ANGIOTENSIN II MODULATION OF NEURONAL CALCIUM CURRENT

By

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I dedicate this dissertation to my family and especially my mother, Barba Lee Evans. They have made countless sacrifices so that I could pursue my dreams.

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TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
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In neurons cultured from neonatal rat hypothalamus and brainstem, angiotensin II (Ang II) caused an increase in Ca^{2+} current. Molecular and pharmacological analyses revealed the presence of all high voltage activated Ca^{2+} current subtypes in the neurons: L, N, P/Q, and R. The Ca^{2+} current could be facilitated with a depolarizing voltage pulse, which is indicative of voltage-dependent Ca^{2+} current inhibition by $G\beta\gamma$ subunits. Recovery of inhibition following a depolarizing voltage pulse occurs with a time constant consistent with rates of $G\beta\gamma$ reassociation. Ang II partially prevented facilitation, consistent with relief of $G\beta\gamma$ inhibition. These data suggest that Ang II, via the Ang II type 1 receptor (AT_1R), may increase Ca^{2+} current by relieving tonic G protein inhibition in a PKC dependent manner.

Embryonic or neonatal rat neurons retain plasticity and are readily cultured, but neurons of the adult brain are thought to be non-replenishable, and therefore difficult to culture. Adult neural cell cultures were prepared from brainstem or hypothalamus. Very

few adherent cells were apparent in cultures for up to one week, at which time the cell population expanded dramatically. The predominant cell type was immunopositive for α-internexin, a neurofilament expressed in developing neurons of the CNS. α-internexin positive cells co-immunostained for neuronal markers including MAP2, β-tubulin III, and tetanus toxin, but were negative for glial markers and for the neurofilaments characteristic of mature neurons. α-internexin positive cells incorporated BrdU, suggesting that the neuron-like cells retain the ability to proliferate. Patch clamp analysis revealed voltage gated Na^+ currents and small Ca^{2+} currents, but the cells were unable to fire action potentials, consistent with an immature phenotype. These results show that the cultures are immunologically and electrophysiologically more similar to neurons than glia. The immature neuronal phenotype makes these cells an attractive model system for several neurobiology applications.

CHAPTER 1
INTRODUCTION

Angiotensin II in Blood Pressure Control

Peripheral Actions of the Renin-Angiotensin System

Angiotensin II (Ang II) is the octapeptide product of a series of enzymatic reactions starting with the cleavage of angiotensinogen by the enzyme renin to produce angiotensin I (Figure 1-1). Angiotensin I is then cleaved by angiotensin converting enzyme (ACE) to produce Ang II, the peptide with the broadest range of activities and highest potency in the cardiovascular system.

Ang II affects cardiovasular function on both the long and short term. The acute, rapid actions of Ang II are a concerted effort to maintain extracellular fluid volume. These pressor actions include vasoconstriction, aldosterone secretion leading to salt retention, water conservation through stimulation of thirst and release of antidiuretic hormone, increased strength of myocardial contraction, and stimulation of the sympathetic nervous system to potentiate the vasoconstrictor and ionotropic actions of Ang II. Chronic elevation of Ang II is associated with vascular smooth muscle hyperplasia, cardiac hypertrophy and fibrosis, endothelial dysfunction, altered arterial contractile sensitivity, and renal insufficiency. Prolonged loss of extracellular fluid volume is not likely to occur in humans, so the chronic Ang II effects are probably pathophysiological rather than protective.

The circulating renin-angiotensin system (RAS) is a classical endocrine system. Angiotensinogen, the only known precursor protein for the family of angiotensin peptides, is produced primarily in the liver. Renin is synthesized and secreted by the kidney. ACE is synthesized primarily in the lung. These proteins come into contact in the circulation to produce circulating levels of Ang II.

In addition to the circulating RAS, several tissue RASs exist and contribute to cardiovascular structure, function, and adaptation to stressors. These tissues can make all of the components of the RAS, but may also pick up components from the circulation. The tissues known to have specific systems are the kidney, vasculature, heart, and brain.

Ang II in Brain Neurons

All of the components of the RAS have been found in the brain. Some effects of Ang II in the brain can be achieved with peripherally administered doses while some occur only with direct injections to the brain. There appear to be significant differences between the effects elicited from these two sources of the hormone, since systemic Ang II can only contact sites that have no blood-brain barrier, such as the circumventricular organs. The central and peripheral actions of Ang II are therefore entirely independent, but are focused toward the ultimate goal of maintaining body fluid homeostasis.

High densities of Ang II receptors are located in the hypothalamus and brainstem (Gehlert et al., 1986; Mendelsohn et al., 1984). Ang II receptor subtypes, the Ang II Type 1 receptor (AT_1R) and the Ang II Type 2 receptor (AT_2R), are found in these areas, but are localized to different nuclei and tracts, overlapping only in the superior colliculus. Radioligand binding, quantitative autoradiography, and *in situ* hybridization shows the AT_1R to be located in the suprachiasmatic nucleus, the median preoptic nucleus, the nucleus tractus solitarius, the dorsal vagal nucleus, the paraventricular nucleus, the

median eminence, the organum vasculosum of the lamina terminalis, the lateral parabrachial nucleus, the supraoptic nucleus, the subfornical organ, and the area postrema, all nuclei in the hypothalamus or brainstem or structures known to be circumventricular organs. The areas that contain the AT_2R are the inferior olive, the ventral septum, the lateral septum, the locus coeruleus, the mediodorsal thalamic nuclei, the medial amygdala, and the medial geniculate nucleus. Activation of the AT_1R in the hypothalamic or brainstem nuclei ultimately leads to physiological changes such as increased blood pressure, altered baroreflex modulation, increased water and sodium intake, and increased vasopressin secretion (Hegarty et al., 1996; Phillips, 1987; Sumners et al., 1994).

Signal Transduction Mechanisms of Ang II in Brain Neurons

Short-term Ang II Effects

Electrophysiological recordings from supraoptic neurons in hypothalamic explants showed that Ang II, via the AT_1R, increased the firing rate and increased outward current. In the presence of tetrodotoxin, which blocks Na^+ current and therefore action potentials (APs) and subsequent neurotransmitter release, as well as in the absence of Ca^{2+}, Ang II was still able to effect a net depolarization of the neurons, suggesting that Ang II acts directly *in situ* to modify resting membrane potential and neuronal excitability (Yang et al., 1992).

Because the depolarization induced by Ang II is observed even in the absence of inward currents Na^+ and Ca^{2+}, it is likely that the effect is due to an inhibition of outward current, commonly carried through voltage gated K^+ channels. Indeed, in neurons cultured from neonatal rat hypothalamus and brainstem, Ang II decreased voltage-gated

K^+ current in a losartan sensitive manner. Additionally, Ang II, via the AT_1R, increased voltage-gated Ca^{2+} current. These effects were inhibited by intracellular application of antibodies to phospholipase C-gamma (PLCγ), calphostin C and protein kinase C inhibitor peptide (PKCIP), and were mimicked by superfusion of 4-α-phorbol myristate acid (PMA), a protein kinase C (PKC) stimulator. Ang II increased IP_3 release as measured by Dowex chromatography, and increased incorporation of labeled phosphate from ATP into histones in a PKC activity assay (Sumners et al., 1996). These results, taken together, suggest that Ang II stimulates a classic Gq coupled receptor pathway to stimulate voltage dependent Ca^{2+} current and inhibit voltage dependent K^+ current.

In the same model system of neurons cultured from neonatal rat hypothalamus and brainstem, Ang II increased tetrodotoxin-sensitive AP-firing rate, concurring with the studies in hypothalamic explants. In addition Ang II increased cadmium (Cd^{2+}) sensitive subthreshold oscillations in the cultured neurons that are likely to be carried through Ca^{2+} channels. In contrast to the explant studies wherein the absence of Ca^{2+} did not alter the Ang II response, a Cd^{2+} block of the Ca^{2+} current decreased the firing rate in the presence of Ang II. In agreement with the putative signal transduction mechanism, stimulation of PKC with PMA as well as tetraethylammonium or 4-aminopyridine inhibition of voltage gated K^+ channels, mimicked the effect of Ang II on the firing rate. These results showed that the substrates underlying the chronotropic effect of Ang II are voltage-gated Ca^{2+} and K^+ channels. The current clamp studies in the cultured neurons also expand on the findings in the explants regarding the depolarizing actions of Ang II and demonstrate that the *in vitro* resting membrane potential is not altered, but the amplitude of the current

stimulus required to reach an AP was decreased, suggesting that the threshold in the neurons was decreased on Ang II stimulation.

Using different methodology in the same model system, a peptide corresponding to the 3rd intracellular loop of the AT_1R was introduced into the neurons via the patch pipette (Zhu et al., 1997). The peptide mimicked the effect of Ang II stimulation on K^+ and Ca^{2+} current, and these effects were inhibited by introduction of antibodies to $G\alpha_{q/11}$. In accord with the other signal transduction studies in this model, PKCIP and BAPTA (a Ca^{2+} chelator) fully alleviated the inhibitory effect of the peptide on the voltage-dependent K^+ current. However, PKCIP and calphostin C, another inhibitor of PKC, blocked the stimulatory effect on voltage-gated Ca^{2+} current. Calphostin C or anti-IP_3 receptor antibodies alone were unable to block the entire effect of the AT_1R peptide on K^+ current, indicating the possibility of another contributing mechanism.

Likely to mediate the PKC-independent effect is calmodulin-dependent kinase Type II (CaMKII) (Zhu et al., 1999). Pretreatment of the neurons with CaMKII inhibitors including CaMKII 281-302 peptide, KN92, and KN93 attenuates the reduction of voltage-gated K^+ (K_v) current caused by Ang II. Western blot analysis of proteins isolated from the neonatal cultured neurons revealed the presence of CaMKII α and β isoforms. The Ang II decrease in K^+ current is mimicked by intracellular application of active CaMKII.

Pharmacological dissection of the K^+ current shows that the non-inactivating current modulated by Ang II was sensitive to TEA, 4-AP, and low doses of quinine. Despite the presence of the mRNA for Kv subunits 2.2 or 3.1b, only Kv2.2 was present at the protein level. Coupling of Kv2.2 and Ang II did occur in oocytes injected with the

appropriate mRNAs, implicating this subtype of K^+ current as a substrate for the Ang II actions (Gelband et al., 1999). Ang II was also shown to decrease the conductance and open probability of A-type single channels in whole-cell, outside-out patch, and cell-attached patch experiments on neuronal cultures (Wang et al., 1997). A-type channels therefore underlie the Ang II modulation of the transient component of the whole cell K^+ current.

Activation of the AT_2R had an apparently opposite effect on K^+ current in the neuronal cultures (Kang et al., 1993). Ang II, via the AT_2R, stimulated TEA-sensitive K_v current and A-current. Charybdotoxin and apamin, which block Ca^{2+} activated K^+ current subtypes did not block the AT_2R-mediated effects of Ang II. No alteration of voltage-gated Ca^{2+} current was observed with AT_2R activation. Although some groups have proposed antagonistic effects of the two Ang II receptor subtypes, recent studies suggest that the AT_2R pathway stimulates AP firing rate, similar to the situation with the AT_1R (Zhu et al., personal communication).

Interestingly, all of the K_v recordings in the AT_1R studies were conducted in the presence of Cd^{2+} which blocks voltage-gated Ca^{2+} current, suggesting that the role of the Ca^{2+} current is not solely to contribute to Ca^{2+}-dependent components of the pathway (such as PKC or CaMKII) resulting in the decrease in K^+ current. Unlike the situation with the K^+ current, all of the acute Ang II effects on Ca^{2+} current seem to require PKC activity. While K^+ current largely determines the resting membrane potential and ultimately the excitability of neurons, Ca^{2+} current generally acts to shape the AP and subsequent signaling pathways. Therefore, Ang II may alter K^+ current for acute chronotropic results and Ca^{2+} current for other consequences.

Long-term Ang II Effects

In addition to the acute effects of AT_1R activation, neuromodulation by Ang II can also involve transcriptional and translational events, and involve signal transduction pathways distinct from those that cause the acute changes in membrane excitability. The dissociation of the G-protein α and $\beta\gamma$ subunits may liberate the $\beta\gamma$ subunits for interaction with membrane-associated factors causing activation of the small G-protein, Ras. Indeed, incubation of neuronal co-cultures with Ang II resulted in the activation of Ras, which is known to require $G\beta\gamma$, as judged by an increase in the ratio of GTP-bound Ras to GDP-bound Ras. The ratio reached a maximum between 5 and 10 minutes after Ang II treatment, and falls back to control levels in an hour. Ang II stimulated activity and phosphorylation of Raf-1 on a similar time scale, consistent with the idea that activated Ras in turn activates Raf-1. The MAP kinase (MAPK) activity is increased on the same timescale, and likely underlies the stimulation of tyrosine hydroxylase (TH) activity and TH mRNA that begins within about an hour of Ang II treatment, and peaks at about 4 hours (Yang et al., 1996). In another study, activation of MAPK by Ang II resulted in its translocation to the nucleus. This translocation has been shown to mediate activation of serum-response element (SRE) binding activity, c-fos gene expression, and AP_1 binding activity (Lu et al., 1996). Initiation of these transcription factors by Ang II may result in their binding to the AP1 binding site on the promoter regions of TH and norepinephrine transporter (NET) genes, explaining the increase in NET and TH mRNA expression in co-cultured neurons after chronic (4-hour) treatment with Ang II (Lu et al., 1996; Yu et al., 1996). These results, taken together, suggest a stimulation of the Ras/Raf/MAPK pathway via the AT_1R, resulting in an increase in production and activity

of the TH enzyme, which is the first enzyme in the production pathway for catecholamines, and in the NET protein. The Ras/Raf/MAPK pathway, then, likely mediates long-term effects of Ang II on NE modulation.

Ca^{2+} Channels

Subtypes

Ca^{2+} influx through voltage-gated Ca^{2+} channels plays a major role in many cellular processes, including muscle contraction, neurotransmitter release, and signal transduction. Distinctions between various types of voltage-gated Ca^{2+} channels have historically been based on functional properties such as distribution, pharmacology, single-channel conductance, and voltage-dependent kinetics. Early studies of cardiac and skeletal muscle electrophysiology pointed to two groups of Ca^{2+} channels: high voltage activated (HVA) and low voltage activated (LVA).

LVA Ca^{2+} channels support pacemaker activity in the heart and Ca^{2+} entry at negative membrane potentials. They are termed T-type, because of their transient kinetics during a voltage pulse caused by rapid activation and slow deactivation. They are less Cd^{2+} sensitive than HVA channels, and more sensitive to nickel, amiloride, and octanol (Tsien et al., 1991).

Functional studies defined the major pathway for voltage gated Ca^{2+} entry in cardiac, smooth muscle, and skeletal muscle cells as through HVA L-type channels. The L-type channels are sensitive to 1,4 dihydropyridines (DHP) such as nifedipine, are sensitive to the Ca^{2+} channel antagonists verapamil and diltiazem, and are found in virtually all excitable tissues and in many non-excitable cells (Tsien et al., 1991).

Molecular and pharmacological tools have revealed a number of DHP-insensitive HVA Ca^{2+} channels expressed predominantly in the nervous system. The N-type Ca^{2+} channels are sensitive to ω-conotoxins MVIIC and GVIA (McDonough et al., 1996). They have a smaller single-channel conductance than the L-type channels and a greater tendency to inactivate with depolarized holding potentials, though the rate of inactivation varies from cell type to cell type. The ω-conotoxin-sensitive channels are found in neurons in various densities, but are absent in Purkinje cells of the cerebellum (Tsien et al., 1991).

The prominent Ca^{2+} channel subtype in the Purkinje cells of the cerebellum is the P/Q-type HVA channel. Qualitatively, these channels show slow inactivation during depolarization, and are widely dispersed in many types of neurons. The P/Q-type channels are sensitive to the funnel web toxin from *Aglenopsis aperta*, ω-agatoxin IVA (Mintz et al., 1992).

The R-type channels are so named because they are resistant to the toxins and antagonists used to block the other HVA channels.

Molecular biology has revealed structural diversity to parallel the functional and pharmacological diversity of voltage-gated Ca^{2+} channels. Immunoprecipitation of the DHP receptors from skeletal muscle showed that the channel exists as a multimeric assembly of α_1, α_2, β, γ, and δ subunits. The α_1 subunit is a single polypeptide chain with four repeated units of homology. Each homologous unit contains six α-helical membrane-spanning segments. The fourth segment in each repeat is considered the voltage sensor of the channel and contains positively charged amino acid residues at every third or fourth residue. The α_1 subunit appears to be necessary and sufficient to

confer the functional, biophysical, and pharmacological attributes to a particular channel subtype (Table 1-1).

Cloning of the α_1 subunits corresponding to the various Ca^{2+} channel subtypes has allowed for analysis of their expression in various mammalian tissues (Table 1-2) as well as an analysis of their relationship to each other molecularly. The P-type channels were first identified in cerebellar Purkinje cells (Hillman et al., 1991), while Q-type currents were first recorded from cerebellar granule cells. The α_{1A} subunit underlies both of these types of Ca^{2+} current, and thus they are commonly lumped together as P/Q type current (Stea et al., 1994). Compared to the α_1 subunits that underlie L-type Ca^{2+} current, the P/Q type protein contains a number of amino acid substitutions in the voltage sensor that may explain its distinct gating properties (Starr et al., 1991). The rat α_{1A} clone differs from the L-type channel in the II/III linker and in the carboxy terminal segment, and is about 33% identical at the amino acid level (Starr et al., 1991). Northern blot analysis of RNA from rat tissues revealed expression in brain, heart, and pituitary, but not in spleen, kidney, or liver. Detailed Northern blot analysis of brain areas showed expression of α_{1A} RNA in virtually every area of the brain: spinal cord, cerebellum, pons/medulla, hypothalamus/thalamus, olfactory bulb, striatum, hippocampus and cortex (Starr et al., 1991).

Antibodies to the protein product of the α_{1B} clone immunoprecipitated w-conotoxin binding sites from rat brain, confirming that this subunit underlies N-type current. Northern analysis of rat RNA exposed the transcript in all brain areas, and exclusively in brain tissues (Dubel et al., 1992). Immunocytochemical analysis of the

expression pattern of α_{1B} at the single neuron level showed that N-type channels are localized primarily in dendrites and their associated synapses (Westenbroek et al., 1992).

Three α subunits are associated with L-type current. The α_{1S} subunit is the equivalent of the skeletal muscle cell DHP-receptor. It was the first α subunit identified, and it was discovered by immunoprecipitation of the DHP sites from skeletal muscle, where it is exclusively located. The cardiac and smooth muscle isoform of the L-type Ca^{2+} channel is α_{1C}. Two splice variants of α_{1C} have been identified, and injection of antisense to either variant is sufficient to block 90% of the Ca^{2+} currents caused by injection of rat heart mRNA into oocytes. Both transcripts are expressed in all regions of the CNS, but in varying proportions as determined by RT-PCR (Snutch et al., 1991). Both are expressed in the tissues examined by Northern blot: heart, adrenal, pituitary, and brain. The α_{1D} subunit, which also produces an L-type current and was first identified in neuroendocrine cells, is expressed in the nervous system, along with α_{1C} (Tomlinson et al., 1993). The subunit diversity underlying L-type current suggests functional diversity, which is further supported by distinct expression patterns.

Recently, it has come to light that α_{1E} is likely to be the molecular equivalent of R-type Ca^{2+} current. The α_{1E} rat clone shows only about a 53-54% identity to the subunits associated with N-type and P/Q-type Ca^{2+} current, and about 23% identity to α_{1C} or α_{1D}, the subunits that underlie the L-type Ca^{2+} current in non-skeletal muscle cells. The polypeptide sequence of the II/III linker and the carboxyl terminal region shows little conservation as compared to the other neuronal α subunits (Soong et al., 1993). Although no specific antagonist has been identified for the R-type current, its high

sensitivity to both nickel and Cd^{2+} distinguish it from the HVA and LVA channels, respectively.

The LVA Ca^{2+} current is carried via α subunits α_{1G}, α_{1H}, and α_{1I}. Two α_{1G} mRNA transcripts were detected by Northern analysis of rat mRNA, and the strongest signals were detected in the brain, with less abundant expression in the heart. Transcripts were detected in all brain areas tested including amygdala, thalamus, subthalamic nuclei, substantia nigra, hippocampus, caudate nucleus, corpus callosum, and cerebellum (Perez-Reyes et al., 1998). The T-type current responsible for pacemaker activity in the heart is carried via α_{1H} subunits. Human tissue Northern blots revealed strong α_{1H} expression in the kidney, relatively higher abundance in heart than in brain, and expression in the liver (Cribbs et al., 1998). The α_{1I} protein is 59.3% identical to human α_{1H} and 56.9% identical to rat α_{1G}, but it is only 13-19% identical to the HVA subunits. The distribution of α_{1I} in various rat tissues was determined by Northern blot to be predominantly in the brain (Lee et al., 1999). In contrast to the fast activating currents generated by α_{1G} and α_{1H}, the T-type current carried by α_{1I} is considered "slow".

Expression of the varied α subunits in oocytes or HEK cells permitted analysis of the biophysical properties associated with single current types (Table 1-2) in isolation. In general, expression of the alpha subunits associated with L-type current (α_{1S}, α_{1C}, and α_{1D}) in Xenopus oocytes yielded a current with a threshold of about –20 mV that peaks at 30 mV. The half-maximal activation occurred at about 6 mV, while the half-maximal inactivation occurred at –37 mV. Single-channel analysis revealed a conductance of about 20 pS. These recordings were done in solutions containing 40 mM Ba^{2+} in the absence of exogenous channel subunits (Tomlinson et al., 1993).

