VGSC. Brevetoxins augment Na^+ influx through VGSC by increasing the mean open time of the channel, inhibiting channel inactivation and shifting the activation potential to more negative values (Jeglitsch et al., 1998).

K. brevis blooms have been implicated in massive fish kills, bird deaths, and marine mammal mortalities (O'Shea et al., 1991: Bossart et al., 1998). In humans, two distinct clinical entities, depending on the route of exposure, have been identified. Ingestion of bivalve mollusks contaminated with brevetoxins leads to neurotoxic shellfish poisoning (NSP), the symptoms of which include nausea, cramps, paresthesias of lips, face and extremities, weakness and difficulty in movement, paralysis, seizures and coma (McFarren et al., 1965; Baden and Mende, 1982; Ellis, 1985). Inhalation of the aerosolized brevetoxins from the sea spray, mainly results in respiratory irritation as well as dizziness, tunnel vision and skin rashes (Pierce 1986; Baden, 1982). Brevetoxins are known to accumulate in the CNS when administered systemically in animals (Cattet and Geraci, 1993) at concentrations sufficient to affect CNS (Templeton et al., 1989). These

findings underscore the importance of studying the cellular consequences of brevetoxin exposure in the CNS.

Our laboratory has previously demonstrated that brevetoxin leads to glutamate release and acute neurotoxicity in cerebellar granule neurons (Berman and Murray, 1999). Brevetoxin induces Ca^{2+} influx in CGC which is responsible for this neurotoxic action (Berman and Murray, 2000). Neocortical neurons in culture differ from CGC in that they exhibit spontaneous synchronous Ca^{2+} oscillations that are mediated by glutamatergic signaling. Oscillations in cytoplasmic Ca^{2+} levels are a common mode of signaling both in excitable and non-excitable cells and can increase the efficiency and specificity of gene expression (Dolmetsch et al., 1998).

Mitogen activated protein kinases constitute a family of serine/threonine kinases, of which the extracellular signal-regulated kinases (ERKs) have been shown to participate in physiologic events such as synaptic plasticity and learning and memory in the CNS (Sweatt, 2001). In neurons a rise in intracellular Ca^{2+} mediated by NMDA receptors, Ca^{2+}-permeable AMPA/kainate receptors and voltage-gated calcium channels (VGCC) has been shown to regulate ERK1/2 activation (Sweatt, 2001). ERK1/2 phosphorylates cAMP responsive element binding protein (CREB) which governs the transcription of a number of genes including brain-derived neurotrophic factor (BDNF). The ERK-CREB-BDNF cascade has been shown to be important for neuronal survival (Xia et al., 1995; Bonni et al., 1999). The regulation of ERK1/2 activation by VGSC has not been studied. In the present study using PbTx-2 as a probe we have characterized the effects of VGSC activation on Ca^{2+}-dependent ERK1/2 activation and the downstream signaling events.

We found that brevetoxin modulates the spontaneous Ca^{2+} oscillations generated by neocortical neurons. PbTx-2-induced Ca^{2+} responses were mediated by both glutamatergic and non-glutamatergic mechanisms, the relative contributions of the two pathways varied considerably as a function of PbTx-2 concentration. The distinct Ca^{2+} response induced by PbTx-2 resulted in distinct temporal pattern of ERK1/2 activation. PbTx-2 also modulated the phosphorylation of CREB and the expression of BDNF. The differential modulation of ERK1/2 depending on the extent of activation of VGSC may be explained by the activation of distinct synaptic versus extrasynaptic NMDA receptors.

Materials and Methods

Neocortical Neuron Culture: Primary cultures of neocortical neurons were obtained from embryonic day 16-17 Swiss-Webster mice. Briefly, pregnant mice were euthanized by CO_2 asphyxiation and embryos were removed under sterile conditions. Neocortices were collected, stripped of meninges, minced by trituration with a Pasteur pipette and treated with trypsin for 25 min at 37˚ C. The cells were then dissociated by two successive trituration and sedimentation steps in soybean trypsin inhibitor and DNase containing isolation buffer, centrifuged and, resuspended in Eagle's minimal essential medium with Earle's salt (MEM) and supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 10% horse serum, 100 I.U./ml penicillin and 0.10 mg/ml streptomycin, pH7.4. For intracellular Ca^{2+} monitoring cells were plated onto poly-L-lysine coated 96-well (9 mm) clear-bottomed black-well culture plates (Costar) at a density of 4.7 X 10^5 cells/cm^2. For western blotting experiments cells were plated on 12 well plates. Cells were incubated at 37 ºC in a 5% CO_2 and 95% humidity atmosphere. Cytosine

arabinoside (10 μM) was added to the culture medium on day 2 after plating to prevent proliferation of nonneuronal cells. The culture media was changed biweekly with MEM supplemented with 2 mM L-glutamine, 5% horse serum, 100 I.U./ml penicillin and 0.10 mg/ml streptomycin, pH 7.4. The cultures were used for experiments between 9 –13 days *in vitro*.

Cytotoxicity Assay: Conditioned medium was collected and saved, and the neocortical neurons were washed 3 times with Locke's buffer containing 154 mM NaCl, 5.6 mM KCl, 1.0 mM $MgCl_2$, 2.3 mM $CaCl_2$, 8.6 mM HEPES, 5.6 mM glucose, and 0.1 mM glycine, pH 7.4. The neurons were then exposed to PbTx-2 in 0.5 ml of Locke's buffer for 2 h at 22°C. At the termination of PbTx-2 exposure, the incubation medium was collected for later analysis of lactate dehydrogenase (LDH) activity, and the neurons were washed twice in Locke's buffer followed by replacement with 0.5 ml of the previously collected conditioned medium. The cell cultures were returned to the 37°C incubator. At 24 h after PbTx-2 exposure, growth medium was collected and saved for analysis of LDH activity. LDH activity was assayed according to the method of Koh and Choi (1987).

Intracellular Ca^{2+} monitoring: For intracellular Ca^{2+} monitoring the cells grown in 96 well plates were first washed four times with Locke's buffer using an automated cell washer (Labsystems, Helsinki, Finland). Cells were then incubated for 1 h at 37˚C with dye loading buffer (100 μl/well) containing 4 μM fluo-3 AM and 0.04% Pluronic acid in Locke's buffer. Fluo-3 AM is taken up by cells and entrapped intracellularly after hydrolysis to fluo-3 by cell esterases. After 1h incubation in dye loading medium, cells were washed 5 times with Locke's buffer. The final volume of Locke's buffer in each well was 100 μl or 150 μl depending on whether antagonist treatment was done prior to

PbTx-2 addition. All the drugs were made at 4-times the final concentration and 50 µl of the drugs were added to the culture wells.

Ca^{2+} monitoring was done using FLIPR (Fluorometric Imaging Plate Reader) Neurons were excited by the 488 nm line of the argon laser and Ca^{2+} bound fluo-3 emission in the 500 nm to 560 nm range was recorded with the CCD camera shutter speed set at 0.4 s. Prior to each experiment, average baseline fluorescence was set between 10,000 and 20,000 fluorescence units by adjusting the power output of the laser. Fluorescence readings were taken either once every sec or once every 6 sec depending on the duration of the recording.

Drug treatment: For western blotting analysis the cells grown in 12 well plates were washed 3 times with Locke's buffer. The cultures were treated with the indicated drugs diluted in Locke's buffer. After the indicated incubation period the plates were transferred to an ice slurry and the cells were washed with ice cold PBS to terminate the treatment. The cells were then scraped in ice cold PBS and centrifuged at 5000rpm for 10 min to collect the cells. Lysis buffer (50 mM Tris, 50 mM NaCl, 5 mM, EGTA, 5 mM EDTA, 2 mM sodium pyrophosphate, 1 mM activated sodium orthovanadate, 1 µg/ µl aprotinin, 10 µg/ µl leupeptin, 10 µg/ µl pepstatin, 0.5 mM PMSF and 1% NP-40) was added to the cell pellet and incubated on ice for 45 min. Thereafter the lysates were centrifuged at 14000 rpm for 15min at $4^{o}C$.

Western Blotting: The supernatant were assayed by Bradford reagent to detect the protein content. Equal amount of protein from the samples were mixed with the Laemmli sample buffer and boiled for 5 min. The protein samples were run on a 10% SDS-PAGE gel and transferred to nitrocellulose membrane by electroblotting. The nitrocellulose

membranes were incubated in the TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween) containing 5% skimmed milk powder for 1 h. After blocking the blots were incubated overnight in primary antibody diluted in TBST containing 5% BSA. The blots were washed with TBST and incubated with the secondary antibody conjugated with horseradish peroxidase for 1h, washed with TBST and exposed to ECL plus for 3 min. Blots were exposed to Kodak hyperfilm and developed. Bands were analyzed using the Alfaimager.

RT-PCR: The neocortical neurons were treated for 4h with the indicated treatments. The cells were then washed with ice-cold PBS and then lysed with TRI reagent (Molecular Research Center, Cincinnati, OH) according to manufacturer's protocol. Reverse-transcriptase polymerase chain reaction (RT-PCR) was used to analyze the expression of mRNA for BDNF and β-actin (internal control). The sense and antisense primers used were 5'-GCGGCAGATAAAAAGACTGC-3' and 5'-CTTATGAATCGCCAGCCAAT-3' for BDNF and 5'-ATGGATGACGATATCGCT-3' and 5'-ATGAGGTAGTCTGTCAGGT-3' for β-actin. The thermal cycles consisted of denaturation at 94 °C for 15 s, annealing at 54 °C for 15 s and extension at 72 °C for 30 s followed by a final extension at 72 °C for 5 min. The numbers of cycles optimized within the linear range of amplification for each primer set were 28 cycles BDNF and 25 cycles for β-actin. The amplification products were fractionated on 2% agarose gel and documented using a Kodak DC290 digital camera. The resulting images were digitized and quantified using UN-SCAN-IT software (Silk Scientific Inc., Orem, UT) normalized to that of β-actin.

Data Analysis: Nonlinear regression analysis, frequency and amplitude analysis and other data analysis was done using GraphPad analysis package (GraphPad Software, San Diego, CA, USA).

Materials: Trypsin, penicillin, streptomycin, heat-inactivated fetal bovine serum, horse serum and soybean trypsin inhibitor were obtained from Atlanta Biologicals (Norcross, GA.). Minimum essential medium (MEM), Deoxyribonuclease (DNAse), poly-L-lysine, cytosine arabinosoide, MK-801, NBQX, cyclothiazide, NMDA, Protein A Sepharose were purchased from Sigma (St. Louis, MO). (S)-4-carboxyphenylglycine ((S)-4CPG) was from Tocris (Ballwin, MO). Nifedipine was obtained from RBI (Natick, MA). Pluronic acid and fluo-3 AM were purchased from Molecular Probes (Eugene, OR). Phospho-ERK1/2 and total-ERK1/2 antibody were obtained from Cell Signaling (Beverly, MA), phospho-CREB antibody from Upstate Biotechnology (Charlottesville, VA) and NR2B antibody from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). PP2 and PP3 were obtained from Calbiochem (La Jolla, CA). ECL plus kit was from Amersham Biosciences (Piscataway, NJ). Materials for RT-PCR were obtained from Invitrogen Life Technologies (Carlsbad, CA).

Results

PbTx-2 modulates Ca^{2+} oscillations in neocortical neurons

Neocortical neurons exhibit spontaneous oscillations in intracellular Ca^{2+}. These oscillations are triggered by the activation of AMPA receptors and involve secondary recruitment of activation of NMDA and metabotropic glutamate receptors (mGluR). We investigated the effect of PbTx-2 on these spontaneous Ca^{2+} oscillations. At low

concentrations (10 nM-100 nM) PbTx-2 significantly increased the amplitude (Fig. 5.2A) and duration of a single Ca^{2+} spike (Fig. 5.1). An increase in the amplitude of Ca^{2+} oscillation was accompanied by a decrease in the frequency (Fig. 2B) and therefore the integrated Ca^{2+} influx indicated by the area under curve analysis during the recording period remained unaltered (Fig. 5.2C). PbTx-2 at concentrations greater than 100nM disrupted the Ca^{2+} oscillations and produced a sustained rise in cytosolic Ca^{2+} concentration (Fig. 5.1). The sustained rise in Ca^{2+} after exposure to 300 nM of PbTx-2 returned to oscillatory state after a period of 25-30 min, while cultures treated with 1 μM PbTx-2 did not exhibit Ca^{2+} oscillations even after a period of 30 min (data not shown). Thus depending on the concentration, PbTx-2 can differentially affect Ca^{2+} dynamics in neocortical neurons which may then lead to distinct downstream effects.

PbTx-2-induced modulation of intracellular Ca^{2+} dynamics mediated by both glutamatergic and non-glutamatergic mechanisms

In CGC the brevetoxin-induced Ca^{2+} response is derived from Ca^{2+} influx through NMDA receptors, L-type Ca^{2+} channels and reversal of Na^{+}/ Ca^{2+} exchanger (Berman and Murray, 2000). It was therefore of interest to determine whether PbTx-2-induced increment in Ca^{2+} response in neocortical neurons engaged the same pathways. We first characterized the Ca^{2+} response induced by 100 nM PbTx-2 which leads to an increase in amplitude and reduction in the frequency of basal spontaneous Ca^{2+} oscillations. The NMDA antagonist MK-801 (1 μM) reduced the 100 nM PbTx-2-induced Ca^{2+} spike amplitude by 70% (Fig. 5.3 and Fig. 5.4B). Although MK-801 alone does affect the amplitude of basal Ca2+ oscillations, the results show that the incremental response of

PbTx-2 was also largely dependent on the activation of NMDA receptors. The basal spontaneous Ca^{2+} oscillations are not affected by the inhibitor of L-type Ca^{2+} channels, nifedipine (1 μM) or by the inhibitor of reverse mode of Na^{+}/Ca^{2+} exchanger KB-R7943 (1 μM) (data not shown). Nifedipine and KB-R7943 significantly reversed the decrease in frequency of Ca^{2+} oscillations mediated by 100 nM PbTx-2 (Fig. 5.3 and Fig. 5.4A). Nifedipine also significantly decreased the amplitude of 100nM PbTx-2 mediated Ca^{2+} oscillations while KB-R7943 did not affect the amplitude of Ca^{2+} oscillations (Fig. 5.4B). A hallmark of spontaneous Ca^{2+} oscillation in neocortical neurons is the control of their frequency by the AMPA receptors (Dravid and Murray, submitted). The AMPA receptor antagonist, NBQX (1 μM), decreases the frequency of basal Ca^{2+} oscillation and produced a similar effect on the Ca^{2+} oscillations induced by 100 nM PbTx-2 (Fig. 5.4A) indicating that the oscillations occurring in the presence of PbTx-2 retain the properties of the synaptically mediated Ca^{2+} oscillations. Treatment of neocortical neurons with NMDA, non-NMDA and mGluR receptor antagonists leads to complete inhibition of spontaneous Ca^{2+} oscillations, similarly the PbTx-2-induced Ca^{2+} oscillations were also completely abolished by such a treatment even though, the initial peak after addition of PbTx-2 remained unaffected by this treatment (Fig 5.3).

