Modeling Schizophrenia by Altering cAMP Signaling in Human Neurons

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Introduction

Schizophrenia is a debilitating mental disorder with patients exhibiting hallucinations, delusions, irrational thinking, and abnormal behavior in social situations (World Health Organization, 2012). It was first coined in 1908 by Eugen Bleuler to literally mean "spitting of the mind" and describe the separation of function between personality, thinking, memory, and perception (Stotz-Ingenlath, 2000). At present, schizophrenia has a worldwide prevalence of twenty-four million with approximately 0.3-0.7% of all people having the disorder at some point in their life (World Health Organization, 2012). Genetic factors are the most important in the etiology of the disease with shared environmental factors modulating the expression of symptoms (Wong and Van Tol, 2003). More interestingly, meta-analysis of twin studies estimated that the heritability of the disease is eighty-one percent, suggesting a strong genetic component (Sullivan et al, 2003).

Positive and Negative Symptoms of Schizophrenia

The symptoms of schizophrenia can be divided into three main categories: psychotic or 'positive' symptoms, deficit or 'negative' symptoms, and cognitive impairment (Kelly et al, 2000). These positive symptoms can be further classified into three main groups: hallucinations, delusions, and thought disorder. Hallucinations are usually in the form of auditory hallucination of human speech: 'hearing voices' which may be single or multiple. These voices comment on the patient's actions or thoughts (Kelly et al., 2000). The negative symptoms consist of severe disturbances in social interaction, motivation, expression of affect, ability to experience pleasure, and spontaneous speech (Wong and Von Tal, 2003). Cognitive impairment affects executive function, attention, memory, and general intellectual functioning.

Symptoms of this disorder usually begin in late adolescence or early adulthood. However, later onset cases are possible. Several studies indicate that men have a higher rate of incidence and usually develop schizophrenia between the ages of eighteen and twenty-five. This is in contrast to women who usually develop between the ages of twenty-five and thirty-five. Women also seem to have two peaks in age of onset for the disease (first after menarche and second after age forty). There are also gender differences in terms of the symptoms: men have a higher presence of negative and disorganization symptoms; women have a higher presence of anxiety, illogical thinking, inappropriate affect, and bizarre behavior (Ochoa et al., 2012).

Drug treatments

The first class of drugs used to treat schizophrenia is conventional neuroleptics. First discovered in the 1950s, these drugs reduced the positive symptoms, decreased the length of hospitalizations, and reduced the risk of re-lapse and re-hospitalization (Wong and Von Tal, 2003). Different chemical families of neuroleptics such as haloperidol, perphenazine, trifluoperiazine, and fluphenazine are identified as antipsychotic drugs. The antipsychotic action is linked to the blockade of dopamine receptors. Conversely, dopamine (DA) receptor agonists have been shown to induce a schizophrenia-like psychosis. While these drugs are effective in reducing psychotic symptoms, they do not improve the negative symptoms and cognitive deficits (Jibson and Tandon, 1995). These neuroleptics cause a variety of neurological, gastrointestinal, and cardiovascular side effects including extrapyramidal or Parkinsonian symptoms (Wong and Von Tal, 2003). These drugs are also not effective in treating roughly 30% of patients and produce intolerable side effects in roughly 5-10% of patients (Brenner et al., 1990).

The current drug treatments, effective in treating 30% of schizophrenic patients, are atypical antipsychotic drugs. The prototypical example of this class of drugs is Clozapine, which is

unique in that it has an affinity for serotonin (5-HT_{2A}) adrenergic (α1), dopamine (D_2), histamine (H_1), and muscarinic receptors (M_1) (Levine and Ruha, 2012). These atypical agents, because of their lower D2 receptor affinity and faster dissociation rate for D2 receptors, may be the reason for having a reduced presence of extrapyramidal side effects. Clozapine not only treats the positive symptoms of schizophrenia, but also reduces negative symptoms and cognitive deficits. However, its use is limited because it causes agranulocytosis in about one percent of those treated (Wong and Von Tal, 2003).

Schizophrenia as a neurodevelopmental disease

A neurodevelopment rather than neurodegenerative process is considered the general explanation of the pathophysiology of schizophrenia. Neuropathology studies documented differences among schizophrenia, other mental disorders, and normal patients (Wong and Von Tal, 2003). Macroscopic post-mortem studies of schizophrenics show decreased brain weight and increased ventricular volume. These studies suggest that the temporal lobe and the corresponding temporal horn of the lateral ventricle are the most affected (Wong and Von Tal, 2003). Many computed tomography (CT) and magnetic resonance imaging (MRI) studies also show deficits in cerebral gray matter as well as increased lateral and third ventricular volume (Kerwin et al., 1990). Meta-analysis twin studies have suggested that these structural changes seen in schizophrenics are due to genetics. The magnitude of these structural changes is correlated with clinical expression of symptoms. In monozygotic twins discordant for schizophrenia, the affected twin tends to have less cortical volume and larger ventricles. Even the normal relatives of schizophrenics have decreased cortical volumes and enlarged ventricles (Sullivan et al., 2003)

Histological pathology findings have shown decreased size of neurons in the cortex and hippocampus as well as an overall fewer number of neurons in the dorsal thalamus for patients

diagnosed with schizophrenia. There have been some studies that even suggest a reduction of synaptic and dendritic markers as well as abnormal distribution of white matter neurons. Specifically, there is a reduction of presynaptic and dentritic markers such as synaptosomal-associated protein 25 (SNAP-25), complexin II (CPLXII), and microtubule associated protein 2 (MAP-2) (Arnold et al., 1991). The most recent histological evidence suggests that schizophrenia is a neurodevelopmental disorder for its lack of presence in gliosis (hypertrophy of several different types of glial cells normally associated with neurodegenerative disorders).

