Single-cell RT-PCR Analysis and CRF Effects on TRPC Channels Expressed in Rat Brain

BY

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THESIS

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This thesis is dedicated to my mother, Mary Ann West-Jones, my grandmother, Mozilla Nicole Watkins, my great-grandmother Mary McCaleb, and my father, Rufus Mims, your unwavering love, guidance and support have allowed me to pursue my dreams.
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<td>ChAT</td>
<td>choline acetyltransferase</td>
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<td>CRF</td>
<td>corticotropin-releasing factor</td>
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<td>SNPC</td>
<td>substantia nigra pars compacta</td>
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<td>TRH</td>
<td>tyrosine hydroxylase</td>
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The canonical transient receptor potential (TRPC) channels comprise a family of nonselective cation channels composed of seven members (TRPC1-7). TRPC channels are widely distributed in the nervous system and contribute to neuronal excitation. In the present study, using the single-cell RT-PCR method, the distribution of TRPC channel mRNA was analyzed in cholinergic neurons in the nucleus basalis (NB), serotonergic neurons in the dorsal raphe nucleus (DRN), noradrenergic neurons in the locus coeruleus (LC), dopaminergic neurons in the substantia nigra (SN), and dopaminergic neurons in the ventral tegmental area (VTA). NB, LC, SN and VTA neurons were cultured from 3-5 day-old and DRN from 10-12 day-old Long Evans rats. Single-cell RT-PCR was performed on these cultured neurons using the previously described method (Kawano et al., 2004, Neuroscience letters, 358:63). Tyrosine hydroxylase primers were used to identify noradrenergic neurons in LC and dopaminergic neurons in SN and VTA. Choline acetyltransferase primers were used to identify cholinergic neurons in NB and tryptophan hydroxylase primers were used to identify serotonergic neurons in DRN. TRPC1
mRNA was most frequently detected in all neuron types. TRPC2, involved in pheromone sensing, was not present in any of the five brain areas examined. TRPC3, 4 and 5 existed most frequently in cholinergic neurons in NB (80-90%). TRPC6 was relatively more frequent in dopaminergic neurons in VTA (58%) and SN (46%). TRPC7 was most frequently found in noradrenergic neurons (80%) in LC and cholinergic neurons (80%) in the NB. Interestingly, cholinergic neurons in the NB showed the highest frequency of TRPC mRNA expression. Present results demonstrate that each type of neuron expresses specific combination of TRPCs, suggesting that the specific TRPCs are responsible for excitation of each type of neurons.

Secondly, I have investigated the effects of corticotrophin-releasing factor (CRF) on nucleus basalis (NB) cholinergic neurons with emphasis on non-selective cation (TRPC) channels. I have identified PKC as a necessary signal transducer in the activation of TRPC channels.

Finally, I have studied the effects of CRF on TRPC5 using HEK293A cell lines that have been transfected with TRPC5 and CRHR1 cDNAs. I found PKC activation a requirement for the opening of TRPC5 channels.
SUMMARY

The focus of this investigation was the study of canonical transient receptor potential channels (TRPC). The main objective was to elucidate the distributional patterns of TRPC channels present in five medically relevant brain regions. The areas studied were the cholinergic neurons of the nucleus basalis (NB), the dopaminergic neurons of the ventral tegmental area (VTA) and substantia nigra pars compacta (SNPC), the noradrenergic neurons of the locus coeruleus (LC) and the serotonergic neurons of the dorsal raphe nucleus (DRN). In order to achieve this, the single-cell reverse transcription-polymerase chain reaction (RT-PCR) procedure was employed. This involved the culturing, harvesting, and analyzing the cellular mRNA contents which unveiled a unique population-specific allocation of TRPC channels.

Secondly, electrophysiological methods were used to study the regulation of TRPC channels by corticotropin-releasing factor (CRF) within the nucleus basalis cholinergic neurons and a heterologous system. This allowed for the identification of PKC as the signal transducer involved in the activation of TRPC channels.

Canonical transient receptor potential (TRPC) channels are important in promoting cellular excitability. TRPC channels are distributed throughout the mammalian central nervous system, where they participate in a multitude of diverse and important functions. Our group has investigated these non–selective cation channels extensively by using electrophysiological methods to measure the responses that are evoked by neurotransmitters. In this study, we performed RT-PCR analysis in order to provide a more precise understanding of the pattern of TRPC channel isoforms that are found in the regions that we have studied electrophysiologically. They are the cholinergic neurons of the nucleus basalis (NB), dopaminergic neurons of the
substantia nigra pars compacta (SNPC) and ventral tegmental area (VTA), noradrenergic neurons from the locus coeruleus (LC), and serotonergic neurons from the dorsal raphe nucleus (DRN).

We discovered that among the five types of brain neurons tested, TRPC1 mRNAs were most recurrently detected compared with the other TRPC channel mRNAs. In the cholinergic neurons of the NB, TRPC3, TRPC4 and TRPC5 were present frequently. In VTA and SNPC dopaminergic neurons, TRPC6 mRNA was comparatively recurrent. In the LC noradrenergic neurons, TRPC7 was frequent. The highest frequency of TRPC mRNA was found in nucleus NB cholinergic neurons. None of the neurons examined contained TRPC2 mRNA.

Also, by using a PKC inhibitor, we were able to identify PKC as the signal transducer necessary for activation of TRPC5 in heterologous systems and also for the non selective (TRPC) channels of the nucleus basalis. We are the first group to report these findings in order to better understand the signaling pathway involved in TRPC channel activation.
I. LITERATURE REVIEW

A. Introduction

The focal point of this study is the examination of the canonical transient receptor potential (TRPC) channels. The transmembrane spanning TRPC channels promote cellular excitation by allowing for the flux of cations, namely, sodium, potassium and possibly calcium, down their electrochemical gradients (Montell, 2005). They are present extensively throughout mammalian organisms and thus participate with a broad range of functions. These tasks include neurite outgrowth, vasodilation, glomerular filtration, proliferation and muscle contraction (Venkatachalam, 2007). TRPC channels may participate in neuronal excitation, when present on neurons (Clapham, 2005).

The proposed mode of activation for TRPC channels is the Gq-coupled pathway (Clapham, 2005). However, the exact signal messengers involved remains elusive. There have been very few studies executed for the purpose of determining the intracellular pathway leading to TRPC opening after Gq-coupled receptor activation.

Alterations in TRPC gene structure or function have been linked with a number of diseases. These channelopathies include exaggerated inflammation responses, vascular disease, respiratory disorders, impotence, glomerular disease, as well as neurological disorders (Nilius et al., 2007). Considering the wide range of disorders that can occur upon alterations of TRPC gene structure, and hence TRPC channel functions, it is of importance to get a precise understanding of the signal transduction pathway involve in the regulation of these channels.

There is much to discover about the functioning of the TRPC channels located within the central nervous system alone. It has been recently discovered that TRPC channels are present
within many distinct populations of neuronal tissue. Their selectivity for cation permeability suggests the possibility that TRPC channels can participate in neuronal excitation. Coexisting with TRPC channels are excitatory neuropeptides such as corticotropin-releasing factor (CRF), which functions by activating G-protein coupled receptors (Navarro-Zaragoza et al., 2010). The key function of corticotropin-releasing factor (CRF) in the CNS is to mediate cognitive processing within the cholinergic neurons of the nucleus basalis. This is achieved through the binding of CRF with one of two receptors, corticotropin-releasing hormone receptor 1 (CRHR1) or corticotropin-releasing hormone receptor 2 (CRHR2). One of the hallmark features of Alzheimer’s disease is the changes in CRF and CRF receptors (Bayetti et al., 2003; Bennett et al., 2005). Thus, clarification of the signal transduction pathway that is initiated with CRF binding, leading to TRPC opening is very important in understanding the role of CRF in cognition.

B. Transient receptor potential (TRP) channels

1. Background

The transient receptor potential (TRP) channels are a group of ion channels that are located on the plasma membrane of numerous animal cell types (Ramsley et al., 2006; Clapham, 2005). Each of the TRP channel subunits form six transmembrane spanning regions which included a putative pore and they group together into tetrameric proteins. TRP channels are relatively non-selectively permeable to cations, including sodium, potassium and calcium (Sergeeva et al., 2003; Strubing et al., 2003). The discovery of TRP channels was made in the fruit fly Drosophila while studying the mutant variant trp gene (Cosens and Manning, 1969). Since then, six TRP channel subfamilies have been discovered to be expressed ubiquitously in vertebrates (Clapham,
2005). They are TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin), TRPP (polycystin) and TRPML (mucolipin) channels, which are grouped based on structural and functional similarities (Ramsley et al., 2006; Clapham, 2005; Montell, 2005).