Similarly, expression of α_{1B} subunits in Xenopus oocytes and recording in 40 mM Ba^{2+}, revealed the biophysical properties of the non-regulated N-type Ca^{2+} current. This current activated near –30 mV and the peak of the current-voltage relationship was at 30 mV. The half maximal activation was found to be near the origin at 1 mV and the half maximal inactivation occurred at –34 mV (Stea et al., 1993). These parameters are very similar to those found for the L-type currents. Small differences in the voltage-dependent properties of the current carried via the α_{1A} subunit were revealed in similar Xenopus oocyte experiments, also in 40 mM Ba^{2+}. The threshold was found to be –20 mV and the current peaked somewhere between 10 and 20 mV. Like the N-type current, the half-maximal activation was just negative at –1 mV, and the half maximal inactivation was slightly less depolarized than that of L or N type current at –29 mV (Stea et al., 1994).

Because of their similar biophysical properties, the current carried by α_{1E} was thought to be an LVA current. Expression in Xenopus oocytes revealed a threshold of –50 mV, significantly hyperpolarized with respect to the other HVA currents, and a peak at –10 mV. The half maximal activation and inactivation, –25 and –65 mV, respectively, are also significantly shifted for the R-type current as compared to those for the N, P/Q and L type currents (Soong et al., 1993). However, molecular analysis showed that the α_{1E} subunit is evolutionarily more related to the HVA currents than the LVA currents that are carried via α_{1G}, α_{1H} and α_{1I} (Cribbs et al., 1998; Lee et al., 1999; Perez-Reyes et al., 1998). The LVA currents expressed in Xenopus oocytes or HEK-293 cells activate near –50 to –60 mV and peak at –25 mV, a significantly more negative potential than that for R-type current. The half maximal activation and inactivation are in the ranges of –68 to –21 mV and –75 to –50 mV, respectively, also significantly shifted as compared to the R-

type current. The single channel conductance is small, ranging from 4 to 11 pS (Cribbs et al., 1998; Lee et al., 1999; Perez-Reyes et al., 1998).

Regulation

As current molecular and imaging techniques give rise to a deep understanding of Ca^{2+} channel subunits, their localization, and their function, a stronger knowledge base of the regulation of Ca^{2+} channels is emerging. A wide variety of neurotransmitters, peptides, and drugs can affect Ca^{2+} channel activity. Additionally, accessory subunit expression can affect the voltage-dependent properties of Ca^{2+} current. Existing studies, although limited, suggest that multiple pathways dynamically regulate Ca^{2+} channels, in a subtype-specific manner (Tsien et al., 1988). Such shifting but orchestrated control provides the opportunity for shaping a given current subtype for a highly specific function or cell type.

As with other proteins including other ion channels, phosphorylation is an important regulatory mechanism. Proteins immunoprecipitated with an antibody to ω-conotoxin binding sites (N-type channels) proved to be *in vitro* substrates for both PKC and PKA phosphorylation as assayed by Western blot analysis and autoradiography (Ahlijanian et al., 1991). Although the electrophysiological effects of PKA on N-type current vary between cell types, in general, PKC phosphorylation results in a stimulation of the current (Gross and MacDonald, 1989; Gross and Macdonald, 1988; Kaneko and Nomura, 1987). Conversely, PKA activity results in stimulation of the cardiac L-type current, while PKC effects on this current subtype are variable (Kamp and Hell, 2000). Multiple G-protein coupled receptors in the heart signal through cAMP/PKA pathways to stimulate or inhibit L-type current, depending on the coupling with either $G\alpha_s$ or $G\alpha_i$.

Stimulation of $G\alpha_q$ coupled receptors and subsequent PKC activation in the heart, however, shows varying effects on I_{Ca}. *In vitro* studies demonstrated that both α_{1C} and β_{2a} subunits are substrates for PKC, so it is possible that phosphorylation of different sites can produce different effects (Kamp and Hell, 2000).

Different splice variants of α_{1A} are susceptible to differential phosphorylation. Immunoprecipitation of α_{1A} subunits and subsequent *in vitro* phosphorylation showed that the 220-kDa polypeptide was most susceptible to PKA phosphorylation, while the 190-kDa form was a substrate for PKC or PKG phosphorylation (Sakurai et al., 1995).

Protein-protein interactions, including the interactions of the α-subunit of a Ca^{2+} channel with other subunits can also regulate the expression and voltage-dependence of the current. Coexpression of α_{1A} with β_{1b}, β_{2a}, β_3, and β_4 subunits resulted in an apparent increase in the number of positive oocytes and also increased the average size of barium current recorded from positive cells, but had no apparent effect on the rate of activation of the current. The different β subunits did have pronounced effects on the inactivation, depending on the particular β subunit expressed. All of the β subunits shifted the I/V relations of the α_{1A} subunit to more hyperpolarized potentials. The sensitivity of the α_{1A} current to the various pharmacological agents, including ω-agatoxin IVA was not changed by expression of the β subunits (Stea et al., 1994).

Expression of α_{1B} subunits in HEK293 cells required expression of a β subunit, but α_{1E} expression did not require any additional subunits for functional expression, although expression of α_{1E} with α_{2b} and β_{1-3} cDNAs did increase current magnitudes.

Expression of the additional subunits also shifted the peak of the current-voltage relationship from +10 to +5 mV (Williams et al., 1994).

Neurotransmitter modulation of neuronal Ca^{2+} current has been extensively documented. The voltage-dependent Ca^{2+} current of many neurons is depressed by many neurotransmitters including NE, possibly as a mechanism to inhibit presynaptic neurotransmitter release. The initial model proposed suggested that a neuronal Ca^{2+} channel can exist in two modes that are in equilibrium with each other: a willing mode and a reluctant mode. In the willing mode, which predominates in the absence of an inhibitory neurotransmitter, channels can be opened by moderate depolarization, while in the reluctant mode, strong depolarizing potentials are required to open the channels (Bean, 1989). It has been shown that stimulation of some G-protein coupled receptors liberates $G\beta\gamma$ that associates with the Ca^{2+} channel α-subunit, switching it to a reluctant state, and thus inhibiting the current. Several groups established that $G\beta\gamma$ interacts with the α-subunit of N, P/Q, and R type Ca^{2+} channels, which are the current types subject to the membrane-delimited voltage-dependent inhibition. The L-type channels do not seem to be substrates for this type of G-protein dependent inhibition. Relief of the $G\beta\gamma$-inhibition of Ca^{2+} current in voltage-clamp mode with a depolarizing prepulse has been termed facilitation. Facilitation by inference is then a technique to measure the inhibition of a channel by a neurotransmitter.

The PKC activator PMA was shown to disrupt the voltage-dependent inhibition of Ca^{2+} current in freshly dissociated central and peripheral rat neurons by baclofen, leutinizing hormone-releasing hormone, Oxo-M (a muscarinic receptor agonist), and an adenosine receptor agonist (Swartz, 1993). Competition studies with peptides

corresponding to the I-II linker loop of the α_{1A} or α_{1B} subunits suggested that the crosstalk between the G$\beta\gamma$ modulation and the PKC modulation were at the level of the channel itself (Zamponi et al., 1997). Further dissection of the linker narrowed the region capable of competing for regulation of the channels and pointed to the QXXER domain in the intracellular loop. Interestingly, this motif overlaps with the consensus sequence for binding of Ca^{2+} channel β subunits, and therefore may be critical for interaction of three different modulatory mechanisms (Herlitze et al., 1997).

Although the various mechanisms regulating Ca^{2+} channels have typically been studied in isolation, in the physiological environment, the dynamic combination of kinases, phosphatases, subunit proteins, etc., regulates the ultimate function of the channel.

Neurotransmitter Release

Role of Voltage Dependent Ca^{2+} Channels

The Ca^{2+} entry via voltage-gated Ca^{2+} channels is essential for neurotransmitter release. Stimulated release of neurotransmitters is principally resistant to dihydropyridines, suggestive of a role for non-L type channels in exocytosis. The K^+ evoked [^{3}H]-NE release from rat sympathetic neurons was markedly reduced by Cd^{2+} and ω−conotoxin GVIA but was largely resistant to nitrendipine, a dihydropyridine antagonist. The Ca^{2+} imaging experiments using Fura-2 showed that nitrendipine strongly reduced calcium influx, despite its lack of effect on neurotransmitter release (Hirning et al., 1988). These results, taken together, suggest a dominant role for N-type Ca^{2+} current in NE release from sympathetic neurons, despite a significant contribution of L-type current to the total Ca^{2+} influx of the cell.

Different types of Ca^{2+} channels mediate central synaptic transmission. Inhibitory postsynaptic currents in cerebellar and spinal neurons and excitatory postsynaptic currents in hippocampal neurons are reduced by ω-agatoxin IVA and to a lesser degree by ω-conotoxin GVIA. Again, nicardipine had no effect (Takahashi and Momiyama, 1993). Studies like these suggested a role for P/Q type Ca^{2+} current in neurotransmitter release and also initiated the idea that multiple current subtypes could be involved with exocytosis. Consistent with this idea, and further revising the function of Ca^{2+} current subtypes in neurotransmitter release, [^{3}H]-glutamate release from synaptosomes was resistant to ω-conotoxin GVIA, but was partially blocked by ω-agatoxin IVA, suggesting that P/Q type and a toxin-resistant (possibly R-type) Ca^{2+} current coexist to regulate release of this excitatory neurotransmitter. In contrast, [^{3}H]-dopamine release from synaptosomes was blocked by ω-conotoxin and ω-agatoxin, suggesting that N-type and P/Q type currents regulate release of this neurotransmitter (Turner et al., 1993). Regulation of transmitter release in different types of cells by different current subtypes which themselves have different biophysical properties and regulatory mechanisms adds to the ways in which synaptic transmission can be modulated.

Immunoprecipitation with an antibody to N-type Ca^{2+} channels revealed a tight interaction with syntaxin, a protein involved in synaptic vesicle docking at the plasma membrane. The N-type Ca^{2+} channel/syntaxin complex also immunoprecipitated with synaptotagmin, the calcium binding protein of synaptic vessels (Leveque et al., 1994). A peptide corresponding to a sequence in the II-III loop of the α_{1B} subunit, can block the binding of syntaxin to α_{1B} (Sheng et al., 1994). This region is termed the “synprint” or synaptic protein interaction site on the Ca^{2+} channel. The corresponding II-III segment of

the α_{1A} subunit did not bind syntaxin, suggesting that P/Q type Ca^{2+} current may not bind to presynaptic transmitter release machinery. However, the II-III loop of the α_{1A} subunit is subject to alternative splicing, and fusion proteins containing the synprint site from one isoform interacted with both syntaxin and SNAP-25 (synaptosome-associated protein of 25 kDa), while fusion proteins from the other isoform bound SNAP-25 alone (Rettig et al., 1996). The physical interaction of the Ca^{2+} channel with the exocytotic machinery coupled with the localization of non-L type channels to the neuron terminals and dendrites is consistent with the tight coupling between Ca^{2+} influx and transmitter release.

Role of Ang II in Neurotransmitter Release: Specific Aims

Ang II has a centrally mediated pressor effect that correlates with levels of NE in the brain (Camacho and Phillips, 1981; Chevillard et al., 1979; Sumners and Phillips, 1983). Studies of K^+ evoked [^{3}H]-NE release from rabbit hypothalamic slices revealed that Ang II facilitated this release in a losartan-sensitive, Ca^{2+}-dependent manner (Garcia-Sevilla et al., 1979). Microdialysis showed that Ang II evokes NE release from the PVN (Stadler et al., 1992).

Evidence for a direct interaction between Ang II and NE neuromodulation includes elimination of Ang II-mediated responses by central injections of 6-hydroxydopamine, a lesioning agent specific to catecholaminergic neurons (Bellin et al., 1987; Cunningham and Johnson, 1991). Also, the AT_1R has been co-localized to catecholaminergic neurons (Gelband et al., 1997; Jenkins et al., 1995; Rowe et al., 1990), providing anatomical evidence for the possibility of a direct interaction.

The neuromodulatory effects of Ang II are exacerbated in the spontaneously hypertensive rat (SHR) model of hypertension. AT_1R levels are increased in neurons cultured from hypertensive rat brain (Raizada et al., 1984). Additionally, regulation of the norepinephrine transport system and expression of the TH gene are increased in the SHR as compared to normotensive animals (Lu et al., 1996; Yu et al., 1996).

Since voltage gated Ca^{2+} channels are required for neurotransmitter release, and Ang II increases voltage gated Ca^{2+} current, the increase in NE release by Ang II may be a result of the increase in Ca^{2+} current. Since NE neuromodulation in the SHR is altered, this NE release pathway may be altered in hypertensive animals, leading to increases in sympathetic outflow, ultimate modification of the baroreflex, and alterations in vasopressin release, among other pathways leading to changes in blood pressure. The specific aims of the study presented in the following chapters are:

1. Determine which subtypes of voltage gated Ca^{2+} current are expressed in neurons co-cultured from neonatal rat hypothalamus and brainstem.
2. Determine which Ca^{2+} current subtypes are modulated by Ang II.
3. Determine the role of PKC at the level of the channel in the modulation of Ca^{2+} current.
4. Develop a model of neurons from adult rat brainstem and hypothalamus to be used in future electrophysiology, signal transduction, or biochemical studies.

Establishment of the pathway in neurons from normotensive animals will provide the reference to compare to hypertensive animals, and will reveal potential central targets for hypertension therapeutics. In addition, determination of the pathway of Ang II modulation of Ca^{2+} current will provide information relevant to other peptide neuromodulators.

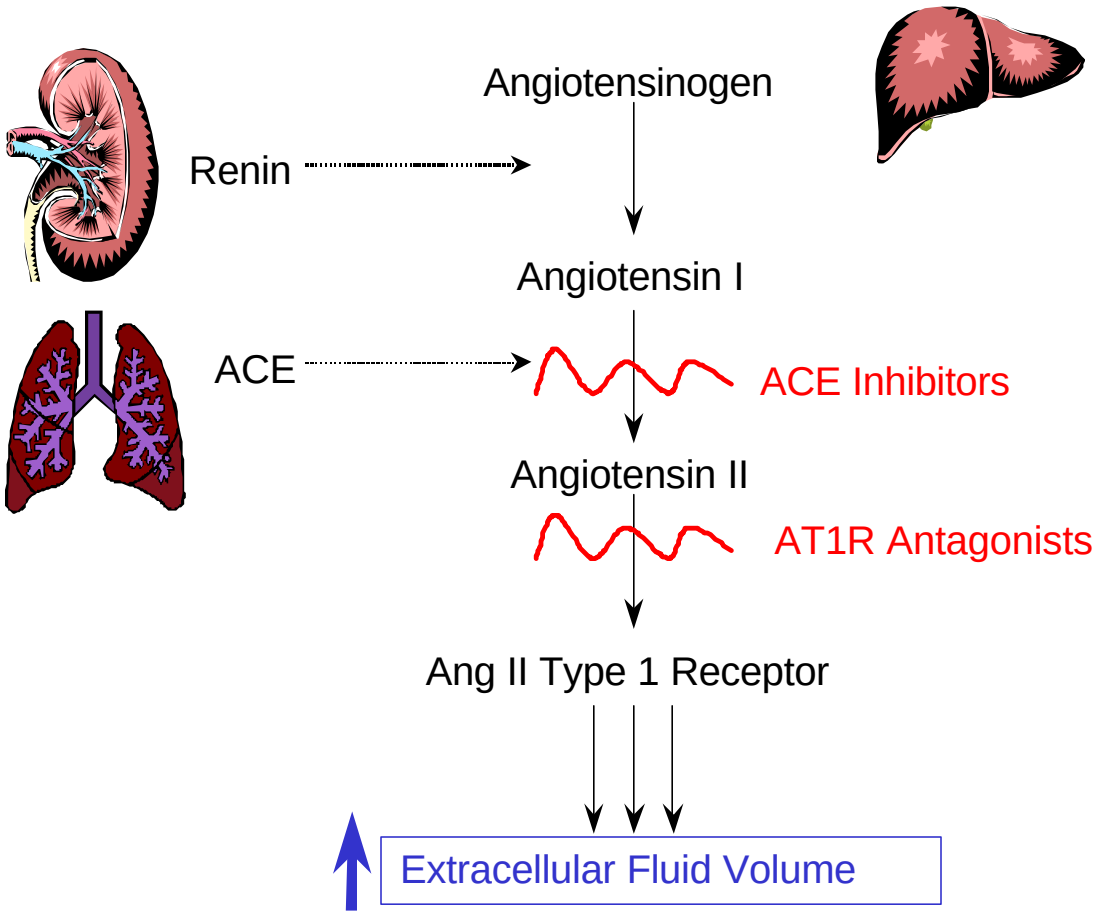


Figure 1-1. Classic renin-angiotensin pathway.

Table 1-1. Ca^{2+} channel subtypes, pharmacology, function, and molecular identities.

Subtype	**Antagonist**	**Putative Function**	**Molecular Equivalent**
L	Dihydropyridines	Signaling	α_{1C}, α_{1D}, α_{1S}, α_{1E}
N	ω-Conotoxins GVIA, MVIIC	Neurotransmitter release	α_{1B}
P/Q	ω-Agatoxin IVA	Neurotransmitter release	α_{1A}
R	?	?	α_{1E}
T	Mibefradil	Pacemaker activity	α_{1G}, α_{1H}, α_{1I}

Table 1-2. Biophysical properties of Ca^{2+} channel subtypes in expression systems.

Current Type	**L**	**N**	**P/Q**	**R**	**T**
Expression system	*Xenopus oocytes*	*Xenopus oocytes*	*Xenopus oocytes*	*Xenopus oocytes*	*Xenopus oocytes*, HEK-293 cells
Charge Carrier	40 mM Ba^{2+}	40 mM Ba^{2+}	40 mM Ba^{2+}	4 mM Ba^{2+}	10-40 mM Ba^{2+}
Threshold (mV)	-20	-30	-20	-50	-50 to -60
Peak of I/V (mV)	30	30	10-20	-10	
V1/2 Activation (mV)	6	1	-1	-25	- 68 to -21
V1/2 Inactivation (mV)	-37	-34	-29	-65	-75 to -50
Conductance (pS)	20				4 to 11
Reference	(Tomlinson et al., 1993)	(Stea et al., 1993)	(Stea et al., 1994)	(Soong et al., 1993)	(Cribbs et al., 1998; Lee et al., 1999; Perez-Reyes et al., 1998)

CHAPTER 2
ANGIOTENSIN II MODULATION OF CA^{2+} CURRENT

Introduction

All of the components of the RAS can be found in the brain. In the brain, like in the periphery, Ang II stimulates a number of mechanisms to net an increase in blood pressure and extracellular fluid volume (Phillips, 1987). One such mechanism that has been studied in detail at the neuronal level is NE neuromodulation. Ang II stimulates NE release on the short term, and, on the longer term, stimulates uptake and transcriptional and translational events leading to synthesis of proteins involved in norepinephrine production and release (Gelband et al., 1998). These actions of Ang II can be reversed with losartan, the AT_1R antagonist. The AT_1R is localized to nuclei in the hypothalamus and brainstem, areas that have been long associated with cardiovascular control, providing anatomical support for the direct role of the brain RAS in blood pressure regulation (Phillips et al., 1993).

In a model system of neurons co-cultured from neonatal rat hypothalamus and brainstem, Ang II has been shown to increase firing rate, decrease delayed rectifier K^+ current, and decrease an A-type K^+ current, in a losartan-sensitive manner (Sumners et al., 1996; Zhu et al., 1997). Detailed studies of the mechanisms involved in the AT_1R-mediated regulation of K^+ current have revealed a role for PKC and CaMKII (Gelband et al., 1999; Wang et al., 1997; Zhu et al., 1999). In contrast, Ang II, via the G_q-coupled AT_1R, causes an increase in voltage-gated Ca^{2+} current. This effect can be completely

inhibited with PKC-inhibitor peptide (PKCIP), and mimicked with superfusion of PMA (Sumners et al., 1996).

There are 5 subtypes of voltage-gated Ca^{2+} current: T, L, N, P/Q, and R (Nooney et al., 1997; Perez-Reyes and Schneider, 1995; Tsien et al., 1991). Since voltage-gated Ca^{2+} influx is required for neurotransmitter release (Miller, 1987; Takahashi and Momiyama, 1993; Turner et al., 1993), the Ang II stimulated increase in Ca^{2+} current may underlie the Ang II stimulated increase in NE release. Several studies have addressed the regulation of Ca^{2+} current subtypes implicated in neurotransmitter release (N, P/Q, and R) (Barrett and Rittenhouse, 2000; Bean, 1989; Currie and Fox, 1997; Herlitze et al., 1996; Mintz and Bean, 1993; Shapiro et al., 1996; Swartz, 1993; Zamponi et al., 1997). PMA activation of PKC increases these current types by alleviating voltage-dependent $G\beta\gamma$ inhibition (Barrett and Rittenhouse, 2000, see figure 2-1). Since the Ang II effect is PKC-dependent, this mechanism may be involved. The study presented in this chapter dissects the current subtypes from the total Ca^{2+} current in the neuronal co-cultures and examines the role of the PKC/$G\beta\gamma$ pathway in the stimulatory effect. We find that Ang II stimulates at least one of the non-L, HVA current subtypes, which have been implicated in neurotransmitter release in other neuron types, and that $G\beta\gamma$ tonically inhibits the Ca^{2+} current. Ang II can alleviate this inhibition, demonstrating, for the first time, a signal transduction pathway stimulation of PKC causing an increase in non-L type Ca^{2+} current. This pathway is therefore important in the physiological regulation of neuronal Ca^{2+} influx.