Experiments with Nissl stains have also found a disorientation of the pyramidal cells in the hippocampus, a decrease in cell density in layers I and II in the amygdala and pes hippocampus, incomplete glomerular clustering in layer II, and abnormal clustering of cells in the deeper cortical layers. It was found that there were in fact more pyramidal cells in deeper layers and an overall decreased in γ-aminobutyric acid (GABA) neurons in the prefrontal cortex (PFC) (Benes, 1993).

Functional Imaging of Schizophrenics

Some studies have also known that the PFC or left frontal cortexes are especially affected. These studies have shown that there is decreased activity in the frontal cortex during various cognitive tasks. Functional magnetic resonance imaging (fMRI) studies also reveal reduced activation of limbic regions such as the amygdala and hippocampus during facial emotion tasks (Quintana et al., 2001).

Positron emission tomography (PET) studies confirm that hypofrontality (less anterior cerebral blood flow) is seen in both chronic and never medicated first-episode patients Treatment with antipsychotic medication seems to increase activity in the basal ganglia, suggesting that

hypofrontality in the resting state is an effect of antipsychotic treatment (Wong and Von Tal, 2003).

Molecular Basis of Schizophrenia: Dopamine Connection

Neurochemical pathology studies in schizophrenia have suggested that increased activity of dopamine receptors is responsible for its positive (psychotic) symptoms (Wong and Von Tal, 2003). Five distinct dopamine receptors, D_1-D_5, have been identified. The D_1 couples to G_s and stimulates cAMP formation. Receptor D_5 is D_1-like in that it also increases cAMP levels. Another dopamine receptor, D_2, has different pharmacology and location within the synapse from the D_1 receptor. D_2 receptors are coupled to $G_{i/o}$ GTP-binding proteins and inhibit cAMP formation. D_3 and D_4 are similar to D_2 in that they decrease cAMP formation. These D_2- like receptors were originally identified by their high affinity for radio labeled antipsychotic drugs such as haloperidol. These receptors are known to be important in schizophrenia treatment as there is a direct relationship between the potency of the binding of antipsychotic drugs to the D_2-like receptor and their clinical potency. D_2 receptors are targeted by anti-psychotic and anti-Parkinson's drugs (Gnegy, 2012). PET studies have also suggested that hyperactivity of dopamine is present in schizophrenia. Amphetamine and cocaine induce the positive symptoms by increasing release or prevented reuptake of dopamine in the striatum (Wong and Von Tal, 2003). However despite this evidence, dopamine hyperactivity in its etiology of schizophrenia is inconclusive as the increase in receptor number and metabolic activity of dopamine may be due to the antipsychotic treatment itself.

Molecular Basis of Schizophrenia: Serotonin Connection

The role of serotonin in schizophrenia has been studied, partly due to the development of newer antipsychotic drugs target that serotonin receptors. There are fourteen subtypes of serotonin

receptors that can be categorized into seven subfamilies. 5-HT_1 receptors are linked to the inhibition of adenylate cyclase and primarily function as inhibitory presynaptic and postsynaptic receptors. The 5-HT_{1A} subtype receptor is known to play a role in depression, specifically involved with antidepressant treatments. These 5-HT_{1A} receptors are involved in the negative coupling to adenylate cyclase. Similarly, another subtype, 5-HT_{1B}, are also presynaptic and postsynaptic receptors. 5-HT_{1B} receptors are located somadendritically on 5-HT neurons, where they regulate serotonin synthesis and release. Postsynaptic 5-HT_{1B} receptors are located mostly in motor control centers such as the basal ganglia, where they control synaptic transmission (Artigas et al., 2012). It has been reported that there is a decreased cortical 5-HT_{2A} receptor density and increased 5-HT receptor density in patients with schizophrenia. Not only has serotonin been hypothesized to play a role in schizophrenia, it is known to be involved in depression.

It has been shown that the clinical efficacy of antidepressants depends on presynaptic serotonergic function. Approximately 90% of all antidepressants target the serotonin transporter: specifically selective serotonin reuptake inhibitors (SSRIs) and dual serotonin and norepinephrine reuptake inhibitors (SNRIs) (Artigas et al., 2012).

Importance of cAMP, CREB, and BDNF to Schizophrenia

While there have been many neuropathological findings, the intracellular molecular mechanisms of schizophrenia have yet to be elucidated. Cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) and its role in mood disorders, neuronal plasticity in learning and memory, and involvement drug treatments for depression and bipolar disorder have been studied (Duman, 2002). CREB is a member of the basic leucine zipper domain (bZIP) super family of transcription factors. It has a c-terminal zipper domain that is responsible for binding to

DNA and a leucine zipper domain that controls dimerization with itself or transcription factors in the CREB family (Chao and Nestler, 2004). CREB dimers bind to consensus cAMP response element (CRE), regulating transcription of downstream genes. Many genes have CREs in their promoters including genes coding for neuropeptides, neurotransmitter synthesizing enzymes, neurotransmitter receptors, signaling proteins, and other transcription factors. These CRE-mediated transcription responses require phosphorylation of CREB. This activation of CREB is involved in many signal transduction cascades including cAMP via protein kinase A (PKA), the Ras/extracellular signal regulated kinase (ERK), phosphoinositide 3-kinase/ protein kinase B (PT3K/Akt) pathway, and stress induced signaling cascades (Chao and Nestler, 2004).

CREB is of interest in neural differentiation. Cells of the nervous system coordinate their metabolic activities through the use of neuropeptides and neurotransmitters. Many of these neuropeptides act on their target cells by activating adenylate cyclase through specific receptor proteins (or G-protein coupled receptors). Once activated, the adenylate cyclase converts adenosine-5'-triphosphate (ATP) to cAMP, allowing cAMP to act as a second messenger (Uhler and Abou-Chebl, 1991). The cAMP then binds to the regulatory subunits of cyclic AMP dependent kinase (PKA) and changes its conformation. The two catalytic subunits of PKA are now able to enter the nucleus where they phosphorylate transcription factors such as the CREB protein (Ren and Uhler, 2008), subsequently altering gene expression (Uhler and Abou-Chebl, 1991).