2. **Function**

These channels have diverse functions ranging from mechanosensation, vasodilation, thermosensation, taste, neurite outgrowth and neuronal cell death, and neuroexcitation (Venkatachalam and Montell, 2007). The channels are activated by diverse stimuli, such as osmolarity, pheromones, touch, pain, temperature and neurotransmitters (Montell, 2005). They are found throughout entire mammalian organism, but most notably in the brain where there is also new emerging data suggesting that TRP channels in the nervous system play an important role in neuronal cone guidance, chemotaxis and neuronal excitation (Greka et al., 2003; Clapham, 2003; Montell et al., 2002). Other cellular events implicated are migration, proliferation, muscle contraction and neuronal excitation (Minke and Cook, 2002; Schilling, 2001). Almost all studies conclude that TRP channels have roles in cellular signaling (Ramsley et al., 2006; Clapham, 2005; Montell, 2005).

**C. Canonical transient receptor potential (TRPC) channels**

1. **Background**

TRPC was the first mammalian TRP channel discovered and coincidently, it is the most similar to *Drosophila trp* in terms of sequence homology (Venkatachalam and Montell, 2007). There are seven mammalian TRPC channel subfamily members. They share the presence of a structural motif in the carboxyl–terminal, which is the TRP box, located on the intracellular edge
of the S6 region and it contains an invariant EWKFAR sequence (Nilius et al., 2006). Located on
the amino-terminal of the TRPC channels, is three or four ankyrin repeats (Nilius et al., 2006).
TRPC channels are non-selectively permeable to cations, however, the ratio of permeability for
sodium, potassium and calcium varies depending on the homomeric or heteromeric combination
that is formed between them.

2. Classification

On the basis of functional comparisons and sequence alignments, the members TRPC
subfamily, all of which are important to excitation, are separated into TRPC 4/5, TRPC3/6/7,
TRPC2 and TRPC1 (Ramsley et al., 2006; Clapham, 2005; Montell, 2005). TRPC1 is most
similar to the group consisting of TRPC4 and TRPC5 however, it is different in its ability to
form a non-conductive homotetramer (Ramsley et al., 2006; Clapham, 2005; Montell, 2005). The
second major group, composed of TRPC3, TRPC6 and TRPC7 are up to 78% identical, and can
form either homomeric or heteromeric complexes (Dietrich et al., 2003). The last and least
similar of the subfamily, TRPC2, codes for a pseudogene in humans and is only expressed in the
rodent vomeronasal organ.

3. Structure

The TRPC subfamily, similar to the other TRP subfamilies, consists of four subunits, each
with six-transmembrane spanning regions and a putative pore. The n-terminus and the c-terminus
are intracellular. Located on the intracellular c- terminal of all TRPC subunits is a conservative
TRP box, containing the sequence: EWKFAR (Clapham et al., 2002). Characteristic of TRPC
(and TRPV), is a section of the n-terminus that contains a series of three to four ankrin repeats (Clapham et al., 2002).

4. **Expression**

In recent years, there have been attempts by several groups to find out the distributional patterns of TRPC channel expression in both the CNS and PNS. Their findings are as follows: Sergeeva et al. (2003) reported that by using single-cell RT-PCR method on aminergic neurons, demonstrated that in Wistar rats, there exists a regional and cell-type specific TRPC channel subtype co-expressional pattern.

By using Taqman real-time quantitative PCR, Fowler et al. (2007), discovered that TRPC4 and TRPC5 are present at very high concentrations within the frontal cortex, dentate gyrus and pyramidal layer of the hippocampus. This group also reported that the distributional pattern varied depending on which layer of the prefrontal cortex was examined.

Zechal et al. (2007) discovered that by using in situ hybridization, Western blotting, and immunocytochemistry, there exists a spatial pattern of expression with TRPC4 mRNA and protein within the cerebellum, hippocampus, septum and cortex of the developing prenatal and postnatal rodent CNS.

5. **TRPC channel Activation**

Currently, the signal transduction pathway leading to the opening of TRPC channels, remain the topic of much debate. However, the two competing theories are that TRPC channels are either receptor-operated or calcium store-operated.
Figure 1. The complete TRPC subunit. All of the seven TRPC subunits contain six transmembrane spanning regions with intracellular N- and C-termini. Between the sixth and seventh transmembrane spanning regions is a putative pore. Located on the C-terminus is a conservative sequence referred to as a TRP box. Located on N-terminus is an ankrin binding domain.

Adapted from Clapham (2002)
a. Receptor activation

TRPC channel receptor activation is believed to occur via Gq activated pathway. Accordingly, the binding of a ligand with its G protein-coupled receptor, causes detachment and subsequent activation of once tethered G proteins: Gqα and Gqβ-Gqγ subunits. The activated Gq protein is then targeted to phospholipase C (PLC). The activated PLC, then hydrolyzes phosphotidylinositol 4, 5- bisphosphate (PIP$_2$) into inositol 1, 4, 5-triphosphate (IP$_3$) and diacylglycerol (DAG). IP$_3$ and DAG goes on to phosphorylate and thereby activate their respective targets.

i. Diacylglycerol

Diacylglycerol (DAG) is the product of phospholipase inositol biphosphate (PIP$_2$) hydrolysis by the phospholipase C (PLC) enzyme and it functions as a second messenger signaling lipid (Leung et al., 2008). After its activation, DAG remains attached to the plasma membrane while it activates its targets. One known target of DAG is protein kinase C (PKC) (Leung et al., 2008).

ii. Protein Kinase C

Protein kinase C (PKC) is a signal transduction enzyme that once activated by DAG, activates its target enzymes by phosphorylation (Mellor and Parker, 1998). The phosphorylation occurs on the hydroxyl groups of the serine or threonine residues (Mellor and Parker, 1998).

b. Store-operated activation

There have been propositions in favor of the role of TRPC proteins as store-operated calcium (SOC) channel candidates. This hypothesis remains highly controversial. However, proponents of this theory believe that this TRPC involvement in store-operated Ca$^{2+}$ entry (SOCE) is
dependent on the heteromeric combination of TRPC1 isoform with other TRPC isoforms (Salido et al., 2009).

6. TRPC1

The first mammalian TRPC channel to be discovered was TRPC1. Although its activation has yet to be fully understood, it is believed to require phospholipase C (Nilius et al., 2007). It was cloned by Craig Montell and Lutz Birnbaumer, while they were researching Drosophila TRP channel proteins (Wes et al., 1995). TRPC1 can be considered to belong to the group 2 of TRPC channels along with TRPC4 and TRPC5, while others consider it to belong in a class of its own (Nilius et al., 2007). To date, there have been few recent studies with the goal of understanding TRPC1 channels.

A recent report by Paez et al. (2011), mentioned a possible link between TRPC1 and the migration of oligodendrocyte precursor cells (OPC). They noticed after endogenous expression of TRPC1 channels had been ablated by the use of the small interfering RNA knockdown approach, the migration of the OPC would cease. They concluded that the calcium entry provided by TRPC1 was mandatory for migration.

Cheng et al. (2011) reported that Orai1 may be required to activate TRPC1. They further postulated that TRPC1 combines with Orai1 to form a distinct class of channels that they consider to be the long sought after store-operated calcium entry channels. By replacing extracellular calcium with 1 μM Gd³⁺, the TRPC1 plasma membrane insertion, triggered by binding with Orai1, was markedly reduced. The same results were obtained if Orai1 was knockdown by using small hairpin RNA interfering technology (Cheng et al., 2011).
7. **TRPC2**

The group 1 of TRPC channels is reserved for TRPC2 alone. TRPC2 has been reported to associate with TRPC6 (Chu et al., 2004). In rodents, TRPC2 is expressed in the vomeronasal area and is important in their ability to process pheromones (Clapham et al., 2005). Therefore, most recent studies on TRPC2 are focused on rodents. According to a study published by Flanagan et al. (2011), TRPC2 appears to be necessary for the pheromone-mediated uterine development in female mice. The group utilized both wild type and mutant (lacking a functional TRPC2 gene) BALB/cJ females. The study ended inconclusively with the inability to accurately identify the pheromones present in the urine of the male mice.

Zhao et al. (2011) released a report that examined the role of TRPC2 in the vomeronasal system of bats. By using 11 species belonging to a suborder of bats, called the Yinpterochiroptera, lacked a functional TRPC2 gene. While another 4 species of the suborder of bats, Yangochiroptera, may contain some TRPC2 functionality. This study also supports complexity in the lineage of TRPC2 as functional or non-functional.

8. **TRPC3**

TRPC3 is a member of the third group of TRPC channels along with TRPC6 and TRPC7. (Clapham et al., 2005). Group three is believed to be activated by phospholipase C receptors (Trebek et al., 2009). It has also been report that TRPC3 can be activated by an analog of diacylglycerol called 1-oleoyl-2-acetyl-sn-glycerol (OAG) (Trebek et al, 2009). TRPC3 is also stated to be inhibited by phorbol 12-myristate 13-acetate (PMA), indicating phosphorylation is
mandatory for TRPC3 activation (Trebak et al., 2009). The exact mechanism of signal transduction of TRPC3 is unknown.