We further studied the routes of Ca^{2+} entry that lead to the sustained rise in intracellular Ca^{2+} after exposure to 1 μM of PbTx-2. The antagonist of NMDA receptors, MK-801 (1 μM), produced significant reduction in the AUC after PbTx-2 treatment (Fig. 5.5). S-4-CPG (500 μM), the mGluR antagonist, and NBQX (1 μM), the AMPA/kainate receptor antagonist, alone did not significantly reduce the Ca^{2+} influx, but were additive to the response produced by MK-801 (Fig. 5.5A and 5.5B). KB-R7943 (1 μM) was most

effective in reducing Ca^{2+} influx and nifedipine (1 μM) also significantly reduced the AUC indicating that L-type calcium channels and reversal of Na+/Ca^{2+} exchanger are important routes of Ca^{2+} entry after PbTx-2 exposure (Fig. 5.5). The rank order of relative contributions of the Ca^{2+} influx pathways are as follows: combination of reversed mode of Na^{+}/Ca^{2+} exchanger and L-type Ca^{2+} channel>reversed mode of Na^{+}/Ca^{2+} exchanger>combination of NMDA, AMPA and mGluR receptors>NMDA receptors>L-type Ca^{2+} channel. Thus the major routes of Ca^{2+} entry after PbTx-2 exposure in neocortical neurons are similar to those earlier reported in CGN (Berman and Murray, 2000).

PbTx-2 does not produce acute neurotoxicity in neocortical neurons

In CGN brevetoxin PbTx-2 produces acute toxicity with an EC_{50} of 80.5 ± 5.9 nM that was prevented by the application of NMDA antagonists (Berman and Murray, 1999). PbTx-2 did not produce acute toxicity in neocortical neurons as demonstrated by the absence of LDH release after 2 h of incubation with PbTx-2 as well as the normal appearance of neurons on microscopic examination. Only a concentration of PbTx-2 of 3 μM produced toxicity after 24 h exposure (Table 5.1). Neocortical neurons thus appear to be less vulnerable than CGN to acute PbTx-2-induced toxicity.

PbTx-2 exposure leads to increase in ERK1/2 phosphorylation

Increase in intracellular Ca^{2+} through NMDA receptors, L-type calcium channel, Ca^{2+} permeable AMPA/kainate receptors and release of intracellular stores of Ca^{2+} after mGluR activation has been shown to induce activation of ERK1/2 (Sweatt, 2001).

Synaptic activity exhibited by the neocortical neurons is responsible for basal ERK1/2 activation. Blocking the synaptic activity by tetrodotoxin (TTX), a VGSC antagonist, leads to a decrease in ERK1/2 activation as indicated by a decrease in the dually phosphorylated (p-ERK1/2) form of the ERK1/2 (Fig. 5.6). This synaptic activity-induced ERK1/2 activation is mediated by Ca^{2+} influx through NMDA receptors since 100 nM MK-801 reduces the synaptic activity-induced ERK1/2 activation which is equivalent to the reduction observed with the TTX treatment (Fig. 5.6). Inasmuch as PbTx-2 exposure alters the Ca^{2+} dynamics in the neocortical neurons and since Ca^{2+} has been shown to be a key regulator of ERK1/2 activation we explored the effect of PbTx-2 on ERK1/2 activation. We first examined the temporal pattern of ERK1/2 activation by two different concentrations of PbTx-2 which differ in their effect on Ca^{2+} oscillations exhibited by neocortical neurons. PbTx-2 at 100 nM, a concentration that only alters the amplitude and frequency of the Ca^{2+} oscillations but does not disrupt the oscillatory state, produced a steep rise (2-fold over control) in ERK1/2 activation within 15 min (Fig. 5.7A and Fig. 5.8) . This ERK1/2 activation was sustained for the entire 2h observation period. On the other hand, 300nM PbTx-2, a concentration that disrupts the oscillations and produces sustained rise in cytosolic Ca^{2+}, produced a steep rise (3-fold over control) in ERK1/2 activation within 15min followed by a decrease in the phosphorylation state, which was reduced beyond the basal level after 1h of treatment (Fig. 5.7B and Fig. 5.8). Though both concentrations of PbTx-2 produced an increase in p-ERK1/2, the temporal patterns of the ERK1/2 activation were distinct in the two cases. No changes in the total ERK1/2 levels were observed by PbTx-2 treatment (Fig. 5.7). We further examined the concentration dependence of PbTx-2-induced ERK1/2 activation at the 15 min time

point, since ERK1/2 phosphorylation peaked at 15min after treatment with 300nM PbTx-2. PbTx-2 produced a concentration-dependent increase in ERK1/2 phosphorylation (Fig. 5.9A). The EC_{50} obtained from the non-linear regression analysis of the concentration-response curve for PbTx-2-induced ERK1/2 activation was 96.8 nM (Fig. 9B).

In hippocampal neurons in culture, CREB dephosphorylation is developmentally regulated (Sala et al., 2000; Hardingham and Bading, 2002). Similarly, influence of 300 nM PbTx-2 on ERK1/2 activation was influenced by development. In contrast to the response in 12 DIV cultures, 300 nM PbTx-2 stimulated ERK1/2 activation in a sustained pattern in 7 DIV cultures (Fig. 5.10). These distinct temporal patterns may be related to developmental regulation of phosphatases involved in the inactivation of ERK1/2.

PbTx-2-induced ERK1/2 activation mediated by Ca^{2+} influx through manifold routes

To determine whether the phosphorylation of ERK1/2 was mediated by a rise in intracellular Ca^{2+} and not Na^{+}, we performed a concentration response study for PbTx-2-induced ERK1/2 activation in the presence and absence of extracellular Ca^{2+}. Removal of extracellular Ca^{2+} led to a decrease in the basal phosphorylation of ERK1/2 (Fig. 5.10) indicating that the synaptic activity mediated ERK1/2 is mediated by Ca^{2+} entry that occurs through the NMDA receptors. PbTx-2 did not induce ERK1/2 activation in the absence of extracellular Ca^{2+} (Fig. 5.11), suggesting that a rise in intracellular Ca^{2+} is necessary for ERK1/2 activation. PbTx-2-induced Ca^{2+} influx occurs through various routes, therefore we pharmacologically characterized the PbTx-2-induced ERK1/2

activation. After a 15 min pretreatment of different antagonists, 1 μM PbTx-2 was added to neocortical neurons. Treatment was terminated after 15 min of PbTx-2 exposure and cell extracts were analyzed for ERK1/2 activation. We found that, similar to the results obtained with Ca^{2+} imaging, the Na^{+}/Ca^{2+} exchanger inhibition by KB-R7943 was most efficacious in inhibiting PbTx-2-induced ERK1/2 activation (Fig. 5.12). Pretreatment with MK-801, nifedipine and S-4-CPG also lead to significant reduction in the PbTx-2-induced ERK1/2 activation. Nonetheless, the relative reduction in PbTx-2-induced ERK1/2 mediated by these antagonists cannot be regressed to the relative ability of these antagonists to reduce PbTx-2-induced Ca^{2+} influx. Therefore, in addition to Ca^{2+} load, the route of Ca^{2+} entry may also dictate ERK1/2 activation.

Role of MEK, CaMKII and PI3K in PbTx-2-induced ERK1/2 activation

ERK1/2 activation is exclusively regulated by MAP kinase kinase1/2 (MEK1/2), the immediate upstream dual-specificity kinases that phosphorylate ERK1/2. Treatment of neurons with PD98059 (50 μM), a specific blocker of MEK1/2 (Alessi et al., 1995) inhibited both the basal and 300 nM PbTx-2-induced ERK1/2 activation (Fig. 5.13). CaMKII has been suggested to be a key mediator of glutamate induced ERK1/2 activation (Vanhoutte at al., 1999). Treatment of neocortical neurons with the specific CaMKII inhibitor KN-93 (40 μM) significantly reduced the PbTx-2-induced ERK1/2 activation (Fig. 5.13). Phosphatidylinositol 3-kinase (PI 3-kinase) has been shown to mediate both NMDA and glutamate induced ERK1/2 activation (Walker et al., 2000; Perkinton et al., 2002). We therefore evaluated the role that PI 3-kinase played in the activation of PbTx-2-induced ERK1/2 activation. Wortmannin (100 nM) a selective

inhibitor of PI 3-kinase lead to a partial reduction in the PbTx-2-induced ERK1/2 activation (Fig. 5.13). These data indicate that other than the classical Ras-Raf-1 kinase/MEK1 pathway (Rosen et al., 1994), both the CaMKII and PI 3-kinase pathway may contribute to the observed activation of ERK1/2 by PbTx-2.

PbTx-2 augments CREB phosphorylation and BDNF expression

To examine whether PbTx-2 induced ERK1/2 phosphorylation is accompanied by the activation of a downstream transcriptional factor and alteration in gene expression, we studied the phosphorylation of the transcription factor CREB by immunoblotting and BDNF expression by RT-PCR. CREB is activated by phosphorylation at Ser 133. Treatment with 300 nM PbTx-2 lead to an increase in CREB phosphorylation which peaked at 30min (Fig. 5.14). PbTx-2 also induced an increase in BDNF gene expression after 4h of treatment (Fig. 5.15). These studies indicate that the effect of PbTx-2 is not restricted to phosphorylation of signaling proteins, but also affects gene expression in neurons.

Discussion

We have used PbTx-2 as a probe to identify the effects of activation of VGSC on Ca^{2+} dynamics and ERK1/2 activation in neocortical neurons. We have previously shown that PbTx-2 induces acute neurotoxicity in CGN which is mediated by glutamate release (Berman and Murray, 1999). The neocortical neurons were resistant to PbTx-2-induced acute neurotoxicity. There could be several reasons for these differences in cell specific susceptibility. The CGN are predominantly glutamatergic neurons whereas

cultures of neocortical neurons also have a considerable number of GABAergic interneurons which have a tonic inhibitory control on the system (Schousboe et al., 1985). In addition the spontaneous synaptic neuronal activity in neocortical neurons may lead to tonic activation of the cell survival elements (Hardingham et al, 2002) that may counteract the cell death signaling enhanced by PbTx-2 treatment.

The Ca^{2+} oscillations occurring in the presence of 100 nM PbTx-2 shared the same properties as the synaptically mediated spontaneous Ca^{2+} oscillations in that the frequency was AMPA receptor dependent. The PbTx-2 induced Ca^{2+} oscillations though mostly mediated by the activation of NMDA receptors, also had contributions from L-type Ca^{2+} channels and reverse mode of Na^{+}/Ca^{2+} exchanger, since blockade of either L-type Ca^{2+} channels or Na^{+}/Ca^{2+} exchanger altered the frequency of these oscillations. It should be noted that the initial peak induced by PbTx-2 was not abolished in the presence of glutamate receptor antagonists, which suggests that activation of L-type Ca^{2+} channels and reversal of Na^{+}/Ca^{2+} exchanger may be responsible for the initial peak. At concentrations greater than or equal to 300 nM and more PbTx-2 disrupted the Ca^{2+} oscillations leading to a sustained rise in intracellular Ca^{2+}, suggesting that at higher concentrations PbTx-2 modulation of Ca^{2+} dynamics may not be restricted to synaptic augmentation but rather generalized to both synaptic and extrasynaptic receptor activation.

Brevetoxin was found to augment ERK1/2 phosphorylation and this response was dependent on an increase in intracellular Ca^{2+} concentration. The different Ca^{2+} response

induced by the two concentrations of PbTx-2 had differential temporal patterns of downstream ERK1/2 activation. The ERK1/2 activation by 300 nM PbTx-2 followed a biphasic response and lead to an apparent dephosphorylation of synaptically mediated basal ERK1/2 activation. Lower concentration of PbTx-2 which augmented the amplitude of synaptic Ca^{2+} oscillations induced a sustained activation of ERK1/2. NMDA receptor-induced ERK1/2 phosphorylation is transient and is regulated by striatal enriched phosphatases (STEP) (Paul et al., 2003). Activation of VGCC on the other hand leads to a sustained activation of ERK1/2 (Paul et al., 2003). Our unpublished observations indicate that the Ca^{2+} response mediated by 55 mM KCl, which activates the VGCC, and 300nM PbTx-2 are similar in the magnitude of the Ca^{2+} response, suggesting that the time course of ERK1/2 activation depends on the source of Ca^{2+} entry and not on the Ca^{2+} load. STEP has been shown to be associated with NMDA receptor protein scaffold complex and responsible for the source specificity of ERK1/2 dephosphorylation in striatal neurons (Pelkey et al., 2002; Paul et al., 2003).

The neuronal localization of NMDA receptors dictates their influence on cellular responses. For instance, activation of synaptic NMDA receptors leads to sustained enhancement of AMPA responses, while extrasynaptic NMDA receptors lead to lasting depression of AMPA receptor response (Lu et al., 2001). CREB phosphorylation has been shown to be positively regulated by Ca^{2+} entry through synaptic NMDA receptors, whereas extrasynaptic NMDA receptors lead to the inactivation of synaptically mediated CREB phosphorylation (Hardingham et al., 2002). A similar mechanism may be invoked to be responsible for the different temporal pattern of ERK1/2 activation produced by the

two concentrations of PbTx-2 in neocortical neurons. An increase in Ca^{2+} influx through the synaptic NMDA receptors and through L-type Ca^{2+} channels by 100 nM PbTx-2 produces a sustained activation of ERK1/2. On the other hand, 300 nM PbTx-2, may lead to a more generalized depolarization and higher levels of glutamate release. This excess glutamate could spillover to activate extrasynaptic NMDA receptors leading to activation of a shut-off mechanism and, therefore, the shorter duration of ERK1/2 activation. In hippocampal neurons ERK1/2 activation does not seem to be regulated differentially by synaptic and extrasynaptic NMDA receptors since bath application of NMDA does not shut-off the synaptically mediated ERK1/2 activation (Sala et al., 2000). In neocortical neurons bath application of NMDA has been found to dephosphorylate basal p-ERK1/2 (Chandler et al., 2001). We have found similar results in our neocortical culture model using NMDA. The apparent differences in hippocampal and neocortical neurons may arise from differences in the post-synaptic density (PSD) proteins linked to NMDA receptors. These observations indicate that, not only receptor specificity, but also receptor localization may dictate the temporal pattern of ERK1/2 activation.

Higher concentrations of PbTx-2 leads to a robust increase in ERK1/2 activation which is mediated by multiple routes of Ca^{2+} entry. In neocortical and striatal neurons NMDA induced ERK1/2 is completely dependent on PI3K activation (Chandler et al., 2001; Perkinton et al., 2002). ERK1/2 activation by 1 μM PbTx-2 treatment was not completely abolished by treatment with wortmannin, indicating that NMDA receptors may not be solely responsible for ERK1/2 activation following exposure to PbTx-2. This correlates with the fact that inhibition of Ca^{2+} entry through other routes, the L-type

channels, reversal of Na^+/Ca^{2+} exchanger and mGluR also reduced the ERK1/2 activation by this high concentration of PbTx-2.

The duration of ERK1/2 activation can modify the cellular responses. In PC12 cells, transient ERK1/2 activation leads to cell proliferation, while sustained activation leads to differentiation (Traverse et al., 1992). The role of sustained versus transient ERK1/2 activation in post-mitotic neurons is not known. Synaptic transmission and NMDA receptor activation is necessary for cell survival in the developing brain (Ikonomidou et al., 1999). In addition, stimulation of synaptic activity by environmental enrichment inhibits spontaneous apoptosis in hippocampus and is neuroprotective (Young et al., 1999). NMDA receptor mediated synaptic activity has been shown to be anti-apoptotic and this is associated with activation of the CREB-BDNF cascade (Hardingham et al., 2002). Since ERK1/2 activation is accompanied by CREB and BDNF activation, the synaptically mediated sustained ERK1/2 activation may serve as a pro-survival signal. Further studies will be needed to directly demonstrate that synaptic activity enhancing concentrations of VGSC activators are anti-apoptotic. ERK activation has been shown to have a central role in LTP induction and is required for activity-dependent membrane insertion of AMPA receptors (English and Sweatt, 1997; Zhu et al., 2002). Moreover, synaptic NMDA receptor activation leads to insertion and potentiation of AMPA receptors, while extrasynaptic NMDA receptor leads to downregulation and depression of AMPA receptors (Lu et al., 2001; Iwakura et al., 2001; Pickard et al., 2001). Our results indicate that augmentation of synaptic NMDA receptor activity leads to sustained ERK1/2 activation, whereas bath application of NMDA which activates both

synaptic and extrasynaptic NMDA receptors leads to rapid shut-off of synaptically mediated ERK1/2 activation. Taken together, it is possible that synaptic and extrasynaptic NMDA receptors, by differentially regulating ERK1/2 signaling, may lead to AMPA receptor potentiation or depression.