Treatments for unipolar depression or bipolar disorder up-regulate CREB and repeated administration of haloperidol (antipsychotic drug) increased phosphorylation of CREB in striatum but not in the hippocampus or cerebral cortex (Duman, 2002). It also has been shown that CREB expression is altered in the cerebral cortex of depressed patients (Duman, 2002). In

patients not receiving medication, levels of CREB in postmortem brains in the cerebral cortex is lower. Conversely, patients treated with antidepressants, showed a significant increase in CREB (Duman, 2002). This suggests that decreased expression or function of CREB contributes to mood disorders and disruption of CREB could alter the normal adaptive plasticity needed in response to stress.

CREB also mediates the calcium dependent regulation of brain-derived neurotrophic factor (BDNF), a neurotrophin that modulates synaptic activity (Tao et al., 1998). CREB is an important regulator of BDNF-induced gene expression as exposure of neurons to BDNF stimulates CREB phosphorylation and activation by calcium/calmodulin dependent kinase (Finkbeiner et al., 1997). BDNF is a secreted protein that is important for survival, expansion, and differentiation of neural stem cells (NSCs). Studies have suggests that neurotrophic factors, such as BDNF, regulate proliferation and differentiation commitment of NSCs during the developing stages and adult regeneration events. BDNF can promote NSC proliferation by binding to Tyrosine-related kinase B (TrkB) receptor and activating intracellular MAP kinase, AKT, and STAT3 signaling pathways (Chen et al., 2012). Although its downstream mechanism is not fully understood, it is evident that BDNF-mediated signaling cascade is critical for neuronal cell survival, progenitor maturation, synapse formation, and neural repair after injury. BDNF is expressed in developing and adult brains and is important for the generation of dopamine neurons (Chen et al., 2012).

It has even been suggested that deficiency of BDNF generation or BDNF signaling dysfunction is implicated in neurodegenerative diseases (Chen et al., 2012). Repeated administration of antidepressants (including norepinepinephrine and serotonin re-uptake inhibitors) increases expression of BDNF in the hippocampus (Duman, 2002). BDNF may also be critical for normal

adaptive responses to stress as stress down-regulates BDNF. For patients with Parkinson's disease, intranigral transplantation of BDNF-secreting human mesenchymal stem cells was beneficial for cell-based therapy. BDNF, when over expressed in NSCs in a stroke model, showed neuroprotection and increased functional recovery (Lee et al., 2010).

FGF Signaling in Schizophrenia

In addition to BDNF, fibroblast growth factor (FGF) also regulates expression of CREB by stimulating transcriptional activity of CREB. FGFs and their receptors are involved in an elaborate signaling system that is involved in many developmental and repair processes. Of the 23 FGF members, 10 have been identified in the brain (Reuss and von Bohlen und Halbach, 2002). The binding of FGFs to fibroblast growth factor receptors (FGFRs) triggers receptor dimerization and tyrosine kinase activation, resulting in autophosphorylation of the intracellular domain of the receptor and recruitment and assembly of signaling complexes. Multiple pathways have been shown to operate downstream of FGFRs. MAPK/Erk signaling cascade is the pathway most commonly employed by FGFRs and results in stimulation of the expression and/or activation of various transcription factors that act as effectors of the pathway, including Ets proteins, GATA proteins, and CREB. The mitogen-activated protein kinase (MAPK)/Erk pathway is particularly important in mediating the proliferative activity of FGFs (Guillemot and Zimmer, 2011).

During central nervous system (CNS) development FGF is involved in neurogenesis, axon growth, and differentiation. It also is a major determinant of neuronal survival both during development and adulthood. Adult neurogenesis depends greatly on FGF-2, FGF-1, and FGFR. These growth factors and receptors are thought to also be involved in the regulation of synaptic plasticity in addition to processes for learning and memory. FGFs are important in the repair

process following different types of brain and peripheral nerve lesions as well as degenerative disorders (Reuss and von Bohlen und Halbach, 2003).

In addition, there have been studies that implicate FGF-2 signaling in the PFC in the behavioral and cellular responses to antidepressant treatments (Elsayed et al., 2012). FGF-2 infusions, specifically in the PFC in male rats, are sufficient to produce significant anxiolytic and antidepressant actions in the novelty suppressed feeding test (NSFT) and forced swim test. Infusions into the dorsal striatum, which also expresses FGF-2 and FGFR1, did not produce behavioral changes in NFST (Elsayed et al., 2012).

Fibroblast growth factor (FGF) signaling has been implicated in schizophrenia. Patients with schizophrenia show altered levels of FGF ligands and/or receptors in the hippocampus and cortex (Stevens et al., 2012). Polymorphism of FGF genes was also found in patients with schizophrenia. Manipulations of both basic fibroblast growth factor 2 (FGF2) and basic fibroblast growth factor 1 (FGFR1) in animals show that FGF signaling in the hippocampus affects anxiety behavior and is correlated with response to antipsychotic and antidepressant treatment (Stevens et al., 2012).

Induced pluripotent stem cells

The advent of patient-derived induced pluripotent stem cells (iPSCs) has allowed researchers to recapitulate disease phenotypes and increase understanding of pathogenesis of many diseases (Ito et al., 2012. These iPSCs generated from adult fibroblasts express embryonic stem (ES) cell markers as well as displaying similar morphology and growth. ES cells that are derived from the inner cell mass of mammalian blastocysts have the ability to maintain pluripotency and differentiate into any cell of the three germ layers. Ethical difficulties regarding ES cells and its

use of human embryos ES cells as well as tissue rejection following transplantation in patients has led to more ethically acceptable alternative iPS cells (Takahashi and Yamanka, 2006). Human induced pluripotent stem cells (hiPSCs) can be developed by taking a skin sample from a biopsy, a skin plug from the core of the skin, and putting the skin plug into culture in order to produce fibroblasts. Fibroblasts can then be reprogrammed with the introduction of viruses expressing transcription factors Oct3/4, SOX2, c-Myc, and Klf4 (Takahashi and Yamanka, 2006).