Materials and Methods

Preparation of Neuronal Cultures

Rat pups less than one day post-natal were euthanized with pentobarbital. The brains were removed and placed in a culture dish containing solution D (in mM: 140 NaCl, 5.4 KCl, 0.17 Na_2HPO_4, 0.2 KH_2PO_4, 5.5 glucose, 58 sucrose, 0.007 streptomycin, and $1x10^5$ U penicillin, and 250 mg/mL fungizone.The hypothalamus and brainstem were dissected from the brains, the pia and meninges were removed, and the tissue was transferred to another dish containing solution D. The tissue was minced, digested with 0.25% trypsin solution (1.0 mL per brain used) at 37°C for 4 min and 45 sec with gentle shaking. The DNase solution (2 mL) was added to the cells, and shaking continued for an additional 5 min. DMEM/10% PDHS (10 mL) was added to stop the enzyme actions, and the volume of the cell suspension was brought to 45 mL with DMEM/10% PDHS. Cells were then pelleted at 600 x g for 4 min. The supernatant was aspirated and the pellet was gently triturated 6 times with 10 mL DMEM/10% PDHS each time. The supernatant from each trituration was filtered through sterile gauze into a sterile bottle. Cells were counted using a hemacytometer and plated at a density of 3 million cells per dish on 35 mm culture dishes that had been coated with poly-L-lysine for three hours before plating and rinsed with Solution D. Cultures were maintained in a 10% CO_2 incubator for 3 days at which time the medium was replaced with DMEM/10% PDHS containing 1% cytosine arabinoside (ARC) for two days. The ARC was replaced with fresh DMEM/10% PDHS, and cells were maintained in culture for an additional 5-13 days before use.

Whole-cell Patch Clamp and Current Analysis

Membrane currents were isolated using the whole-cell patch-clamp technique. Patch electrodes had resistances of 2-5 MΩ when filled with internal solution that contained (in mM): 110-tetraethylammonium chloride (TEA-Cl), 9 HEPES, 9 EGTA, 4.5 $MgCl_2$, 0.3 GTP, and 4 ATP, and 14 phosphocreatine (pH 7.4 with TEA-OH). The standard external solution contained (in mM): 0.001 PD123, 319 (AT_2R antagonist), 142 TEA-Cl, 10 $BaCl_2$, 10 HEPES, and 0.003 tetrodotoxin (pH 7.3 with TEA-OH). All experiments were performed at room temperature. For all experiments except current-voltage relationships and where otherwise noted, currents were evoked by stepping from a holding potential of -80 mV to a test potential of -10 mV for 80 ms. Composite data is expressed as the mean ± s.e.m. All chemicals were purchased from Sigma (St. Louis, MO).

Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

Specific oligonucleotides were synthesized against 3' portions of rat brain Ca^{2+} channel α–subunit cDNAs (Table 2-1). BLAST analysis was used to ensure the specificity of the primers, and initial products were sequenced to further guarantee amplification of the desired transcript.

RT was performed using RNA isolated from 5 dishes of co-cultured neurons via Trizol extraction and ethanol precipitation. 1 μg of DNase I-digested RNA was added to the RT reaction using random hexamers. As a control for the PCR, a parallel reaction was carried out in the absence of the RT enzyme. The reaction was carried out at 25°C for 10 min, 42°C for 45 min, 99°C for 5 min, and 5°C for 5 min. The PCR step was carried out in a reaction volume of 50 μL with 2 mM $MgCl_2$ and 10 pmoles of each

primer specific to the desired product. The reaction was performed with an initial 2 min denaturation followed by 35 cycles with the following profile: 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The final step was an extension at 72°C for 10 min.

Reaction products (25 μL) were electrophoresed through a 1.3% agarose gel in TBE buffer (90 mM Tris base, 90 mM boric acid, and 2 mM EDTA), and visualized with ethidium bromide.

Protein Isolation

Proteins from cultured neurons (10-18 days post-culture) were isolated for immunoblotting by the following procedure: First the culture dishes were washed with 1 mL cold PBS containing 5 mg/mL leupeptin and 8 mg/mL calpain inhibitors. After this wash was aspirated, the cells were solubilized for 20-40 min at 4°C in 100 μL of a sterile solution of 1.2% digitonin, 300 mM KCl, 150 mM NaCl, 10 mM $NaPO_4$ buffer, pH 7.4 containing the following protease inhibitor cocktail: pepstatin A (1 μg/mL), leupeptin (1 μg/mL), aprotinin (1 μg/mL), AESFB (0.2 mM), benzamidine (0.1 mg/mL), and calpain inhibitors (8 μg/mL). Dishes were then scraped and the cells centrifuged at 10,000 rcf for 20 min at 4°C. The protein concentration in the supernatant was determined via Biorad assay using BSA for the standard curve.

Immunoblotting

Each sample (20 μg) was prepared and denatured with Laemmli sample buffer in a boiling water bath for 3 min. 16 μL of each sample mixture per well was electrophoresed in 4-15% gradient SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was blocked with 10% non-fat dried milk in PBST-BSA for 1 hour followed by incubation overnight at 4°C with anti-α_{1A}, anti-α_{1B}, anti-α_{1C}, anti-α_{1D},

or anti-α_{1E} (Alomone Labs). Protein-bound antibody was detected by incubation of the membrane with horseradish peroxidase-labeled secondary antibody for 1 hour and enhanced by chemiluminescent assay reagents. The bands recognized by the primary antibody were visualized by exposure to film.

Results

Using 10 mM Ba^{2+} as the charge carrier, a voltage pulse from –80 mV to +10 mV for 80 ms was used to assay the inward current carried via voltage-gated Ca^{2+} currents. 100 nM Ang II caused a dramatic increase in the inward current as compared to the control current, and 10 μM losartan reversed this effect (Figure 2-2, A). Composite data from a number of experiments is shown in the form of a current-voltage (I/V) relationship, where Ang II caused a significant increase in the peak current density, but did not cause a shift of the peak. Losartan, again, reversed the effect and returned the current to control levels (I/V, Figure 2-2, B).

Holding the cells at a membrane potential at –40 mV inactivates any LVA current present in the cells, resulting in a decrease in the total current as compared to that with a holding potential of –80 mV. Holding at –40 mV did not reduce the inward current, demonstrating the lack of functional LVA, T-type, current in the co-cultured neurons (Figure 2-3).

10 μM nifedipine was used to block the L-type calcium current. Some cells had a significant amount of L-type current (Figure 2-4, left), while some had little or no nifedipine-sensitive current (Figure 2-4, right). Composite data suggests that at least 60.8±6.3% (n=10) of the Ca^{2+} current in the neurons is non-L type, and the residual non-

L type current was completely blocked with 10 μM Cd^{2+}, confirming that it is carried via voltage gated Ca^{2+} channels.

Using nifedipine to isolate the HVA Ca^{2+} current subtypes that have been implicated in neurotransmitter release, 100 μM Ang II caused an increase in the non-L type component of Ca^{2+} current in the neurons, in a losartan-reversible manner (data not shown). This suggests that at least one non-L Ca^{2+} current subtype is potentiated by Ang II.

Exploiting the unique α-subunits underlying the various Ca^{2+} current types (see Table 1-1), RT-PCR analysis was used to determine which current subtypes' transcripts were expressed in the neuronal co-cultures. Primers specific for α_{1A}, α_{1B}, α_{1E}, α_{1C}, α_{1D}, and α_{1G} amplified products of the predicted sizes (Table 2-1 and Figure 2-5), suggesting that the mRNAs for P/Q, N, R, L, and T type Ca^{2+} current were all actively transcribed in the neurons. Western blot analysis revealed the protein α-subunits corresponding to P/Q, N, L, and R type Ca^{2+} current (Figure 2-6). Antibodies to the α-subunits associated with T-type current were unavailable, and no α_{1C} protein expression was detectable by Western (Figure 2-7), though the antibody recognized two bands of appropriate molecular weights in proteins isolated from heart. Prominent bands of lower molecular weight were recognized with the α_{1C} antibody but correspond with non-specific binding by the secondary antibody alone (data not shown).

Further pharmacological dissection of the non-L, nifedipine insensitive current revealed the presence of all HVA current types (Figure 2-8). A significant portion of the non-L type component could be blocked with 3 μM ω-agatoxin IVA, showing the presence of functional P/Q type current. The remaining non-L, non-P/Q type current was

sensitive to 1 μM ω-conotoxin GVIA, revealing the presence of functional N-type channels. The residual current was Cd^{2+} sensitive and was likely to be carried via R-type Ca^{2+} channels.

To test the hypothesis that the PKC-mediated increase in non-L type HVA currents occurs via relief of Gβγ inhibition, it was first necessary to establish tonic Gβγ inhibition of the Ca^{2+} current in the cultured neurons. Gβγ inhibition is voltage-dependent, and as such, can be relieved with strong depolarizations. Using a triple pulse protocol (see Figure 2-9), a control test pulse was given (P1). The current in this test pulse was then compared to the current in the second test pulse (P2) that immediately followed a strong depolarizing pulse. If the current in the second test pulse was larger than the current in the first test pulse, the current has been facilitated, and, by inference was inhibited by Gβγ subunits. A representative trace (Figure 2-9) shows that the current in the hypothalamus and brainstem neurons can be facilitated.

This data is expressed as the ratio of the current in the second test pulse to the current in the first test pulse, the facilitation ratio. A facilitation ratio greater than one suggests that the current was inhibited by Gβγ subunits and that the depolarizing prepulse alleviated the inhibition. Both the total Ca^{2+} current as well as the non-L type component have facilitation ratios greater than 1 (Figure 2-10), suggesting that the Ca^{2+} current in the co-cultures is tonically inhibited by Gβγ subunits.

To rule out the possibility that the depolarizing pulse caused any artifactual increases in the current, the time between the depolarizing prepulse and the second test pulse was varied to assay the recovery from facilitation (Figure 2-11). The recovery from facilitation or the recovery of inhibition occurred with a time constant of about 39±0.01

ms, consistent with values of Gβγ association with non-L type Ca^{2+} channels in published reports (Currie and Fox, 1997).

If Ang II is acting by stimulating PKC to phosphorylate the channel and prevent inhibition by Gβγ, Ang II should shift the voltage-dependence of activation of the Ca^{2+} current leftward, at least at less depolarized potentials. 100 nM Ang II did not significantly shift the activation curve, while the prepulse did cause a significant leftward shift (Figure 2-12). As determined by a fitting the data with a simple Boltzmann function, the membrane potential necessary for half-maximal activation ($V_{1/2}$) in the presence of Ang II (-38.7±1.57 mV) was not significantly different from that of control (-42.9±3.6 mV) or losartan (-39.0±1.6 mV) treated currents, but the prepulse caused a significant change in the $V_{1/2}$ activation (-51.6±1.3 mV).

In contrast to the large increase in the peak of the I/V curve in the presence of Ang II (Figure 2-1), the depolarizing prepulse only doubled the current density at the peak of the I/V (Figure 2-13). This suggests that Ang II may be affecting an increase by yet another PKC-dependent pathway.

Consistent with this idea, superfusion of Ang II not only increased the current elicited by a control test pulse, but it also alleviated a portion of the Gβγ inhibition of the non-L type current as assayed by facilitation (Figure 2-14). The difference between the control current and the current following a prepulse in the presence of Ang II is smaller than that in the absence of Ang II demonstrating that Ang II decreased the facilitation ratio. Less facilitation is consistent with a PKC phosphorylation of the Gβγ association site on the channel, causing an apparent increase in Ca^{2+} current. Addition of PMA completely prevents facilitation of the current, confirming that this pathway is present in

the co-cultures and that PKC phosphorylation is able to totally alleviate the Gβγ inhibition.

Discussion

The results presented here shed light not only on the Ang II modulation of neuronal Ca^{2+} current, but also on the physiological role of the dynamic regulation of Ca^{2+} current by Gβγ/PKC systems. Ang II increased non-L type Ca^{2+} current via the AT_1R, and Ang II stimulation of norepinephrine release is also AT_1R-mediated. Since non-L voltage gated Ca^{2+} current subtypes have been implicated in neurotransmitter release in a variety of neuron types (Hirning et al., 1988; Takahashi and Momiyama, 1993; Turner et al., 1993), this modulation of Ca^{2+} current may be directly related to the modulation of NE release.

RT-PCR analysis of RNA isolated from the neuronal cultures revealed transcripts for the α-subunits representing all of the Ca^{2+} channel subtypes. This was in contrast to the Western blot analysis, which failed to detect the α_{1C} protein, and to the biophysical analysis that did not reveal any T-type Ca^{2+} current, despite the presence of the mRNA for α_{1G}. There are two possible explanations for these discrepancies. The first is that the neuronal cultures contain glia, although only 10% of the total cells are glial. Regardless, the glia may be actively transcribing the mRNAs for these channels. RT-PCR analysis of RNA isolated from glia remaining after high-KCl elimination of the neurons did reveal the same expression pattern as with the RNA isolated from the total population of the neuronal cultures (data not shown). The other possibility is that the channels transcribe the mRNA for these channels, but they are post-transcriptionally regulated so they are not translated to functional channels. In either case, it is difficult to determine which

subtypes of channels exist in a population of neurons using these analyses, but these methods can be effective to rule out particular subtypes if they are not revealed molecularly. Also, as is evident with the differing nifedipine sensitivity between cells, the molecular analysis does not paint an accurate picture of the expression pattern in a given neuron. We have made efforts to optimize single cell RT-PCR methods, but this methodology is again only useful to confirm the electrophysiological analysis, not to make statements about functional expression, since the cell may be transcribing mRNAs for channels it does not express as functional currents. Fortunately, the selective antagonists are effective in dissecting the current and uncovering functional channel subtypes, here revealing the presence of all HVA current types: L, N, P/Q, and R.

Previous studies from our group have shown the Ang II effect on Ca^{2+} current can be completely inhibited by PKCIP, but this was not the case for K^+ current, which also involves CaMKII activity (Zhu et al., 1999). Since PKC is required for the effect of Ang II, but seems to alleviate $G\beta\gamma$ inhibition only partially, it is likely that another PKC dependent mechanism is involved in the stimulation. The α-subunits of Ca^{2+} channels contain a number of phosphorylation sites, and many are yet uncharacterized. In addition, there are a number of accessory subunits that may be associated with the α-subunits in the neurons which themselves could be substrates for PKC phosphorylation. Finally, PKC could be phosphorylating and activating another kinase or accessory protein fostering another pathway that then goes on to effect the Ca^{2+} channel's ability to pass current. A similar mechanism could explain the unexpected lack of shift in the voltage-dependence of activation in the presence of Ang II. Perhaps the other mechanism causes a right shift that masks the shift caused by relieving G-protein inhibition. Additionally,

the activation curve was constructed with total Ca^{2+} currents, not just with non-L type current isolated. Ang II may have an effect on the L-type current that is masking the anticipated shift of the activation curve.

Now that the current subtypes expressed in the neurons have been deciphered, it will be possible to isolate the L-type component and examine its modulation by Ang II, but because the individual non-L type currents are relatively small (about 100-200 pA) it is difficult to isolate them and determine their regulation individually. An expression system such as stably transfected HEK cells carrying the AT_1R and the calcium channel of interest may be an appropriate alternative system to study the intricacies of the signaling mechanism.

Regulation studies have shown PMA alleviating Gβγ inhibition, often with Gβγ inhibition initiated by an inhibitory neurotransmitter cascade, such as in the case with NE or somatostatin (Bean, 1989; Currie and Fox, 1997; Golard and Siegelbaum, 1993). This is the first reported case, to our knowledge, using a ligand-receptor interaction to stimulate PKC to alleviate Gβγ inhibition. The origin of the tonic Gβγ inhibition is unknown, but is likely due to βγ subunits liberated from endogenous spontaneously activated G-protein coupled receptors or due to an excess of Gβγ subunits in the cell. The fact that activation of PKC via the AT_1R is not sufficient to alleviate the tonic Gβγ inhibition in the neurons raises interesting possibilities regarding subtype specificity both on the part of the PKC isozyme as well as on the part of the βγ subunits. PMA, which has been the PKC activator used in the Ca^{2+} channel regulation studies, activates all isoforms of PKC, so it is possible that its effects are more potent. This idea also explains the ability of PMA to alleviate facilitation to a greater degree than Ang II in the

experiments presented here. The Ang II inhibition of K^+ current is mediated by PKCα, so it is possible that this isoform of PKC mediates the Ca^{2+} current increase as well. Further studies addressing the PKC isoform involved, as well as studies directly linking this mechanism to neurotransmitter release will provide the groundwork to study the role of the pathway in the development or maintenance of hypertension.

Table 2-1. Primers designed to amplify transcripts of specific Ca^{2+} channel α-subunits.

Target	Sense primer (5'-3')	Antisense primer (5'-3')	Predicted product size (bp)
α_{1A}	CCCTCCTCAACTCCATGAAA	AAAGTGTCGAAGTTGGTGGG	144
α_{1B}	ATGAGGCCAGAGCACCTCTA	TGTGTTGCAAAGCTGAGTCC	266
α_{1C}	ACGGCACCCTCTTACCTTTT	TGCTGACATAGGACCTGCTG	141
α_{1D}	ACTGGTCTATTCTGGGGCCT	CTTGATCTTGAGAGCCGTCC	260
α_{1E}	CAGCTCCCTGATGAGACACA	GCAAGGAGTTGGAAGACTCTG	128
α_{1G}	ACTGTGACCAGGAGTCCACC	TTGCCTCTTTGTTGCTTTCT	144

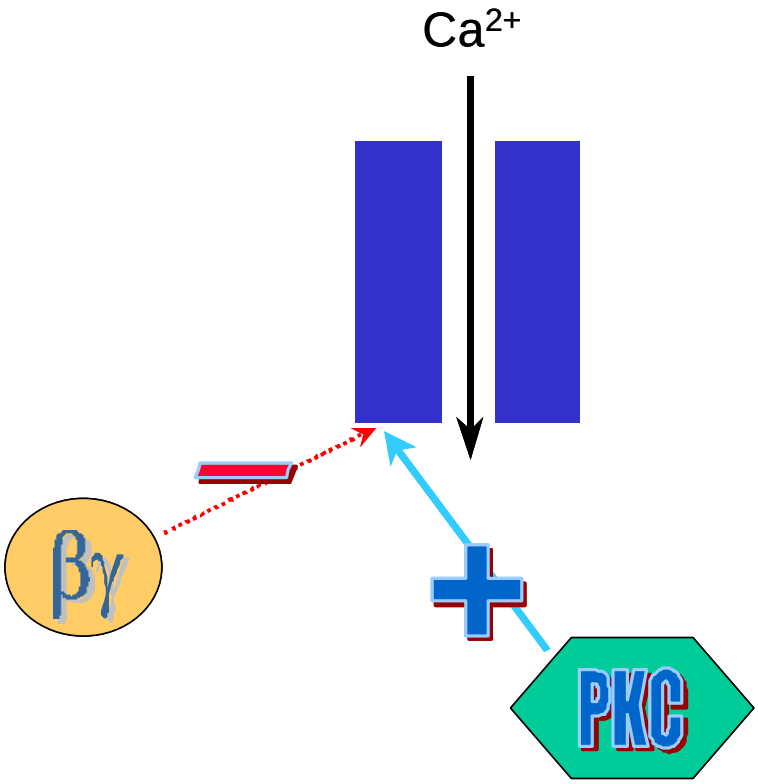


Figure 2-1. Putative regulation mechanism for non-L type Ca^{2+} channels.

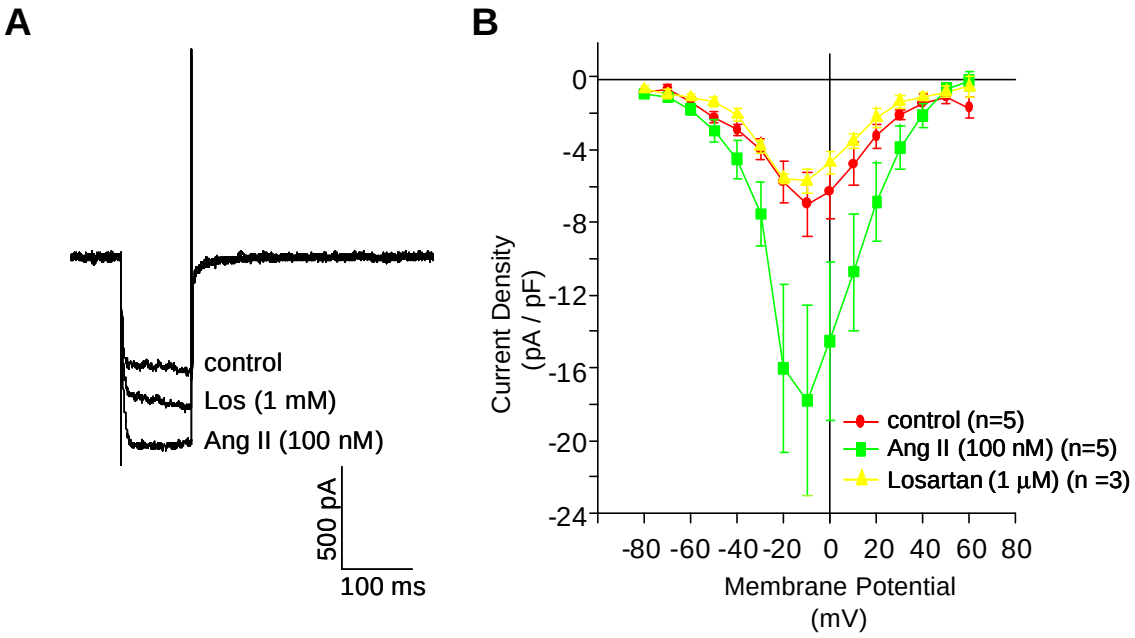


Figure 2-2. Ang II increases voltage-gated Ca^{2+} current via the AT_1R. A: Representative traces elicited from with an 80 ms voltage pulse from –80 mV to –10 mV in the presence or absence of Ang II or Ang II and losartan. B: Mean current-voltage relationship from a number of cells in the presence or absence of Ang II or Ang II and losartan.