VGSC are vital for normal CNS functioning and abnormal gating of these ion channels may lead to pathophysiological conditions exhibited as epileptiform diseases (Kohling, 2002). VGSC are also molecular targets for numerous neurotoxins (Cestele and Catterall, 2000). The results from the present study represent the initial molecular signaling events triggered by exposure to a VGSC activating neurotoxin, and may have implications for neurological diseases such as epilepsy. As mentioned previously, ERK1/2 have been shown to regulate important physiological functions such as learning, memory, growth and survival in neurons (Grewal et al., 1999; Sweatt, 2001). The results of the present study indicate that the cellular effects of brevetoxin may not always be manifested by neurotoxic sequelae (Berman and Murray, 1999) but by modulating key signaling pathways in the CNS may also lead to positive influences on neuronal plasticity and survival.

References:

Alessi D. R., Cuenda A., Cohen P., Dudley D. T., and Saltiel A. R. (1995) PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. *J Biol Chem* **270,** 27489-27494.

Baden D. G. and Mende T. J. (1982) Toxicity of two toxins from the Florida red tide marine dinoflagellate, Ptychodiscus brevis. *Toxicon* **20,** 457-461.

Baden D. G. (1989) Brevetoxins: unique polyether dinoflagellate toxins. *FASEB J* **3,** 1807-1817.

Berman F. W. and Murray T. F. (1999) Brevetoxins cause acute excitotoxicity in primary cultures of rat cerebellar granule neurons. *J Pharmacol Exp Ther* **290,** 439-444.

Berman F. W. and Murray T. F. (2000) Brevetoxin-induced autocrine excitotoxicity is associated with manifold routes of Ca2+ influx. *J Neurochem* **74,** 1443-1451.

Bonni A., Brunet A., West A. E., Datta S. R., Takasu M. A., and Greenberg M. E. (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science* **286,** 1358-1362.

Bossart G. D., Baden D. G., Ewing R. Y., Roberts B., and Wright S. D. (1998) Brevetoxicosis in manatees (Trichechus manatus latirostris) from the 1996 epizootic: gross, histologic, and immunohistochemical features. *Toxicol Pathol* **26,** 276-282.

Cattet M. and Geraci J. R. (1993) Distribution and elimination of ingested brevetoxin (PbTx-3) in rats. *Toxicon* **31,** 1483-1486.

Cestele S. and Catterall W. A. (2000) Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. *Biochimie* **82,** 883-892.

Chandler L. J., Sutton G., Dorairaj N. R., and Norwood D. (2001) N-methyl D-aspartate receptor-mediated bidirectional control of extracellular signal-regulated kinase activity in cortical neuronal cultures. *J Biol Chem* **276,** 2627-2636.

Dolmetsch R. E., Xu K., and Lewis R. S. (1998) Calcium oscillations increase the efficiency and specificity of gene expression. *Nature* **392,** 933-936.

Ellis S. (1985) Brevetoxins: chemistry and pharmacology of 'red tide' toxins from Ptychodiscus brevis (formerly Gymnodinium breve). *Toxicon* **23,** 469-472.

English J. D. and Sweatt J. D. (1997) A requirement for the mitogen-activated protein kinase cascade in hippocampal long term potentiation. *J Biol Chem* **272,** 19103-19106.

Grewal S. S., York R. D., and Stork P. J. (1999) Extracellular-signal-regulated kinase signalling in neurons. *Curr Opin Neurobiol* **9,** 544-553.

Hardingham G. E., Fukunaga Y., and Bading H. (2002) Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. *Nat Neurosci* **5,** 405-414.

Hardingham G. E. and Bading H. (2002) Coupling of extrasynaptic NMDA receptors to a CREB shut-off pathway is developmentally regulated. *Biochim Biophys Acta* **1600,** 148-153.

Ikonomidou C., Bosch F., Miksa M., Bittigau P., Vockler J., Dikranian K., Tenkova T. I., Stefovska V., Turski L., and Olney J. W. (1999) Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. *Science* **283,** 70-74.

Ishida H., Nozawa A., Totoribe K., Muramatsu N., Nudaya H., Tsuji K., Yamaguchi K., Yasumoto T., Kaspar H., Berkett N., Kosuge T. (1995) Brevetoxin B1, a new polyether

marine toxin from the New Zealand shellfish, *Austrovenus stutchburyi. Tetrahedron Lett.* **36** 725-728.

Iwakura Y., Nagano T., Kawamura M., Horikawa H., Ibaraki K., Takei N., and Nawa H. (2001) N-methyl-D-aspartate-induced alpha-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) receptor down-regulation involves interaction of the carboxyl terminus of GluR2/3 with Pick1. Ligand-binding studies using Sindbis vectors carrying AMPA receptor decoys. *J Biol Chem* **276,** 40025-40032.

Jeglitsch G., Rein K., Baden D. G., and Adams D. J. (1998) Brevetoxin-3 (PbTx-3) and its derivatives modulate single tetrodotoxin-sensitive sodium channels in rat sensory neurons. *J Pharmacol Exp Ther* **284,** 516-525.

Koh J. Y. and Choi D. W. (1987) Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. *J Neurosci Methods* **20,** 83-90.

Kohling R. (2002) Voltage-gated sodium channels in epilepsy. *Epilepsia* **43,** 1278-1295.

Lu W., Man H., Ju W., Trimble W. S., MacDonald J. F., and Wang Y. T. (2001) Activation of synaptic NMDA receptors induces membrane insertion of new AMPA receptors and LTP in cultured hippocampal neurons. *Neuron* **29,** 243-254.

McFarren E. F., Silva F. J., Tanabe H., Wilson W. B., Campbell J. E., and Lewis K. H. (1965) The occurrence of a ciguatera-like poison in oysters, clams, and Gymnodinium breve cultures. *Toxicon* **3,** 111-123.

O'Shea T.J., Rathbun G.B., Buergelt C.D., Odell D.K. (1991) An epizootic of Florida manatees associated with a dinoflagellate bloom. *Marine Mammal Sci.* **7** 165-179.

Paul S., Nairn A. C., Wang P., and Lombroso P. J. (2003) NMDA-mediated activation of the tyrosine phosphatase STEP regulates the duration of ERK signaling. *Nat Neurosci* **6,** 34-42.

Pelkey K. A., Askalan R., Paul S., Kalia L. V., Nguyen T. H., Pitcher G. M., Salter M. W., and Lombroso P. J. (2002) Tyrosine phosphatase STEP is a tonic brake on induction of long-term potentiation. *Neuron* **34,** 127-138.

Perkinton M. S., Ip J. K., Wood G. L., Crossthwaite A. J., and Williams R. J. (2002) Phosphatidylinositol 3-kinase is a central mediator of NMDA receptor signalling to MAP kinase (Erk1/2), Akt/PKB and CREB in striatal neurones. *J Neurochem* **80,** 239-254.

Pickard L., Noel J., Duckworth J. K., Fitzjohn S. M., Henley J. M., Collingridge G. L., and Molnar E. (2001) Transient synaptic activation of NMDA receptors leads to the insertion of native AMPA receptors at hippocampal neuronal plasma membranes. *Neuropharmacology* **41,** 700-713.

Pierce R. H. (1986) Red tide (Ptychodiscus brevis) toxin aerosols: a review. *Toxicon* **24,** 955-965.

Rosen L. B., Ginty D. D., Weber M. J., and Greenberg M. E. (1994) Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras. *Neuron* **12,** 1207-1221.

Sala C., Rudolph-Correia S., and Sheng M. (2000) Developmentally regulated NMDA receptor-dependent dephosphorylation of cAMP response element-binding protein (CREB) in hippocampal neurons. *J Neurosci* **20,** 3529-3536.

Schousboe A., Drejer J., Hansen G. H., and Meier E. (1985) Cultured neurons as model systems for biochemical and pharmacological studies on receptors for neurotransmitter amino acids. *Dev Neurosci* **7,** 252-262.

Sweatt J. D. (2001) The neuronal MAP kinase cascade: a biochemical signal integration system subserving synaptic plasticity and memory. *J Neurochem* **76,** 1-10.

Templeton C. B., Poli M. A., and Solow R. (1989) Prophylactic and therapeutic use of an anti-brevetoxin (PbTx-2) antibody in conscious rats. *Toxicon* **27,** 1389-1395.

Traverse S., Gomez N., Paterson H., Marshall C., and Cohen P. (1992) Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor. *Biochem J* **288 (Pt 2),** 351-355.

Vanhoutte P., Barnier J. V., Guibert B., Pages C., Besson M. J., Hipskind R. A., and Caboche J. (1999) Glutamate induces phosphorylation of Elk-1 and CREB, along with c-fos activation, via an extracellular signal-regulated kinase-dependent pathway in brain slices. *Mol Cell Biol* **19,** 136-146.

Walker E. H., Pacold M. E., Perisic O., Stephens L., Hawkins P. T., Wymann M. P., and Williams R. L. (2000) Structural determinants of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin, and staurosporine. *Mol Cell* **6,** 909-919.

Xia Z., Dickens M., Raingeaud J., Davis R. J., and Greenberg M. E. (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* **270,** 1326-1331.

Young D., Lawlor P. A., Leone P., Dragunow M., and During M. J. (1999) Environmental enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective. *Nat Med* **5,** 448-453.

Zhu J. J., Qin Y., Zhao M., Van Aelst L., and Malinow R. (2002) Ras and Rap control AMPA receptor trafficking during synaptic plasticity. *Cell* **110,** 443-455.

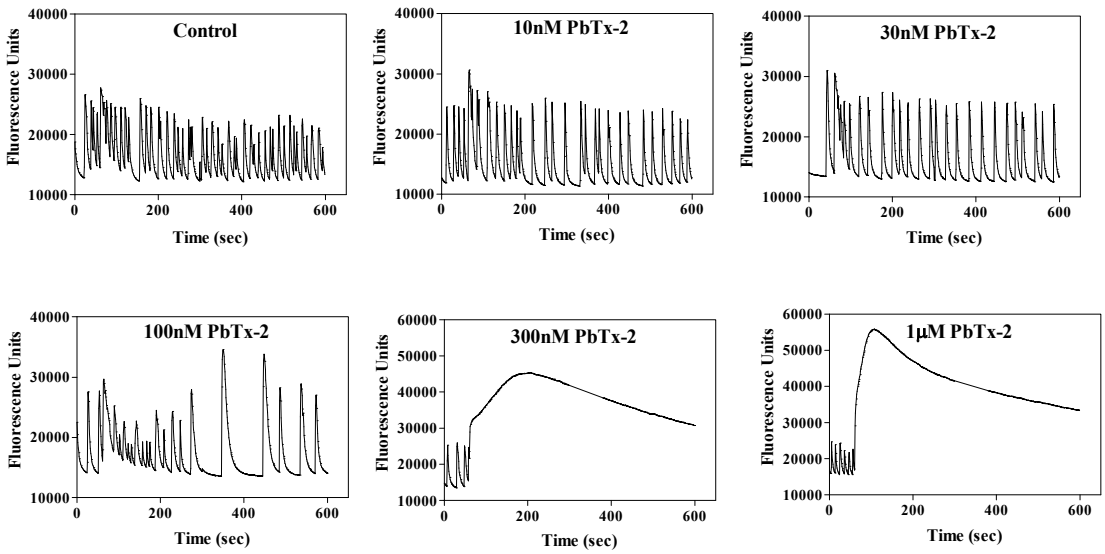


Figure 5.1. The effect of PbTx-2 on Ca^{2+} dynamics in neocortical neurons.

Ca^{2+} imaging was done in mature neocortical neurons exhibiting spontaneous Ca^{2+} oscillations treated with PbTx-2.

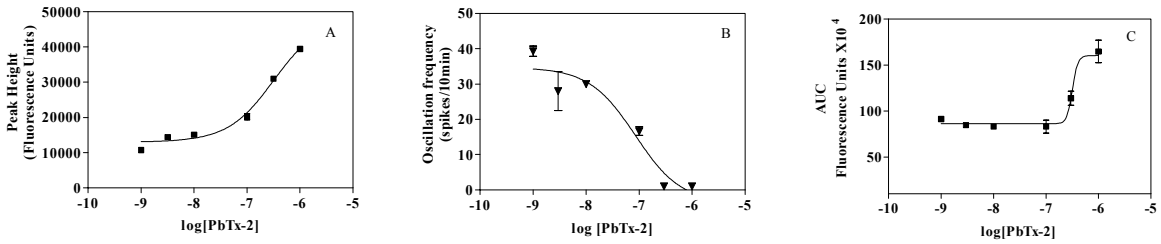


Figure 5.2. Effect of PbTx-2 on peak height, oscillation frequency and area under the curve (AUC) of spontaneous Ca^{2+} oscillations in neocortical neurons.

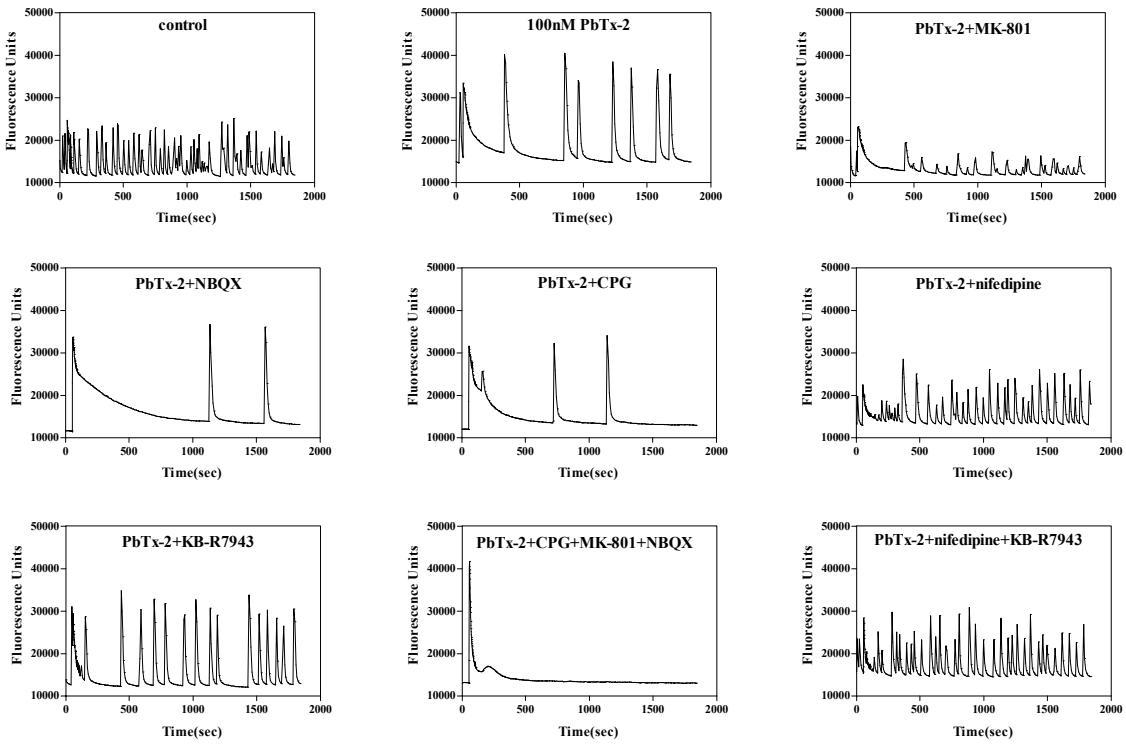


Figure 5.3. Pharmacological evaluation of 100 nM PbTx-2 induced Ca^{2+} oscillations in neocortical neurons. All antagonists were used at 1 μM concentration except CPG which was used at 500 μM concentration.