Huang et al. (2011) sought-after to discover a possible link with TRPC3 and hypoxia-reoxygenation (H-R). They believed that TRPC3 is responsible for allowing a significant influx of calcium, as well as nitric oxide production which is critical for the regulation of endothelial cells. It was hypothesized by this group that the presence of H-R could compromise TRPC3 function. In order to test this theory, the group utilized patch-clamp, RT-PCR and western blot methods with primary cultured porcine endothelial cells under the influence of a TRPC 1-7 subunit inhibiting substance, SKF96365 (a potent blocker of low-voltage-activated T-type calcium channels), or PyR3, a TRPC3-specific inhibiting reagent and measured the effects. They found that the effects of the inhibitors, lead to a significant reduction in bradykinin-induced vasorelaxation of the cells which was similar to cells treated with H-R.

9. **TRPC4**

TRPC4 is a member of the second group of TRPC channels along with TRPC5 and possibly TRPC1 (Clapham et al., 2005). It is believed to be activated via the Gq-receptor pathway (Clapham et al., 2005). Both TRPC4 and TRPC5 are unique in part because they both contain PDZ motifs, which allows for regional protein-protein binding of the C-termini, and also in their ability to form either homomers and heteromers (Clapham et al., 2005). There have been few investigations on the molecular regulations of TRPC4 channels.

Wu et al. (2007) investigated the possible role that TRPC4 channels may have in neuronal development and cellular plasticity. By using primary cultures of dorsal root ganglia from rats,
they were able to monitor axonal regeneration and neurite outgrowth that were induced by adding dibutyryl-cAMP directly to the culture. They had measured TRPC1-7 mRNA during that process and discovered that TRPC4 mRNA was the only one that had significantly increased. They furthered discovered that once TRPC4 levels were reduced by using small hairpin RNA technology, the length by which the neuritis would extend was markedly diminished.

Crousillac et al. (2003) investigated the localization of TRPC4 and TRPC1 in the chicken retina. By using polyclonal antibodies raised against the mammalian TRPC1 and TRPC4 and injecting them into the retina of chickens, they were able to localize both TRPC1 and TRPC4 intensities to distinct layers in the retina. They concluded that the two subunits have distinct distributional expression patterns that are layer-specific.

10. TRPC5

TRPC5 is a TRPC subunit that is able to form either homomeric complexes or heteromeric complexes with TRPC1. This combination similarly allows for the permeation of cations. However, TRPC5 has recently been implicated in unique cell-type specific functions, some of which are discussed below.

TRPC5 has been recently shown to participate in cell motility and actin remodeling by combining with Rac1. Data presented by Tian et al. (2010), had shown that in opposition with TRPC6-RhoA complex, receptor activated TRPC5 can combine with Rac1, to allow for the selective permeation of Ca^{2+} ions sufficient for motility. The TRPC5-Rac1 complex works by directly releasing the TRPC6-RhoA complex cell mobility inhibitory effect (Tian et al., 2010).
Tai et al. (2010) had demonstrated electrophysiologically and pharmacologically, that in hippocampal tissue, the activation muscarinic receptors had the ability to enhance translocation of TRPC5 to the plasma membrane. This group had also noticed that increase of TRPC5 on the surface of hippocampal tissue was not accompanied by TRPC1 or TRPC4. The work was performed under the premise that TRPC5 had a significant role in generating non selective cation currents associated with after depolarization currents and plateau potentials.

Shi et al. (2010) studied the interactions between TRPC5, TRPC6 and angiotensin in native rabbit mesenteric artery vascular smooth muscle cells (2010). They reported that while applications of angiotensin II (100 nM) increased TRPC5 activity, it simultaneously decreased levels of TRPC6 activity. They concluded by this that TRPC5 and TRPC6 are antagonists to one another.

There is also supporting evidence that oxidative phosphates, 1-palmitoyl-2- glutaroyl-phosphatidylcholine (PGPC) and 1-palmitoyl-2-oxovaleroyl- phosphatidylcholine (POVPC), which are key players in inflammation, may also have a role in TRPC5 channel activation (Al-Shawaf, et al., 2010). Al-Shawaf, et al. reported that even micromolar concentrations of PGPC and POVPC has the effect of increasing intracellular calcium concentrations in HEK cells that are over-expressing TRPC5 channels, which subsequently had the effect of promoting migration in these cells. They stated that the oxidized phosphates had no effect on G protein-coupled receptors, but on the G(o/i) proteins themselves.

Grekka et al. (2003) reported that TRPC5 is important in axonal growth cone morphology and neurite outgrowth in the hippocampus. They concluded that TRPC5 is present in the hippocampal tissue of young rats and that it combines with statmin 2 to form a complex that are
trafficked in vesicles to the location of filopedia. They believed that the traditional role of TRPC5 in allowing for the receptor-mediated influx of calcium aids in its very important function in hippocampal tissue.

Flemming et al. (2006) tested whether activation of TRPC5 could be due to endogenous lysophospholipids. They believed that once a G protein-coupled receptor was activated, molecular players would simultaneously activate both lysophospholipids as well as G proteins and that it is the activation of the lysophospholipids, independently of the G proteins that leads to TRPC5 channel activation. They further concluded that TRPC5 contains a lysophospholipid-sensing region, and that it may be possible for TRPC5 to function by lipid-ionotropic mechanisms as well.

The pH of the extracellular environment is also reported to play a role in the regulation of TRPC5 channel activity. Semtner et al. (2007) reported that in the presence of La$^{3+}$ and Gd$^{3+}$, in concentrations that lower the pH of the extracellular medium from 7.4 to 6.5, had the effect of increasing the activity of G proteins leading to an increase in spontaneous TRPC5 currents upon stimulation of G protein-coupled receptors. Mutagenesis experiments revealed that two exposed glutamate residues on TRPC5 served as binding sites for the cations. They concluded that TRPC5 may have an additional role as a pH sensor which works in conjunction to its activation and governs its participation in cellular depolarizations.

Kim et al. (2008) tested the role of another suspected molecular player in TRPC5 regulation. They believed that phosphotidylinositol 4,5 biphosphate (PIP$_2$) is the molecule responsible for the rapid desensitization of TRPC5 channels after its activation by a G protein-coupled receptor.
It was also indicated that PIP_2 is unique in this ability after testing other phosphotidylinositides, including PI_{3,4}P_{2}, PI_{3,4,5}P_{3} and PI_{4}P, which all failed to elicit the same response.

11. TRPC6

TRPC6 belongs to the third group of TRPC channels along with TRPC3 and TRPC7 (Clapham et al., 2005). The members of the third group are said to share a 75% sequence homology (Clapham et al., 2005). They are also believed to have a higher preference for sodium over calcium (Hofmann et al., 2000). Very little is known about TRPC6, however, mutations of the TRPC6 gene has been linked with familial focal segmental glomerulosclerosis (Winn et al., 2005), psoriasis (Muller et al., 2008), as well as diabetes (Graham et al., 2011).

Graham et al. (2011) sought to understand the role of TRPC6, PKC and reactive oxygen species (ros) in a diabetic rat model as well as cultured mesangial cells. They discovered that when the glucose concentration is increased, the TRPC6 expression is decreased in response to that change. This effect was reversed by antioxidant treatment. Also, it was discovered that in the diabetic condition, there is a decrease of TRPC6 expression in the glomeruli, but not in the heart nor in the aorta. Graham et al also noticed that a PKC activator, phorbol 12-myristate 13-acetate (PMA), had the effect of diminishing TRPC6 expression. Taken together, the group concluded that the hyperglycemia that is commonly associated with diabetes has the effect of decreasing TRPC6 expression.

Muller et al. (2008) discovered a possible link to TRPC6 dysfunction and psoriasis. They sought to understand the role that calcium influx through TRPC6 homotetrameric channels had on cellular differentiation and proliferation. By using HaCaT cells and primary cultured human
keratinocytes as models, and using a TRPC6 activator, hyperforin, they were able study calcium influx through TRPC6 channels. They discovered that TRPC6 knockdown, diminished calcium influx and halted differentiation. This effect was reversed and enhanced by application of hyperforin.

Fuchs et al. (2011) discovered a connection between hypoxic pulmonary vasoconstriction (hpv) and TRPC6 channel regulation. The group used both wild type mice and a TRPC6-deficient mouse model and treated the ventilated lungs from the aforementioned mice with either DAG or phospholipase C inhibitors, R59949 and U73122, respectively. The results were that inhibition of DAG by R59949 had no effect, whereas inhibition of phospholipase C created suppression of cellular excitation.

Kinosita et al. (2010) investigated a link between TRPC6, cardiac hypertrophy and a natriuretic peptide, ANP. They discovered that ANP-induced phosphorylation of a single residue, threonine 69, on a TRPC6 subunit, had the effect of restricting TRPC6 activation in cardiac myocytes. In contrast, a substitution of threonine for alanine at the same site on TRPC6 created a resistance to this inhibitory effect.

12. TRPC7

TRPC7 belongs to the same group of TRPC channels as TRPC6 and TRPC3 (Clapham et al., 2005). It is believed to function through DAG-mediated activation (Clapham et al., 2005). Also, studies have shown that TRPC7 is selective for sodium over calcium (Clapham et al., 2005).

Vazquez et al. (2006) sought to identify the signal transducer involved in the regulation of TRPC7 channels in DT40 B lymphocytes. Prior work revealed that activation of TRPC occurs
through activation of inositol trisphosphate receptors (IP3R). While attempting to replicate the previous findings, they noticed that TRPC7 channels were not responsive to IP3R stimulation, but they were responsive to endogenous DAG stimulation, independently of IP3R.