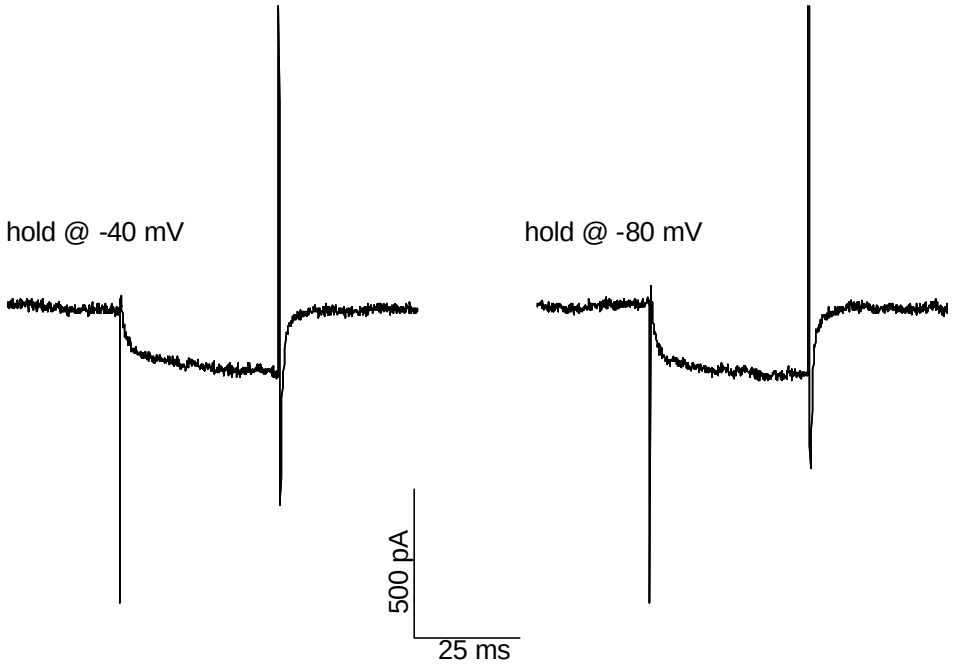


Figure 2-3. Changing the holding potential from –80 mV to –40 mV does not significantly alter the current elicited by a voltage pulse to 0 mV. Since T-type Ca^{2+} current is the LVA current that inactivates at –40 mV, a lack of difference signifies a lack of LVA current in the co-cultured neurons.

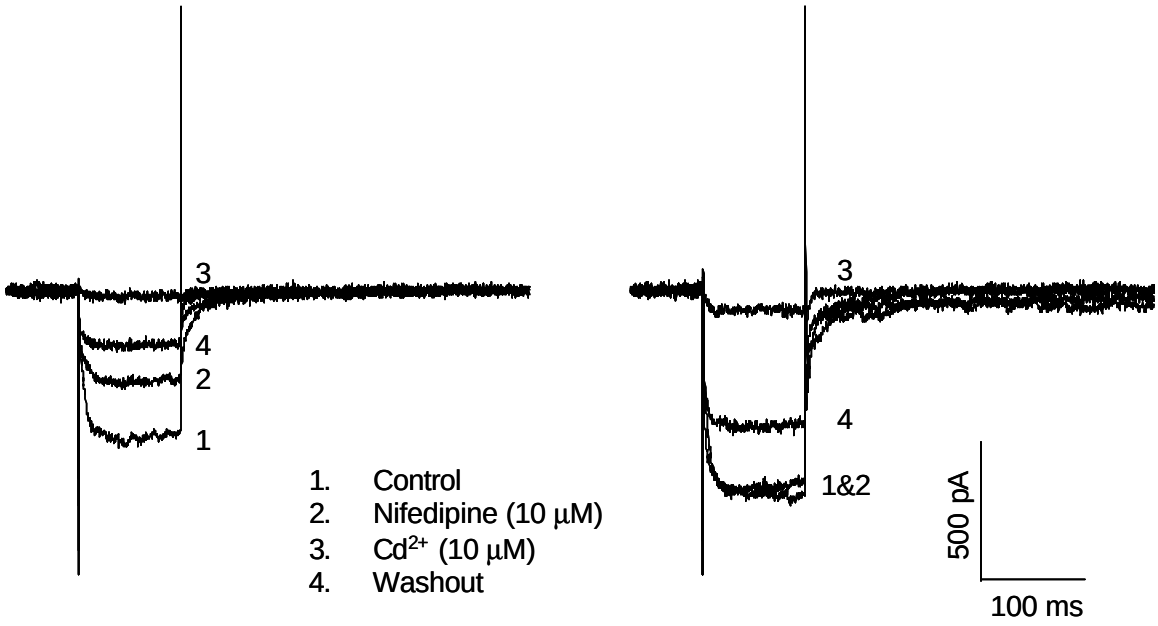


Figure 2-4. Most of the current in the neurons is non-L type, nifedipine insensitive current. The traces on the left are from a cell with significant L-type current, while the traces on the right are from a cell with little or no nifedipine sensitivity.

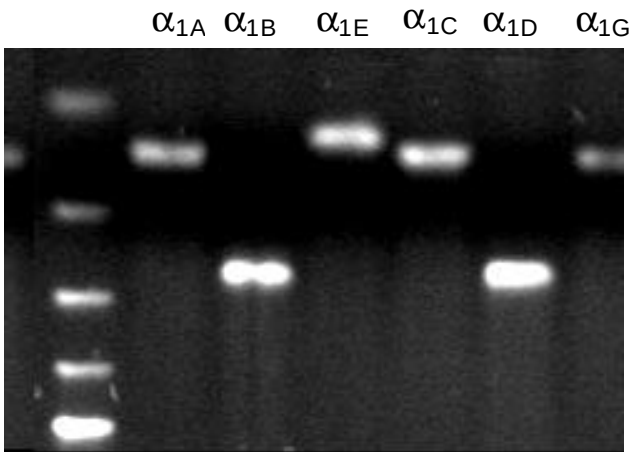


Figure 2-5. RT-PCR analysis of RNA isolated from co-cultured neurons reveals the presence of transcripts encoding all Ca^{2+} current subtypes.

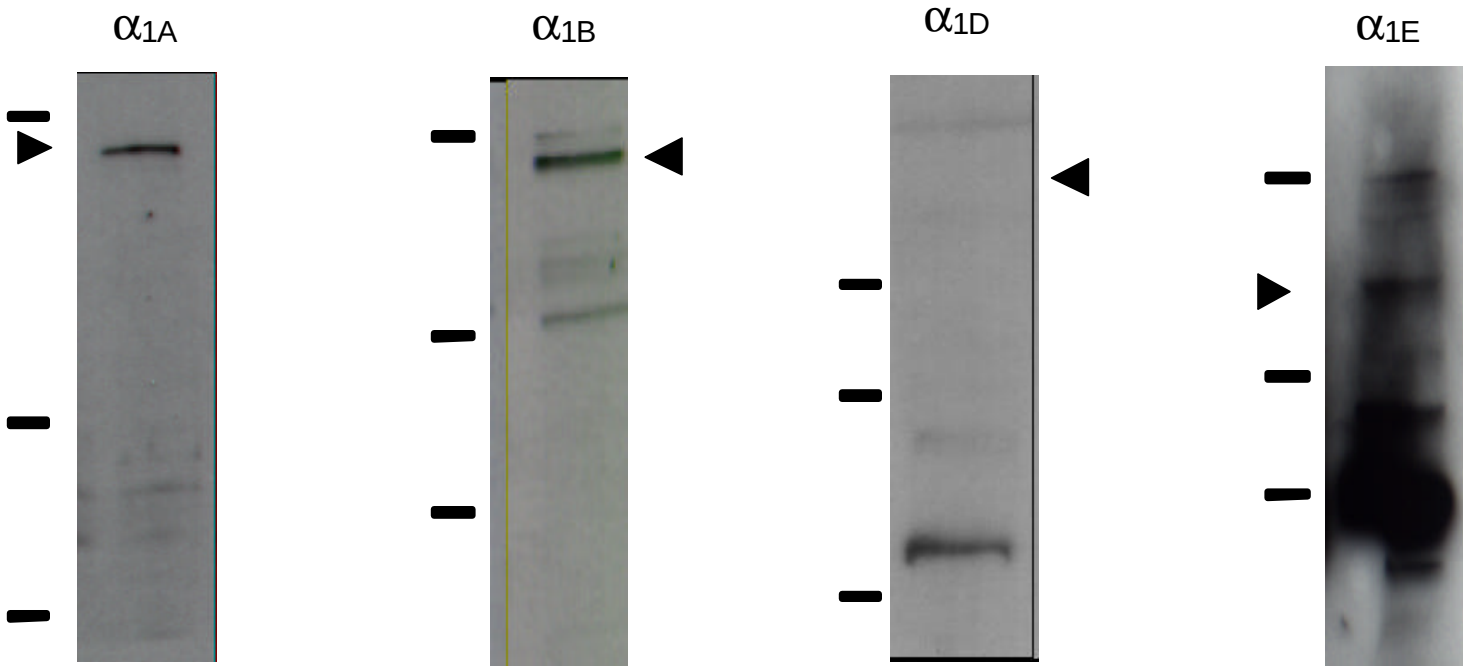


Figure 2-6. Western blot analysis of proteins extracted from neuronal co-cultures reveals the α-subunits corresponding to each of the subtypes of HVA Ca^{2+} current. The markers denoting the molecular weight standards designate 205 kDa, 121 kDa, and 78 kDa, top to bottom.

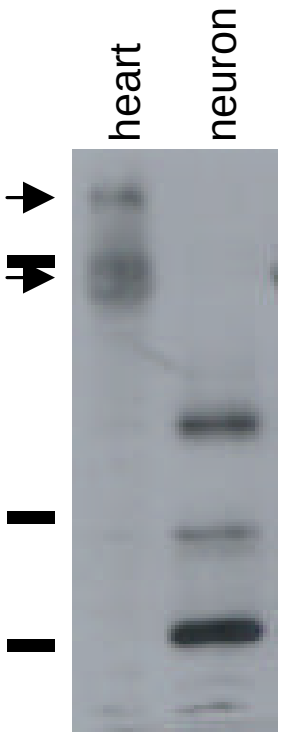


Figure 2-7. Western blot analysis using an antibody to α_{1C} failed to recognize a protein band of the correct molecular weight in the neuronal protein isolates. Heart protein was run as a positive control and two isoforms of α_{1C} were identified.

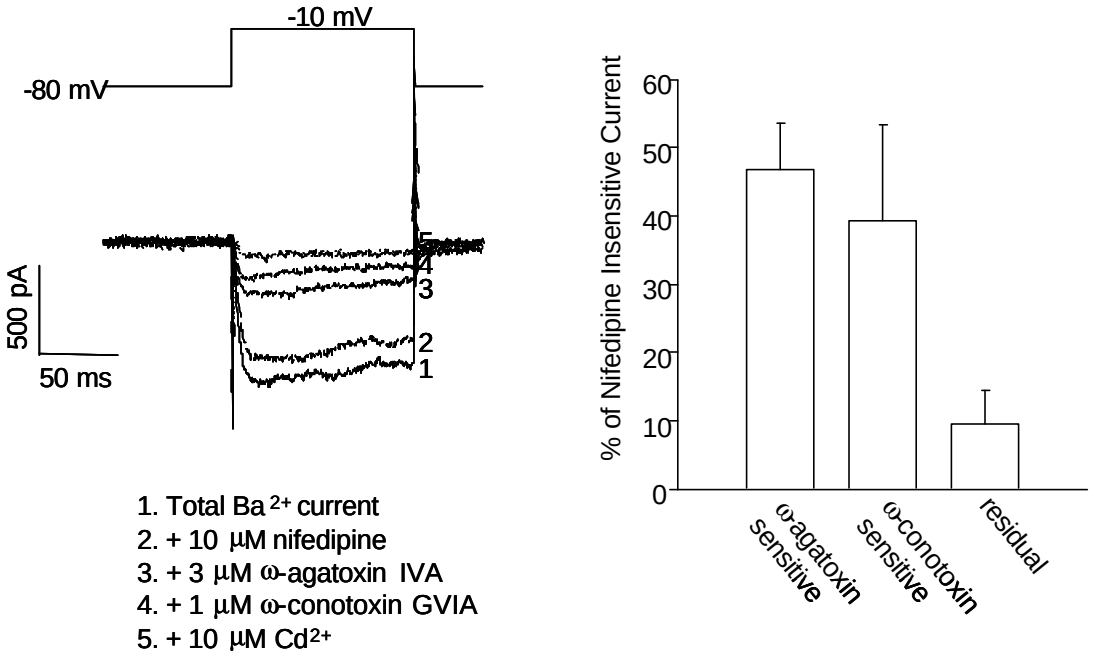


Figure 2-8. Pharmacological dissection of Ca^{2+} current in co-cultured neurons reveals the presence of all HVA current subtypes. Composite data shows that the major components of the non-L type current are sensitive to ω-agatoxin and ω-conotoxin (n=3).

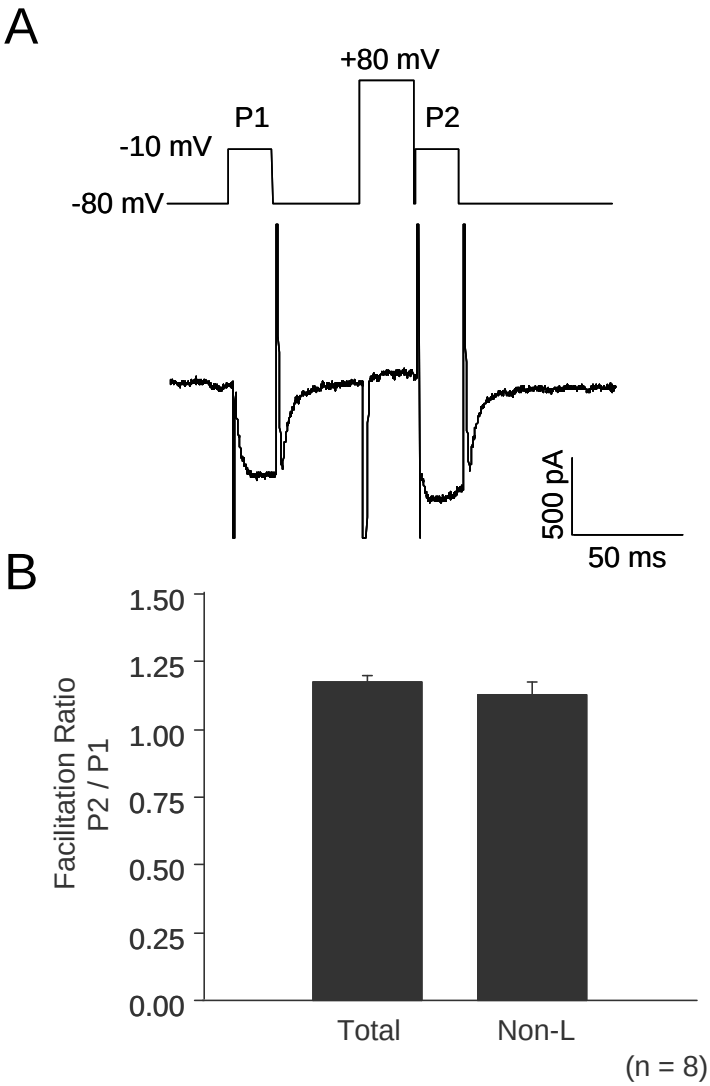


Figure 2-9. Ca^{2+} current in neuronal co-cultures can be facilitated. A: The depolarizing prepulse (-80 mV) relieves the voltage dependent inhibition by Gβγ subunits, rendering the current in the second test pulse (P2), larger than the current in the first test pulse (P1). B: The facilitation ratio of both the total and the non-L Ca^{2+} currents are greater than one, indicating the tonic inhibition by Gβγ subunits.

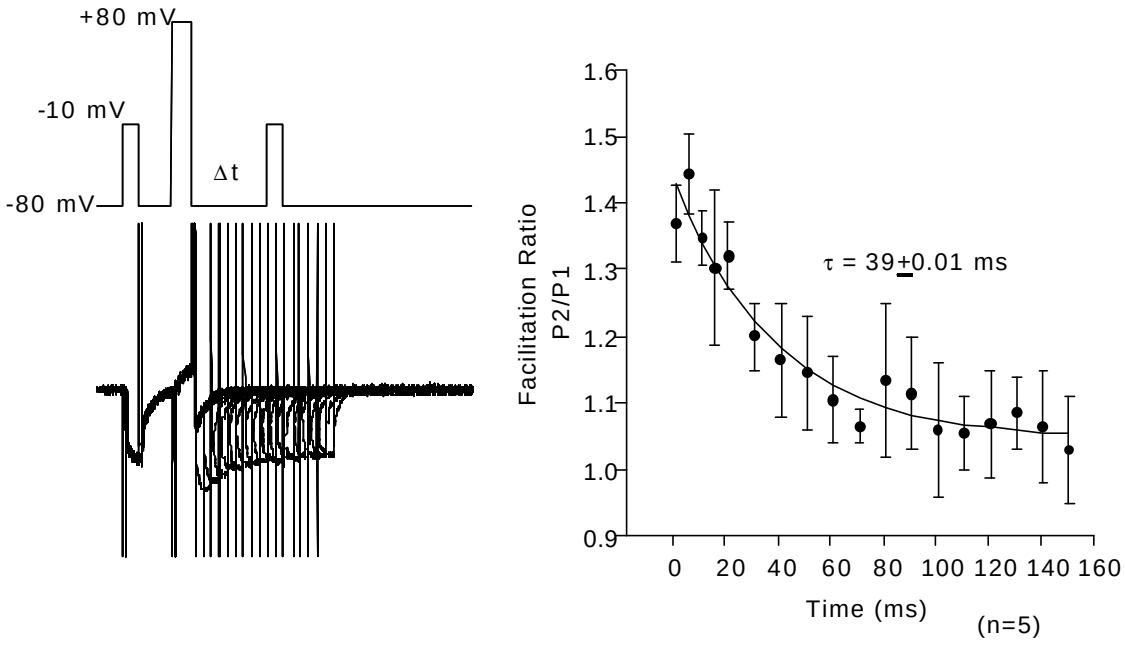
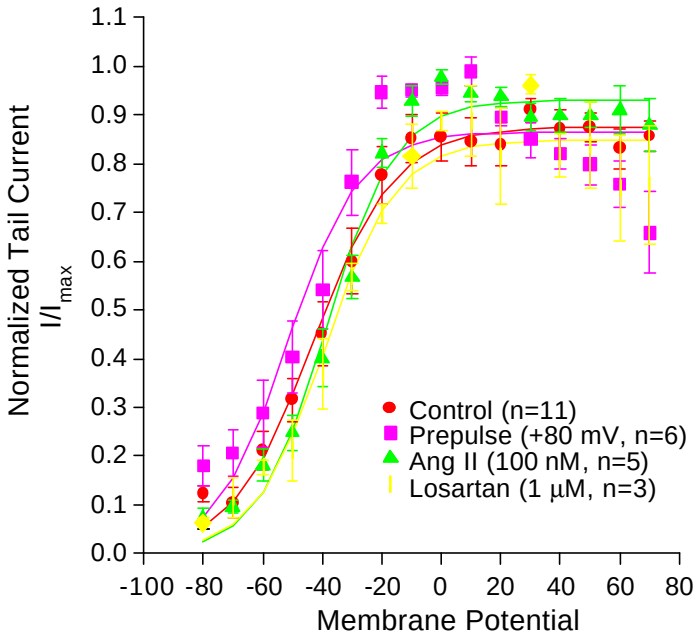


Figure 2-10. Recovery from facilitation is consistent with recovery of Gβγ subunit association. On the left is a representative experiment showing the decay of the facilitation in the current elicited by the second test pulse (P2). Composite data from several experiments is shown on the right. The facilitation is decaying with a time constant of 39±0.01 ms.



		V1/2	Std. Err.
Control		-42.87	3.622
Prepulse	*	-51.64	1.322
Ang II		-38.65	1.568
Losartan		-38.99	2.248

*$_{p<0.05}$

Figure 2-11. Voltage-dependence of activation. Ang II did not significantly shift the activation curve, but a depolarizing prepulse did cause a significant leftward shift.

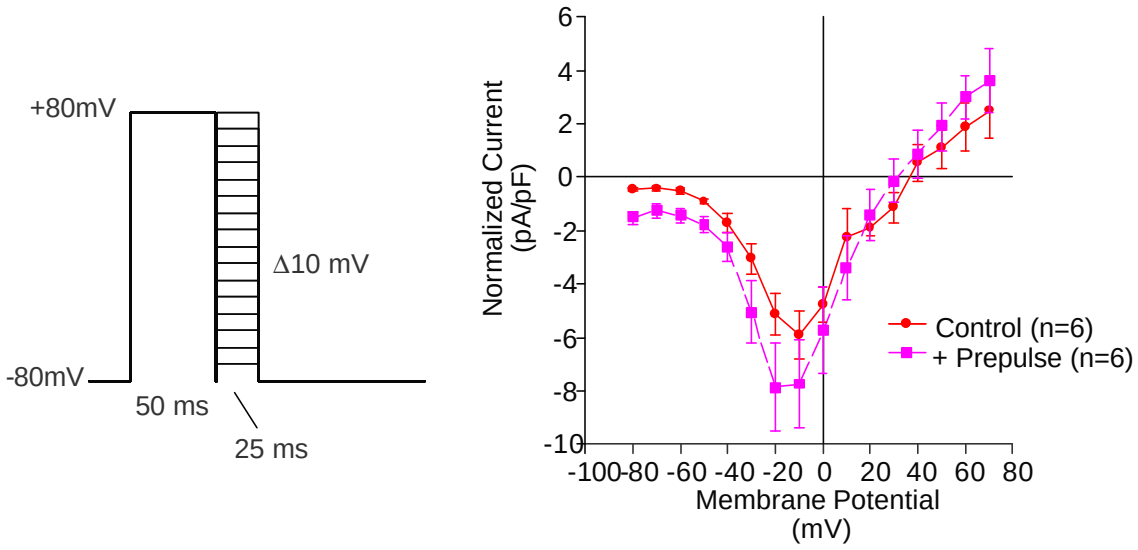


Figure 2-12. Ang II and a depolarizing prepulse have differing effects on the current-voltage relationship. The prepulse increases the peak of the current-voltage relationship less than Ang II does (Figure 2-1) and shifts it leftward as compared to that of the control.