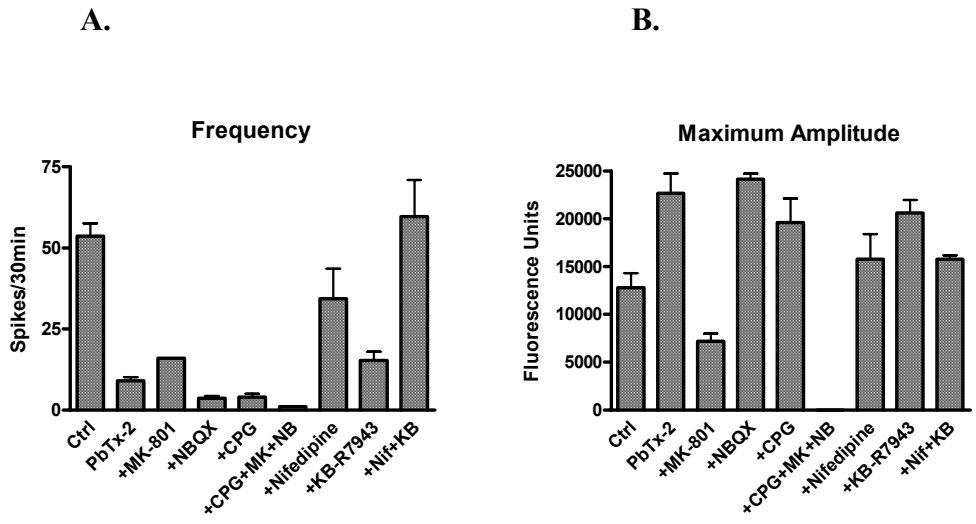


Figure 5.4. Oscillation frequency and maximum amplitude analysis of the raw data characterizing the 100 nM PbTx-2 induced Ca^{2+} oscillations. The initial Ca^{2+} peak induced by PbTx-2 was excluded from the data.

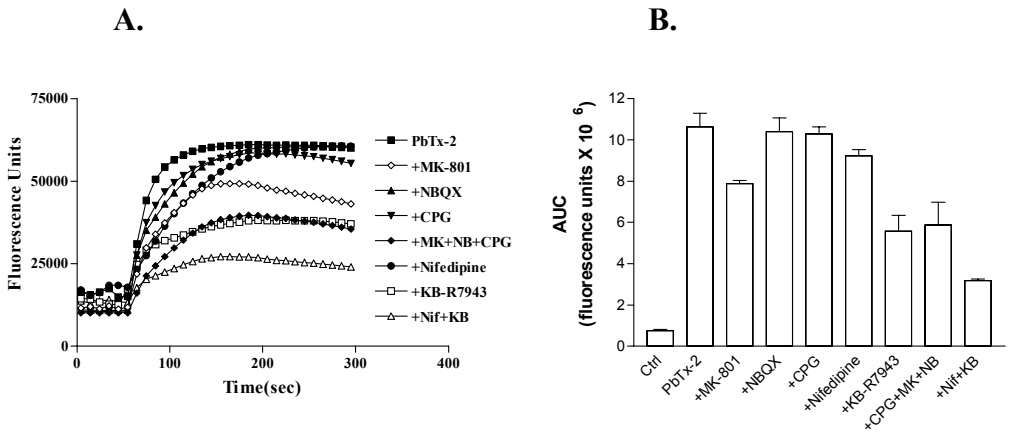


Figure 5.5. The pharmacological characterization of 1µM PbTx-2 induced Ca^{2+} influx in neocortical neurons. All antagonists were used at 1 µM concentration except CPG which was used at 500 µM concentration.

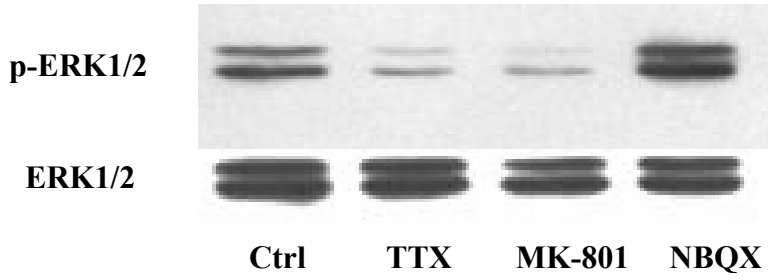
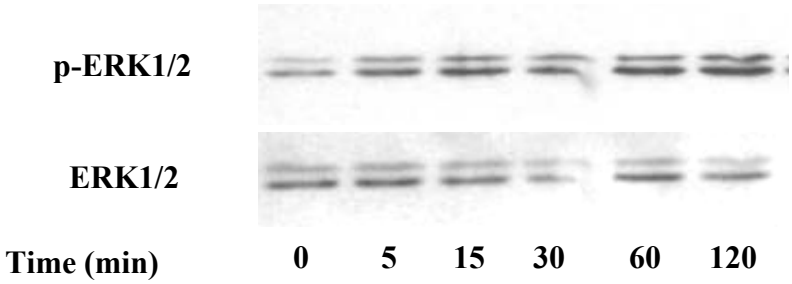


Figure 5.6. Basal synaptic activity mediated ERK1/2 phosphorylation in neocortical neurons. Inhibition by VGSC antagonist, tetrodotoxin (TTX) and NMDA receptor antagonist, MK-801.

A.



B.

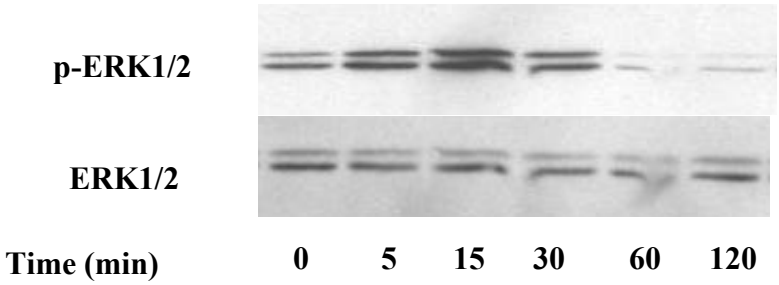


Figure 5.7. PbTx-2 induces ERK1/2 phosphorylation in neocortical neurons.

A. Neocortical neurons treated with 100 nM PbTx-2 for the indicated times. B. Neocortical neurons treated with 300 nM PbTx-2 for the indicated times.

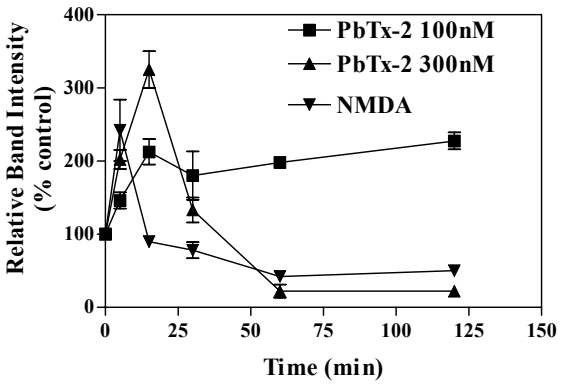


Figure 5.8. Temporal pattern of PbTx-2 and NMDA induced ERK1/2 activation.

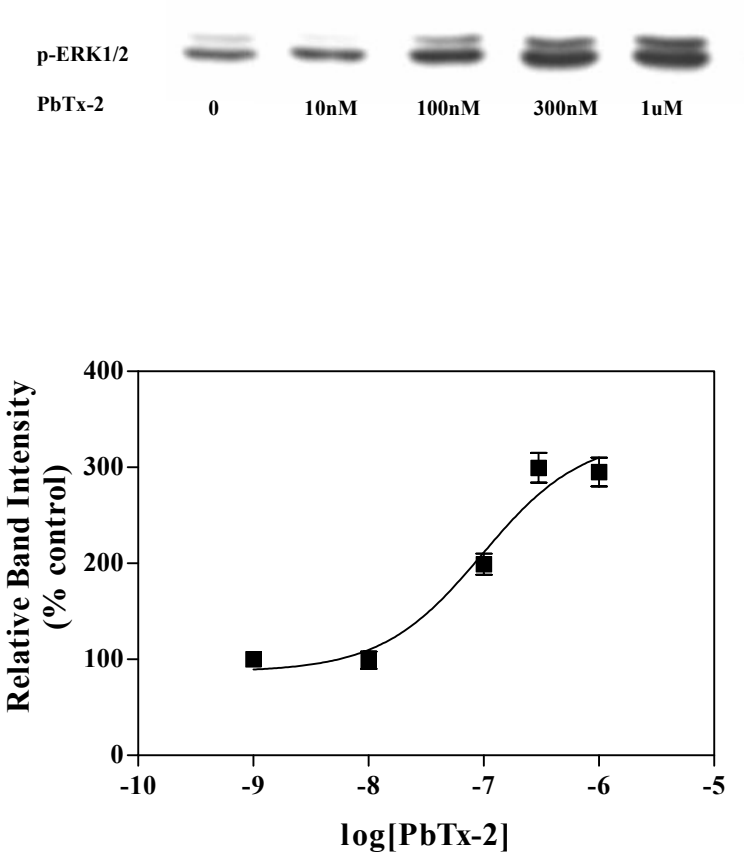


Figure 5.9. Concentration-dependent activation of ERK1/2 by PbTx-2. Neocortical neurons were treated for 15 min with different concentrations of PbTx-2.

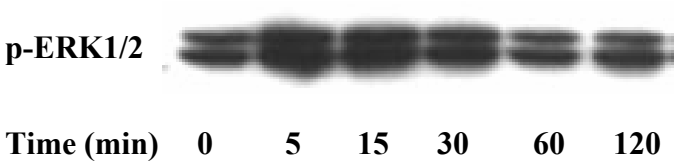


Figure 5.10. PbTx-2 induced dephosphorylation of synaptically activated ERK1/2 is developmentally regulated.

Immature neocortical neurons (7DIV) were treated with 300 nM PbTx-2 and the ERK1/2 activation was evaluated after the indicated times.

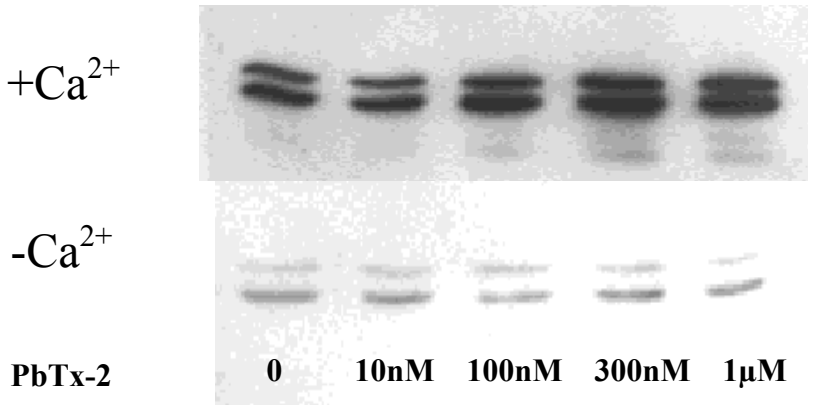


Figure 5.11. Extracellular Ca^{2+} dependence of PbTx-2 induced ERK1/2 activation. Neocortical neurons were treated with the indicated concentration of PbTx-2 for 15min, in the presence ($+Ca^{2+}$) or absence ($-Ca^{2+}$) of extracellular Ca^{2+}.

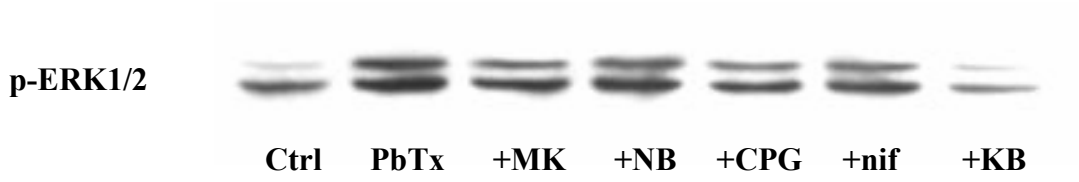


Figure 5.12. Pharmacological characterization of PbTx-2 induced ERK1/2 activation.

The neocortical neurons were treated with 1 µM PbTx-2 after a 15min pre-incubation with the different antagonists. All antagonists were used at a concentration of 1 µM except CPG which was used at 500 µM concentration.

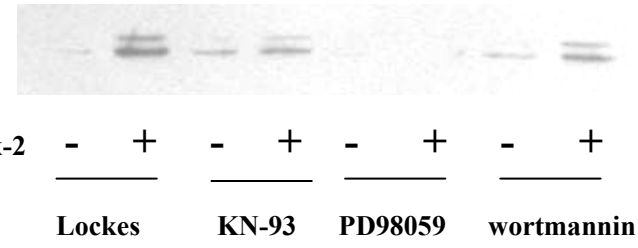


Figure 5.13. The effect of CaMKII, MEK and PI3K inhibitors on PbTx-2 induced ERK1/2 activation.

The neocortical neurons were treated with 1 µM PbTx-2 after a 15 min pre-incubation with the different inhibitors. The concentrations of the inhibitors are indicated in the text.

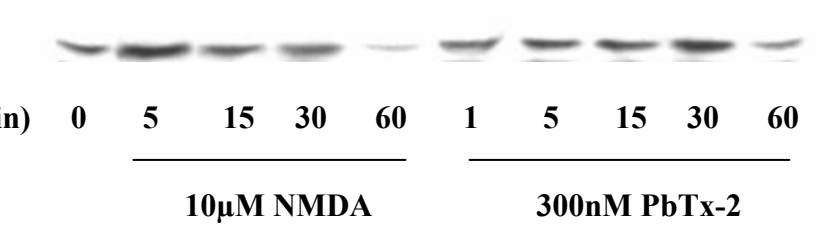


Figure 5.14. The effect of NMDA and PbTx-2 on CREB phosphorylation. Phosphorylation of CREB was assessed using a phospho-CREB specific antibody that detects CREB phosphorylated at Ser 133 site. NMDA and PbTx-2 produce distinct temporal pattern of CREB activation.

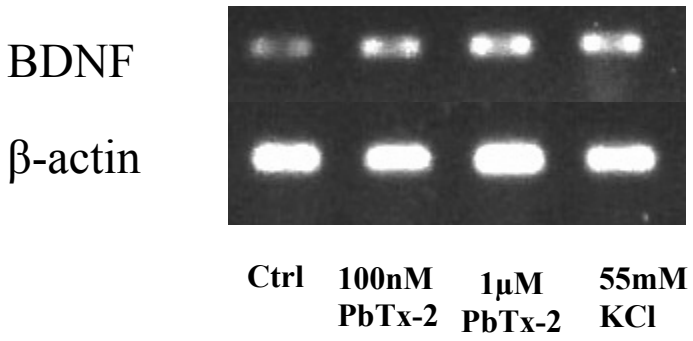


Figure 5.15. PbTx-2 increases BDNF expression in neocortical neurons.

The neocortical were exposed for 4 h with the indicated treatments and BDNF and β-actin (internal control) expression levels were detected by RT-PCR.