These iPS cells can subsequently be induced to differentiate into neuronal cells to be used in pathogenesis research, drug screening, diagnosis and regenerative medicine. iPS cells and ES cells can be directed to differentiate into cortical stem and progenitor cells by combining retinoid signaling with inhibition of SMAD signaling (Shi et al., 2012). Cortical stem and progenitor cells were identified by their expression of the transcription factors Foxg1, Pax6, Otx1/2 and Tbr2. Retinoid signaling with inhibition of SMAD produced cells in culture that were Pax6- and Otx1/2- (Shit et al., 2012). This allowed for neurological disease-specific iPS cells analysis for diseases such as amyotrophic lateral sclerosis (ALS), sporadic Parkinson's disease (PD), Duchenne/Becker muscular dystrophy, and Huntington disease (Ito et al., 2012).

A pioneering study of iPSC-derived neurons in schizophrenia

It has been recently demonstrated that there is altered cAMP signaling in patients with schizophrenia (SCZD). Brennand et al. selected four schizophrenia patients: one patient had childhood onset schizophrenia; the remaining patients were from families in which all children and one parent were diagnosed. In addition they also had four control subjects. The fibroblasts from these patients were reprogrammed using inducible lentiviruses. In both the control and schizophrenia neurons these induced pluripotent stem cells expressed pluripotency genes and

reprogramming genes such as NANOG, NESTIN, and SRY (sex determining region Y)-box 2 (SOX2). The SCZD neurons, while displaying normal neuronal activity (normal transience for inward Na^+ currents and outward K^+ currents), showed an overall decrease in neuronal connectivity. By using fluorescence activated cell sorted (FACS) and trans neuronal tracing, decreased neuronal connectivity was confirmed. Treatment with antipsychotic drugs (clozapine, loxapine, olanzapine, risperidone and thioridazine) for a period of three weeks and found that only loxapine (high affinity antagonist of serotonin 5-HT_2, dopamine D_1, D_2, D_4 receptors) significantly increased neuronal connectivity in human induced pluripotent stem cells (hiPSCs) from all patients. Loxapine also increased NRG1, glutamate receptors (GRIK1, GRM7, GRIN2A) and decreased expression of ADCY8, PRKCA, WNT7A, and TCF4.

A difference found in SCZD hiPSCs was a decreased number of neurites and synaptic protein levels. In terms of gene expression, the comparison of schizophrenia and control cells showed lower levels of adenylate cyclase (ADCY8), PKA catalytic subunits (PRKACA), and greater levels of the PKA regulatory subunits (PRKAR2A) (Brennand et al. 2011). This is all consistent with the idea that there is less cAMP signaling to regulate gene transcription in SCZD neurons.

Current Study

In this study we sought to model the molecular mechanisms underlying schizophrenia. In particular, we employed a system with human induced pluripotent stem cell-derived neurons and different levels of cAMP signaling to identify whether PKA could cause the different levels of gene expression observed in schizophrenic patients. We pharmacologically altered cAMP signaling by increasing PKA activity using an analogue of cAMP, CPT-cAMP, and inhibiting protein kinase A with H89 and KT-5720. By observing changes in the neuron morphology and gene expression changes amongst the different treatments, we sought to determine whether a

decrease in PKA activation is consistent with gene expression changes associated with schizophrenia.

Objectives

The goal of the study is to perturb cAMP signaling in order to observe gene expression changes seen in human iPS cell-derived neurons. We increased PKA activity using CPT-cAMP and decreased PKA activity using H89 and KT-5720. By comparing the gene expression changes found after perturbing PKA activity and the changes reported in neurons derived from patients with SCZD, we hypothesize these altered gene expressions are secondary to changes related in PKA signaling. The overall goal is to understand molecular mechanisms of SCZD and whether or not drugs that alter cAMP dependent kinase protein could lead to treatments for patients with schizophrenia.

Methodology

Maintenance of iPS cells

iPSCs were maintained on mouse embryonic fibroblasts (MEFs) in human embryonic stem (hES) cell media (DMEM/F12 with 20% knockout serum replacement, 6 ng/mL FGF2, 1 mM glutamine, 100 μM non-essential amino acids, 100 μM β-mercapatoethanol, and 1X penn/strep). Cells were dissociated with accutase and pre-plated on gelatin coated dishes in hES media with 10 ng/ml FGF2 and 10 μM rho-associated protein kinase (ROCK) inhibitor for one hour. Non-adherent PSCs were then collected and plated on matrigel coated plates in MEF-conditioned hES media with 10 ng/ml FGF2. Neural induction began with 3N media with 1 μM dorsomorphin and 10 μM SB431542 once cells reached 95% confluence. The treatment continued for eleven days.

Neuronal Progenitor Cells

Neuroepithelial cells were collected by dissociation with dispase and plated in 3N media (equal parts of DMEM/F12 with N2 supplement, 5 microgram/mL insulin, 1mM glutamine, 100 micromolar non-essential amino acids, 100 micromolar β-mercaptoethanol, 1X penn/strep and an equal part of neurobasal media, B27 supplement, 200 mM glutamine, 1X penn/strep) and 20ng/mL FGF2. Following collection of neuroepithelial cells, FGF2 was withdrawn to promote differentiation. These cells were maintained on 10 cm diameter plates (coated with matrigel at 1:200) with 10 mL of 3N media, which supports neural induction, neurogenesis, and neuronal differentiation (Shi, et al).

Treatment of neuronal progenitor cells with CPT-cAMP, KT-5720, H89

There were three separate experiments with different combinations of cAMP activators and inhibitors. The first experiment consisted of a control, CPT-cAMP (Sigma-Aldrich, St. Louis, MO, USA) treatment (200 μM), and KT-5720 (Cayman Chemical Company, Ann Arbor, MI, USA) treatment (3 μM). The second experiment was identical. The third experiment consisted of a control, CPT-cAMP treatment (200 μM), and H89 (Cayman Chemical Company, Ann Arbor, MI, USA) treatment (200 μM). Each treatment period was seven days.