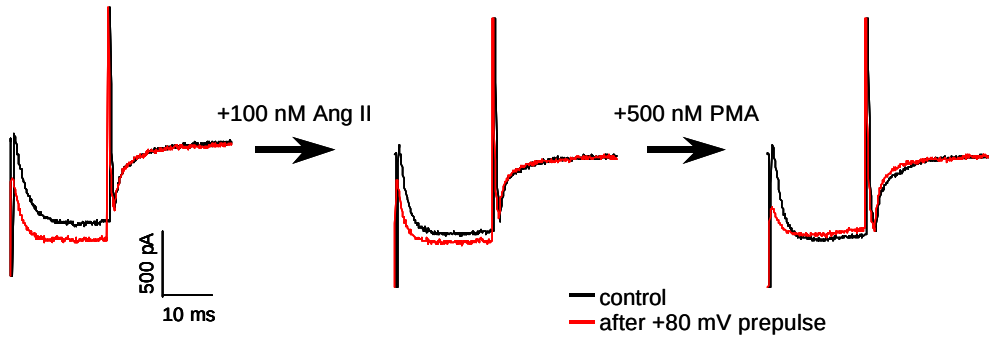


Figure 2-13. Ang II and PMA treatment prevent facilitation of Ca^{2+} current in neuronal co-cultures. Not only did Ang II increase the non-facilitated current as compared to control, but the difference between the currents in the absence and presence of a prepulse was much smaller after superfusion of Ang II. Superfusion of PMA completely eliminated the increase in current caused by the prepulse as compared to the control conditions.

CHAPTER 3
DEVELOPMENT OF A NEW MODEL

Introduction

Ca^{2+} entry via voltage-gated Ca^{2+} channels is essential for neurotransmitter release, and Ang II has been shown to increase spontaneous and evoked NE release both *in vivo* and *in vitro* (Bellin et al., 1987; Cunningham and Johnson, 1991; Garcia-Sevilla et al., 1979; Stadler et al., 1992). The central Ang II-mediated pressor effect correlates with levels of NE in the brain (Camacho and Phillips, 1981; Chevillard et al., 1979; Sumners and Phillips, 1983). Establishment of the pathway connecting Ang II modulation of Ca^{2+} current to NE release in neurons from normotensive animals will provide the reference for comparison to hypertensive animals, and will reveal potential central targets for hypertension therapeutics. In addition, determination of the pathway of Ang II modulation of NE release will provide information relevant to other peptide neuromodulators.

Different types of Ca^{2+} current mediate synaptic transmission. In general, studies suggest that L-type Ca^{2+} current plays little or no role in neurotransmitter release (Hirning et al., 1988; Takahashi and Momiyama, 1993). Varying combinations of N, P/Q, or R type Ca^{2+} current are necessary for neurotransmitter release, depending on both the neuron type and the transmitter type (Takahashi and Momiyama, 1993; Turner et al., 1993).

Since non-L type Ca^{2+} influx is required for neurotransmitter release, and Ang II increases non-L type Ca^{2+} current (Chapter 2), the increase in NE release by Ang II may be a result of the increase in Ca^{2+} current. Several mechanisms of NE neuromodulation are altered in the SHR (Lu et al., 1996; Raizada et al., 1984; Yu et al., 1996), therefore the NE release pathway may be altered in hypertensive animals, leading to increases in sympathetic outflow, ultimate modification of the baroreflex, and alterations in vasopressin release, among other pathways leading to changes in blood pressure. The specific aim of the study presented in the following chapter was originally to examine the role of the Ca^{2+} current subtypes in Ang II facilitation of both spontaneous and K^+ evoked release. Preliminary studies suggested that the model system and the assay used were not sufficient to study this mechanism, and the effects of Ca^{2+} modulators on release generally could not be dissected from their effects on uptake. Therefore, also presented in this chapter is the characterization of a new model system of neuron-like cells cultured from adult rat brain that is currently under development for future studies.

Materials and Methods

Preparation of Neuronal Cultures

Rat pups less than one day post-natal were euthanized with pentobarbital. The brains were removed and placed in a culture dish containing solution D (in mM: 140 NaCl, 5.4 KCl, 0.17 Na_2HPO_4, 0.2 KH_2PO_4, 5.5 glucose, 58 sucrose, 0.007 streptomycin, and $1x10^5$ U penicillin, and 250 mg/mL fungizoneThe hypothalamus and brainstem were dissected from the brains, the pia and meninges were removed, and the tissue was transferred to another dish containing solution D. The tissue was minced, digested with 0.25% trypsin solution (1.0 mL per brain used) at 37°C for 4 min and 45 seconds with

gentle shaking. DNase solution (2 mL) was added to the cells, and shaking continued for an additional 5 min. DMEM/10% PDHS (10 mL) was added to stop the enzyme actions, and the volume of the cell suspension was brought to 45 mL with DMEM/10% PDHS. Cells were then pelleted at 600 x g for 4 min. The supernatant was aspirated and the pellet was gently triturated 6 times with 10 mL DMEM/10% PDHS each time. The supernatant from each trituration was filtered through sterile gauze into a sterile bottle. Cells were counted using a hemacytometer and plated at a density of 3 million cells per dish on 35 mm culture dishes that had been coated with poly-L-lysine for three hours before plating and rinsed with Solution D. Cultures were maintained in a 10% CO_2 incubator for 3 days at which time the medium was replaced with DMEM/10% PDHS containing 1% cytosine arabinoside (ARC) for two days. The ARC was replaced with fresh DMEM/10% PDHS, and cells were maintained in culture for an additional 5-13 days before use.

Norepinephrine Release and Uptake

Initial uptake experiments were performed in triplicate with 4-5 dishes per sample per run in the absence and presence of 10 μM maprotiline, as described previously (Sumners et al., 1985). For uptake experiments that correspond directly to release experiments, cells were dissolved with NaOH after the release experiment, and the lysate was collected and diluted into 4 mL of Scintiverse I. Radioactivity was estimated using an LKB 1215 LS counter. Data were expressed as the mean dpm per dish ± s.e.m. Statistical differences were tested using one-way analysis of variance followed by a pairwise multiple comparison method.

For release experiments, DMEM was removed, and the dishes were washed 3 times with physiological Krebs-Hensleit (KH) buffer, pH 7.4, containing in mM: 140 NaCl, 3.9 KCl, 1.8 $CaCl_2$, and 1.2 $MgCl_2$, 1.5 KH_2PO_4, 5.5 glucose, 10 HEPES. The neurons were incubated with 1 mL of KH buffer containing pargyline (100 μM), ascorbic acid (200 μM) and 0.1 μM L-[^{3}H]-NE for 20 min at 37°C. This mixture was aspirated and the dishes were washed four times with modified PBS at 37°C. Cells were then incubated in 0.75 mL of KH or high K^+ KH (containing, in mM: 74.4 NaCl, 69.5 KCl, 1.8 $CaCl_2$, and 1.2 $MgCl_2$, 1.5 KH_2PO_4, 5.5 glucose, 10 HEPES) with or without the appropriate Ca^{2+} modulator for 1 min at 37°C. The PBS was removed and placed in a scintillation vial containing 4 mL of Scintiverse I. Radioactivity was estimated using an LKB 1215 LS counter. Data are expressed as mean dpm per dish ± s.e.m. Statistical differences were tested using one-way analysis of variance followed by a pairwise multiple comparison method.

Ang II Binding Studies

Media was removed from culture plates and cells were washed twice with PBS. Cultures were incubated for 1 hour at room temperature with reaction mix containing ~800,000 cpm ^{125}I-Ang II in PBS with 0.004% heat-inactivated BSA. To assess non-specific binding, 1 nM cold-Ang II was added to the reaction mix, and to assess specific binding to the AT_1R or AT_2R, 1 nM losartan or PD 123,319 was added to the reaction mix, respectively. The reaction mix was removed and culture plates washed twice with ice-cold PBS containing 0.8% heat-inactivated BSA. 1 mL of 1N NaOH was added to each dish to dissolve the cells. The dissolved cells were placed in a 12 X 75 mm glass tube and radioactivity was measured on a gamma counter.

Protein Isolation

Culture dishes containing cells were rinsed with ice-cold PBS. 100 µL of boiling125 mM Tris-HCl lysis buffer containing 2% SDS, 5% glycerol, and 1% β-mercaptoethanol was added to each 35 mm culture dish. 5 dishes were scraped into a sterile tube using a rubber policeman. After boiling the dissolved cells in the microcentrifuge tubes for 3 min, the samples were sonicated for 15 seconds and centrifuged at 12,000 x g for 5 min. The supernatant was transferred to a fresh microcentrifuge tube and stored at -80°C until needed. Protein concentrations were determined via Bio-Rad protein assay.

Immunoblotting

Each sample (20 µg) was prepared and denatured with LaemmLi sample buffer in a boiling water bath for 3 min. 16 mL of each sample mixture was electrophoresed per well in 7.5% SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was blocked with 10% non-fat dried milk in PBST-BSA for 1 hour followed by incubation overnight at 4°C with anti-TH antibody (Sigma). Protein-bound antibody was detected by incubation of the membrane with horesradish peroxidase-labeled secondary antibody for 1 hour and enhanced by chemiluminescent assay reagents. The bands recognized by the primary antibody were visualized by exposure to film.

Preparation of Adult Rat Brain-Derived Cultures

Cultured adult neurons were prepared as described by Brewer *et al*, with exceptions noted (Brewer, 1997). Fifteen week or older Sprague-Dawley female rats or Wistar Kyoto male rats were injected with 3 mL of euthanasia solution and decapitated.

The brain was rapidly dissected in a cell culture hood into 7 mL of cold Hibernate A supplemented with 2% B27 defined media supplement and 0.5 mM glutamine (Hibernate A/B27). The hypothalamus or brain stem was dissected and the meninges removed in 7 mL of cold Hibernate A/B27. Tissue was transferred to a culture dish containing 5.5 mL of cold Hibernate A/B27 and chopped into small pieces using fine dissecting scissors. Tissue pieces in Hibernate A medium were transferred to a centrifuge tube, digested with papain (2 mg/mL in Hibernate A medium), and triturated with a fire-polished glass Pasteur pipet. The suspended cells were added to the top of an Optiprep gradient made with four 1 mL steps of 35, 25, 20 and 15%, and the cell suspension was centrifuged for 15 min at 800g. Debris was removed and fractions containing cells were diluted into Hibernate A/B27 and centrifuged for 1 min at 200g. Cell pellets were resuspended in Neurobasal A/B27. 2 mL of cell suspension was added to 35 mm Nunc tissue culture dishes coated with poly-D-lysine. One hour after plating, the media was aspirated, replaced with 1 mL of Hibernate A/B27 that was aspirated and replaced with Neurobasal A/B27 containing 5 ng/mL FGF_2. Every 3 days, half of the media was removed and replaced with fresh Neurobasal A/B27 containing twice the original concentration of FGF_2. Neurobasal A, Hibernate A, B27, FGF_2, and glutamine were obtained from Gibco Life Technologies (Grand Island, NY). Poly-D-lysine was purchased from Sigma (St. Louis, MO).

Passaging of Cultured Cells

Media was aspirated from dishes roughly 3 weeks after initial plating. Dishes were treated with 0.5 mL warm trypsin solution (1g/400 mLs in Hibernate A at 37°C) per dish for 4 min while swirling the dish to loosen cells. Neurobasal A containing 10%

serum was added to the dish to quench the trypsin activity, and cells were removed from the dish with a glass Pasteur pipette. Cells were spun at low speed (250 x g) for 4 min and the supernatant was aspirated and discarded. The pellet was diluted into Neurobasal A/B27/glutamine and replated at half the initial density onto poly-D-lysine coated 35 mm culture dishes. Cultures were permitted to recover for 2 weeks before fixing and staining.

Cryopreservation of Cultured Cells

Cells were trypsinized and pelleted cells as in the passaging procedure. The pellet was resuspended in a minimal amount of Neurobasal A/B27/glutamine medium containing 7.5% sterile DMSO. The cells were aliquotted into 1 mL cryotubes and frozen slowly in a –70°C freezer. 10 days post-freezing, aliquots were removed from storage and thawed quickly in a 37°C water bath. 1 mL of preserved cells was diluted in 45 mL of Neurobasal A/B27/glutamine and centrifuged at low speed for 5 min. Supernatant was discarded and the pellet resuspended in fresh medium and plated on poly-D-lysine coated 35 mm dishes.

Infection of Cultured Cells

The virus used was an HIV-1 derived, replication-incompetent, self-inactivating vector produced by transient transfection of 293T cells. Virus was concentrated by ultracentrifugation at 50,000 x g for 2.5 hours and resuspended in 1/500th of the original volume in Hank's balanced salt solution. The pellet was resuspended by orbital shaking at 200 rpm for 5 hours. The titer of the virus was 1 +/- 2 X 10^8 infectious units per milliliter. Cells were infected in a minimal amount of media (~ 1 mL) with 1 µl (~ 10 MOI) of concentrated HIV-1 vector pseudotyped with the VSV-G envelope and encoding the gene for enhanced green fluorescent protein (EGFP, for methods review see

Huentelman, et al. *Methods in Enzymology*, in press) expressed from the human elongation factor 1 alpha promoter. 24 hours later approximately half the media was replaced. At this time EGFP expression was evident. Half the media was replaced every three days for the following ten days. After ten days the cells were fixed in 4% paraformaldehyde solution for 10 min, rinsed with PBS, and mounted in Fluoromount-G (Gibco). GFP expression was documented via epifluorescent photography using flourescein filters.

Immunocytochemistry

Cultures were washed briefly with Dulbecco's phosphate buffered saline (PBS) and then fixed for 1 min with PBS containing 0.1% Tween 20 (PBS/Tween) and 10% of a 37% w/w formaldehyde solution (Fisher). Dishes were then washed briefly with PBS/Tween. For all antibody combinations, except those with anti-tetanus toxin or anti-GalC, dishes were then fixed with -20°C methanol for 1 min, followed by an additional wash with PBS/Tween. 10% Goat serum in PBS/Tween was added to the dish for 30 min at 37°C to block non-specific binding, followed by a further wash with PBS/Tween. Primary antibodies, diluted in a 1 mL total volume of PBS/Tween, were added to the dish and incubated for 30 min to 1 hour at 37°C. Following two 30-min washes with PBS/Tween, the dishes were incubated with secondary antibodies, washed twice, 30 min each time, with PBS/Tween, and mounted with anti-bleaching medium and a glass coverslip. Primary antibodies were titrated for use to give robust staining and were as described in Table 1. Secondary antibodies were extensively cross-adsorbed goat anti-mouse and goat anti-rabbit IgGs coupled to either ALEXA 594 or ALEXA 488 and were obtained from Molecular Probes (Eugene, OR 97402). These were used at 1:2000

dilutions corresponding to a final concentration of 1μg/mL. All other chemicals were purchased from Sigma (St. Louis, MO).

Current Recording and Analysis

Membrane currents were measured using the whole-cell patch-clamp technique (Hamill et al, 1981). When filled with internal solution, electrode resistances measured 1-4 MΩ. Voltage-clamp command potentials were applied to the cells and membrane current recorded using an Axopatch 200A patch-clamp amplifier (Axon Instruments, Burlingame, CA). Membrane current was digitized on-line (10.0 kHz) with an analog-to-digital interface and filtered at 5.0 kHz. All experiments were performed at room temperature. Pipette solution for Na^+ currents contained (in mM) 140 CsCl, 2 $MgCl_2$, 10 HEPES, 10 EGTA, 20 TEA-Cl, 0.2 GTP, and 1 ATP (pH 7.2 with CsOH). Bath solution for Na^+ recordings contained (in mM) 140 NaCl, 1 $MgCl_2$, 10 HEPES, and 10 EGTA (pH 7.4 with TEA-OH). Pipette solution for Ca^{2+} current recordings contained (in mM) 110 TEA-Cl, 9 HEPES, 9 EGTA, 4.5 $MgCl_2$, 4 ATP, 0.3 GTP, and 14 phosphocreatine (pH 7.3 with TEA-OH). Bath solution for Ca^{2+} recordings contained (in mM), 142 TEA-Cl, 10 HEPES, and 10 $BaCl_2$ (pH to 7.3 with TEA-OH). Cell capacitance measurements ranged from 9 to 90 pF. Composite data are expressed as the mean ± s.e.m. All chemicals were purchased from Sigma (St. Louis, MO).

Proliferation Assay via BrdU Incorporation

Bromodeoxyuridine (BrdU) was added to the cell culture medium to a final concentration of 30 μg/mL. After 24 hours of incubation in the dark at 37°C, cells were collected from the culture plates by treatment with trypsin (0.25 g/L in Hibernate A) for 3 min. Four plates were used for each sample. The cells were centrifuged at 300 x g for 3

min. The cell pellet was resuspended in PBS and fixed in 10 mL of ice-cold methanol for 1 min. The cells were collected by centrifugation and resuspended in 25 mL of ice-cold 0.1% Triton X-100/0.1M HCl. Following 1 min incubation on ice, cells were again centrifuged and collected. The pellet was resuspended in 1 mL of DNA denaturation buffer (0.15 mM NaCl and 15 µM trisodium citrate dihydrate) using a Pasteur pipette. The cell suspension was heated for 5 min at 90°C, and then placed on ice for 5 min prior to the addition of 10 mL of antibody diluting buffer (100 mL PBS containing 100 µL Triton X-100, and 1 g BSA) and subsequent centrifugation. The resulting pellet was incubated with 0.005 µg/µL of FITC-labeled anti-BrdU antibody (clone BMC9318, Chemicon) in the dark for 30 min at room temperature prior to addition of 20 mL of antibody diluting buffer and centrifugation until a pellet formed (about 5 min at 300 x g). The supernatant was discarded and the pellet was resuspended in 2 mL of PBS containing 20 µg of propidium iodide and 0.2 mg RNase A for 30 min in the dark. The samples were then analyzed using a FACScan (BD Biosciences, San Jose, CA) flow cytometer. The instrument illuminated the cells with a 15 mW argon-ion laser, emitting 488 nm. Signals collected were forward light scatter, side light scatter, green fluorescence emission (515-545 nm) and red fluorescence emission (>650 nm). Data was acquired for 30,000 cells per sample. The resulting computer files were transferred to a Macintosh G3-350 computer running Cell Quest Software (BD Biosciences) or a PC running WinMDI Version 2.8 (courtesy of J. Trotter, Scripps Institute, La Jolla, CA) for routine plotting and percentage calculations. Cell cycle analysis was performed on the Macintosh using ModFit LT, version 2.0 (Verity Software House, Topsham, ME).

For dual staining with α-internexin antibodies and BrdU antibodies, cells were treated with BrdU as above for 36 hours, and fixed and stained as detailed in the dual staining procedure, using the R35 antibody to α-internexin and a FITC-tagged anti-BrdU antibody.

Results

In preparation for studies of NE release from co-cultured neurons, it was necessary to optimize conditions for uptake of [^{3}H]-NE. A time course was run, allowing cells to take labeled neurotransmitter up for up to 45 min. Maprotiline, a drug with specifically blocks the neuronal NE transporter, was used to assay the proportion of specific uptake. In averaged data from 3 sets of neurons cultured from Sprague-Dawley neonatal rat pups, [^{3}H]-NE uptake plateaued at 13,000 dpm/dish at about 20 min (Figure 3-1, A). More than half of this uptake was insensitive to 100 μM maprotiline, suggesting it was glial or non-specific. In contrast, uptake in neuronal cultures from 3 sets of WKY rat pups, does not plateau during the time course of the experiment (Figure 3-1, B), and was significantly greater than the uptake in the Sprague Dawley cultures. The maprotiline-insensitive uptake was only a small portion of the uptake at 45 min, implying about 35,000 dpm/dish of [^{3}H]-NE uptake was by the neuronal uptake 1 mechanism (i.e. via the NET), and was available for exocytotic release. The specific uptake in both culture types was also assayed by omitting sodium. Since maprotiline-sensitive NE uptake is sodium-dependent, it was anticipated that the same results would be obtained, but omitting sodium apparently had a non-specific osmotic effect, and produced unreliable results (data not shown).

Because both WKY and Sprague Dawley rats are normotensive and because the cultures are prepared in the same way, it was unanticipated that there would be such a significant difference between them in terms of maprotiline-sensitive uptake. The difference cannot be attributed to glial uptake because both culture types have roughly the same amount of non-specific uptake. One possibility is that the WKY cultures contain more catecholaminergic neurons capable of transporting NE. To address this possibility, Western blot analysis was performed using an antibody to the TH enzyme necessary for production of all catecholamines. Surprisingly, each of two sets of Sprague-Dawley derived cultures expressed significantly more TH protein than each of three sets of WKY cultures (Figure 3-2). Assuming that the level of TH expression is relative to the number of catecholaminergic neurons, it appears that WKY cultures actually have fewer such neurons than do the Sprague-Dawley cultures.