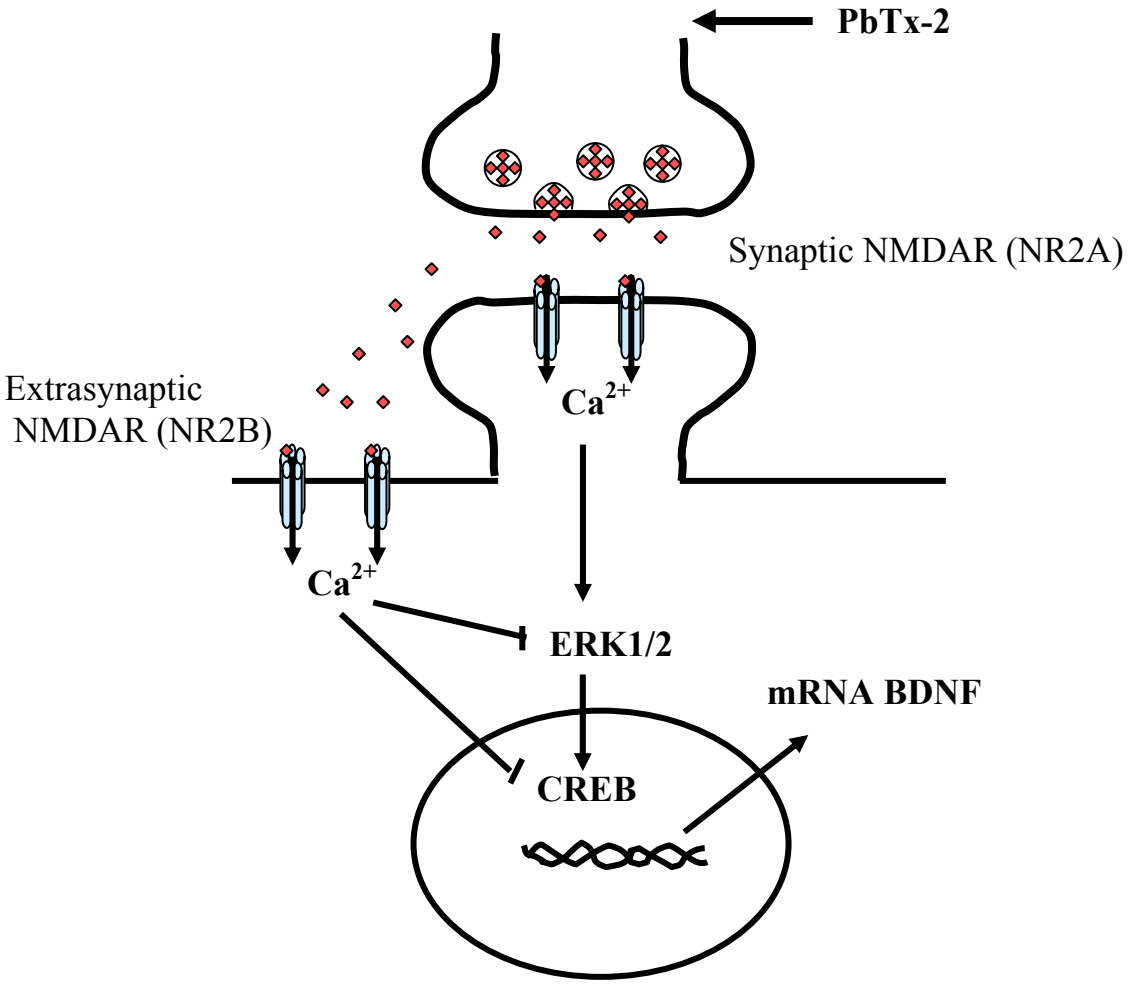


Figure 5.16 Schematic depicting the proposed mechanism of differential effects of PbTx-2 mediated by activation of synaptic and extrasynaptic NMDA receptors.

Table 5.1: PbTx-2 does not produce acute neurotoxicity in neocortical neurons.

Treatment	2 h LDH	24 h LDH
control	12±2	130±10
1 μM PbTx-2	13±1	135±12
3 μM PbTx-2	24±2	350±24
cell lysis	872±42	1197±63

CHAPTER 6

BREVETOXIN AUGMENTS NMDA RECEPTOR SIGNALING IN MURINE NEOCORTICAL NEURONS[1]

[1]Dravid S.M. and Murray T.F., to be submitted to Journal of Neurochemistry

Abstract

Brevetoxins (PbTx) are potent allosteric enhancers of voltage-gated sodium channel (VGSC) function and are associated with the periodic "Red Tide" blooms. These neurotoxins produce neuronal injury and death in cerebellar granule cells (CGC) following acute exposure. In murine neocortical neurons, brevetoxin induces Ca^{2+} influx which is mediated through both glutamatergic and non-glutamatergic pathways. Inasmuch as Src kinase is capable of upregulating the NMDA subtype of glutamate receptors, we determined whether Src kinase was involved in PbTx-2 induced Ca^{2+} influx. Inhibition of Src kinase blocked the PbTx-2 induced Ca^{2+} influx. Src kinase inhibition also inhibited basal, spontaneous Ca^{2+} oscillations exhibited by neocortical neurons, which is consistent with the role of Src kinase in the epileptiform activity exhibited by hippocampal neurons (Sanna et al., 2000). PbTx-2 treatment increased the tyrosine phosphorylation state of the NR2B subunit. Src kinase mediated tyrosine phosphorylation of NMDA receptors and rise in intracellular Na^{+} is known to increase NMDA receptor activity. We therefore explored the effect of brevetoxin on NMDA receptor functioning. We found that PbTx-2 augments NMDA receptor mediated Ca^{2+} influx both in spontaneously oscillating mature neurons and in non-oscillatory immature neurons. PbTx-2 also enhanced the effect of bath applied NMDA on extracellular signal-regulated kinase 2 (ERK2) activation. These results suggest that brevetoxin augments NMDA receptor signaling in neocortical neurons, and this upregulation may be mediated by coincidence of an elevation in intracellular [Na^{+}] and Src kinase activation.

Keywords: Brevetoxin, Ca^{2+}, Src kinase, NMDA, ERK, neocortical neurons, Na^{+}, PP2.

Introduction

Brevetoxins (PbTx-1 to PbTx-10) are potent lipid soluble polyether neurotoxins produced by the marine dinoflagellate *Karenia brevis* (formerly known as *Gymnodinium breve* and *Ptychodiscus brevis*) an organism linked to periodic red tide blooms in Gulf of Mexico, along the western Florida coastline (Baden, 1989) and New Zealand (Ishida et al., 1995). Brevetoxins interact with the site 5 of the α-subunit of the VGSC. Brevetoxins augment Na^+ influx through VGSC by increasing the mean open time of the channel, inhibiting channel inactivation and shifting the activation potential to more negative values (Jeglitsch et al., 1998).

K. brevis blooms have been implicated in massive fish kills, bird deaths, and marine mammal mortalities (O'Shea et al., 1991: Bossart et al., 1998). In humans, two distinct clinical entities, depending on the route of exposure, have been identified. Ingestion of bivalve mollusks contaminated with brevetoxins leads to neurotoxic shellfish poisoning (NSP), the symptoms of which include nausea, cramps, paresthesias of lips, face and extremities, weakness and difficulty in movement, paralysis, seizures and coma (McFarren et al., 1965; Baden and Mende, 1982; Ellis, 1985). Inhalation of the aerosolized brevetoxins from sea spray mainly results in respiratory irritation as well as dizziness, tunnel vision and skin rashes (Pierce 1986; Baden, 1982). Brevetoxins are known to accumulate in the CNS when administered systemically in animals (Cattet and Geraci, 1993) at concentrations sufficient to affect CNS (Templeton et al., 1989). Multiple-die offs of manatee following prolonged inhalation of brevetoxin laden aerosol, exhibit neuropathological sequelae (Bossart et al, 1998). These findings underscore the importance of studying the cellular consequences of brevetoxin exposure in the CNS.

Our laboratory has previously demonstrated that brevetoxin leads to glutamate release and acute neurotoxicity in cerebellar granule neurons (Berman and Murray, 1999). Brevetoxin induces Ca^{2+} influx in CGC which is responsible for the neurotoxic action of brevetoxin (Berman and Murray, 2000). Brevetoxin also affects the ERK-CREB-BDNF cascade in neocortical neurons (Dravid and Murray, submitted) that is involved in growth and survival in neurons (Grewal et al., 1999; Sweatt, 2001). The effect of PbTx-2 on NMDA receptor signaling has not been full explored.

NMDA receptors are regulated by voltage-dependent Mg^{2+} block; relief of the Mg^{2+} blockade by the depolarization incurred by brevetoxin may lead to an increase in NMDA receptor mediated Ca^{2+} influx. VGSC activators may also influence the NMDA channel activity by another mechanism which emanates from their ability to increase intracellular Na^{+}. In 1998 Yu and Salter reported that increases in intracellular Na^{+} increases the NMDA receptor mediated whole-cell currents and NMDA-receptor single-channel activity by increasing the open probability and mean open time of the channel. Using veratridine, a plant derived alkaloid and a potent VGSC activator, these investigators demonstrated that influx of Na^{+} through the TTX-sensitive VGSC is sufficient to produce potentiation of NMDA-channel activity. This augmentation was shown to be dependent on the coincidence of Src kinase activation.

In the present study we explored the effect of PbTx-2, the most commonly occurring brevetoxin in the red tide (Baden, 1989), on NMDA receptor mediated Ca^{2+} influx and signaling. We also tested the involvement of Src kinase in the ability of PbTx-2 to modulate Ca^{2+} dynamics in neocortical neurons. We found that PbTx-2 induced Ca^{2+} influx was dependent on Src kinase activity. PbTx-2 moreover increased the tyrosine

phosphorylation of NMDA receptor NR2B subunit. In addition PbTx-2 at subthreshold concentration was found to augment NMDA-induced Ca^{2+} influx and ERK1/2 activation. These results suggest that brevetoxin exposure leads to augmentation of NMDA receptor signaling in neocortical neurons which may be mediated by a coincidence of increase in Src kinase activity and rise in intracellular Na^{+}.

Materials and Methods

Neocortical Neuron Culture: Primary cultures of neocortical neurons were obtained from embryonic day 16-17 Swiss-Webster mice. Briefly, pregnant mice were euthanized by CO_2 asphyxiation and embryos were removed under sterile conditions. Neocortices were collected, stripped of meninges, minced by trituration with a Pasteur pipette and treated with trypsin for 25 min at 37˚ C. The cells were then dissociated by two successive trituration and sedimentation steps in soybean trypsin inhibitor and DNase containing isolation buffer, centrifuged and, resuspended in Eagle's minimal essential medium with Earle's salt (MEM) and supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 10% horse serum, 100 I.U./ml penicillin and 0.10mg/ml streptomycin, pH7.4. For intracellular Ca^{2+} monitoring cells were plated onto poly-L-lysine coated 96-well (9mm) clear-bottomed black-well culture plates (Costar) at a density of 4.7 X 10^5 cells/cm^2. For western blotting experiments cells were plated on 12 well plates. Cells were incubated at 37 ºC in a 5% CO_2 and 95% humidity atmosphere. Cytosine arabinoside (10 μM) was added to the culture medium on day 2 after plating to prevent proliferation of nonneuronal cells. The culture media was changed biweekly with MEM supplemented with 2 mM L-glutamine, 5% horse serum, 100 I.U./ml penicillin and 0.10

mg/ml streptomycin, pH 7.4. The cultures were used for experiments between 9 –13 days *in vitro*.

Intracellular Ca^{2+} monitoring: For intracellular Ca^{2+} monitoring the cells grown in 96 well plates were first washed four times with Locke's buffer (154 mM NaCl, 5.6 mM KCl, 1.0 mM MgCl2, 2.3 mM CaCl2, 8.6 mM HEPES, 5.6 mM glucose, and 0.1 mM glycine, pH 7.4) using an automated cell washer (Labsystems, Helsinki, Finland). Cells were then incubated for 1 h at 37˚C with dye loading buffer (100 µl/well) containing 4 µM fluo-3 AM and 0.04% Pluronic acid in Locke's buffer. Fluo-3 AM is taken up by cells and entrapped intracellularly after hydrolysis to fluo-3 by cell esterases. After 1h incubation in dye loading medium, cells were washed 5 times with Locke's buffer.

Ca^{2+} monitoring was done using FLIPR (Fluorometric Imaging Plate Reader) Neurons were excited by the 488 nm line of the argon laser and Ca^{2+} bound fluo-3 emission in the 500 nm to 560 nm range was recorded with the CCD camera shutter speed set at 0.4 s. Prior to each experiment, average baseline fluorescence was set between 10,000 and 20,000 fluorescence units by adjusting the power output of the laser. Fluorescence readings were taken either once every sec or once every 6 sec depending on the duration of the recording.

Drug treatment: For western blotting analysis cells grown in 12 well plates were washed 3 times with Locke's buffer. The cultures were then allowed to equilibrate in Locke's buffer for 15 min. The cultures were treated with the indicated drugs diluted in Locke's buffer. After the indicated incubation period, the plates were transferred to an ice slurry and the cells were washed with ice cold PBS to terminate the treatment. The cells were then scraped in ice cold PBS and centrifuged to at 5000 rpm for 10 min at 4^{o}C to collect

the cells. Lysis buffer (50 mM Tris, 50 mM Nacl, 5 mM, EGTA, 5 mM EDTA, 2 mM sodium pyrophosphate, 1 mM activated sodium orthovanadate, 1 µg/µl aprotinin, 10 µg/µl leupeptin, 10 µg/µl pepstatin, 0.5 mM PMSF and 1% NP-40) was added to the cell pellet and incubated on ice for 45 min. Thereafter the lysates were centrifuged at 14000 rpm for 15 min at 4^{o}C.

Immunoprecipitation: After treatment with PbTx-2 cells were washed with ice-cold PBS. The cells were then collected by centrifugation at 5000 rpm for 10 min at 4^{o}C. Lysis Buffer (additionally containing 0.5% SDS in order to solublize NMDA receptor subunits) was added to the pellet and boiled for 5 min. Fresh lysis buffer was added 9 times the volume of the cell lysates. The samples were then centrifuged at 14000 rpm for 25 min. The supernatant were immunoprecipitated with NR2B rabbit polyclonal antibody overnight at 4˚C. The immunocomplexes were recovered using Protein A sepharose beads. The protein was eluted by boiling in Laemmli sample buffer. Western blotting was performed using the biotin conjugated monoclonal phospho-tyrosine antibody and visualized using horseradish peroxidase conjugated streptavidin.

Western Blotting: The supernatant obtained after centrifugation of lysates were assayed by Bradford reagent to detect the protein content. Equal amount of protein from the samples were mixed with the Laemmli sample buffer and boiled for 5 min. The protein samples were run on a 10% SDS-PAGE gel and transferred to nitrocellulose membrane by electroblotting. The nitrocellulose membranes were incubated in the TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween) containing 5% skimmed milk powder for 1 h. After blocking the blots were incubated overnight in primary antibody diluted in TBST containing 5% BSA. The blots were washed with TBST and incubated with the

secondary antibody conjugated with horseradish peroxidase for 1 h, washed with TBST and exposed to ECL plus for 3 min. Blots were exposed to Kodak hyperfilm and developed. Bands were analyzed using the Alfaimager.

Data Analysis: Nonlinear regression analysis, frequency and amplitude analysis and other data analysis was done using GraphPad analysis package (GraphPad Software, San Diego, CA, USA).

Materials: Trypsin, penicillin, streptomycin, heat-inactivated fetal bovine serum, horse serum and soybean trypsin inhibitor were obtained from Atlanta Biologicals (Norcross, GA.). Minimum essential medium (MEM), Deoxyribonuclease (DNAse), poly-L-lysine, cytosine arabinosoide, MK-801, NBQX, cyclothiazide, NMDA, Protein A Sepharose were purchased from Sigma (St. Louis, MO). Pluronic acid and fluo-3 AM were purchased from Molecular Probes (Eugene, OR). Phospho-ERK1/2 antibody was obtained from Cell Signaling (Beverly, MA), phospho-tyrosine antibody from BD Transduction Laboratories (San Diego, CA) and NR2B antibody from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2) and 4-Amino-7-phenylpyrazol[3,4-d]pyrimidine (PP3) were obtained from Calbiochem (La Jolla, CA). ECL plus kit was from Amersham Biosciences (Piscataway, NJ).