D. TRPC channels in specific types of brain neurons

TRPC channels are present in neuronal types with functional and medical significance. However, prior to this study, specific information on the TRPC channel distribution patterns were missing. Here, we identified the distribution pattern of TRPC channel mRNAs that are expressed in these five neuronal types, cholinergic NB, dopaminergic SNPC, dopaminergic VTA, noradrenergic LC, and serotonergic DRN neurons, by using the single-cell RT-PCR method.

E. Nucleus basalis of Meynert (structure and function)

The nucleus basalis of Meynert, also referred to as the nucleus basalis (NB), consists of a group of neurons that are located in the substantia innominata of the basal forebrain (Bennett et al., 2005). Projections from this group of neurons have been found to widely innervate the neocortex and serve as a major supplier of acetylcholine.

Among the neuronal populations of the NB region, acetylcholine transferase and acetylcholinesterase immunocytochemistry have revealed that approximately 75% of magnocellular neurons (20-25 micrometers in diameter) are cholinergic (Nakajima et al., 1985). A trademark of Alzheimer’s disease is the degeneration of cholinergic neurons of the NB (Whitehouse et al., 1981). Research has shown that there is a substantial reduction within the neurons of the nucleus basalis with Alzheimer’s disease (AD) patients compared with the brains
of people that did not exhibit AD symptoms, but were of the same age and sex (Whitehouse et al., 1981).

**F. Substantia Nigra Pars Compacta (structure and function)**

The substantia nigra pars compacta (SNPC) resides within the midbrain and is easily located due to its identifiable darker, striped, melanin containing neurons (Francois et al., 1999). The SNPC is further subdivided into the ventral and dorsal regions which both receive inhibitory signals from the neighboring pars reticulata region (Francois et al., 1999). From there, the axons of the SNPC regions migrate along the nigrostriatal pathway, targeting the striatum where they release the neurotransmitter dopamine (DA) (Francois et al., 1999). Besides the striatum, other targets of the include other nuclei within the basal ganglia such as the substantia nigra pars reticulata, the subthalamic nucleus, the lateral pallidum, and the medial pallidum (Schultz, 1992).

Although it was previously assumed that the main function of the pars compacta was to participate in the movement process, it remains unknown as to the complexity of the SNPC actions. It is believed that the main function of the dopaminergic neurons, once activated by unknown stimuli, is to become involved in processes of learning and reward motivation (Schultz, 1992). This belief has led many researchers to study this region in order to understand the addictiveness of drugs of abuse and alcoholism. Among the currently studied is the loss of pigmented neurons in the SNPC region corresponding with the progression of Parkinson’s disease (Alasog et al., 2004).

**G. Ventral Tegmental Area (structure and function)**
The ventral tegmental area (VTA) is located in the midbrain, intertwined and indistinguishable from neighboring nuclei, giving neuroanatomists great difficulty in identifying this area from the substantia nigra (Olson and Nestler, 2007). Within the VTA, Oades and Halligay (1987), categorized four areas consisting of tyrosine hydroxylase-positive, dopaminergic neurons.

Projections from the VTA are very extensive. They include the prefrontal cortex, the insular cortex, the anteromedial striatum, the limbic cortex, the amygdala, the superior colliculus, the reticular formation, the periaqueductal gray, the thalamus and hypothalamus (Ikemoto, 2007). It is believed that the extensive interactions participate in functions such as cognition, emotions, motivation and drug addiction (Ikemoto, 2007).

**H. Locus Coeruleus (structure and function)**

The location of the locus coeruleus (LC) nucleus is in within the brain stem in the lateral floor of the forth ventricle (Ramos and Arnsten, 2007). In the 18th century, it was discovered by Felix Vicq-d’Azyr, due to its notable bluish tint within the dorsal wall of the rostral pons (Moulon et al., 1994). The locus coeruleus is said to be the prime site for synthesis of noradrenaline (NE) within the central nervous system (Moulon et al., 1994). It consists of medium sized neurons, most containing melatonin granules, that gives the nucleus its characteristic blue appearance (Moulon et al., 1994). The polymerization of the NE is responsible for the formation of these granules (Moulon et al., 1994). The approximate count of pigmented neurons within the LC for adult humans is between 22,000 and 51,000 cells (Moulon et al., 1994).
Projections from the locus coeruleus innervates the regions of the spinal cord, the brain stem, the thalamic relay system, the basal telencephalon, the hypothalamus, the cerebellum, as well as the cortex (Jasmin et al., 2003). Due to the excitatory effect of NE, the targets of the innervations from the LC generally become aroused. An example of this occurs during stress. According to Bernarrock (2009), once the LC becomes activated by stress, it responds by increasing secretion of NE, thereby activating targets in the cortex, resulting in increased motivation. Also excited in this stress pathway by NE, is the hypothalamic-pituitary adrenal cortex that reacts by secreting corticotropin-releasing factor from the hypothalamus that targets the adrenal cortex leading to secretion of cortisol (Bernarrock, 2009). Throughout this pathway, the NE from the LC will serve as a positive feedback mechanism to control its production (Bernarrock, 2009).

I. Dorsal Raphe Nucleus (structure and function)

The location of the dorsal raphe nucleus (DRN) is within the brainstem along the midline where it is considered to be the largest serotonergic nucleus in the CNS (Ma et al., 1991). It is divided into two subdivisions, rostral and caudal, that are then further divided (Janusonis et al., 1999). Oftentimes, located on the pre-synaptic terminal of the serotonergic (5-HT) neurons of the DRN are 5-HT1A autoreceptors in which selective serotonin reuptake inhibitor (SSRIs) antidepressants are thought to react upon (Hetrick et al., 2007).

Innervations from the DRN have been shown to target the lateral hypothalamus, amygdala, olfactory bulb as well as the caudate and putamen (Janusonis et al., 1999). In the lateral hypothalamus, the DRN serotonergic innervations have been demonstrated to be involved in the pathophysiology of narcolepsy. Wu et al. (2004) reported that since SE, which is secreted primarily by the DRN, are most active during the wake phase of the sleep-wake cycle, decreases
during the non-REM phase of sleep, and nearly ceases during REM sleep, there could be a disruption of SE secretion. It was discovered by this group that during cataplexy, where the person is aware of the environment, but all muscle tone is lost, the DRN-SE neurons did not stop firing, and contrarily, increased even at levels higher than what is typically seen in REM sleep.

**J. Corticotropin-releasing factor**

Corticotropin-releasing factor (CRF) is a 41- amino acid residue single chain peptide that is derived from a 191-amino acid preprohormone (Bayatti et al., 2003). CRF functions as both a neurotransmitter as well as a hormone. In the cerebral cortex, CRF-positive cells have been shown to be important in cognitive processing; consequently, dysfunction of these cells may contribute to CNS disorders (Bayatti et al., 2003).

1. **CRF production in the CNS**

Morphological data has shown that the major site of CRF synthesis resides in the paraventricular nucleus of the hypothalamus, although, CRF-containing neurons have been found throughout the central nervous system (Navarro-Zaragoza et al., 2010). The extrahypothalamic expression of CRF has also been found in cell groups within the olfactory bulb as well as the Barrington’s nucleus (Liaw et al., 1997). In the progression of Alzheimer’s disease, the concentration of CRF in the cerebrospinal has been found to markedly decrease (Bayatti et al., 2003).

2. **CRF receptors in the CNS**

The central effects of CRF are mediated through two receptors, the higher affinity binding corticotropin-releasing hormone receptor 1 (CRHR1) and the lower-affinity binding
corticotropin-releasing hormone receptor 2 (CRHR2). The two receptors are encoded by a different gene and have an approximate 70% sequence identity (Liaw et al., 1997). In the progression of Alzheimer’s disease, it has been found that the levels of CRF receptors were markedly increased on the remaining neurons (Bayatti et al., 2003), possibly to compensate for the decrease in available CRF.

**a. Corticotropin-releasing hormone receptor 1 (CRHR1)**

CRHR1 is a G-protein coupled receptor that binds CRF with between 10 to 100 fold greater affinity than with CRHR2. It is thought that CRHR1 activation evokes the Gq activation pathway and subsequent activation of PKC. However, it has been also linked with other intracellular pathways including adenylate cyclase c-AMP-protein kinase A and MAPK (Bayatti et al., 2003). The binding of CRF to CRHR1 has been found to elicit a neuroprotective effect against oxidative stress in the CNS (Bayatti et al., 2003). Interestingly, in AD affected brains, the levels of CRHR1 remains constant in the minimally affected cerebellum (Bayatti et al., 2003).