Isolation of RNA

Following neuronal differentiation RNA was purified from the iPSC neurons. For each plate, the 3N media was removed and washed with 1X PBS. In order to isolate the RNA, 1 mL of TRIzol (Invitrogen) was added to cells, scraped, and transferred to a 1.7 mL centrifuge tube. Next 200 μL of chloroform was added and subsequently centrifuged at 13,000 rpm for 15 minutes to separate the aqueous and organic phases. The aqueous phase was then transferred to a new 1.7 centrifuge tube with the addition of 1 μL glycogen and 500 μL of isopropanol. The solution was

vortexed and placed in the -20°C freezer overnight to precipitate the RNA and glycogen. The following day the tubes were spun at 13,000 rpm for 15 minutes, forming a RNA pellet. The pellet was washed with 80% and dried in a speed-vac. The RNA pellet was re-suspended in 100 μL DEPC-treated water. The total RNA was purified using the RNeasy Kit (Qiagen Sciences, Germantown, MD, USA).

Table 1. RNA Concentrations

Exp #	Treatment	Concentration (μg/μL)	A230	A260	A280	260/280
1	Control	0.2092	++++++	0.105	0.013	8.070
	CPT-cAMP	0.2041	0.061	0.102	0.056	1.810
	KT-5720	0.0734	0.038	0.037	0.025	1.480
2	Control	1.5277	0.321	0.764	0.416	1.840
	KT-5720	1.4215	0.582	0.711	0.406	1.750
3	Control	0.3423	0.019	0.171	0.103	1.660
	CPT-cAMP	0.2959	0.057	0.148	0.092	1.610
	H89	0.8369	0.112	0.418	0.231	1.810

It should be noted in experiment 1 that following TRIzol treatment, 500 μL dH_2O was mistakenly added in to 500 μL and 1 μL glycogen. This diluted the samples and complicated the extraction of RNA. Additionally, the A230 reading for the control sample suggests there are protein impurities in the RNA sample. The KT-5720 sample from experiment 1 had too low of a concentration to make cDNA. For experiment 2, the cell plate treated with CPT-cAMP became contaminated and did not allow for RNA extraction.

cDNA Synthesis

cDNA for each sample was synthesized from 2 μg of RNA by following the addition of 1 μL of random primers, 1 μL of dNTPs and DEPC H_2O (to bring the total volume to 12 μL) in a PCR

tube. Next the samples were heated to 65 °C for 5 minutes in the PCR machine and subsequently placed on ice to cool down. Following addition of 4 μL of 5X first strand buffer, 2 μL of 0.1 M DTT and 1 μL of RNasin, the solution was incubated at 25 °C for 2 minutes in the PCR machine. Next 1 μL of SuperScriptII Reverse Transcriptase was added and then placed in the PCR machine to run for 25°C for 10 minutes, 42°C for 50 minutes, and 70°C for 15 minutes. Finally the cDNA was diluted with 180 μL of dH_2O.

Quantitative Real-Time PCR (qRT-PCR)

Next the levels of cDNA for an individual gene was measured by quantitative real time PCR (qRT-PCR) using either the rEVAlution (Syzygy, Grand Rapids, MI, USA) or SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and MyiQ singlecolor real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). Human specific PCR primers were ordered from Invitrogen for the qRT-PCR analyses. The first set of primers was identical to those used by Brennand et al. (Brennand et al., 2011) those analyzed in the Brennand et al. paper: ADCY8, PRKCA, GRIK1, PRKAR2A, and PRKGI. The second set of primers is CamKII, VGlut1, VGlut2, GAD1, GAD2, DLG4, MAPT, MAP2, and DCX. The primer sequences can be found in Table 2. If using the SYBR Green PCR Master mix, the 96-well plate was prepared by adding 10 μL of 0.5 μM diluted primer stock, 2.5 μL of cDNA, and 12.5 μL of SYBR Green PCR Master mix. If using the rEVAlution, the 96-well plate was prepared by adding 8 μL of 0.5 μM diluted primer stock, 2 μL of cDNA, and 10 μL of rEVAlution. We then verified the specificity of the amplification with a heat dissociation protocol after the final cycle of PCR (Huang et al. 2011). To calculate expression levels, we used the delta-delta CT method, with Rp130 as the normalization control. A paired Student t-test was performed and data was

presented as means ± standard deviation. The formula for calculating the fold changes in gene expression levels is shown below.

$$Fold\ Change = \frac{2^{CT_{exp}^{gene} - CT_{control}^{gene}}}{2^{CT_{exp}^{Rplao} - CT_{control}^{Rplao}}}$$

Table 2. Human specific primer sequences

	Forward	Reverse
Rpl30	GCAAAGCGAAATTGGTCATT	GATGGACACCAGTTTTAGCCA
ADCY8	TGCTGACTTCGATGAGTTGC	ATGTCCCCACTTGTCTTCAC
PRKCA	CACCATTCAAGCCCAAAGTG	CATACGAGAACCCTTCAAAATCAG
GRIK1	AAAGGTTACGGAGTGGGAAC	TCTTTGTTGTCTTCCTCGGG
PRKAR2A	TTCCTCTTCCTCATCAGGGTT	AGCGAGTCGGAGGAGGAC
PRKG1	GATCAATGGCCCAGAGTTTT	TACCATGGGTCCAGGAAAAG
CamKII	CGCTTCACGGAAGAGTACCA	TGATCTTGGCAGCATACTCCT
MAP2	AACCGAGGAAGCATTGATTG	TTCGTTGTGTCGTGTTCTCA
MAPT	AAGATCGGCTCCACTGAGAA	CACACTTGGACTGGACGTTG
DCX	AGACCGGGGTTGTCAAAAA	TCAGGACCACAGGCAATAAA
DLG4	TCCTCACAGTGCTGCATAGC	TGTCTTCATCTTGGTAGCGG
vGlut1	GAGTTTCGGAAGCTAGCGG	ACTCAGCTCCAGCGTCTCC
vGlut2	AGATTCCGGGAGGCTACATC	ACTCTGGCTGCTGATGGAAT
Gad1	CAACGTACGATACCTGGTGC	CTTCTCTTCCAGGCTGTTGG
Gad2	GATTGGAACAGACAGCGTGA	CCCTTTCTGTTTGGCTTCAA