Results

Src kinase inhibitor, PP2, inhibits PbTx-2-induced rise in intracellular Ca^{2+}.

Neocortical neurons exhibit spontaneous oscillations of intracellular Ca^{2+}. These oscillations are triggered by the activation of AMPA receptors and involve secondary

activation of NMDA receptors and metabotropic glutamate receptors (mGluR). At a concentration of 100 nM PbTx-2 significantly increases the amplitude and duration of a single Ca^{2+} spike while at the same time decreasing the frequency of the Ca^{2+} oscillations. PbTx-2 at concentrations more than 100 nM disrupts Ca^{2+} oscillations and produces a sustained rise in cytosolic Ca^{2+} concentration (Fig. 6.1). The increase in amplitude of individual Ca^{2+} spikes is mediated by augmentation of NMDA receptor Ca^{2+} influx and Ca^{2+} influx through L-type Ca^{2+} channels. The sustained increase in the intracellular Ca^{2+} concentration is mediated by NMDA receptors, L-type Ca^{2+} channels and reversal of Na^{+}/Ca^{2+} exchanger. The involvement of Src kinase in PbTx-2-induced augmentation of Ca^{2+} oscllation amplitude was assessed using the specific Src kinase inhibitor PP2 (10 μM). Simultaneous addition of PP2 and PbTx-2 significantly reduced the amplitude of Ca^{2+} oscillations induced by 100 nM PbTx-2, and also significantly reduced the Ca^{2+} influx mediated by 300 nM PbTx-2 (Fig. 6.1). These results suggest that Src kinase plays an important role in PbTx-2 mediated modulation of Ca^{2+} dynamics. When PP2(10 μM) is preincubated with neocortical neurons for 10 min, it significantly reduced both frequency and amplitude of basal Ca^{2+} oscillations (Fig. 6.2). In contrast PP3 (10 μM) the inactive congener of PP2 did not affect the basal Ca^{2+} oscillations indicating that the effect of PP2 is specifically due to inactivation of Src kinase (data not shown).

PbTx-2 augments tyrosine phosphorylation of NMDA receptor NR2B subunit.

It is well known that Src kinase tyrosine phosphorylates the NMDA receptor and leads to augmentation of NMDA channel activity (Salter, 1998). Since the Src kinase inhibitor, PP2, inhibited PbTx-2-induced Ca^{2+} influx, we determined whether PbTx-2

leads to an increase in the tyrosine phosphorylation of the NMDA receptor. Neocortical neurons were treated with 100 nM PbTx-2 for 5 and 15 min. The cells were collected and immunoprecipitation of NR2B subunit was assessed following by immunoblotting with a phoshpo-tyrosine specific antibody. The tyrosine phosphorylation of NR2B subunit was found to be enhanced by PbTx-2 treatment (Fig. 6.3). Thus PbTx-2 induced Ca^{2+} influx may be in part due to an upregulation of NMDA receptor activity by tyrosine phosphorylation of the receptor.

PbTx-2 augments NMDA-induced Ca^{2+} influx in neocortical neurons.

We further examined whether a subthreshold concentration of PbTx-2, which by itself does not produce an increase in Ca^{2+} load in the neocortical neurons, could augment Ca^{2+} influx mediated by NMDA receptors. A concentration-response analysis for PbTx-2 was performed by determining the integrated fluo-3 fluorescence for the recording period which represents the Ca^{2+} load. A concentration of 30 nM PbTx-2 was found to be subthreshold in producing an increase in Ca^{2+} load in neocortical neurons (Fig. 6.4). The concentration-response profile for NMDA on Ca^{2+} influx was monitored in the presence and absence of 30 nM PbTx-2. Nonlinear regression analysis was performed on the concentration-response curves obtained using the integrated fluo-3 fluorescence response (AUC). PbTx-2 was found to augment NMDA-induced Ca^{2+} influx and significantly increased the total Ca^{2+} influx as reflected by the greater maximal response of NMDA in the presence of 30 nM PbTx-2 (Fig. 6.5). The PbTx-2 treatment did not alter the EC_{50} of NMDA.

Tetanus and epileptiform activity lead to an increase in Src kinase activity (Brown and Cooper, 1996; Sanna et al., 2000). Immature neocortical cultures do not exhibit spontaneous Ca^{2+} oscillations and presumably have lower basal Src kinase activity. We therefore determined whether PbTx-2 can induce augmentation of NMDA receptor mediated Ca^{2+} influx in immature neurons. Immature neurons at 2 DIV possess the cellular components required to exhibit tyrosine kinase mediated upregulation of NMDA receptor (Takasu et al., 2002). In this early developmental model 30nM PbTx-2 did not produce an increment in Ca^{2+} influx and neurons were also less sensitive to NMDA treatment. Concentration-dependent effects of NMDA on Ca^{2+} influx were monitored, in the presence and absence of 30 nM PbTx-2. The presence of 30 nM PbTx-2 produced a dramatic potentiation of NMDA-induced Ca^{2+} influx in immature neocortical neurons. This potentiation is best exemplified through the comparison of the response to 3 μM NMDA. In the absence of PbTx-2 the 3 μM concentration of NMDA did not affect the cytoplasmic Ca^{2+}, whereas in the presence of 30 nM PbTx-2 this concentration of NMDA produced a robust increase in Ca^{2+} influx (Fig. 6.6A). The concentration-response curve was leftward shifted by PbTx-2 (30 nM) (Fig. 6.6B). The EC_{50} values for NMDA-induced Ca^{2+} influx in the absence and presence of 30nM PbTx-2 were 17.8 μM and 1.4 μM respectively. These results indicate that PbTx-2 at subthreshold concentrations can sensitize neocortical neurons to NMDA induced Ca^{2+} influx.

PbTx-2 augments NMDA induced ERK1/2 activation.

NMDA receptor activation has been shown to intricately regulate ERK1/2 activation (Hardingham et al., 2001). We therefore explored whether the PbTx-2

augmentation of NMDA induced Ca^{2+} influx was paralleled by an increase in ERK1/2 activation. Upon activation ERK1/2 is dually phosphorylated on the Thr and Tyr residues on the TEY catalytic domain. We used the dually phosphorylated ERK1/2 antibody to evaluate ERK1/2 activation by NMDA. NMDA produced a robust increase in ERK1/2 phosphorylation after 5min of exposure after which it was rapidly dephosphorylated (Fig. 6.7). The NMDA concentration-response for ERK1/2 activation was performed in the presence or absence of 30 nM PbTx-2. The NMDA concentration-response curve for ERK1/2 activation follows an inverted U shaped curve (Chandler et al., 2001). In this study NMDA produced a similar bidirectional curve which peaked at 10 μM and returned to or exceded the basal level at 100 μM (Fig. 6.8A). In the presence of 30 nM PbTx-2 the NMDA induced ERK2 activation was significantly increased at the 3 μM and 10 μM concentrations of NMDA which represent the upward component of the bidirectional curve (Fig. 6.8B). This effect was not seen with the activation of ERK1. At 30 and 100 μM NMDA, which represent the downward portion of the bidirectional curve, 30 nM PbTx-2 further enhanced dephosphorylation of both ERK1 and ERK2 (Fig. 6.8B). Considered together PbTx-2 treatment augmented both the ascending and descending components of the ERK1/2 activation response reflecting, respectively, phosphorylation and dephosphorylation events. These results indicate that mild activation of VGSC is sufficient to augment NMDA receptor-mediated Ca^{2+} influx, which is followed by a corresponding augmentation in the activation of the downstream signaling pathways.

Discussion

We have shown that brevetoxin leads to increase in intracellular Ca^{2+}, which is in part regulated by Src kinase activation and may involve augmentation of NMDA receptor functioning through tyrosine phosphorylation of the receptors. PbTx-2 at a subthreshold concentration leads to augmentation of NMDA receptor mediated Ca^{2+} influx and ERK1/2 activation. An increase in intracellular Na^{+} has been shown to increase NMDA receptor function (Yu and Salter, 1998). The exact mechanism of the Na^{+} mediated increase in NMDA receptor activity is not known but it has been shown that Na^{+} may act synergistically with Src kinase to augment NMDA receptor function (Yu and Salter, 1999). The fact that brevetoxin produced an increase in tyrosine phosphorylation of NMDA receptor, and a substantial fraction of Ca^{2+} influx after PbTx-2 treatment was inhibited by PP2 indicates that Src kinase activity may be upregulated by PbTx-2 treatment. In addition PbTx-2 increases intracellular Na^{+} by altering VGSC function. We propose, that the synergistic consequence of the above mentioned effects of brevetoxin may be responsible for the observed augmentation of NMDA receptor function. As mentioned earlier NMDA receptors are inhibited by extracellular Mg^{2+} which produces a voltage-dependent blockade of the channel (Mayer et al., 1987). PbTx-2 at 30nM did not lead to an increase in Ca^{2+} load in the neocortical neurons; however at this concentration PbTx-2 may lead to slight depolarization and reduction in the threshold potential required for the removal of NMDA receptor Mg^{2+} block. This effect may also serve as a contributing factor for upregulation of NMDA receptor function by PbTx-2. Further studies will be required to distinguish between the two mechanisms through which PbTx-2 can influence NMDA receptor functioning.

Zn^{2+} has been shown to increase NMDA receptor function and tyrosine phosphorylation (Manzerra et al., 2001; Kim et al., 2002). Zn^{2+} increases the intracellular Na^+ in neurons which may be due to its ability to block the Na^+/K^+ ATPase (Manzerra et al., 2001). In effect, Zn^{2+} by simultaneously increasing Src kinase activity and increasing intracellular Na^+ leads to augmentation of NMDA receptor function. Upregulation of NMDA receptors by the synergistic activation of Src kinase activity and rise in $[Na^+]_i$, may therefore serve as a common mechanism of action for diverse modulators of NMDA receptor functioning.

Src kinase has been shown to be involved in the epileptiform activity induced by removal of extracellular Mg^{2+} (Sanna et al., 2000). We found that Src kinase also has a role in the regulation of spontaneous Ca^{2+} oscillations since the Src kinase inhibitor, PP2, reduced the frequency and amplitude of the Ca^{2+} oscillations in neocortical neurons. It is difficult to say whether this effect is a direct consequence of decrease in NMDA receptor activity since MK-801, a non-competitive antagonist of NMDA receptor, reduces only the amplitude of basal Ca^{2+} oscillations without affecting the frequency of the Ca^{2+} oscillations (Murphy et al., 1992). The AMPA receptor antagonist NBQX concentration-dependently reduces the frequency of Ca^{2+} oscillations and inhibition of AMPA receptor desensitization leads to increase in the frequency of oscillations (unpublished data). This indicates that the frequency of Ca^{2+} oscillations in neocortical neurons is regulated by AMPA receptors. Activation of Src kinase also induces potentiation of non-NMDA receptors in an NMDA receptor and Ca^{2+}-dependent manner (Salter, 1998). The reduction in frequency of Ca^{2+} oscillation induced by PP2 may therefore be due to an indirect effect of PP2 on AMPA receptors.

PbTx-2 exposure was found to increase the tyrosine phosphorylation of NMDA receptor NR2B subunit. NR2B tyrosine phosphorylation has been shown to upregulate NMDA receptor activity (Ali and Salter, 2001). Activation of Src kinase leads to tyrosine phosphorylation of NMDA receptor (Salter, 1998). Src kinase activity is regulated by a number of biochemical pathways (Della Rocca et al., 1997; Dikic et al., 1996). Stimulation of metabotropic receptors, including mGluR receptors, leads to increase in tyrosine phosphorylation of NMDA receptors through a PKC-dependent pathway (Lu et al., 1999: Heidinger et al., 2002). NMDA receptor mediated Ca^{2+} influx can also induce protein tyrosine phosphorylation (Bading and Greenberg, 1991) and could serve as another mechanism by which PbTx-2 may be inducing its effect. Future studies will be directed to determine whether brevetoxin induced NMDA receptor tyrosine phosphorylation is a Na^{+}, Ca^{2+} or glutamate dependent mechanism.

Mitogen activated protein kinases constitute a family of serine/threonine kinases, among which the extracellular signal-regulated kinases (ERKs) have been extensively studied. In neurons, rises in intracellular Ca^{2+} mediated by NMDA receptors, Ca^{2+}-permeable AMPA/kainate receptors and voltage-gated calcium channels (VGCC) have been shown to regulate ERK1/2 activation (Sweatt, 2001). A cytoplasmic Ca^{2+} microdomain near NMDA receptors has been shown to serve as an on switch for ERK1/2 activation (Hardingham et al., 2001). We found that PbTx-2 treatment lead to an augmentation of NMDA receptor mediated effect on ERK2 activation. NMDA receptor mediated ERK1/2 activation is entirely dependent on Ca^{2+} influx through the channel. It is therefore reasonable to conclude that the PbTx-2 upregulation of NMDA-induced ERK2 activation is a direct effect of PbTx-2 increasing Ca^{2+} influx through NMDA

receptors. The C-terminus of NMDA receptor subunits are integrated to a protein network of the postsynaptic density (PSD); the components of which mediate many aspects of postsynaptic signaling by NMDA receptors (Sheng and Kim, 2002). Brevetoxin exposure by influencing Ca^{2+} influx through NMDA receptors can therefore modulate the activation of these signaling proteins and alter the cellular responses mediated by NMDA receptors.

NMDA receptor mediated Ca^{2+} influx plays a critical role for physiological functions such as synaptic plasticity and survival of neurons (Hardingham and Bading, 2003). Excessive activation of NMDA receptor leads to excitotoxicity (Choi et al., 1987). Excessive NMDA receptor mediated excitotoxicity has been implicated in several neurodegenerative disorders. Human exposure to brevetoxin is documented in many regions throughout the world and in particular on the coastline of Florida and Gulf of Mexico (Baden, 1989). The human population exposed to brevetoxin includes a geriatric population. A large portion of the geriatric population is affected by neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease which involve excessive NMDA receptor activation. The present study shows that brevetoxin exposure can induce augmentation of NMDA receptor function and, therefore, may lead to an increase in the NMDA receptor-mediated pathologic sequelae associated with neurodegenerative diseases.

References:

Ali D. W. and Salter M. W. (2001) NMDA receptor regulation by Src kinase signalling in excitatory synaptic transmission and plasticity. *Curr Opin Neurobiol* **11,** 336-342.

Baden D. G. and Mende T. J. (1982) Toxicity of two toxins from the Florida red tide marine dinoflagellate, Ptychodiscus brevis. *Toxicon* **20,** 457-461.

Baden D. G. (1989) Brevetoxins: unique polyether dinoflagellate toxins. *FASEB J* **3,** 1807-1817.

Berman F. W. and Murray T. F. (1999) Brevetoxins cause acute excitotoxicity in primary cultures of rat cerebellar granule neurons. *J Pharmacol Exp Ther* **290,** 439-444.

Berman F. W. and Murray T. F. (2000) Brevetoxin-induced autocrine excitotoxicity is associated with manifold routes of Ca2+ influx. *J Neurochem* **74,** 1443-1451.

Bossart G. D., Baden D. G., Ewing R. Y., Roberts B., and Wright S. D. (1998) Brevetoxicosis in manatees (Trichechus manatus latirostris) from the 1996 epizootic: gross, histologic, and immunohistochemical features. *Toxicol Pathol* **26,** 276-282.