Becausee WKY cultures have significantly higher maprotiline-sensitive uptake levels and therefore a larger releasable pool of NE, these cultures were used for the release studies. After optimizing the number of washes necessary prior to collecting the sample (Figure 3-3), agents altering voltage gated Ca^{2+} current were assayed for effects on both spontaneous and high K^+ evoked NE release. In all cases, high K^+ appeared to evoke significantly greater NE release as compared to that occurring spontaneously in the control samples (Figure 3-4, A). Surprisingly, Ang II, which causes significant increases in the Ca^{2+} currents in the cultured neurons (Chapter 2), had no significant effect on either evoked or spontaneous release, as compared to control. Nifedipine, which blocks L-type Ca^{2+} current also had no effect, nor did Cd^{2+}. The Cd^{2+} is expected to block the high K^+ effect, since the high K^+ increases voltage gated Ca^{2+} current by depolarizing the

neurons. As a control, uptake was measured, to ensure the agents were not acting by affecting transport of NE into the cells. It appears that nifedipine had a significant effect on uptake (Figure 3-4, B), but Cd^{2+} and Ang II did not.

Because the effects of agents which alter voltage-gated Ca^{2+} current were expected to have a much larger effect on the Ca^{2+} dependent neurotransmitter release, the Ca^{2+} dependence of release and uptake were assayed by omitting Ca^{2+} when collecting the samples (0 Ca^{2+}), omitting Ca^{2+} throughout the procedure (0 Ca^{2+} through), and omitting and buffering Ca^{2+} throughout the procedure to eliminate any contaminating Ca^{2+} in the solutions (5 mM EGTA and 10 mM EGTA). While all alterations in Ca^{2+} eliminated the significant differences between spontaneous and high K^+ stimulated release, only 10 mM EGTA had a significant effect on release overall (Figure 3-5, A). Eliminating and buffering Ca^{2+} did not significantly reduce the amount of high K^+ stimulated release, suggesting that it was not Ca^{2+} dependent. Buffering Ca^{2+} did significantly reduce uptake of [^{3}H]-NE (Figure 3-5, B), which may have caused artifactual effects on release.

Because the results from these studies are unexpected and the effects of agents on uptake and release cannot be dissected in this system, the sensitivity of the assay is not sufficient to examine the effects of antagonizing specific Ca^{2+} current subtypes. Efforts were then focused on development of a new model system to use in future experiments. A cell culture procedure optimized to isolate and culture neurons from adult rat hippocampus, specifically the dentate gyrus, was modified for brain areas of interest for the Ang II modulation of NE studies. The target tissues were initially locus coeruleus and dorsal vagal nucleus, since these areas are rich in Ang II receptors in the adult

animal. The dissections were difficult and yielded very few culture dishes, so cultures of total hypothalamus or total brainstem were used for initial studies of the properties of the neurons resulting from the procedure.

Immediately after the culture procedure only a few adherent cells derived from the adult rat brainstem or hypothalamus were apparent, although there was non-adherent material, which contained live cells as well as debris. However, after 7 days in culture many adherent cells were visible in cultures derived from either brain area. Most of these cells showed a generalized neural morphology with rounded cell bodies and short processes (Figure 3-6). Cultures 10 days of age have significantly more adherent cells with longer processes and neuronal and glial-like morphology. By two weeks after plating a variety of different cell types were seen including interesting islands of smaller cells with a stellate morphology that appear in clusters over about half of the culture dish. Cells of this type continued to divide and formed continually expanding monolayers for at least 35 days in culture (data not shown). Since these cells formed a regular, reproducible patterned network we named them “patterning cells”. Visual observation made it clear that the patterning cells we noted in our cultures were dividing (Figure 3-6). Further confirmation of this came from flow cytometry of BrdU incorporation and DNA content demonstrated that at least 7% of the cells in 20-day-old brainstem-derived cultures are incorporating BrdU during a 24-hour period (Figure 3-7).

To further characterize the phenotype of this population of proliferating cells, we initially utilized antibodies to intermediate filament proteins and intermediate filament associated proteins, convenient and widely used markers of CNS cell types. Immunocytochemical analysis of the hypothalamus-derived cultures with such antibodies

revealed both neuronal and glial cell types. A few cells per dish were stained with antibodies to the neurofilament subunits of mature neurons NF-L and NF-M (Figure 3-8 A and B, green channel). These presumably correspond to the mature neurons described in cultures derived from the adult hippocampus by the same procedure [Brewer, 1993 #29], although we do not see many cells of this type. In initial experiments, we stained these cultures with monoclonal NF-H neurofilament antibody NE14, as described for the hippocampal cultures, and noted prominent nuclear staining in essentially all cells (Figure 3-8, C). This staining is probably artifactual, and was not replicated with other NF-H antibodies.

Antibodies raised against α-internexin, a neurofilament subunit expressed in developing neurons of the CNS, revealed strong staining of the islands of patterning cells (Figure 3-8, A-C, red channel). This finding was made with both monoclonal and polyclonal antibodies to this protein and suggested that such antibodies and therefore these neurofilament subunits were good markers for cells of this type in these cultures. Staining cultures exposed to BrdU for 36 hours showed that the α-internexin positive cells incorporate BrdU, and are thus proliferating (Figure 3-9). Further double labeling showed that some α-internexin positive cells in the hypothalamus derived cultures expressed nestin, a marker for neuronal stem cells, and all expressed some level of vimentin, an intermediate filament found in developing cells (Figure 3-10). However both these proteins are found in both developing neurons and glia so are not useful for cell type classification. The patterning cells were therefore stained for markers that are specific for different types of neural cells. Interestingly, the α-internexin positive cells showed clear staining for type III β-tubulin, tetanus toxin, MAP2 and tau, showing that

they express several typical neuronal markers (Figure 3-11). There was no overlap of α-internexin staining with glial fibrillary acidic protein (GFAP, Figure 3-12, A), the astrocyte marker. Additionally, there was no overlap of α-internexin expression in these cells with the oligodendrocyte marker galactocerebroside (Gal-C, Figure 3-12, B). There was also no overlap of α-internexin with isolectin B4, a microglial marker. Similar staining patterns were obtained with brainstem-derived cultures. The patterning cells therefore have several immunocytochemical properties expected of neuronal cells.

The hallmark characteristic of cells committed to a neuronal fate versus other cells of the mature CNS is the functional ability to fire action potentials and release quanta of neurotransmitter. Action potential initiation and propagation requires Na^+ channel expression while neurotransmitter release requires Ca^{2+} influx through voltage gated Ca^{2+} channels. Patch clamp analysis of patterning cells reveals the presence of Cd^{2+} sensitive Ca^{2+} current in the cells cultured from the brainstem with a peak current-voltage relationship of -0.5±0.1 pA/pF at a membrane potential of -30 mV (n=5, Figure 3-13). Fast inward currents were recorded in solutions lacking Ca^{2+} and K^+ ions. These currents, likely to be carried via voltage gated Na^+ channels, show a peak current voltage relationship of -154.5±49.8 pA/pF at a membrane potential of -10 mV (n=5, Figure 3-14). Similar results were obtained via voltage-clamp recordings from hypothalamic cultures. Despite the presence of voltage-gated currents, neither spontaneous nor evoked APs were detected in current clamp analyses, consistent with an immature neuronal phenotype.

Exploiting the proliferative nature of these cells, the cultures were passaged and split. Post-passage, the cells continued to express α-internexin and maintained their proliferative nature and stellate morphology (Figure 3-15). The cells also survived

cryopreservation for up to 15 days. Finally the cells were tested for infectability using a lentiviral vector containing a GFP construct. Expression of GFP was evident in infected stellate cells one-day post infection and was maintained for up to ten days post infection (Figure 3-16).

To assess the potential of these cells to use as a model for Ang II studies on NE release, binding studies using [^{125}I]-Ang II were carried out in the absence and presence of losartan, the AT_1R antagonist, and/or PD123,319, the AT_2R antagonist. Results from binding studies on 3 different culture sets showed no specific binding of labeled Ang II, suggesting a lack of expression of either receptor subtype. Additionally, Western blot analysis of TH expression revealed no expression in cultures derived from the hypothalamus, implying a lack of catecholaminergic neurons and the capacity to produce catecholamines (Figure 3-17). Although these data preclude immediate use of the cultures for NE release studies, they are consistent with the immature phenotype revealed by the immunocytochemical and electrophysiological profile.

Stem cells by definition can be converted to other cell types. To assay the ability of the patterning cells to be driven to differentiate to a non-neuronal phenotype, cultures were incubated with the thyroid hormone triiodothyronine (T3, 3 ng/mL), which has been shown to convert stem cells to oligodendrocytes and astrocytes [Johe, 1996 #19]. The astrocyte cell number after T3 treatment was compared to the astrocyte cell number in control cultures relative to the total cell number, and despite the presence of a trend in both the hypothalamic and brainstem cultures toward a greater astrocyte number, the differences were not significant (Figure 3-18).

Discussion

Efforts to optimize the procedure to study NE release produced several unexpected results. First, cultures derived from WKY rat pups had significantly greater [^{3}H]-NE uptake via the specific neuronal uptake mechanism, as determined by maprotiline sensitivity and as compared to Sprague-Dawley derived cultures. Since both rat strains are normotensive, this result was surprising, and could not be explained by a larger expression of TH in the WKY cultures. Since dopamine β-hydroxylase is the enzyme responsible for NE production, this enzyme may have been a better marker to assay for noradrenergic neurons, which may be elevated in the WKY with respect to the Sprague-Dawley cultures. However, at the time of the experiment, a reliable antibody to DBH was unavailable. Also, since TH is required for production of all catecholamines, it may be a better indicator of cells that would be anticipated to take NE up, since there is a possibility of some uptake into dopaminergic neurons.

None of the Ca^{2+} modulators significantly altered NE release, spontaneous or evoked, with respect to control. Ang II has been shown to increase release in several systems, *in vivo* and *in vitro*. Additionally, Ang II, in neurons, increases voltage gated Ca^{2+} current, which has been shown to mediate neurotransmitter release. Surprisingly, Ang II had no effect on spontaneous or evoked NE release from the neuronal cultures. High K^+ concentration is used to evoke release. The increased concentration of external potassium is expected to depolarize the neurons beyond the threshold for Ca^{2+} channel activation, therefore causing neurotransmitter release. Because Ca^{2+} current mediates this effect, Cd^{2+}, which blocks all Ca^{2+} current subtypes, should prevent the evoked release. However, 10 μM Cd^{2+} had no effect on the evoked release suggesting that the high K^+

concentration caused an increase in NE release by a Ca^{2+} independent mechanism. Nifedipine also had no significant effect on spontaneous or evoked release. This can be explained by the fact that nifedipine sensitive L-type current is not generally associated with neurotransmitter release. Distinct from the other Ca^{2+} modulators, nifedipine also had a significant effect on the NE uptake.

The results from these preliminary studies suggest that the [^{3}H]-NE release from the neurons is not Ca^{2+} dependent. In the subsequent experiments, eliminating and buffering Ca^{2+} eliminated the significant differences between the control and high K^+ evoked release. While superficially this suggests that the high K^+-evoked release *does* require Ca^{2+}, in reality, the spontaneous NE release is seemingly affected by the omission of Ca^{2+}, which is causing the loss of significant difference between the control and high K^+ samples. Additionally, EGTA has a significant effect on NE uptake, which may cause artifactual results in the release experiments.

The results from the release and uptake studies suggest that the assay in this model system is not sufficiently sensitive to detect changes in neurotransmitter release by varying the sources of Ca^{2+} influx. In addition, the effects of Ca^{2+} on release cannot be dissected from its effects on uptake in this assay. Carbon fiber amperommetry may be a better assay system to measure the effects of Ang II on release of NE, since it allows study of a single cell that can be voltage clamped to evoke release and monitor Ca^{2+} current. Another way to make the assay more sensitive is to somehow enrich the cultures for neurons containing the desired attributes. Since neurons do not survive automated cell sorting, it may be necessary to use molecular techniques to optimize the system artificially.

Cultures derived from adult rat hypothalamus and brainstem generated a class of dividing cell that formed a distinctive two-dimensional network that were therefore dubbed “patterning cells”. α-internexin antibodies were excellent markers for this type of cell. Since α-internexin positive cells incorporated BrdU, it was clear that they were proliferative. Significantly, these α-internexin positive cells also stained with antibodies to several other well-accepted neuronal markers, namely type III β tubulin, MAP2 and tau. Incubation with tetanus toxin and subsequent antibody staining also revealed the presence of tetanus toxin binding sites, another well accepted neuronal marker. In contrast, these cells lacked GalC staining and GFAP staining, showing a lack of well accepted oligodendrocyte and astrocyte markers. Because the patterning cells appeared to divide, yet expressed neuronal markers, it seemed likely that they could be classified as a neuronal progenitor or precursor cell. The presence of vimentin and nestin is as expected for early neuronal lineage cells, and both were found to be expressed in the α-internexin positive cells. The electrophysiological data presented here indicates the expression of Na^+ channels and voltage gated Ca^{2+} channels. Neurons generally acquire the ability to generate APs relatively late in neurogenesis, so the inability to detect action potentials in patterning cells is not inconsistent with the properties expected of early differentiating neuronal cells [Liu, 1999 #47]. It seems the patterning cells belong to the neuronal lineage but have the unusual property of retaining the ability to divide in tissue culture.

The patterning cells are morphologically similar to a class of dividing cells found in similar cultures derived from adult rat hippocampus that were described while the present work was in progress, though it remains to be seen if the cells are identical

[Brewer, 1999 #23]. It will be interesting to see how far it is possible to expand these cells in tissue culture and if these cells can be induced to differentiate into more mature neuronal phenotypes by appropriate adhesion and growth factor treatments. Preliminary studies with T3 caused a trend toward glial differentiation in the presence of this growth factor, but due to the fact that the culture medium is optimized to promote survival of neurons over glia, it will be necessary to put the cells into less-selective medium to determine the ability for them to differentiate to cell types other than neuronal cells. It is somewhat surprising that these cells are able to divide, as previous studies have suggested that α-internexin is normally expressed post-mitotically [Kaplan, 1990 #34], as are tau, MAP2, type III β tubulin and tetanus toxin binding sites. Even after 35 days in tissue culture these α-internexin positive cells do not express NF-L or NF-M, the neurofilament subunits characteristic of more mature neurons, suggesting that these cells maintain immature neuronal characteristics over several divisions. Possibly the FGF_2 treatment or other aspects of the culture conditions select for an immature neuron type, suppressing terminal differentiation of the cells. Interestingly, and in contrast to previous stem and progenitor cell findings, omission or withdrawal of FGF_2 results in cell death or lack of population expansion [Kaplan, 1990 #34].

The α-internexin positive cells may resemble cells extracted from mature brain tissues by several other groups [Reynolds, 1992 #50]. These cells generally develop "neurospheres" in tissue culture under the influence of growth factors such as epidermal growth factor and FGF_2. The present procedure, while distinct in many ways from that used to generate these progenitor cells, does use FGF_2 and may also select for the proliferation of cells of this type. The α-internexin positive patterning cells may

therefore have the same origin as so-called neuronal progenitors currently being studied by many other groups. In support of this conclusion, sometimes what appeared to be spontaneous neurospheres were observed in these cultures, some cells in which stained with α-internexin antibodies.

The origin of the α-internexin positive cells described here is an interesting subject for future study. Perhaps they derive from a mature neuron type that de-differentiates in tissue culture, or they may derive from a population of stem or progenitor cells such as those described by other research groups. It is now generally accepted that there are two regions of relatively active neurogenesis in the adult mammalian brain, namely the subgranular zone of the dentate gyrus of the hippocampus and the subventricular zone lining the ventricles [Gage, 1998 #44]. One group has provided evidence that a subpopulation of ependymal cells in the adult brain can generate colonies in tissue culture which contain both neurons and glia [Johansson, 1999 #52], while another has shown that a distinct class of GFAP positive cells can generate both neurons and glia [Doetsch, 1999 #41]. The starting material for hypothalamic cultures includes the third ventricle, which is expected to include subventricular zone neuronal progenitor cells. The brainstem cultures were derived from tissue that includes the cerebral aqueduct also lined with ependymal cells and a small subventricular zone. These α-internexin positive cells could therefore be derived either from ependyma or subventricular zone. However, although a detailed immunocytochemical analysis was not performed on these cultures, patterning cells were also derived from locus coeruleus or dorsal vagal nucleus, which were dissected carefully so as not to contain ventricle. In any case it is apparent that a neuron-like dividing cell type can be harvested from adult

brain, identified with an α-internexin antibody, and made to divide and differentiate to an early neuronal phenotype. The culture procedure used for these studies provides a cell type useful for studies of neurogenesis relating to brain injury and repair. Because the cultures proliferate and can be split and passaged, it should also be possible to grow clonogenic cultures. Finally, as demonstrated here, the ability to infect these cells and cause them to express exogenous proteins can be exploited in further differentiation studies and also to track the movement and differentiation of these cells in future transplantation studies.

Despite the lack of Ang II receptors and TH expression, these cells may still prove to be a good model for NE release studies. Exploiting the proliferative nature of these cells, they can be infected and cloned and made to express the appropriate components of a system, therefore becoming a “designer” expression system with a neuronal background. In addition, it will be possible to determine if expression of Ang II receptors is sufficient to convey a particular neurotransmitter phenotype or to cause terminal differentiation of the cells.

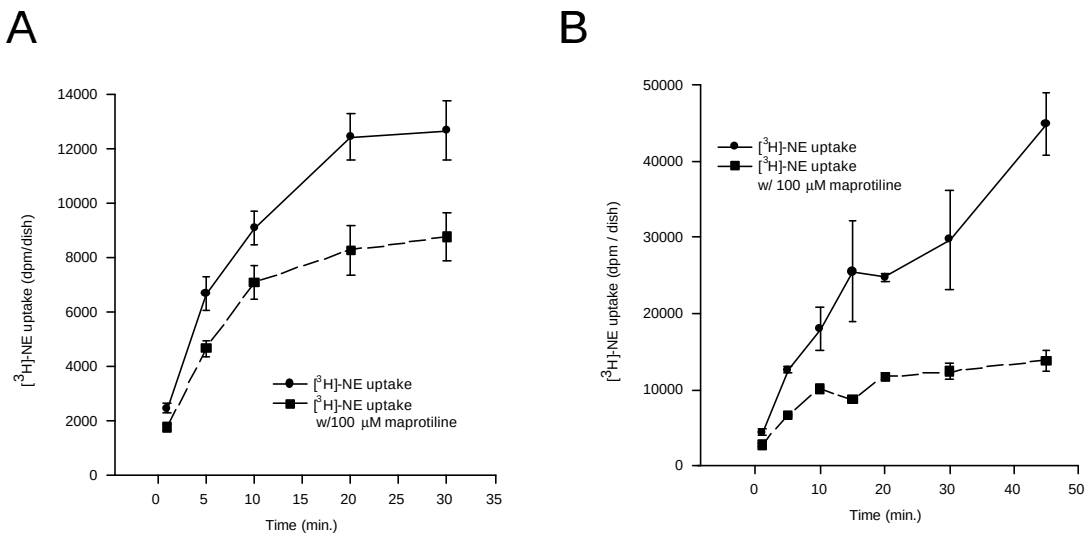


Figure 3-1. Optimization of [^{3}H]-NE uptake in neuronal co-cultures. A. More than half of the NE taken up by Sprague-Dawley derived neurons is not mediated by the maprotiline-sensitive norepinephrine transport protein. B. The NE uptake in WKY-derived neurons is not saturable during the course of the experiment, but a large portion of the uptake is maprotiline-sensitive.

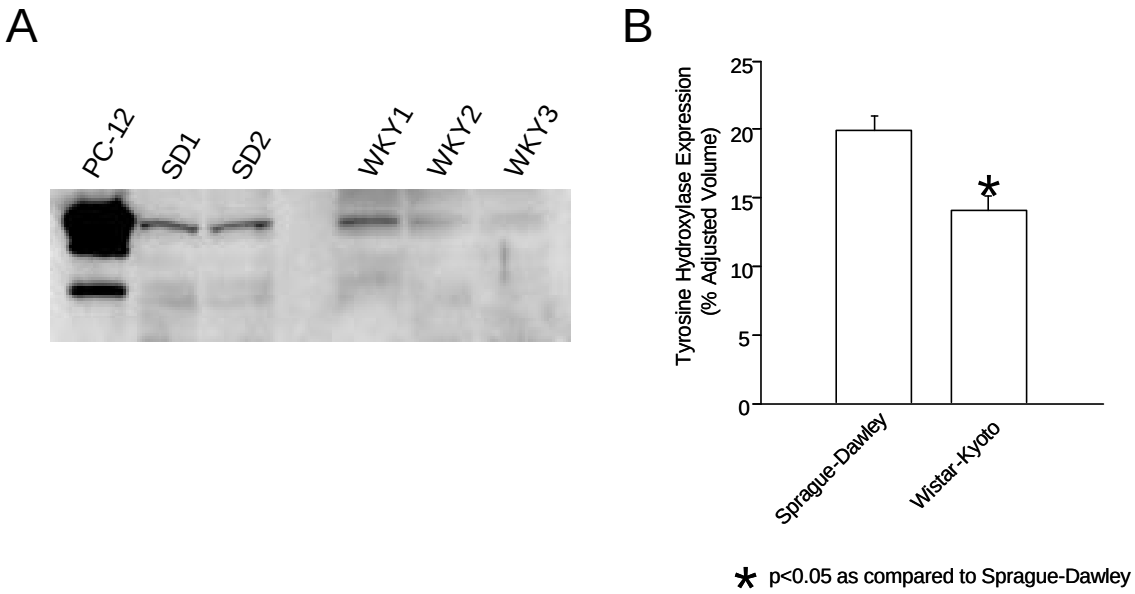


Figure 3-2. Analysis of TH expression in Sprague Dawley and WKY derived neuronal cultures. A. Western blot analysis with an antibody recognizing TH protein shows that WKY cultures generally express less TH per 20 μg of protein than those derived from Sprague Dawley animals. B. Densitometry analysis of TH expression shows that the expression in WKY derived cultures is significantly less than in Sprague Dawley cultures. Data are expressed as the mean ± s.e.m.