Results

Morphological Changes

Human neuronal progenitor cells (hNPCs) treated for seven days either with CPT-cAMP, H89, or KT-5720 were observed for their changes in number of nuclei and length of neuronal processes. As shown in Figure 1, in experiment 1, cells treated with CPT-cAMP displayed more rounded cell bodies relative to the control. Treatment with KT-5720 in experiment 1 reduced the overall number of cell bodies and increased distance between the cells.

In experiment 2, treatment with KT-5720, similar to experiment 1, decreased the number of cell bodies. Relative to the control, the cells treated with the inhibitor appeared more rounded. It should be noted that the image for treatment with CPT-cAMP in experiment 2 is not displayed due to yeast contamination.

The cells following treatment with CPT-cAMP in experiment 3 did not show significant differences in number of nuclei or length of neuronal processes from the control cells. However treatment with H89 did reduce the number of cell bodies and presence of neuronal processes.

In all three experiments, treatment with the inhibitors appeared to significantly reduce the number of cell bodies and length of dendrites.

There was significant variation in starting cell populations for each experiment. In experiment 3, relative to experiment 1 and 2, there appeared to be greater cell density on day 0 for all three cell plates. As shown in figure 1, the control plate in experiment 3 also appeared more neuronal than either of the two previous experiments. The length of neuronal processes increased with each

consecutive experiment. Thus it is possible that these differences in basal conditions could have contributed to inter-experimental variability.

qRT-PCR Results

When performing qRT-PCR analysis, there were two sets of treatments. Set #1 consisted of the control and KT-5720 treated cells from experiment 2 as well as the CPT-cAMP treated cells from experiment 1. Set #2 consists of the control, CPT-cAMP, and H89 treated cells from experiment 3.

As shown in figure 2B, in experiment 1 treatment with CPT-cAMP significantly altered the expression of adenylate cyclase 8 (ADCY8) and PRKG1, while treatment with KT-5720 significantly reduced the expression of PRKCA, GRIK1, and PRKG1. The expression of ADCY8 was increased by a fold change of 2.2 in the presence of CPT-cAMP whereas treatment with KT-5720 insignificantly decreased the expression of ADCY8 (p-value 0.357). The large standard deviation in the KT-5720 values did not allow for a statistically significant decrease in expression of ADCY8 relative to the control. The expression of PRKCA following treatment with CPT-cAMP showed only a slight decrease (.77 fold decrease) relative to the control. This is in contrast to KT-5720 which showed a fold decrease of 0.48. The expression of GRIK1 following treatment with CPT-cAMP was nearly identical to the control sample (1.1 fold increase). However treatment with KT-5720 drastically reduced its expression (0.04 fold decrease). Fold increases or decreases for the expression of PRKAR2A could not be analyzed for significant changes because the fold changes were calculated by taking the average of only two delta CT values for PRKAR2A.There was a bad trace value for the third value when performing qRT-PCR. However, the two values suggest that CPT-cAMP down-regulate the expression of

PRKAR2A. The expression of PRKG1 was significantly decreased in both the treatments of CPT-cAMP and KT-5720 (0.29 and 0.19 fold decrease, respectively).

Repeating cell culture treatment in experiment 2 displayed similar results to experiment 1, as shown in figure 3. In both experiments, ADCY8 was up-regulated in the presence of CPT-cAMP (fold increase of 2.2 for experiment 1 and 3.0 for experiment 2). In contrast to figure 2, presence of the PKA inhibitor significantly decreased the expression of ADCY8 (fold decrease 0.65). The expression of PRKCA, in contrast to experiment 1, showed a slight increase in expression (1.4). It was not a significant increase because there was a large standard deviation amongst the values. Treatment with the H89 decreased expression of PRKCA (0.41 fold decrease); this is similar to KT-5720 in experiment 1. The expression of GRIK1 was similar in the CPT-cAMP sample and control (0.92 and 1.06, respectively). However, like experiment 1, the expression of GRIK1 following treatment of H89 decreased expression 0.25 fold. The normalized expression of PRKAR2A was not significantly altered by the presence of either CPT-cAMP or H89; there was great variation in the CT values for the control. Treatment with CPT-cAMP and H89 significantly decreased the expression of PRKGI (0.34 and 0.44, respectively); this trend is identical to experiment 1 as shown by figure 2 and 3.

Repetition of qPCR analysis for primers ADCY8, GRIK1, PRKAR2A showed slight variations in overall fold changes between the two sets of experiments (Figure 4B and 4C). Both sets of experiments and previous qPCR results all indicate that CPT-cAMP up-regulated expression of ADCY8 by roughly 2-3 fold relative to the control. Gene expression changes for GRIK1 in the presence of CPT-cAMP displayed significant increases in expression in only one qRT-PCR experiment for set#1 (1.3 fold). Set #2 did not display a significant increase in expression (Figure 4C). In the presence of inhibitors H89 and KT-5720, the expression of GRIK1 was down

regulated for both sets of data. The fold change was 0.3 and 0.06 in set#1 and set #2, respectively. PRKAR2A gene expression changes were inconclusive. In only one qRT-PCR analysis in set #2, there was a significant fold decrease of .47 fold in the presence of KT-5720 (Figure 4B).