**b. Corticotropin-releasing hormone receptor 2 (CRHR2)**

CRHR2 is also G protein coupled receptor, but differs from CRHR1 by having both a lower ligand binding affinity and weaker cortical and hippocampal expression (Bayatti et al., 2003). A distinguishing feature of CRHR2 seems to be its involvement in regulating appetite by somehow prolonging the feeding suppression (Coste et al., 2000).
II. MATERIALS AND METHODS

A. Primary cultured neurons of five physiologically relevant brain regions isolated from postnatal rat brains.

The forebrains (for nucleus basalis (NB)) and brainstems (for locus coeruleus (LC), dorsal raphe nucleus (DRN), substantia nigra pars compacta (SNPC) and ventral tegmental area (VTA)) of 2-3 day old Long Evans rats (Charles Rivers) were removed (approved by the animal care committee of the University of Illinois at Chicago). The forebrains and brainstems were immersed in an oxygenated ice-cold salt solution consisting of (in mM) NaCl, 130; KCl, 4.5; CaCl$_2$, 2; glucose, 33; and piperazine-$N,N'$-bis [2-ethanesulfonic acid] (PIPES), 5 buffer (pH 7.4). They were embedded in 3.5% agar in the salt solution and sliced into a thickness of approximately 400 micrometers with a vibratome (Lancer 1000). The regions of the NB, VTA, SNPC, LC and DRN were dissected under a dissecting microscope by using hypodermic needles.

For the single-cell RT-PCR experiments, the fragments of tissue were incubated with 12 units/mL of papain (Worthington Biochemical Corporation), at 37°C, 10% CO$_2$/90% carbogen, for 20 minutes. For electrophysiological experiments, the tissue fragments were treated with 0.25% trypsin in Ca$^{2+}$–, Mg$^{2+}$–free balanced salt solution (GIBCO), in place of papain, at 37°C, 10% CO$_2$/90% carbogen, for 15 minutes. The dissociated tissue fragments were then washed with modified Eagle’s minimum essential culture medium with Earle’s salt (MEM) (GIBCO) with 2% rat serum and 10% horse serum (collectively termed 2R10H) and dissociated using a fire-polished Pasteur pipette. The dissociated neurons were plated onto a feeder layer composed of glia cells that were previously plated on Aclar fluorohalocarbon plastics (Allied Fibers and Plastics, Morristown, NJ) treated with rat tail collagen. The glia, consisting of mostly astroglia,
were used as a feeder layer, was previously treated with 5-fluoro-2'-deoxyuridine (15 µg/ml) and uridine (35µg/ml) for two days in order to suppress proliferation of the glia. Finally, 2R10H conditioned medium (incubated overnight with glia and filtered) was added to the newly plated neuron cultures three hours after they were added to the feeder layer (Nakajima and Masuko, 1996). The cultures remained at 37°C, 10% CO₂/90% air until use.

B. **HEK293A cell line culture and transfection**

Human embryonic kidney 293A (HEK293A) cells were used to study the isolated effects of corticotropin-releasing factor receptor activation on TRPC5 opening. HEK cells were ideal for TRPC channel investigation because of their very low endogenous expression of these channel types. One day prior to transfection, 40,000 cells (purchased from Qiagen, Germantown, MD) were plated onto a 6 cm culture dish in DMEM medium (Invitrogen –GIBCO (Carlsbed, CA) with 10% fetal bovine serum (Invitrogen – GIBCO, Carlsbed, CA). The cells were stored at 37°C, 5% CO₂/95% carbogen and saturated humidity.

On the day of transfection, Effectene (Qiagen, Germantown, MD) was used as an introductory chemical for cDNA entry consisting of 0.2 micrograms of human corticotropin-releasing hormone receptor 1 (in a pCMV5 expression vector), 0.6 µg of human TRPC5 (in a pCMV5 expression vector) and 0.1 µg of n-terminal conjugated green fluorescent protein, into the plated HEK cells (~4 x 10⁴ cells) contained on a 6 cm culture dish. As a negative control, pCMV5 (0.6 micrograms) was substituted for TRPC5.

One day after transfection, the cells were replated onto 3.5 cm dishes and electrophysiological experiments were performed on the following day. GFP–positive cells, with
moderate fluorescent intensity, were chosen as an indicator of the cells also being concurrently transfected with CRHR1 and TRPC5 cDNAs.

C. Electrophysiological method whole-cell voltage-clamp method

Electrophysiological experiments were performed at room temperature while the cells were bathed in an external solution consisting of (in mM): NaCl, 141; KCl, 4; CaCl, 1; MgCl, 1.3; D-glucose, 11; TTX (Worthington Biochemical), 0.0005; and Na$_2$HEPES, 5 (pH 7.4). The cells were perfused for 90 seconds before the application of CRF in an external solution consisting of (in mM): CsCl, 10; NaCl, 141; MgCl$_2$, 1.3; D-glucose, 11; Na$_2$HEPES, 5; CaCl$_2$, 2.4 (pH 7.4) (collectively termed TRP solution). Corticotropin-releasing factor (CRF) (Peptides International, Louisville, KY) was made consisting of a stock solution of 1 mM. The 2 µM working solution was made by further diluting the CRF stock solution with the TRP solution. A 1 µM stock solution of Bisindolylmailemide I (BIS) (Peptides International, Louisville, KY) was used for both nucleus basalis and HEK cell experiments. For the BIS experiments, the cells were incubated at room temperature for between 15-20 minutes prior to recording. The solution inside of the patch pipette contained (in mM): D-gluconate, 141; NaCl, 10; KHEPES, 5; K$_2$EGTA, 0.5; CaCl$_2$, 0.1; MgCl, 4; Na$_2$ATP, 3; and GTP, 0.2 (pH 7.2). CRF was bath perfused (ALA Scientific Instruments, Westbury, NY).

The cells were clamped at -78 mV to measure whole-cell capacitance and series resistance. The electrode was clamped at -65 mV. The liquid junction potential was measured as -13 mV. Membrane conductance was monitored using recurrent voltages of 20 mV/100 ms followed by 50 mV/100 ms command potentials relative to the holding potential.
Nucleus basalis cells were chosen based on their magnocellular morphology with a diameter of 20 micrometers or larger (Nakajima et al., 1985). NB cultures were maintained for 17 – 42 days prior to recording. HEK cells were used 1 day post-transfection and preference was for larger sized cells.

D. Statistical Analysis

A one-tailed Student’s t-test was use to measure statistical significance for electrophysiology data. The statistical values were shown as mean +/- S.E.M.

E. RT-PCR analysis

In order to understand the distributional pattern of TRPC isoform mRNAs representing five medically relevant brain regions, we have cultured, harvested and analyzed the expression patterns of the cells cultured from rat brain. The RT–PCR procedure consisted of the aspiration of cellular contents of a single neuron using a whole-cell configuration in an electrophysiological setting into a patch pipette containing (in mM): KHEPES, 5 (pH 7.2); KCL, 149; MgCl₂, 3; and K₂EGTA, 5, at a volume of 8 µL. The electrophysiological set-up was used both to visualize the cell and to test the membrane integrity. The cytoplasm/pipette contents were then emptied into 7 µL solution with 20 units of RNasin (Promega, Madison, WI) and 50 µg of random hexamer primers (Roche, Indianapolis, IN). To denature the RNA, the samples were treated to 70°C for 10 m. Afterwards, 10 m of 25°C was used to anneal the primers.

The primers for this experiment were designed by Dr. Takeharu Kawano and are shown in table 1. The primers for the cell-type markers were: ChAT to identify cholinergic neurons in the
NB, TPH primers to identify the serotonergic neurons of the DRN, TH to identify the
dopaminergic neurons of the SNPC and the VTA as well as the noradrenergic neurons of the LC.

The TRPC primers designed by Dr. Kawano were constructed to accommodate the splice
variants which are present for all TRPC subtypes (Hofmann et al., 1999; Saura and Ashcroft,
1997; Satoh et al., 2002; Schaefer et al., 2002; Vannier et al., 1999; Walker et al., 2001; Xie et
al., 2007; Yan and Surmeier, 1996; Yildirim et al., 2005; and Zhang and Saffen, 2001).
Therefore, due to the conserved sequences of the variants being targeted, only one PCR product
was obtained for each TRPC subtype primer, with an exception for TRPC6. In the case of
TRPC6, a set of primers produced three different PCR products, a 425 bp product for TRPC6A
or TRPC6B and a 221 bp product for TRPC6C.

The first strand of cDNA was synthesized at 42°C for 50 m in a solution containing (in mM):
Tris-HCl, 50 (pH 8.3); KCl, 75; MgCl₂, 3; each dNTPs, 0.5; DTT, 1; and SuperScript TM II
RNase H reverse transcriptase (Invitrogen, Carlsbad, CA), 200 units. Termination of the reaction
was done by heating the sample at 70°C for 15 minutes. RNase H (Invitrogen, Carlsbad, CA)
was used at 2 units to eliminate the RNA of the 20 μL final sample.