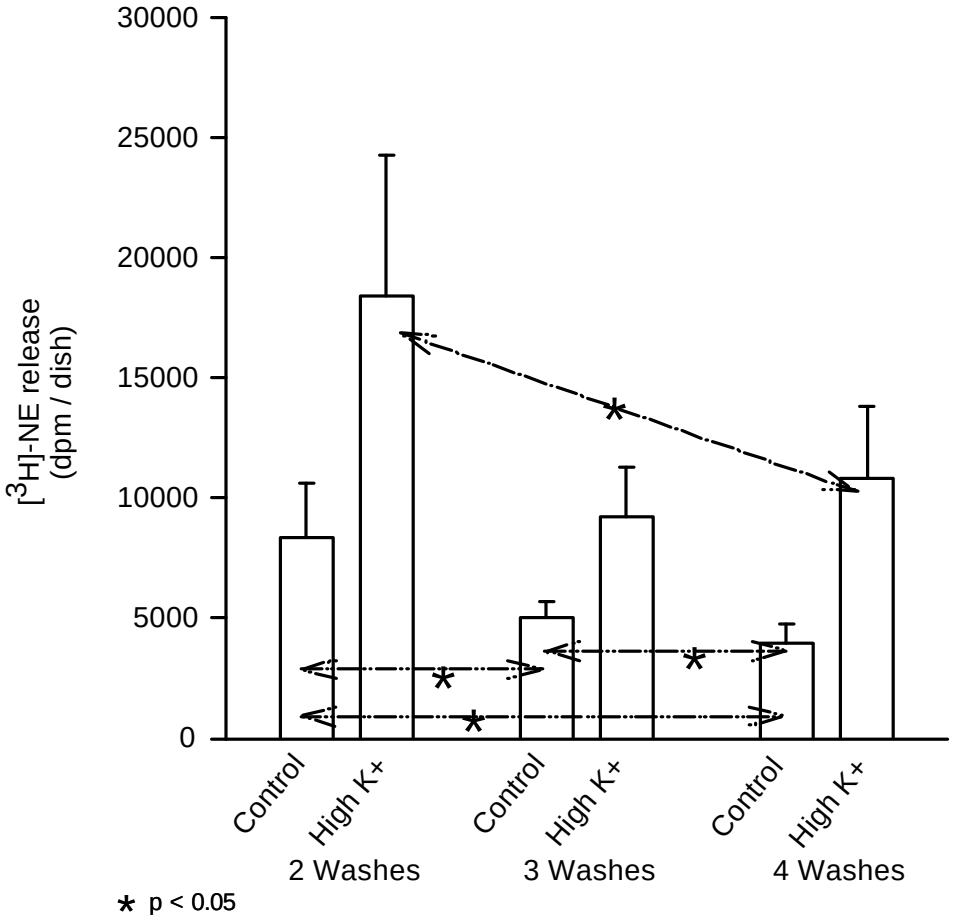


Figure 3-3. Optimization of the number of washes prior to stimulation of NE release.

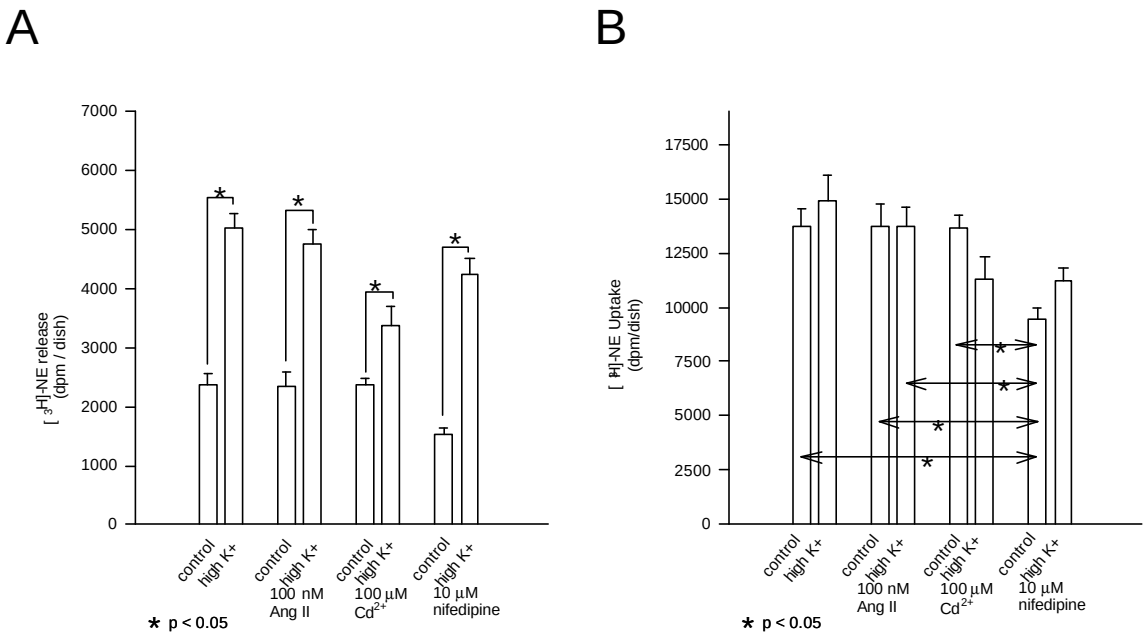


Figure 3-4 Effect of Ang II, Cd^{2+}, or nifedipine on [^{3}H]-NE release and uptake. A. Neither Ang II, which increases voltage gated Ca^{2+} current, nor Cd^{2+} or nifedipine which block all or L-type Ca^{2+} current, respectively, have significant effects on NE release. B. Nifedipine does significantly alter NE uptake.

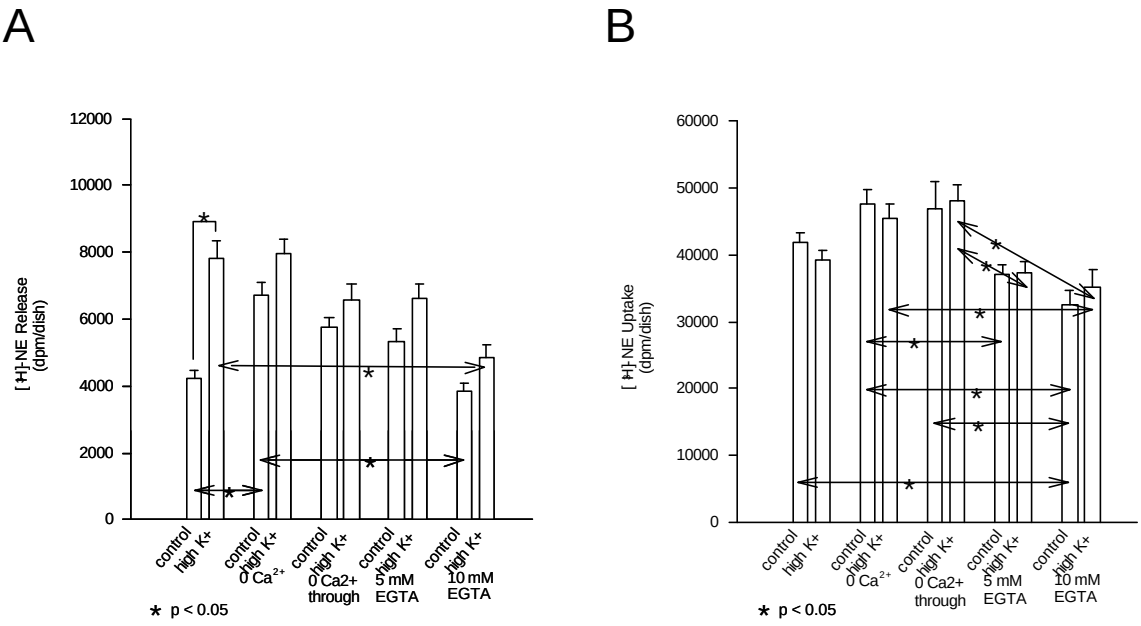


Figure 3-5. Ca^{2+} dependence of [3H]-NE release and uptake. A. 0 Ca^{2+} during the release and 10 mM EGTA appear to have significant effects on NE release. B. Buffering Ca^{2+} with EGTA appears to affect uptake, suggesting the effects of 10 mM EGTA on release may be a result of altered uptake.

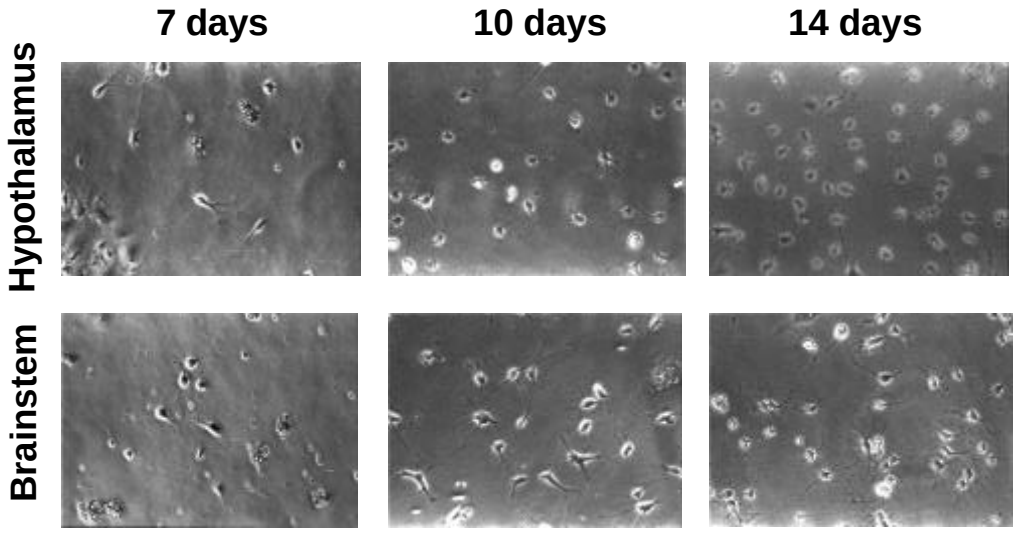


Figure 3-6. Population expansion of cells cultured from adult rat hypothalamus and brainstem photographed in phase contrast microscopy. Cells cultured from adult rat hypothalamus (top row) or brainstem (bottom row) develop a stellate morphology and form extended monolayer networks as shown in the photomicrographs of live cells. Magnification is 100X.

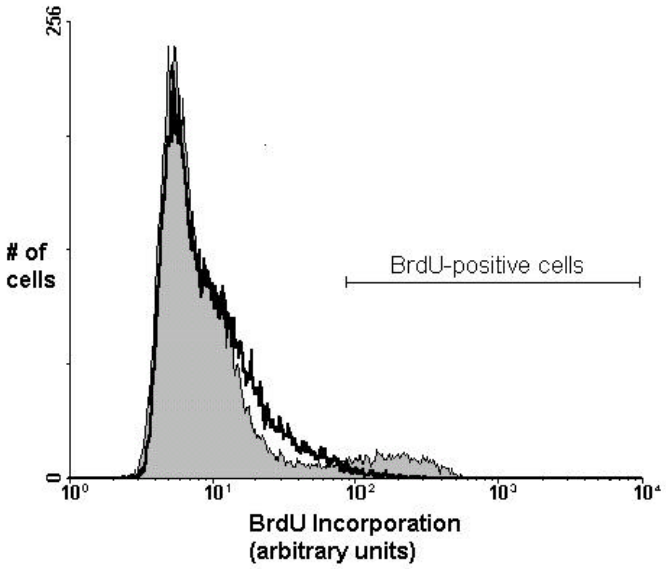


Figure 3-7. Flow cytometric analysis of BrdU incorporation in adult rat brain-derived cultures. A second peak is observed for brainstem-derived cultures which have been incubated with BrdU for 24 hours (gray) when compared to the histogram obtained for control cultures not treated with BrdU (black line). The second peak represents a population of cells that incorporated BrdU into DNA and thus was proliferating. The fraction of cells proliferating was taken as the number of cells in the region marked "BrdU-positive cells" divided by the total number of cells. Data representative of 4 different culture dishes.

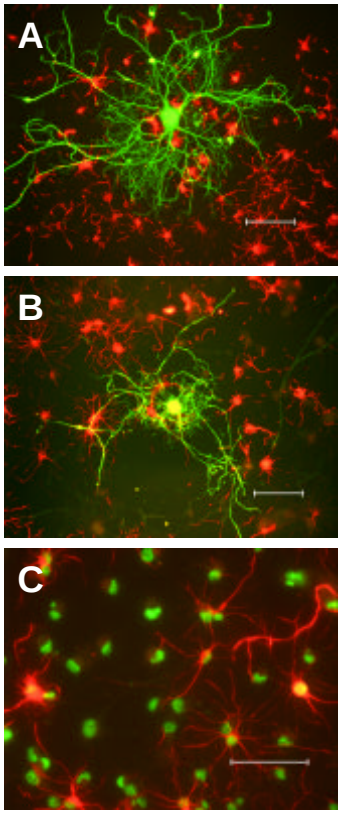


Figure 3-8. Presence of α-internexin in the absence of neurofilament triplet protein expression in patterning cells. *A.* Hypothalamic culture fixed after 23 days in vitro and stained for neurofilament NF-L (DA2 monoclonal antibody, green channel). This antibody stains large relatively rare cells with an obviously neuronal morphology. A network of α-internexin positive cells, as revealed with polyclonal antibody R35 (red channel), do not stain for NF-L. *B.* Hypothalamic culture fixed after 23 days in culture and stained with NF-M as revealed with monoclonal antibody 3H11 (green channel) is also not expressed in α-internexin positive cells as revealed with R35 antibody (red channel). *C.* Monoclonal antibody NE14 (green channel) stains nuclei found in all cell types (hypothalamic culture 22 days in vitro). No filamentous staining of NF-H is present in α-internexin positive cells (red), though some staining was seen in the processes of NF-L/NF-M positive cells such as those shown in figure 4a and 4b. Scale bars: 50 μm.

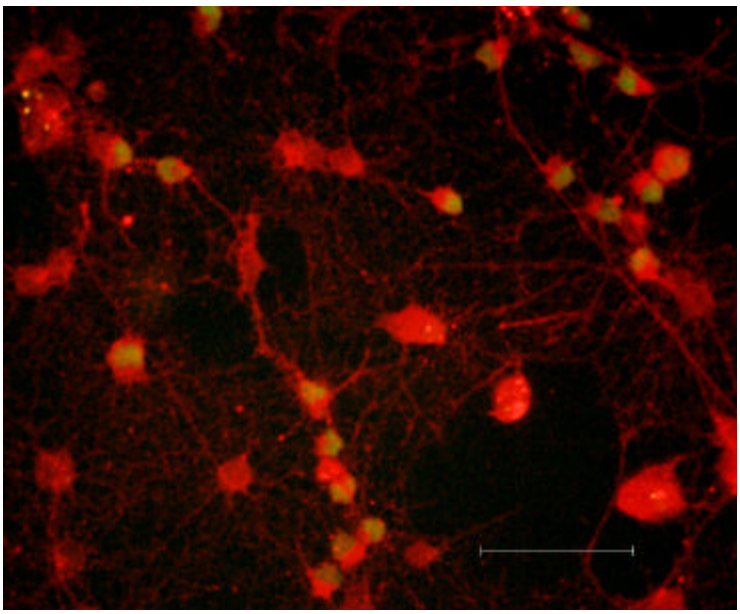


Figure 3-9. BrdU incorporation by α-internexin positive cells. Monoclonal antibody to BrdU (green channel) reveals BrdU incorporation in nuclei of α-internexin positive hypothalamic cells (R35 antibody, red channel). Scale bar: 50 μm.

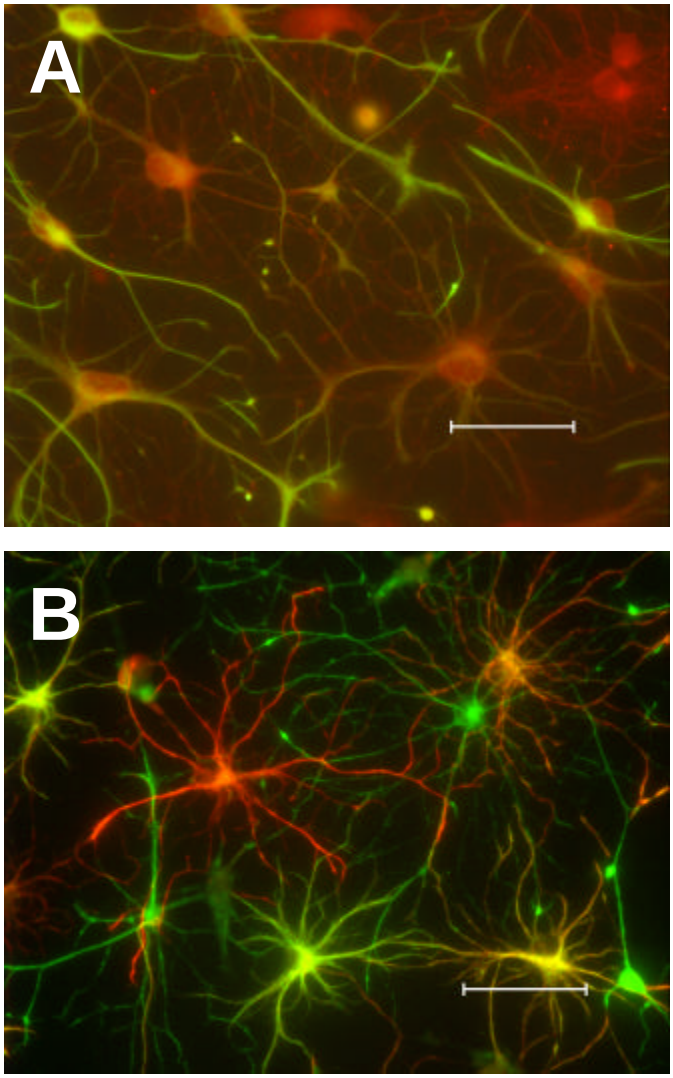


Figure 3-10. Markers indicative of an early neuronal phenotype are expressed in the α-internexin positive patterning cells. *A.* Some α-internexin positive cells (R35 antibody, red channel) co-express nestin (Rat 301 antibody, green channel), a neuroepithelial stem cell marker. Hypothalamic cultures after 21 days in culture. *B.* Vimentin (V9 antibody, green), an intermediate filament found in many cell types, is co-expressed with α-internexin (R36, red) in some cells. All α-internexin positive cells revealed some vimentin staining, but many vimentin positive cells, presumably glia, showed no α-internexin staining. Scale bars: 25 μm.

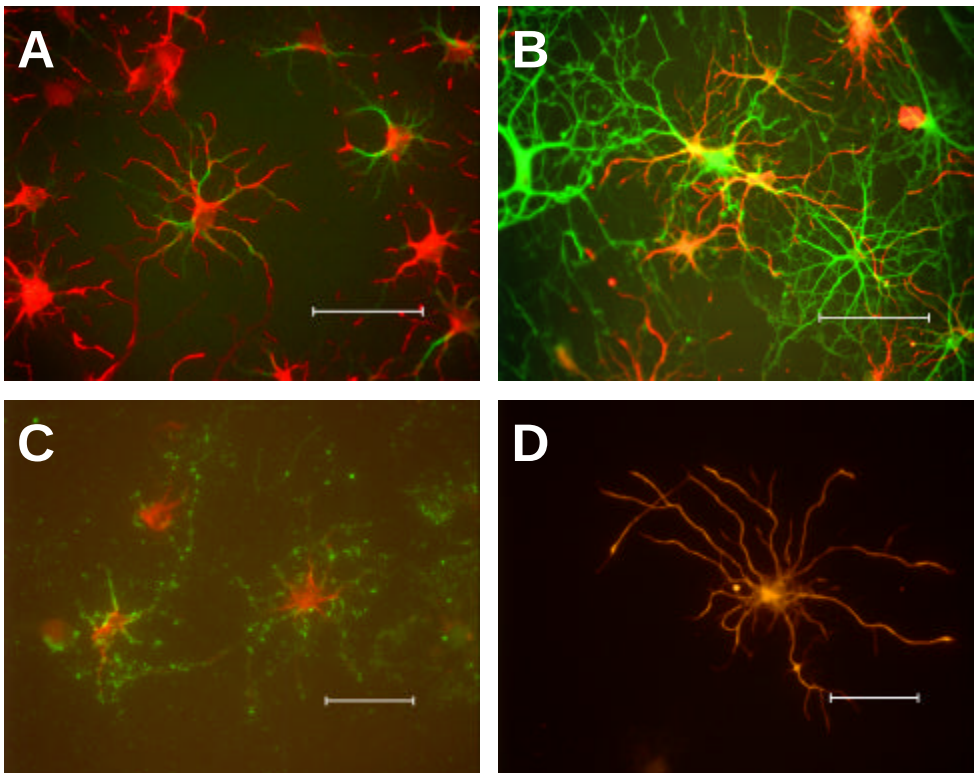


Figure 3-11. The α-internexin positive cells co-express several well-accepted neuronal markers. *A.* α-internexin positive cells (R35 antibody, red) co-express MAP2 (AP20 monoclonal antibody, green) in dendrite like processes. Hypothalamic cells 18 days in tissue culture. *B.* Tau expression (Tau monoclonal antibody, green) overlaps with α-internexin expression (R35 antibody, red). Some other stellate cells, apparently astrocytes also show tau expression. Hypothalamic cultures 24 days in culture. *C.* Tetanus toxin binding sites (revealed with Boerhinger-Mannheim kit, in green) are present on the processes of the α-internexin positive cells (R35 antibody, red). Hypothalamic cultures 29 days in tissue culture. *D.* β-tubulin III expression (Sigma antibody, green) overlaps with α-internexin expression (R35, red) in a cell in a hypothalamic culture after 26 days in culture almost completely. Scale bars: A and B, 50 μm; C and D, 25 μm.