Cattet M. and Geraci J. R. (1993) Distribution and elimination of ingested brevetoxin (PbTx-3) in rats. *Toxicon* **31,** 1483-1486.

Chandler L. J., Sutton G., Dorairaj N. R., and Norwood D. (2001) N-methyl D-aspartate receptor-mediated bidirectional control of extracellular signal-regulated kinase activity in cortical neuronal cultures. *J Biol Chem* **276,** 2627-2636.

Choi D. W., Maulucci-Gedde M., and Kriegstein A. R. (1987) Glutamate neurotoxicity in cortical cell culture. *J Neurosci* **7,** 357-368.

Della Rocca G. J., van Biesen T., Daaka Y., Luttrell D. K., Luttrell L. M., and Lefkowitz R. J. (1997) Ras-dependent mitogen-activated protein kinase activation by G protein-

coupled receptors. Convergence of Gi- and Gq-mediated pathways on calcium/calmodulin, Pyk2, and Src kinase. *J Biol Chem* **272,** 19125-19132.

Dikic I., Tokiwa G., Lev S., Courtneidge S. A., and Schlessinger J. (1996) A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. *Nature* **383,** 547-550.

Ellis S. (1985) Brevetoxins: chemistry and pharmacology of 'red tide' toxins from Ptychodiscus brevis (formerly Gymnodinium breve). *Toxicon* **23,** 469-472.

Grewal S. S., York R. D., and Stork P. J. (1999) Extracellular-signal-regulated kinase signalling in neurons. *Curr Opin Neurobiol* **9,** 544-553.

Hardingham G. E., Arnold F. J., and Bading H. (2001) A calcium microdomain near NMDA receptors: on switch for ERK-dependent synapse-to-nucleus communication. *Nat Neurosci* **4,** 565-566.

Hardingham G. E. and Bading H. (2003) The Yin and Yang of NMDA receptor signalling. *Trends Neurosci* **26,** 81-89.

Heidinger V., Manzerra P., Wang X. Q., Strasser U., Yu S. P., Choi D. W., and Behrens M. M. (2002) Metabotropic glutamate receptor 1-induced upregulation of NMDA receptor current: mediation through the Pyk2/Src-family kinase pathway in cortical neurons. *J Neurosci* **22,** 5452-5461.

Jeglitsch G., Rein K., Baden D. G., and Adams D. J. (1998) Brevetoxin-3 (PbTx-3) and its derivatives modulate single tetrodotoxin-sensitive sodium channels in rat sensory neurons. *J Pharmacol Exp Ther* **284,** 516-525.

Kim T. Y., Hwang J. J., Yun S. H., Jung M. W., and Koh J. Y. (2002) Augmentation by zinc of NMDA receptor-mediated synaptic responses in CA1 of rat hippocampal slices: mediation by Src family tyrosine kinases. *Synapse* **46,** 49-56.

Lu W. Y., Xiong Z. G., Lei S., Orser B. A., Dudek E., Browning M. D., and MacDonald J. F. (1999) G-protein-coupled receptors act via protein kinase C and Src to regulate NMDA receptors. *Nat Neurosci* **2,** 331-338.

Manzerra P., Behrens M. M., Canzoniero L. M., Wang X. Q., Heidinger V., Ichinose T., Yu S. P., and Choi D. W. (2001) Zinc induces a Src family kinase-mediated up-regulation of NMDA receptor activity and excitotoxicity. *Proc Natl Acad Sci U S A* **98,** 11055-11061.

Mayer M. L., Westbrook G. L., and Guthrie P. B. (1984) Voltage-dependent block by Mg2+ of NMDA responses in spinal cord neurones. *Nature* **309,** 261-263.

McFarren E. F., Silva F. J., Tanabe H., Wilson W. B., Campbell J. E., and Lewis K. H. (1965) The occurrence of a ciguatera-like poison in oysters, clams, and Gymnodinium breve cultures. *Toxicon* **3,** 111-123.

Murphy T. H., Blatter L. A., Wier W. G., and Baraban J. M. (1992) Spontaneous synchronous synaptic calcium transients in cultured cortical neurons. *J Neurosci* **12,** 4834-4845.

Pierce R. H. (1986) Red tide (Ptychodiscus brevis) toxin aerosols: a review. *Toxicon* **24,** 955-965.

Salter M. W. (1998) Src, N-methyl-D-aspartate (NMDA) receptors, and synaptic plasticity. *Biochem Pharmacol* **56,** 789-798.

Sanna P. P., Berton F., Cammalleri M., Tallent M. K., Siggins G. R., Bloom F. E., and Francesconi W. (2000) A role for Src kinase in spontaneous epileptiform activity in the CA3 region of the hippocampus. *Proc Natl Acad Sci U S A* **97,** 8653-8657.

Sheng M. and Kim M. J. (2002) Postsynaptic signaling and plasticity mechanisms. *Science* **298,** 776-780.

Sweatt J. D. (2001) The neuronal MAP kinase cascade: a biochemical signal integration system subserving synaptic plasticity and memory. *J Neurochem* **76,** 1-10.

Takasu M. A., Dalva M. B., Zigmond R. E., and Greenberg M. E. (2002) Modulation of NMDA receptor-dependent calcium influx and gene expression through EphB receptors. *Science* **295,** 491-495.

Templeton C. B., Poli M. A., and Solow R. (1989) Prophylactic and therapeutic use of an anti-brevetoxin (PbTx-2) antibody in conscious rats. *Toxicon* **27,** 1389-1395.

Yu X. M. and Salter M. W. (1998) Gain control of NMDA-receptor currents by intracellular sodium. *Nature* **396,** 469-474.

Yu X. M. and Salter M. W. (1999) Src, a molecular switch governing gain control of synaptic transmission mediated by N-methyl-D-aspartate receptors. *Proc Natl Acad Sci U S A* **96,** 7697-7704.

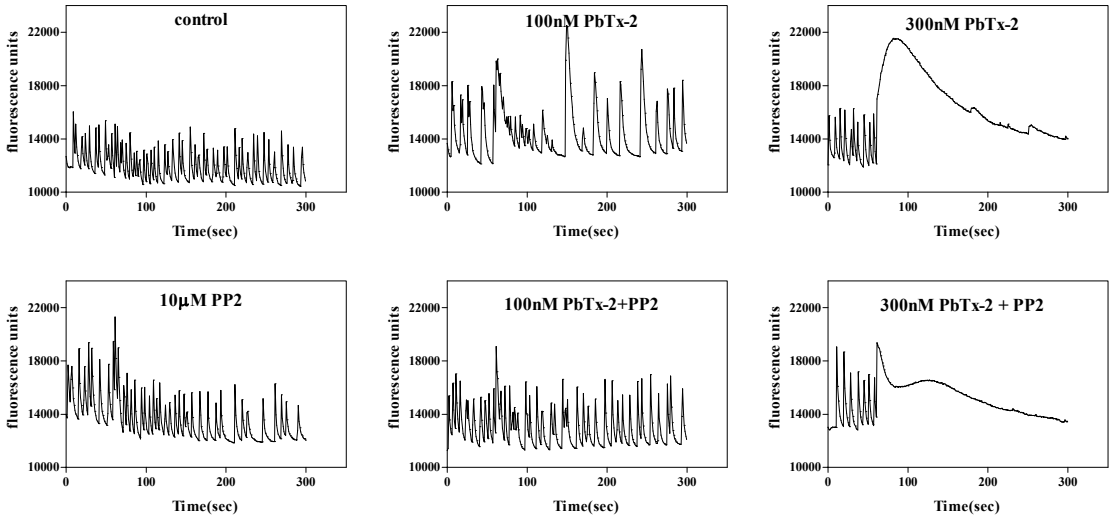


Figure 6.1. Role of Src kinase in PbTx-2 induced Ca^{2+} influx.

PP2 a Src family kinase (SFK) inhibitor reduced Ca^{2+} influx induced by PbTx-2. PbTx-2 and PP2 were added simultaneously to the neocortical cultures.

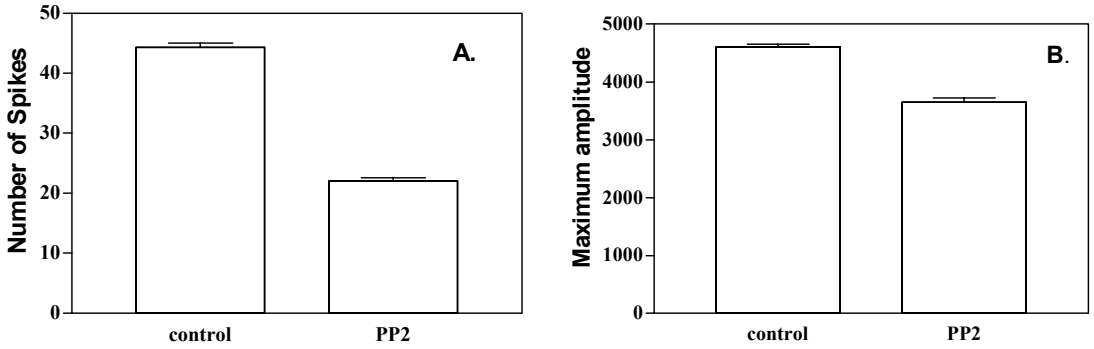


Figure 6.2. Src kinase inhibitor, PP2, inhibits the basal Ca^{2+} oscillations.

The neocortical neurons were treated with 10μM PP2 for 10min and the frequency and amplitude of the Ca^{2+} oscillations were analyzed.

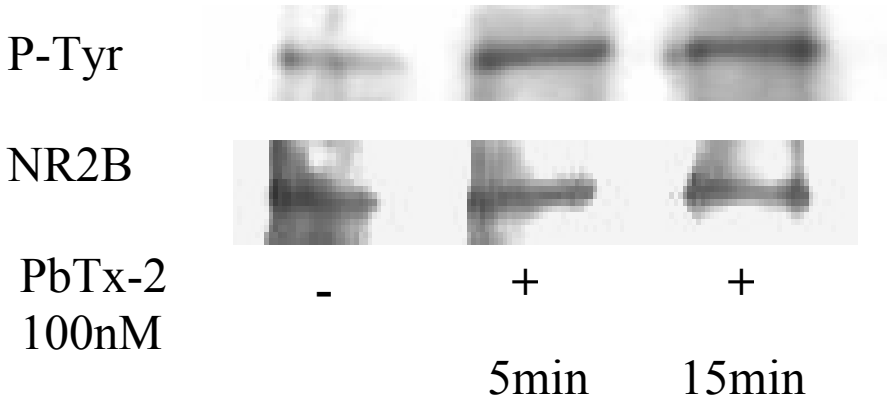


Figure 6.3. PbTx-2 increases tyrosine phosphorylation of NMDA receptor NR2B subunit.

The NR2B subunit was immunoprecipitated and immunoblotting was done using a phospho-tyrosine specific antibody.

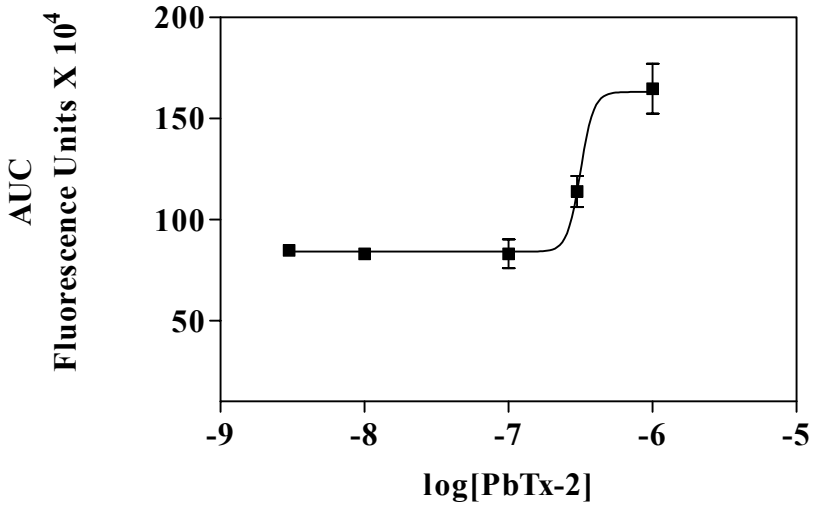


Figure 6.4. PbTx-2-induced Ca^{2+} influx in neocortical neurons.

The integrated increase in fluo-3 fluorescence [area under the curve (AUC)] versus PbTx-2 concentration was plotted.

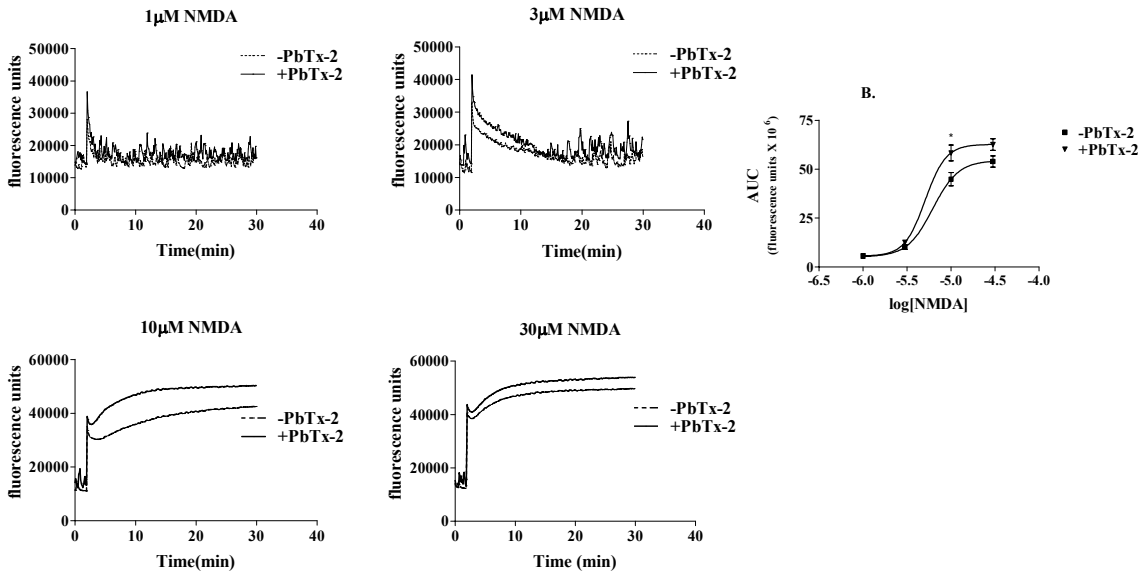
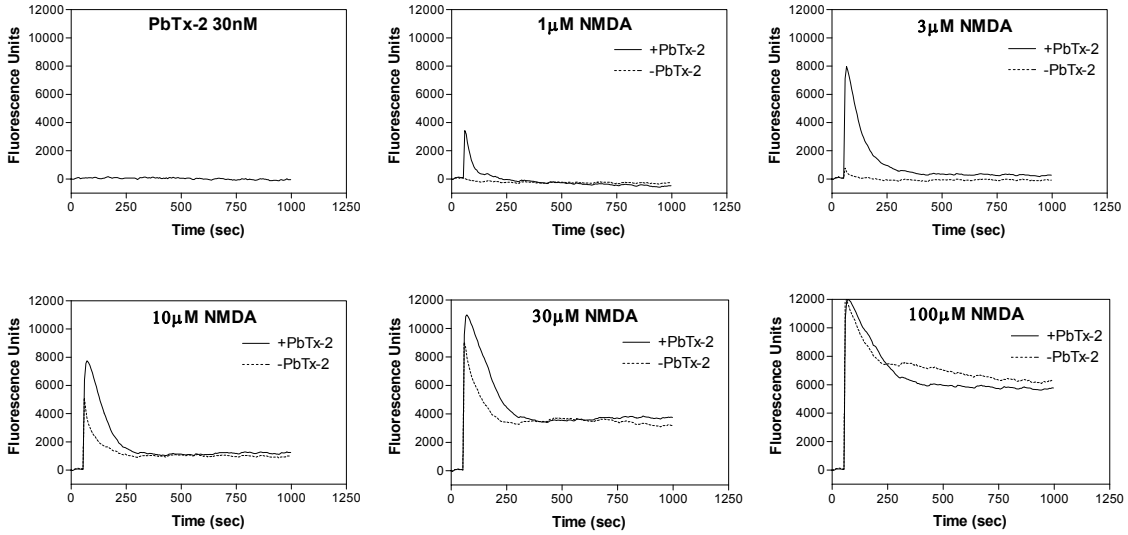


Figure 6.5. PbTx-2 augments Ca^{2+} influx through NMDA receptor.