For the first round multiplex PCR, 20 μL of reverse transcription product taken from each
neuron and added into a PCR tube containing (in mM) Tris-HCl, 10 (pH 9.5); KCl, 50; MgCl₂,
2.5; dNTPs, 0.2 each; the set of primer pairs, and Taq-polymerase or GoTaq Flexi DNA
Polymerase (Promega, Madison, WI) (total volume, 100 μL), 2.5 units. Each primer pair consists
of 100 pmol of outer forward and reverse primers. The following cycling protocol was conducted
in the multiplex PCR: 94°C for 1 min, 45 cycles (94°C, 30 sec; 58°C, 1 min; 72°C, 2 min), and
72°C for 7 min.
The second round nested PCR was performed separately for each gene type. For each reaction, 2 µl of the first PCR product was added to a tube containing a specific primer pair (20 pmol) and PCR Master Mix (Promega, Madison, WI) or GoTaq Green Master Mix (Promega, Madison, WI) (total volume, 50 µl). The following cycling protocol was conducted in the multiplex PCR: 94°C for 1 min, 40 cycles (94°C, 30 sec; 58°C, 1 min; 72°C, 2 min), and 72°C for 7 min. Five µl aliquots of the PCR products were separated and visualized in ethidium bromide-stained, 1.5% agarose gels by electrophoresis.

An aspirated neuronal content was also processed without reverse transcriptase for PCR in order to confirm that genomic DNA contamination did not occur with our primers. This test consisted of three DRN, two LC, three NB, three SNPC, and four VTA neurons, in which nothing was detected.
Table I. TRPC subtype primer sequences; listed from 5’ to 3’. Genebank’s accession numbers are indicated below the gene names. There are two TRPC6 PCR products: TRPC6A/B and TRPC6C.
<table>
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<th>Gene</th>
<th>Length</th>
<th>PCR</th>
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<tr>
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<td></td>
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<tr>
<td>TH</td>
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III. INVESTIGATIONS OF THE TYPES OF TRPC CHANNELS EXPRESSED IN CHOLINERGIC, SEROTONERGIC, DOPAMINERGIC, AND NORADRENERGIC NEURONS IN THE RAT BRAIN

A. Introduction

Here, we have reported a simultaneous examination of all seven subtypes of the TRPC channel mRNAs in cholinergic (ChAT-positive) neurons in the nucleus basalis and in dopaminergic (th–positive) neurons in the substantia nigra pars compacta. Also examined, is the expression of seven subtypes of TRPC mRNA in dopaminergic neurons in the VTA, in noradrenergic neurons in the LC, and serotonergic neurons in the DRN. The known roles of these neuronal groups are as follows: The cholinergic neurons of the NB are important for memory and cognition and their deterioration has been linked with Alzheimer’s disease (Zaborszky et al., 2010). The dopaminergic neurons of the VTA are important in motivation and addictive behaviors (Zhang et al., 2010). The dopaminergic neurons of the SNPC have been implicated in movement and their dysfunction has been linked to Parkinson’s disease (Bajaj et al., 2010). The noradrenergic neurons of the LC are important for arousal in the sleep/wake cycle (Carter et al., 2010). The serotonergic neurons of the DRN have been implicated in the pathology of depression (Boldrini et al., 2005). Before this study, our laboratory had been investigating some of these neuronal types electrophysiologically by using neurotransmitters to evoke TRP-like non-selectively cation channels. Yet, precise information was lacking about the types of TRPC channels present in the above-described functionally important neurons.
B. RT-PCR results for cholinergic NB neurons

Figure 2 shows the RT-PCR results of cholinergic neurons of the NB which was performed on cells 13-33 days after culture. The marker gene for identifying cholinergic neurons was choline acetyltransferase (ChAT). Figure 2B shows that mRNAs for TRPC1, TRPC3, TRPC4, TRPC5 and TRPC7 were detected in over 80% of the 30 cholinergic neurons tested. While only 23.3% or seven cholinergic neurons expressed TRPC6 mRNA. Out of those seven that expressed TRPC6, none of them had TRPC6C mRNA. None of the cholinergic neurons expressed TRPC2 mRNA. The CH-NB mRNA frequencies for TRPC1, 3, 4, 5, 6 and 7 remain relatively constant over time spent in culture (supplemental data S1).

Figure 2. Expression of TRPC subtype mRNAs in cholinergic NB neurons detected by single-cell RT-PCR. ChAT positivity was used to identify cholinergic neurons. A) Six representative neurons out of 30 ChAT-positive neurons are shown. PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide staining. (B) Summary of single-cell RT-PCR experiments of 30 cholinergic neurons. “[+]” represents that the gene of interest was positive and “[-]” represents negative.
C. RT-PCR results for the dopaminergic SPNC neurons

In figures 3A and 3B, the RT-PCR results from dopaminergic neurons that were cultured for 16-23 days from the SNPC are shown. The gene marker to identify the 39 dopaminergic neurons is tyrosine hydroxylase (TH). TRPC1 was found most frequently at 71.8% of cells among the 39 dopaminergic SNPC neurons. The second most frequently found was TRPC6 at 46.2%. Out of the 46.2% of cells that contained TRPC6 mRNA, all of them had TRPC6A and/or TRPC6B mRNA, but only one had TRPC6C mRNA. TRPC3 mRNA was found in 6 (15.4%) cells. In 5 cells (12.8%), TRPC7 mRNA was present. None of the dopaminergic neurons of the SNPC contained TRPC2 mRNA. The TRPC mRNA frequencies fluctuate only slightly during the time in culture for DA-SNPC neurons (supplemental data S2).
Figure 3. Expression of TRPC subtype mRNAs in dopaminergic SNPC neurons detected by single-cell RT-PCR. TH positivity was used to identify dopaminergic neurons. (A) Six representative results out of 39 TH-positive neurons are shown. (B) Summary of single-cell RT-PCR experiments on 39 dopaminergic neurons.
D. RT-PCR results for dopaminergic VTA neurons

Figures 4A and 4B contains the RT-PCR data from 33 VTA dopaminergic neurons (tyrosine hydroxylase-positive) that were cultured for 22-37 days. The most frequently found TRPC mRNA was TRPC1 at 97% (32 cells). TRPC5 mRNA was detected in 66.7% (22) cells. In 19 cells (57.6%), TRPC6 was present, two of which contained TRPC6C. Only 4 cells (12.1%) had TRPC4. TRPC3 was found in only 3 cells (9.1%). One cell (3.0%) had TRPC7 mRNA. TRPC2 was not present in any of the dopaminergic cells examined. TRPC mRNA expression in DA-VTA neurons undergo slight fluctuations during the time in culture (supplemental data S3).

![Figure 4](image)

Figure 4. Expression of TRPC subtype mRNAs in dopaminergic VTA neurons detected by single-cell RT-PCR. TH positivity was used to identify dopaminergic neurons. (A) Six representative results out of 33 TH-positive neurons are shown. (B) Summary of single-cell RT-PCR experiments on 33 dopaminergic neurons.
E. RT-PCR results for noradrenergic neurons of LC

Figures 5A and 5B illustrate the single-cell RT-PCR results for the 35 TH-positive noradrenergic neurons of the LC, which was cultured for 16-25 days. The TRPC mRNA that was found most frequently was TRPC1 at 94.3% (33 cells), and TRPC7 at 80% (28 cells). TRPC3 mRNA was found in 34.3% (12 cells). The same percentage, 34.3%, was found for TRPC6 cells; all of the 12 cells had TRPC6A and/or TRPC6B, only one had TRPC6C mRNA. To a lesser extent, were TRPC4 (6 cells, 17.4%) and TRPC5 (3 cells, 8.6%) mRNA containing cells. TRPC2 mRNA was not present in any of the cells tested. The TRPC mRNA expression in NE-LC neurons changes very slightly during the time in culture (supplemental data S4).
Figure 5. Expression of TRPC subtype mRNAs in noradrenergic LC neurons detected by single-cell RT-PCR. TH positivity was used to identify noradrenergic neurons. (A) Six representative results out of 35 TH-positive neurons are shown. (B). Summary of single-cell RT-PCR experiments on 35 noradrenergic neurons.
F. RT-PCR results for the serotonergic DRN neurons. Shown in figures 6A and 6B, is the data obtained from single-cell RT-PCR on 31 tryptophan hydroxylase-positive serotonergic neurons of the dorsal raphe nucleus. This group of neurons was cultured for 18-25 days prior to analysis. The most frequently detected TRPC mRNA was TRPC1 (21 cells, 67.7%). Also, TRPC5 was frequent at 58.1% (18 cells). TRPC7 was found in 10 cells (32.3%). TRPC3 was detected in 5 cells (16.1%). Only one cell contained TRPC4 (3.2%). TRPC6A and/or TRPC6B mRNA was found in only one cell (3.2%). Neither TRPC6C nor TRPC2 mRNA were found in any of the cells tested. The TRPC mRNA expression in SE-DRN neurons changes very slightly during the time in culture (supplemental data S5).