In addition to observing changes in expression for genes involved in cAMP signaling, GRIK1 and PRKGI, qRT-PCR analysis for general neuronal markers demonstrated significant variability between the two sets of experiments and their controls (Figure 5B). In comparing the controls from experiment 1 and 2, the control from the first experiment showed a fold increase of 1.6 for CamKII and 1.3 for MAPT expression relative to the control from the second experiment (Figure 5B). Conversely, MAP2, DCX, and DLG4 showed significantly less expression in control #1 (0.47, 0.36, and 0.74, respectively). Treatment with CPT-cAMP also caused significant changes in gene expression. In comparison to the control from experiment 2, CamKII expression increased to 3.08 fold. In contrast, MAP2, MAPT, DCX, and DLG4 expression decreased (0.66, 0.57, 0.59, and 0.39 fold, respectively). Treatment with KT-5720 significantly decreased expression for the genes analyzed (.05 fold for CamKII, 0.27 fold for MAP2, 0.57 fold for MAPT, 0.08 fold for DCX, and 0.26 fold for DLG4) (Figure 5B).

In addition to examining genes previously reported to differ in schizophrenic iPS cells, we sought to determine if PKA signaling altered global neuronal gene expression. As shown in figure 6B, the two sets of controls from both experiment 2 and 3 showed significant differences in expression (control #1 showed a fold increase of 3.1 for VGlut1 and 2.9 for Gad2). There also significant increases in expression between the control #2 and CPT-cAMP sample for VGlut1, VGlut2, and Gad2 (2.1, 1.8, and 2.1, respectively). However, there was a 0.60 fold decrease in expression for Gad1. In the presence of inhibitor H89, there were significant decreases in

VGlut1, VGlu2, Gad1, and Gad2 relative to control #2 (0.76, 0.43, 0.60, and 0.26, respectively). The similar trend was observed in the second inhibitor, KT-5720; there were significant decreases in CamKII, VGlut1, VGlut2, Gad1, Gad2 (0.01, 0.24, 0.12, 0.58, and 0.39), also relative to the control from the second experiment.

In all, not only were there alterations in gene expression following treatment with CPT-cAMP, KT-5720, and H89, but also significant variability between the control samples from experiment 1 and 2. This is observed for global neuronal genes as well as general neuronal markers.

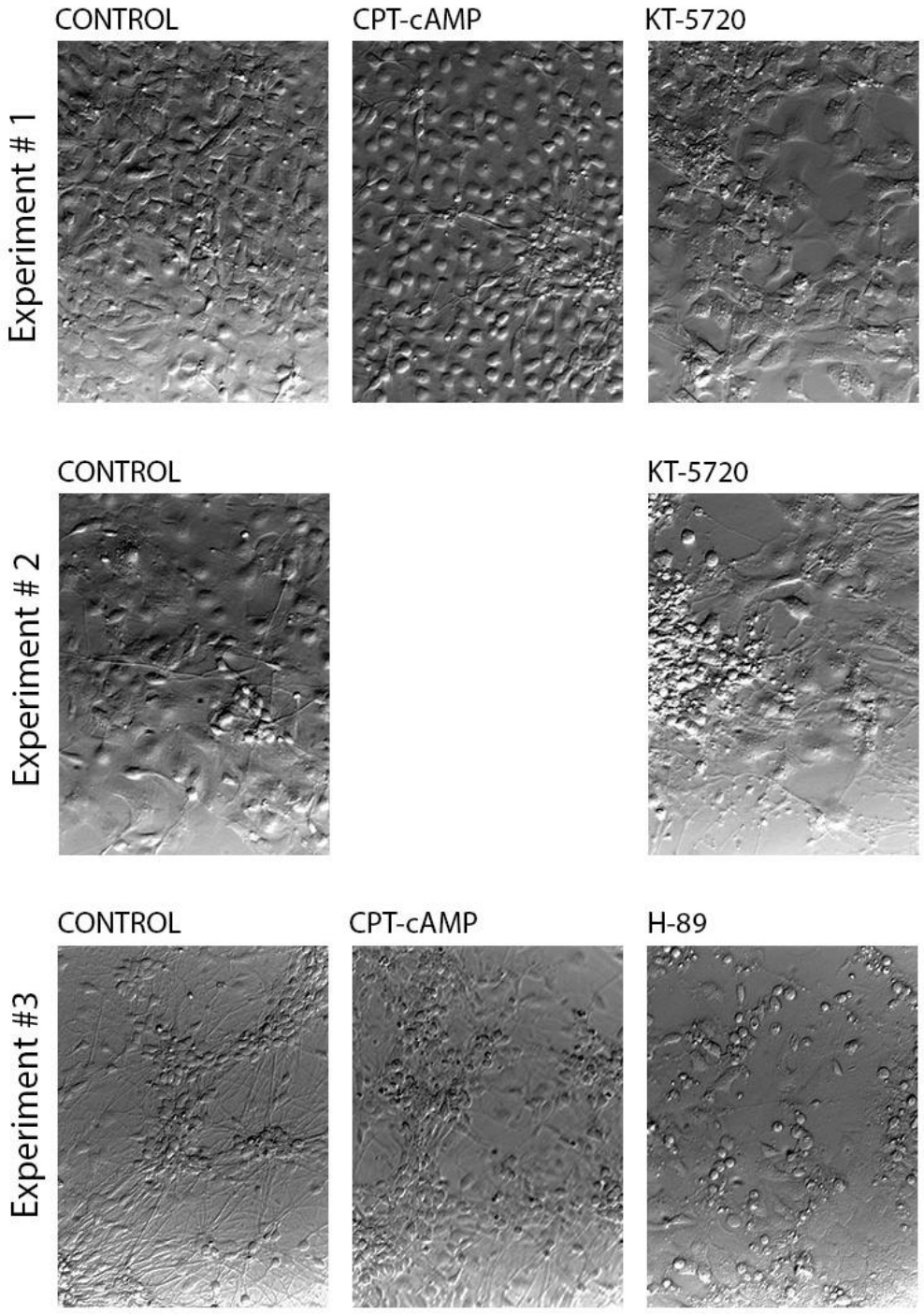
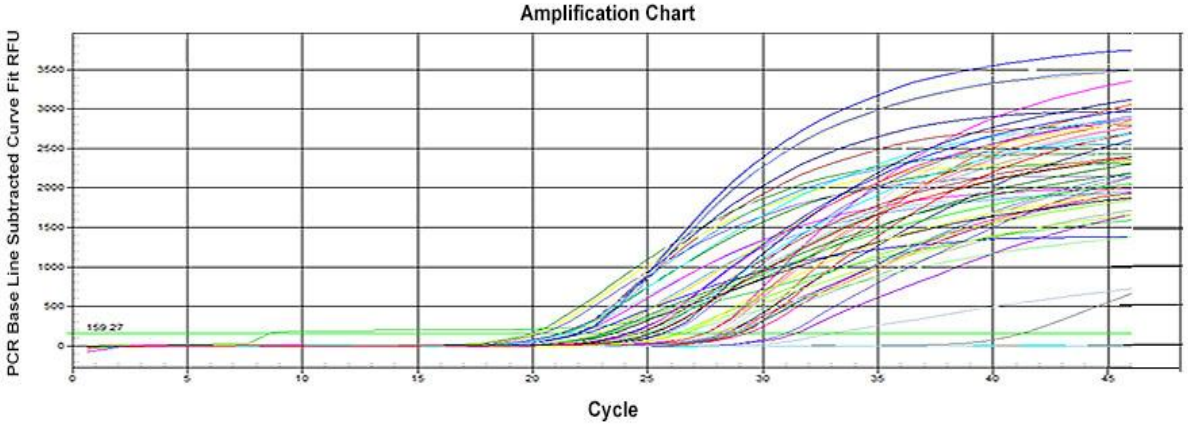