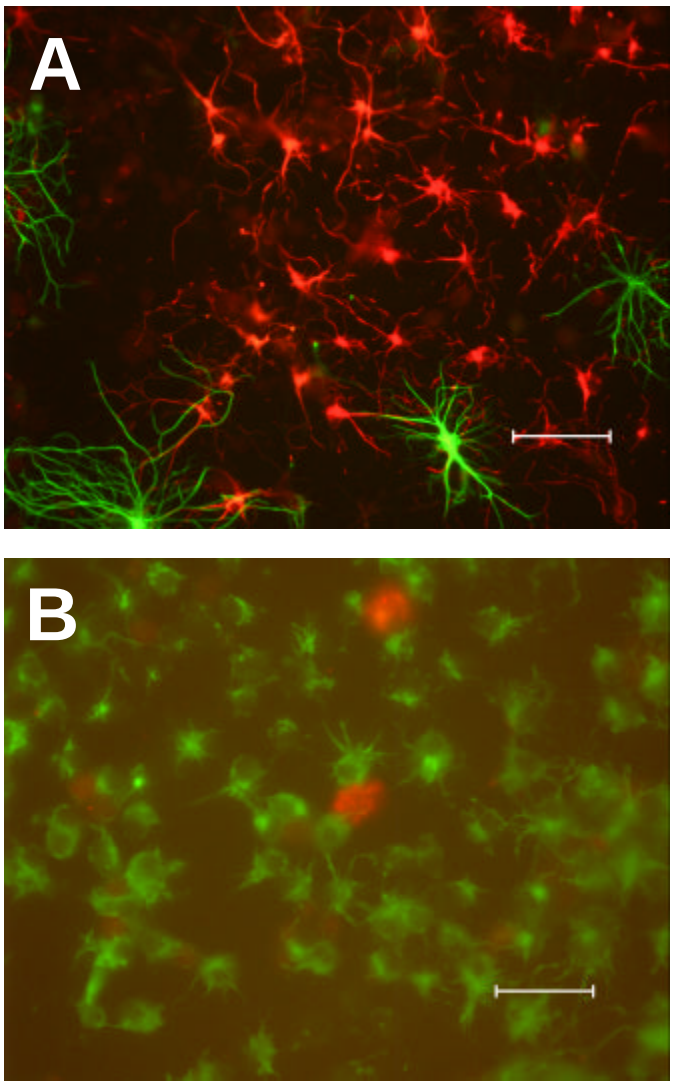


Figure 3-12. α-internexin expression does not overlap with expression of glial markers. *A.* α-internexin (R35 antibody, red) expression of patterning cells does not overlap with GFAP expression (GA5 antibody, green) demonstrating that the lack of the astrocytic marker in these cells. *B.* Cells expressing Gal-C (Sigma antibody, red) are fewer in number and distinct from α-internexin positive patterning cells (2E3 antibody, green) showing the absence of the oligodendrocyte marker in these cells. Scale bars: 50 μm.

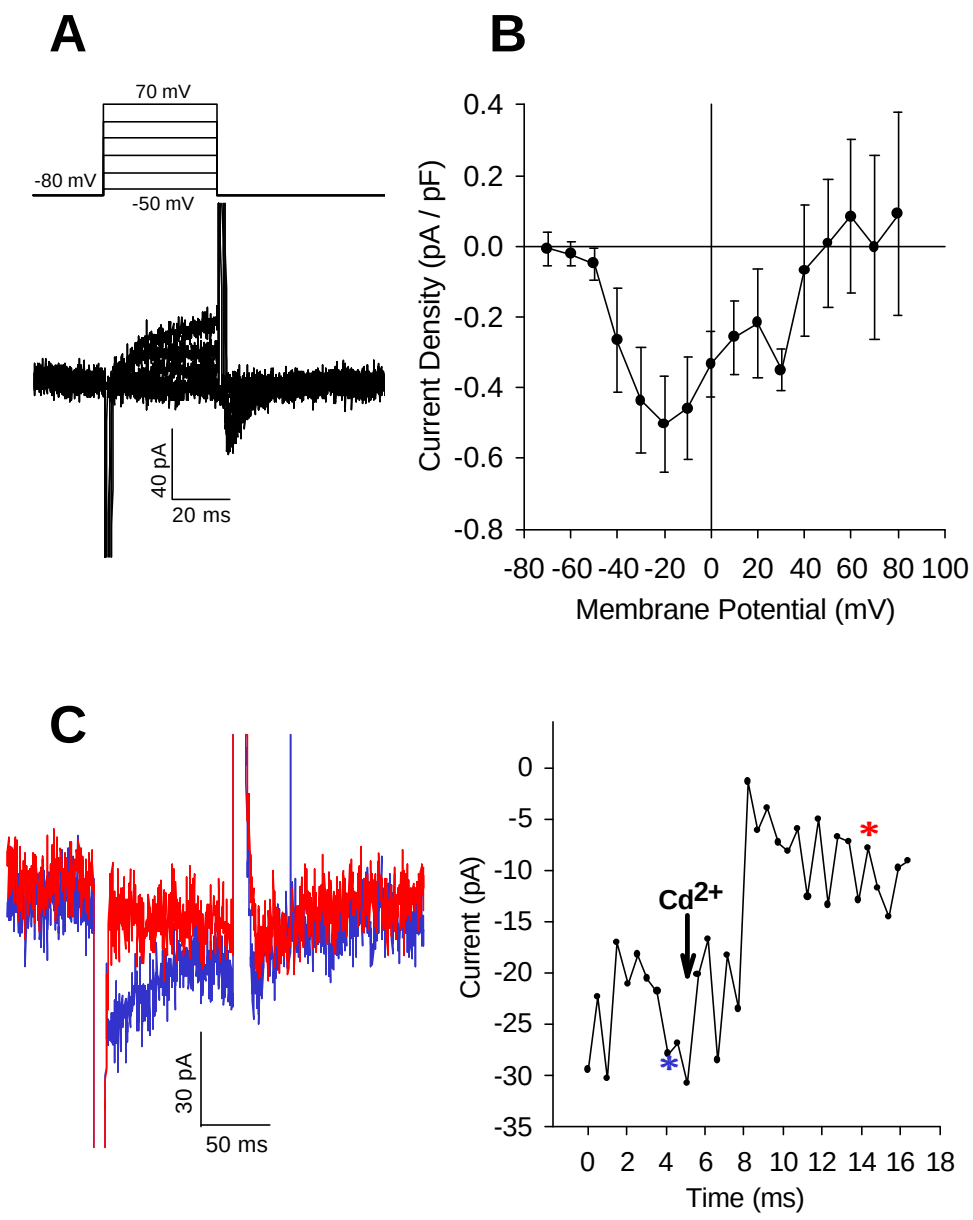


Figure 3-13. Patterning cells cultured from adult rat brain express Cd^{2+} sensitive Ca^{2+} current. *A.* A representative family of Ca^{2+} currents elicited by step depolarizations from a holding potential of -80 mV. *B.* Current voltage relationship with a peak of 0.5 pA/pF at -20 mV (n=5). *C.* Ca^{2+} current elicited by a step depolarization to -20 mV was blocked by superfusion of 10 μM Cd^{2+}.

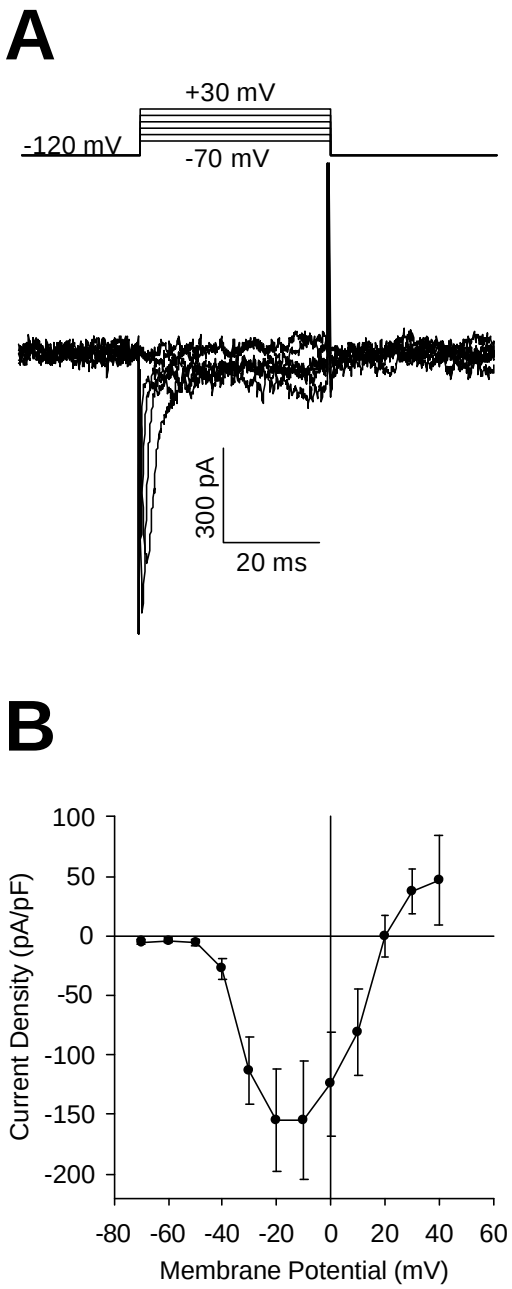


Figure 3-14. Patterning cells from adult rat brain express Na^+ current. *A.* A representative family of Na^+ currents elicited by step depolarizations from a holding potential of -120 mV. *B.* Stellate cells cultured from adult rat brain express Na^+ current with a peak of the current voltage relationship of 150 pA/pF at -15 mV (n=5). Cell capacitance ranged from 9-90 pF.

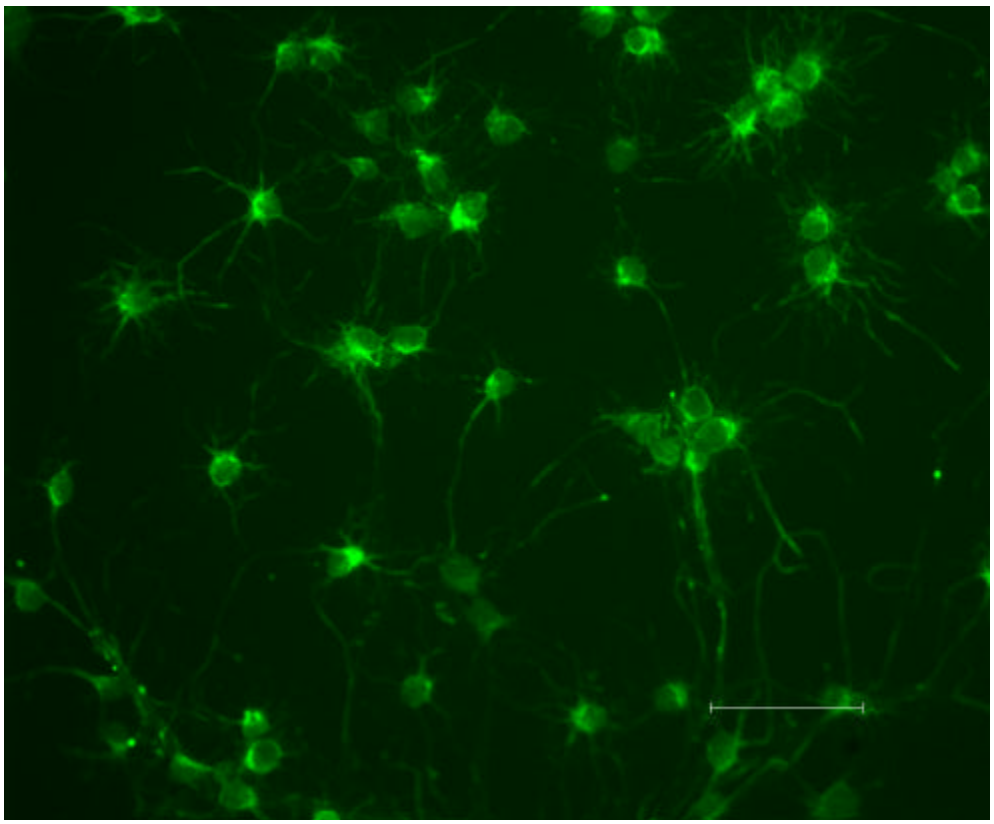


Figure 3-15. Patterning cells survive passaging in tissue culture and retain α-internexin expression. The α-internexin positive cells (green) continue to proliferate and maintain their stellate morphology after being split. Scale bar: 50 μm.

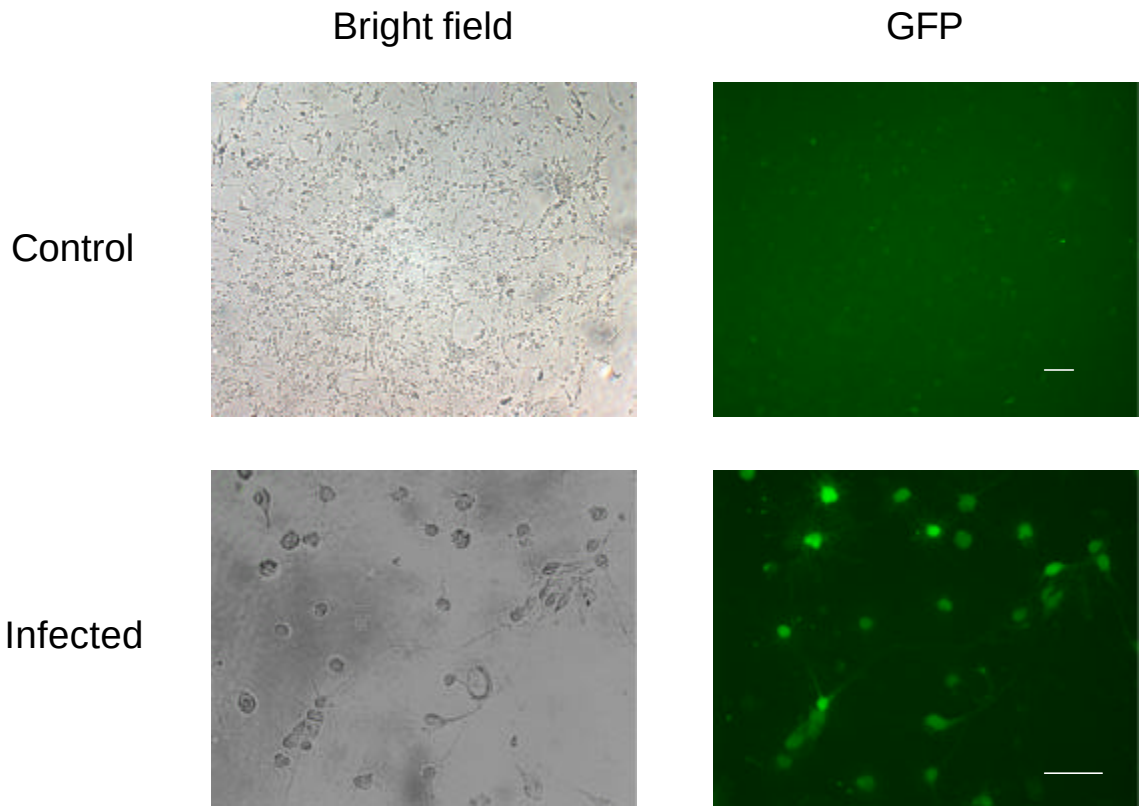


Figure 3-16. Patterning cells express GFP after infection with a lentivirus construct. Stellate cells express GFP (green) 24 hours post infection and continue to express up to 10 days post infection. Control, non-infected cells do not show GFP fluorescence. Scale bars: top, 200 μm; bottom, 50 μm.

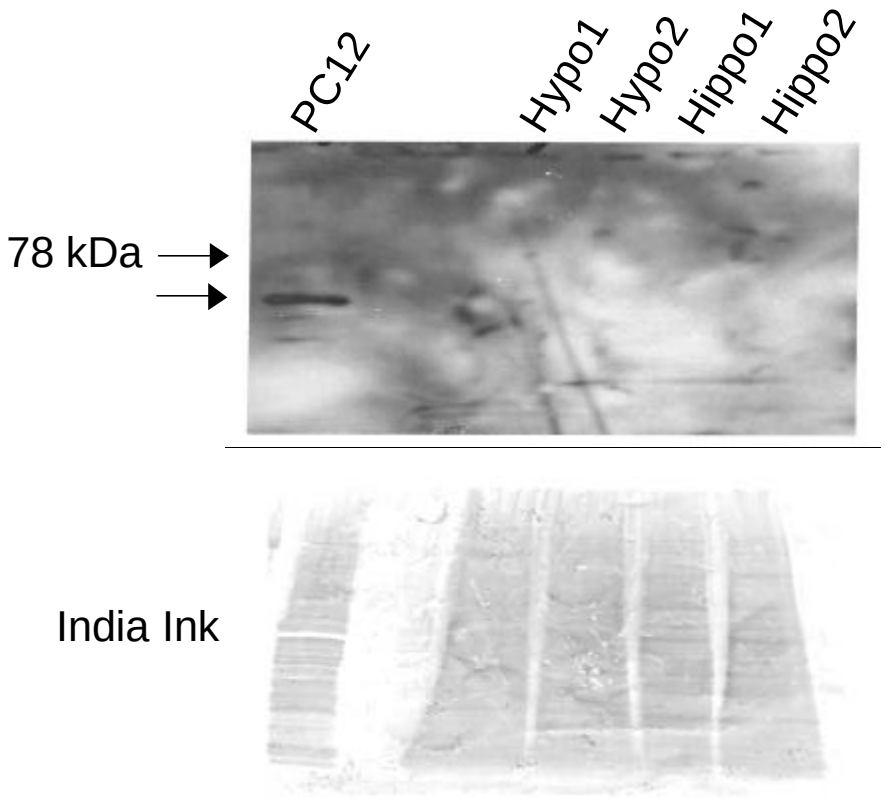


Figure 3-17. Western blot analysis of TH expression in adult rat-derived cultures.

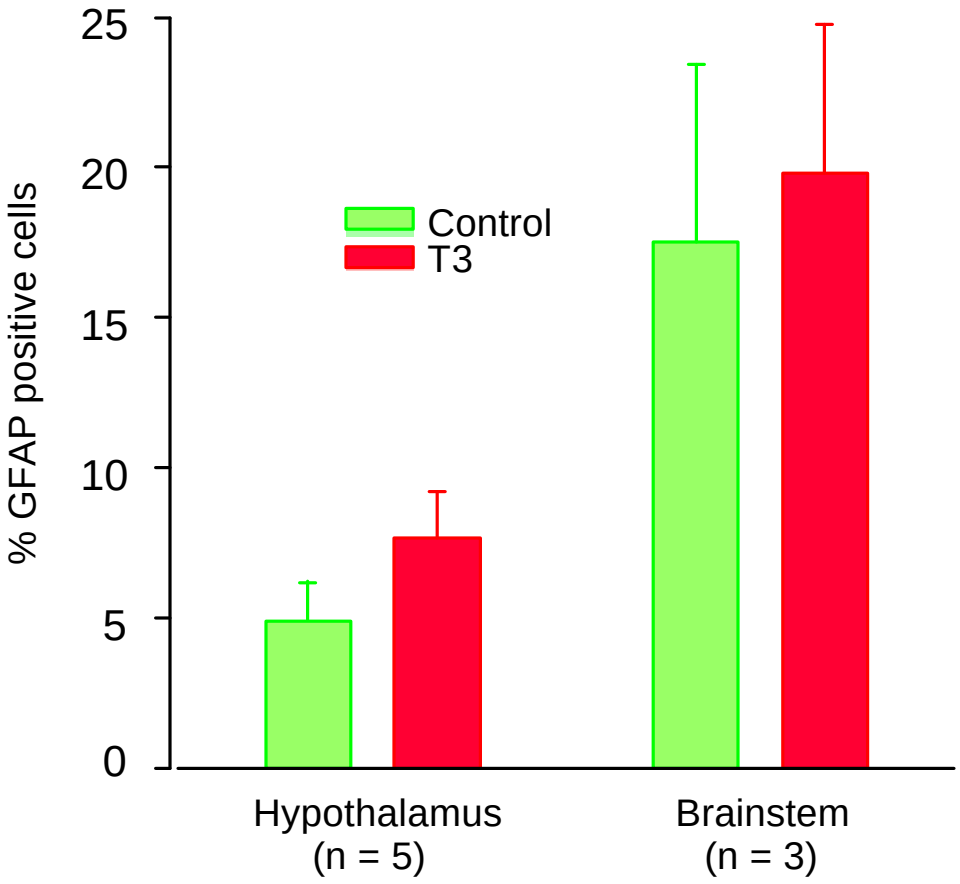


Figure 3-18. Effect of T3 on astrocyte expression in adult rat brain derived cultures.

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BIOGRAPHICAL SKETCH

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