Figure 6. Expression of TRPC subtype mRNAs in serotonergic DRN neurons detected by single cell RT-PCR. TPH positivity was used to identify serotonergic neurons. (A) Six representative data out of 31 TPH-positive neurons are shown. (B) Summary of single-cell RT-PCR data on 31 serotonergic neurons.
G. Summary of RT-PCR results on TRPC subtypes by brain neuronal types

Shown in Figure 7 is a summary of the distribution of TRPC subtypes among various brain neurons. The omission of TRPC2 was due to it not being present in any of the samples tested. The cholinergic neurons of the NB (CH-NB) contained an extremely high frequency of expression for all TRPC subtypes, at over 80%, with the exception of TRPC6. In comparison, the dopaminergic neurons of the SNPC (DA-SNPC) had a fairly sparse TRPC subtype expression. Among the DA-SNPC neurons, TRPC1 mRNA was expressed at the highest frequency (71.8%), followed by TRPC6 (46.2%), while TRPCs 3, 4, 5 and 7 were at frequencies of less than 23.1%. In the dopaminergic neurons of the VTA (DA-VTA), the most frequently found TRPC mRNAs were TRPC1 (97.0%), TRPC5 (57.6%) and TRPC6 (57.6%). In the noradrenergic neurons of the LC (NE-LC), the most frequently expressed TRPC mRNAs were TRPC1 (94.3%) and TRPC7 (80.0%). In the serotonergic neurons of the DRN (SE-DRN), the most frequently found TRPC mRNAs were TRPC1 (67.7%), TRPC5 (58.1%), and TRPC7 (32.3%).

Figure 7. Summary histogram showing the single-cell RT-PCR results by brain neuronal types. CH-NB, cholinergic neurons in the NB; DA-SNPC, dopaminergic neurons in the SNPC; DA-VTA, dopaminergic neurons in the VTA; NE-LC, noradrenergic neurons in the LC; and SE-DRN, serotonergic neurons in the DRN.
H. RT-PCR summary of the frequencies of TRPC subtypes among five different neuronal types

Figure 8 illustrates the distributional frequency of TRPC mRNA subtypes among the different neuronal types. The most frequently expressed TRPC subtype in most neuronal types tested was TRPC1. The DA-VTA contained the most abundant expression of TRPC1 mRNA (97.0%). In contrast, the SE-DRN contained the most sparsed TRPC1 expression (67.7%). None of the cell types tested contained TRPC2 mRNA, therefore TRPC2 mRNA section was omitted. Also, while TRPC3 mRNA was discovered in over 80% of CH-NB cells tested, it was discovered at a frequency of less than 34.3% for the other four neuronal types. The CH-NB cells also contained the greatest frequency of TRPC5 mRNA (90.0%) in contrast to the rest of the neuronal types at less than 67%. TRPC4 mRNA was also found at a higher frequency in CH-NB cells, at 83.3%, in comparison to the other types at less than 17.1%. The dopaminergic neurons of both the VTA and the SNPC contained the greatest frequency of TRPC6 mRNA. In contrast, SE-DRN cells contained the TRPC6 mRNA more sparsed. The TRPC7 mRNA subtype was found at a high frequency of 80% for both CH-NB and NE-LC.

Figure 8. Summary histogram showing the single-cell RT-PCR data according to TRPC subtypes. CH-NB, cholinergic neurons in the NB; DA-SNPC, dopaminergic neurons in the SNPC; DA-VTA, dopaminergic neurons in the VTA; NE-LC, noradrenergic neurons in the LC; and SE-DRN, serotonergic neurons in the DRN.
I. **Discussion**

Using single-cell RT-PCR, we were able to determine the expression pattern of seven TRPC channel mRNAs in five types of physiologically relevant brain neurons. We have demonstrated in these data that for each type of neuron, there is a different pattern of TRPC mRNA expression. The data presented is the first report of examination of all seven subtypes of TRPC mRNA with CHAT mRNA in NB cholinergic neurons. It is also the first account of the seven TRPC subtypes being investigated with TH mRNA in the SNPC. Also reported here, is the investigation of the seven TRPC subtypes present in VTA dopaminergic neurons, LC noradrenergic neurons and DRN serotonergic neurons.

We discovered that TRPC1 mRNA was present at the highest frequency in four out of the five neuronal types tested. It has been reported that TRPC1 alone as a homotetramer produces no current (Strubing et al., 2001). Only as a heterotetramer with either TRPC4 or TRPC5 does it produce a functional current (Strubing et al., 2001). Supporting this report, we found TRPC4 and TRPC5 also at very high frequencies in all neuronal types. We also investigated the three splice variants of TRPC6, which are TRPC6A, TRPCB and TRPC6C. In the present investigation, we found that only a small number of the cells that we tested contained TRPC6C mRNA, all of which were TH-positive cells. However, all of the five neuronal types contained TRPC6A and/or TRPC6B at some variations.

The discovery that I found to be most interesting, was that the highest frequencies of TRPC channel mRNA expression was found in the cholinergic neurons of the NB. TRPC1, TRPC3, TRPC4, TRPC5 and TRPC7 mRNAs were found at frequencies of 80% or higher in this neuronal group. Even more unusual about this particular group of neurons was that the
cholinergic neurons of the NB were the only group found to have a TRPC subtype other than TRPC1 as its predominant TRPC isoform expressed, TRPC5. Therefore, the remainder of my project was devoted to studying the cholinergic neurons of the NB as well as TRPC5.
IV. SIGNAL TRANSDUCTION MECHANISM OF CRF EFFECTS

A. Introduction

The exact molecular elements involved in the signal transduction mechanism of TRPC channel opening remain unknown. Currently, there are two theories pertaining to TRPC channels, which may or may not be mutually exclusive. The first theory suggests TRPC channels are the long sought-after channels responsible for the regulation of intracellular calcium storage (Clapham, 2005; Venkatachalam, 2007). The second theory states TRPC channels function via receptor operated activation (Clapham, 2005; Venkatachalam, 2007). We found the second theory, of receptor operated activations, would explain the experimental findings very well. The work presented here utilized the activation of a G protein-coupled receptor and the effects this had on non-selective (TRP) channels in the nucleus basalis.

Here, we investigated the signal transduction mechanism of TRPC channels in the cholinergic neurons of the nucleus basalis. This group of neurons was chosen based on their unusually high occurrence of TRPC channel mRNAs (more than 80%), with the exception of TRPC2 and TRPC6, a finding shown in part 1 above. Based on previous work (Floreani et al., 2008), we hypothesized the signal transducer PKC, was involved in the activation of non selective cation channels. Floreani et al. had shown that activation of the neurotensin receptor leads to TRPC6 conductance increase in transfected Chinese hamster ovary (CHO-K1) cells. Treatment with a PKC inhibitor, bisindolylmaleimide I (BIS), at 3\(\mu\)M for 20 minutes was sufficient to abolish TRPC6 activation (Floreani et al., 2008). Therefore, I have studied the possible role of PKC as a signal transducer of the CRF effects on non-selective cation TRP-like
activation in NB cholinergic neurons. Corticotropin-releasing factor (CRF) was used in this research as the excitatory neurotransmitter.

**B. CRF effects on cholinergic neurons of the NB and their signal transduction**

The primary cultures of rat nucleus basalis were maintained for 34–60 days. The cells were clamped at -78 mV to measure whole-cell capacitance and series resistance. The electrode was clamped at -65 mV. The liquid junction potential was measured as -13 mV. Membrane conductance was monitored using recurrent voltages of 20 mV/100 ms followed by 50 mV/100 ms command potentials relative to the holding potential. Figure 9A shows an inward current induced by a 10 sec application of 2 μM CRF, 180.1 +/- 37.1 pA/pF. Based on controls which suppressed sodium and potassium channels, we believe that this current is from non-selective cation (possibly TRPC) channel activation. Figure 9B shows that in the presence of 1 μM PKC inhibiting BIS, a PKC inhibitor, CRF has almost no effect on cholinergic neurons of the nucleus basalis, 15.7 +/- 4.8 pA/pF. Statistical values are expressed as mean +/- S.E.M.

The PKC inhibitor, Bisindolylmaleimide I (BIS), works by competing with ATP for binding on PKC (Toullec et al., 1991). The inability of ATP to bind with PKC prevents the ability of PKC to phosphorylate its target substrates (Toullec et al., 1991).
Figure 9. BIS inhibits CRF-induced activation of TRPC-like conductance in NB cholinergic neurons. The cells were clamped at -78 mV to measure whole-cell capacitance and series resistance. The electrode was clamped at -65 mV. The liquid junction potential was measured as -13 mV. Membrane conductance was monitored using recurrent voltages of 20 mV/100 ms followed by -50 mV/100 ms command potentials relative to the holding potential. A (control): Response of NB cholinergic neurons to 2 μM CRF. CRF induced a peak TRPC-like conductance of 180.1 +/- 37.1 pA/pF (n=11). B (BIS): Incubation of the cells with 1 μM BIS for 15-20 min inhibited the CRF-induced conductance, 15.7 +/- 4.8 pA/pF (n=11). C: A graphical depiction of the mean +/- S.E.M. Student’s t-test analysis was statistically significant *p=0.000139). Arrowheads indicate the 0 current level.
C. CRF effect on HEK293A cells transfected with TRPC5 and CRHR1 cDNAs and PKC inhibition