The neocortical neurons were treated with different concentrations of NMDA with or without 30nM PbTx-2. B. Nonlinear regression analysis of the integrated fluo-3 fluorescence response [area under the curve (AUC)] versus NMDA concentration.

A.



B.

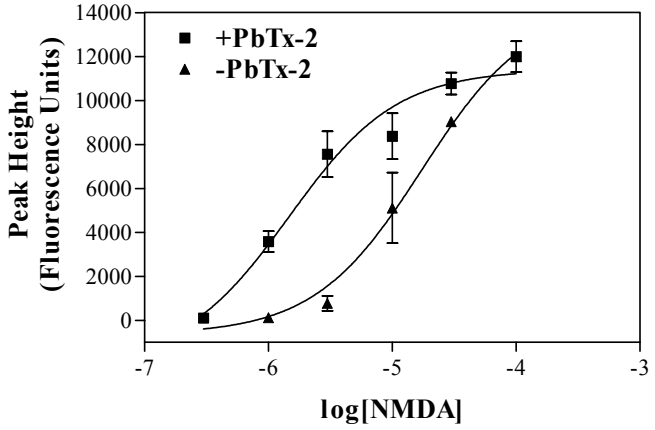


Figure 6.6A. PbTx-2 augments NMDA-induced Ca^{2+} influx in immature (2DIV) neocortical neurons. Raw data. B. PbTx-2 increases the potency of NMDA to induce Ca^{2+} response in immature neocortical neurons. Nonlinear regression analysis of the peak height of the Ca^{2+} response versus log NMDA concentration.

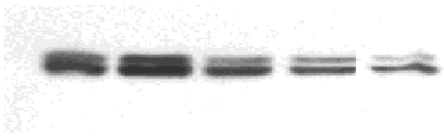
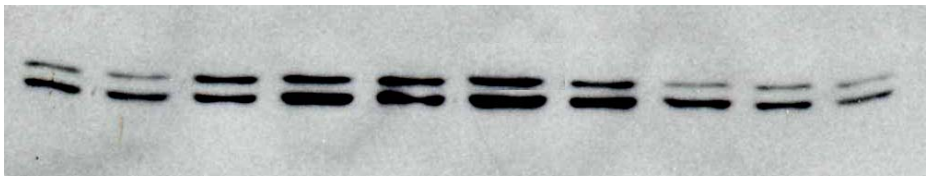


Figure 6.7. NMDA (10μM) induced ERK1/2 activation in neocortical neurons. NMDA bath application leads to an increase in activated ERK1/2 within 5 min followed by rapid dephosphorylation.



PbTx-2 30nM	-	+	-	+	-	+	-	+	-	+
NMDA (μM)	0	0	3	3	10	10	30	30	100	100

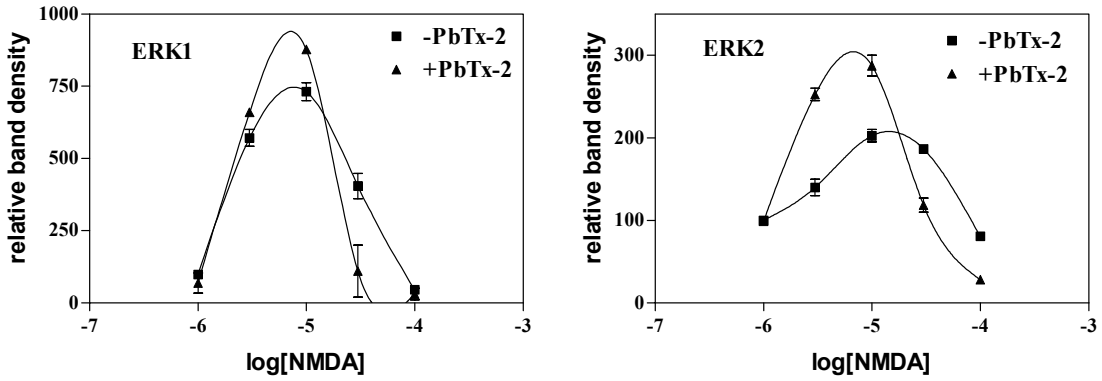


Figure 6.8 A. Effect of 30nM PbTx-2 treatment on NMDA concentration-response profile for ERK1/2 activation. The neocortical neurons were treated simultaneously with the indicated concentrations of NMDA and PbTx-2 for 5 min and ERK1/2 activation was examined. B. Effect of 30nM PbTx-2 on NMDA induced ERK1 and ERK2 activation.

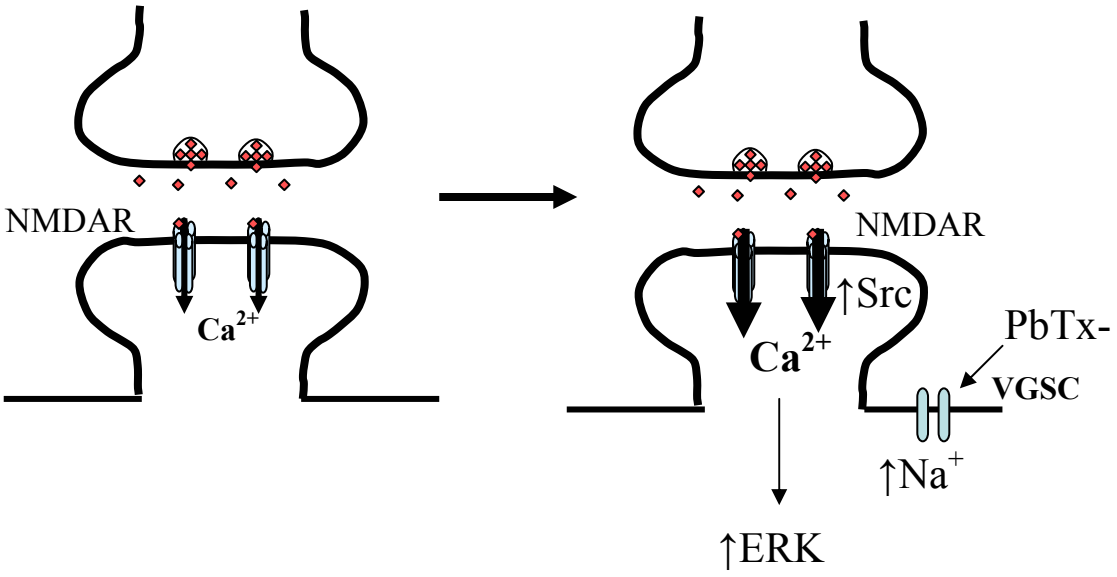


Figure 6.9 Schematic representing the proposed mechanism of PbTx-2-induced augmentation of NMDA receptor

CHAPTER 7

SUMMARY AND CONCLUSIONS

Glutamate activates AMPA receptors with lower potency than NMDA receptors. The EC_{50} value of glutamate is approximately 500μM for AMPA receptors whereas, depending on receptor subunit composition, it is approximately 400nM to 2μM for NMDA receptors. AMPA receptors however usually mediate fast excitatory neurotransmission in the CNS while NMDA receptors are activated only in times of strong synaptic signaling. This is due to the characteristic Mg^{2+} blockade of the NMDA receptors by physiologic submillimolar extracellular concentrations of Mg^{2+}. This Mg^{2+} block of NMDA receptors is voltage-dependent and requires a membrane depolarization to -50mV or more for its removal. Removal of Mg^{2+} from the extracellular compartment therefore biases glutamatergic neurotransmission in favor of NMDA receptors. Accordingly, removal of Mg^{2+} from the extracellular compartment leads to generation of epileptiform activity and cytosolic Ca^{2+} oscillations in hippocampal, cerebellar granule and neocortical neurons that are mediated by NMDA receptors and L-type voltage-gated calcium channels (VGCC). When we explored the mechanism of Ca^{2+} oscillations in the presence of extracellular Mg^{2+}, we determined that the contribution of NMDA receptors to Ca^{2+} spike amplitude was approximately 22% and Ca^{2+} spikes were completely independent of L-type VGCC activation. We also determined that the increase in intracellular Ca^{2+} during a Ca^{2+} spike is partly mediated by release of Ca^{2+} from the endoplasmic reticulum (ER) stores. We also noted that inhibition of metabotropic glutamate receptors (mGluR) reduced the amplitude of Ca^{2+} spike. This was in accordance with earlier studies resorting a role of mGluR receptor activation in the generation of Ca^{2+} oscillations both in primary culture systems and heterologously expressed systems. MGluR induced release of ER Ca^{2+} stores is mediated by phospholipase C. We therefore tested whether inhibition of this enzyme would affect the Ca^{2+} oscillations. We determined that inhibition of PLC inhibited the spontaneous Ca^{2+} oscillations. Thus, we concluded that activation of mGluR by the endogenously released glutamate leads to release of ER Ca^{2+} stores by activation of phospholipase C and contributes to the increase in intracellular Ca^{2+} during a Ca^{2+} spike.

AMPA receptors were traditionally considered to be only permeable to Na^{+}. Pioneering work by several laboratories revealed that AMPA receptors may also be permeable to Ca^{2+}. This occurs when the heteromeric receptor is devoid of the GluR2

subunit. Selected neuronal populations in certain regions of the CNS possess these Ca^{2+}-peremable AMPA receptors. Since excessive Ca^{2+} influx and disregulation leads to cytotoxicity, these Ca^{2+}-permeable AMPA receptors are considered to be responsible for neurological diseases such as Alzheimer's and amyotrophic lateral sclerosis which are characterized by selective neurodegeneration. The Ca^{2+}-permeable AMPA receptors are therefore considered to be important molecular targets for prevention of certain neurological diseases. Fluorescent imaging plate reader (FLIPR) is a high-throughput instrument that utilizes fluorometric assays and is widely used for screening drug candidates. We therefore set out to develop a method for detection of activation of Ca^{2+}-permeable AMPA receptors using FLIPR instrumentation. Ca^{2+}-permeable AMPA receptors exhibit characteristic permeability for Co^{2+} which is not seen among Ca^{2+}-impermeable AMPA receptors, NMDA receptors or VGCC. We exploited this characteristic conductance property by using a fluorescent dye calcein, whose fluorescence is quenched by Co^{2+} but not affected by Ca^{2+}, to develop an assay for detection of Ca^{2+}-permeable AMPA receptors.

Oscillations in intracellular Ca^{2+} regulate activation of Ca^{2+} sensitive proteins and drive gene expression. We explored whether modulation of the Ca^{2+} dynamics by an external stimulus can affect the downstream activation of signaling proteins and gene expression. We are interested in brevetoxins which are potent allosteric enhancers of voltage-gated sodium channel (VGSC) function that induce depolarization in neurons. Brevetoxins are produced by a dinoflagellate *Karenia brevis* that are associated with red tide which leads to death of fish, marine mammals and birds. Human exposure through ingestion of contaminated mollusks and inhalation of aerosolized neurotoxins leads to neurotoxic shellfish poisoning and respiratory irritation, respectively. The cellular effects of brevetoxin on the CNS are not well understood. We explored the effect of brevetoxin on Ca^{2+} dynamics and associated signaling in neocortical neurons. Brevetoxin altered Ca^{2+} dynamics in neocortical neurons with lower concentrations increasing the synaptically mediated Ca^{2+} spike amplitude and higher concentrations leading to a generalized depolarization and activation of synaptic as well as extrasynaptic receptors with an attendant disruption of Ca^{2+} oscillations. We further studied the effect of brevetoxin exposure on a Ca^{2+} sensitive signaling pathway, extracellular-regulated

receptor kinase (ERK1/2). The correlation between the modulation of Ca^{2+} oscillations and activation of ERK1/2 was noteworthy. Brevetoxin concentrations that increased the synaptic activity induced a sustained phosphorylation of ERK1/2. In contrast, concentrations producing a sustained increase in intracellular Ca^{2+} induced ERK1/2 activation for a shorter duration. We propose that these differential temporal patterns of ERK1/2 phosphorylation may result from the activation of synaptic versus extrasynaptic NMDA receptors. It is accordingly proposed that, similar to cAMP responsive element binding protein (CREB) regulation in hippocampal neurons, activation of synaptic NMDA receptors leads to ERK1/2 activation whereas the activation of extrasynaptic NMDA receptors leads to a shut-off of synaptically activated ERK1/2. We also determined that brevetoxin exposure alters the activation of the transcription factor CREB which is downstream of ERK1/2 activation. The effect of brevetoxin was not restricted to activation of signaling molecules but also affected the expression of brain derived neurotrophic factor (BDNF). Thus we concluded that brevetoxin, rather than producing cytotoxicity, can alter the activation and expression of such signaling pathways that are necessary for growth, survival, learning and memory in neocortical neurons.

Tyrosine phosphorylation and augmentation of NMDA receptor activity by Src tyrosine kinase has been extensively studied. We addressed the role of Src kinase in Ca^{2+} influx mediated by brevetoxin and determined whether brevetoxin can produce tyrosine phosphorylation of NMDA receptor and augment its activity. We determined that inhibition of Src kinase reduced brevetoxin induced Ca^{2+} influx, and moreover that brevetoxin exposure increased tyrosine phosphorylation of the NMDA receptor NR2B subunit. Intracellular Na^{+} levels have been shown to regulate NMDA receptor activity. A rise in intracellular Na^{+} by veratridine a VGSC activator leads to increase in mean open time and open probability of NMDA receptors. This effect of intracellular Na^{+} is observed when there is concomitant Src kinase activation. We therefore explored whether brevetoxin treatment could lead to increase in Ca^{2+} influx and signaling mediated by NMDA receptors. We found that concentrations of brevetoxin which by itself do not lead to an increase in Ca^{2+} load in neurons can produce augmentation of NMDA receptor mediated Ca^{2+} influx and ERK1/2 activation. We therefore concluded that brevetoxin upregulates NMDA receptor signaling. We further propose that NMDA receptor

upregulation by brevetoxin is due to concurrent activation of Src kinase induced tyrosine phosphorylation of the NMDA receptor and an increase in intracellular Na^+ due to the action of brevetoxin on VGSC.

These studies provide initial insights into the cellular effects of brevetoxin in particular and VGSC activators in general. Depending on the neuronal cell phenotype, brevetoxin may produce a spectrum of pharmacologic actions ranging from potentiation of signaling mechanisms underlying neural plasticity to cytotoxicity. In this regard brevetoxins resemble the endogenous excitatory transmitter glutamate. Brevetoxins are environmental toxicants and human exposure through ingestion and inhalation is well documented. The results of the present study suggest that the effects of brevetoxin exposure to humans may be subtle and not characterized by acute toxicity. Geriatric populations are also exposed to brevetoxins. Inasmuch as a large section of the geriatric population suffers from neurodegenerative diseases that involve neurotoxicity due to excessive activation of NMDA receptors, our studies suggest that exposure to chronic low levels of brevetoxin may lead to augmentation of NMDA receptor activation and therefore may exacerbate the neurodegenerative processes.