The administration of 2 μM CRF for 10 seconds was sufficient to evoke an inward current, 295.5 +/- 56.0 pA/pF (n= 11), in a HEK cell that was transfected with corticotropin-releasing hormone receptor 1 CRHR1(0.2 μM), TRPC5 (0.6 μM) and GFP (0.1 μM) cDNAs (figure 10A). In figure 10B, incubation of the PKC inhibitor, Bis at 1 μM was sufficient to inhibit the depolarizing current evoked by 2 μM CRF application, 17.0 +/- 9.3 pA/pF (n=11). Based on controls which suppressed any endogenous sodium and potassium channels, we believe that this current is from transfected TRPC5 channel activation.
Figure 10. BIS inhibits CRF-induced activation of TRPC5 in HEK293A cells. HEK293A cells were transfected with CRHR1 receptor (0.2 μg), TRPC5 (0.6 μg), and GFP (0.1 μg) in a dish 6 cm in diameter. The cells were clamped at -78 mV to measure whole-cell capacitance and series resistance. The electrode was clamped at -65 mV. The liquid junction potential was measured as -13 mV. Membrane conductance was monitored using recurrent voltages of 20 mV/100 ms followed by 50 mV/100 ms command potentials relative to the holding potential. Statistical values are expressed as mean +/- S.E.M. Command voltages of +20 mV/100 ms followed by -50 mV/100 ms were applied. A (control): TRPC5 response to CRF (2 μM). CRF elicited a peak TRPC5 conductance of 295.5 +/- 56.0 pA/pF (n=11). B (BIS): Incubation of the cells with 1 μM BIS for 15-20 min inhibited the CRF-induced conductance, 17.0 +/- 9.3 pA/pF (n=11). C: A graphical depiction of the mean +/- S.E.M. Student’s t-test analysis was statistically significant (*p= 0.000043). Arrowheads indicate the 0 current level.
D. **Negative Control**

In order to make certain that the current that was being measuring is indeed that of TRPC5, we simultaneously prepared two sets of transfected cells. The first set consisted of CRHR1 (0.2 μM), TRPC5 (0.6 μM), and GFP (0.1 μM) cDNAs in 6 cm culture dishes. The second set contained cDNAs of CRHR1 (0.2 μM), pCMV5 (0.6 μM) and GFP (0.1 μM) in 6 cm culture dishes. The TRPC5 cDNA that is used for HEK cell experiments had previously been inserted into pCMV5 vectors. The cells that had been transfected with the TRPC5 insert responded to 2 μM CRF, 240.1 +/- 50 pA/pF (n=9). In contrast, there was almost no response, 9.7 +/- 5.8 pA/pF (n=9), from the cells transfected with pCMV5 vector without the TRPC5 insert to 2 μM CRF. An explanation for the negligible response to CRF from the empty vector group is that HEK293A cells could contain a small amount of endogenous TRPC channels.
Figure 11. Cells transfected with empty vector, without the presence of TRPC5, do not respond to CRF. HEK293A cells were transfected with corticotropin-releasing hormone receptor 1 (CRHR1) (0.2 μg), GFP (0.1 μg), and either TRPC5 (0.6 μg) or pCMV5 (empty vector) (0.6 μg) in a 6 cm dish. The cells were clamped at -78 mV to measure whole-cell capacitance and series resistance. The electrode was clamped at -65 mV. The liquid junction potential was measured as -13 mV. Membrane conductance was monitored using recurrent voltages of 20 mV/100 ms followed by 50 mV/100 ms command potentials relative to the holding potential. A (TRPC5): response to CRF (2μM). CRF elicited a peak TRPC5 conductance of 240.1 +/- 50.2 pA/pF (n=9). B (empty vector): Elimination of heterologous induction of TRPC5 expression almost completely eliminated the CRF-induced conductance, 9.7 +/- 5.8 pA/pF (n=9). C: A graphical depiction of the mean +/- S.E.M. Student’s t-test analysis was statistically significant (*p= 0.000161). Arrowheads indicate the 0 current level.
E. Discussion

The above-described data provide supporting evidence for the involvement of PKC as the signal transducer involved in non selective cation (TRPC) channel regulation. Bisindolylmaleimide I (BIS) is a potent and selective PKC inhibitor, with a structure similar to staurosporine (Toullec et al., 1991). The absence of current by BIS (Figure 10), suggests that blocking PKC phosphorylation prevents channel activation. The conductance induced by CRF seemed to evoke similar responses from both TRPC5 transfected HEK293A cells and the non-selective cation (TRP) containing nucleus basalis neurons.

Characterizing the mechanisms by which TRPC channels function has a significant impact on understanding the signaling pathway in the brain. The reasoning is that TRPC channels are present extensively throughout the central nervous system. Data presented early in this work has shown that brain regions with very diverse functions contain region-specific TRPC channel isoforms. The latter portion of this works investigated the mechanism on how TRPC channels are activated by identifying the signal transducer that appeared critical to TRPC channel activation. Perhaps discovering more on this pathway can lead to the development of treatments that can target signal transducers to serve as therapeutic agents for diseases caused by TRPC channelopathies.
APPENDIX

CH-NB

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Supplemental Data S1. TRPC mRNA expression as a function of time in culture for the cholinergic neurons of the nucleus basalis (CH-NB). The data shows that TRPC1, 3, 4, 5, 6 and 7 mRNA frequencies remain relatively constant over the time period spanning 13 days through 33 days in primary culture for CH-NB neurons.

DA-SNPC

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Supplemental Data S2. TRPC mRNA expression as a function of time in culture for the dopaminergic neurons of the substantia nigra pars compacta (DA-SNPC). The data shows that TRPC1, 3, 4, 5, 6 and 7 mRNA frequencies fluctuate slightly during the period spanning 16 days through 23 days in primary culture for DA-SNPC neurons.
## APPENDIX (continued)

### DA-VTA

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Supplemental Data S3. TRPC mRNA expression as a function of time in culture for the dopaminergic neurons of the ventral tegmental area (DA-VTA). The data shows that TRPC1, 3, 4, 5, 6 and 7 mRNA frequencies undergo subtle changes throughout the period spanning 22 days through 37 days in primary culture for DA-VTA neurons.

### NE-LC

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<td>43%</td>
<td>86%</td>
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<tr>
<td>20 days-4 cells</td>
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<td>0%</td>
<td>25%</td>
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<td>50%</td>
</tr>
<tr>
<td>21 days-10 cells</td>
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<td>50%</td>
<td>0%</td>
<td>60%</td>
<td>70%</td>
</tr>
<tr>
<td>22 days-8 cells</td>
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<td>13%</td>
<td>25%</td>
<td>88%</td>
</tr>
<tr>
<td>25 days-5 cells</td>
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<td>0%</td>
<td>80%</td>
<td>20%</td>
<td>20%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Supplemental Data S4. TRPC mRNA expression as a function of time in culture for the noradrenergic neurons of the locus coeruleus (NE-LC). The data shows that TRPC1, 3, 4, 5, 6 and 7 mRNA frequencies undergo very slight variations in the period spanning 16 days through 25 days in primary culture for NE-LC neurons.
APPENDIX (continued)

SE-DRN

<table>
<thead>
<tr>
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<th>TRPC1</th>
<th>TRPC3</th>
<th>TRPC4</th>
<th>TRPC5</th>
<th>TRPC6</th>
<th>TRPC7</th>
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<td>18 days-23 cells</td>
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<tr>
<td>25 days-8 cells</td>
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<td>0%</td>
<td>88%</td>
<td>0%</td>
<td>36%</td>
</tr>
</tbody>
</table>

Supplemental Data S5. TRPC mRNAs expression as a function of time in culture for the serotonergic neurons of the dorsal raphe nucleus (SE-DRN). The data shows that TRPC1, 3, 4, 5, 6 and 7 mRNAs frequencies undergo only subtle changes in the period spanning 18 days through 25 days in primary culture for (SE-DRN) neurons.
CITED LITERATURE


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T. Kawano¹, N. M. Jones¹, C. V. Floreani¹, A. M. Albsoul-Younes¹, P. Yongsatirachot¹, S. Nakajima² and Y. Nakajima¹. Types of canonical transient receptor potential (TRPC) channels expressed in cholinergic, serotonergic, dopaminergic and noradrenergic neurons in the rat brain Poster presented at the 2009 Society for Neuroscience 39th annual meeting, 2009.

T. Kawano¹, N. M. Jones¹, C. V. Floreani¹, A. M. Albsoul-Younes¹, P. Yongsatirachot¹, S. Nakajima² and Y. Nakajima¹. Single-cell RT-PCR analysis of TRPC channels expressed in rat cholinergic, dopaminergic, noradrenergic, and serotonergic neurons in the brain 1. Department of Anatomy and Cell Biology, 2. Department of Pharmacology, University of Illinois at Chicago, Chicago, IL. Poster presented at the 2009 Chicago Chapter Society for Neuroscience annual meeting.
T. Kawano¹, N. M. Jones¹, C. V. Floreani¹, A. M. Albsoul-Younes¹, P. Yongsatirachot¹, S. Nakajima² and Y. Nakajima¹. Single-cell RT-PCR analysis of TRPC channels expressed in rat cholinergic, dopaminergic, noradrenergic, and serotonergic neurons in the brain. 1. Department of Anatomy and Cell Biology, 2. Department of Pharmacology, University of Illinois at Chicago, Chicago, IL. Poster presented at the 2009 Biophysics meeting.


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