Figure 1. Bright field photomicrograph of human iPS cells treated for 1 week as indicated. Each experiment is labeled on left. Magnification at 200X

A.



B.

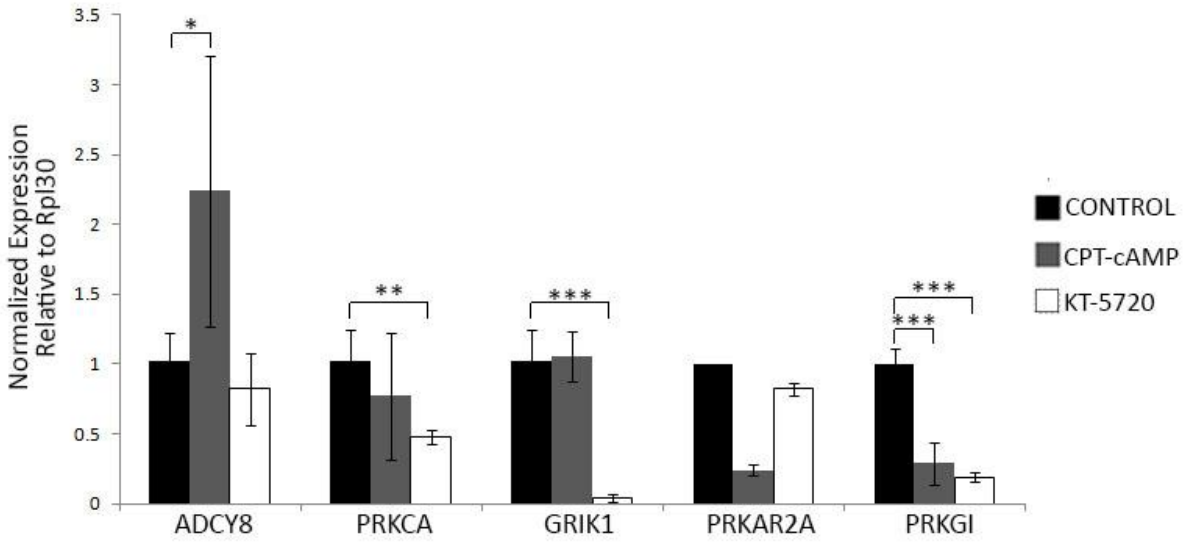
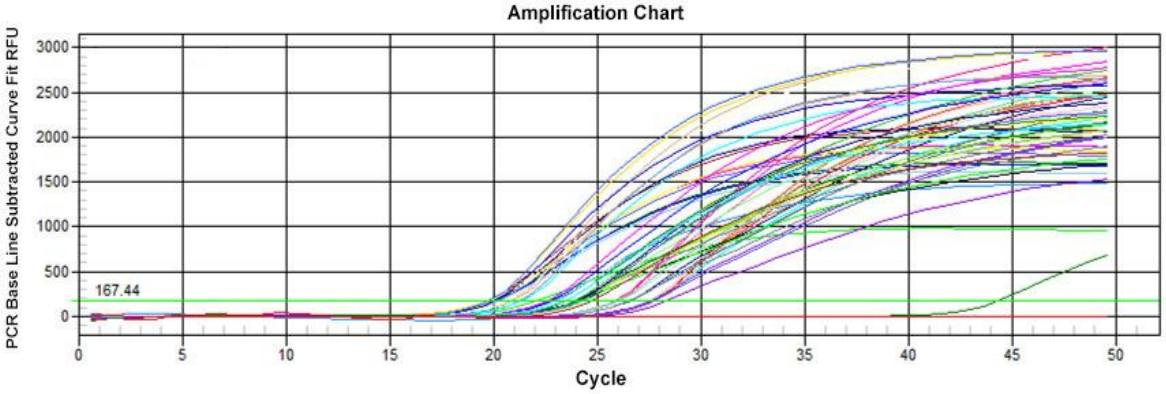


Figure 2. qPCR analysis of cDNA from Experiment 1. A) Representative qPCR results for experiment showing SYBR Green Fluorescence as a function of cycle number. B) Quantitation of results shown in panel A and other assays for ADCY8, PRKCA, GRIK1, PRKAR2A, PRKG1 genes normalized to Rpl30 transcription levels. *p<0.1, **p<0.05, ***p<0.01.

A)



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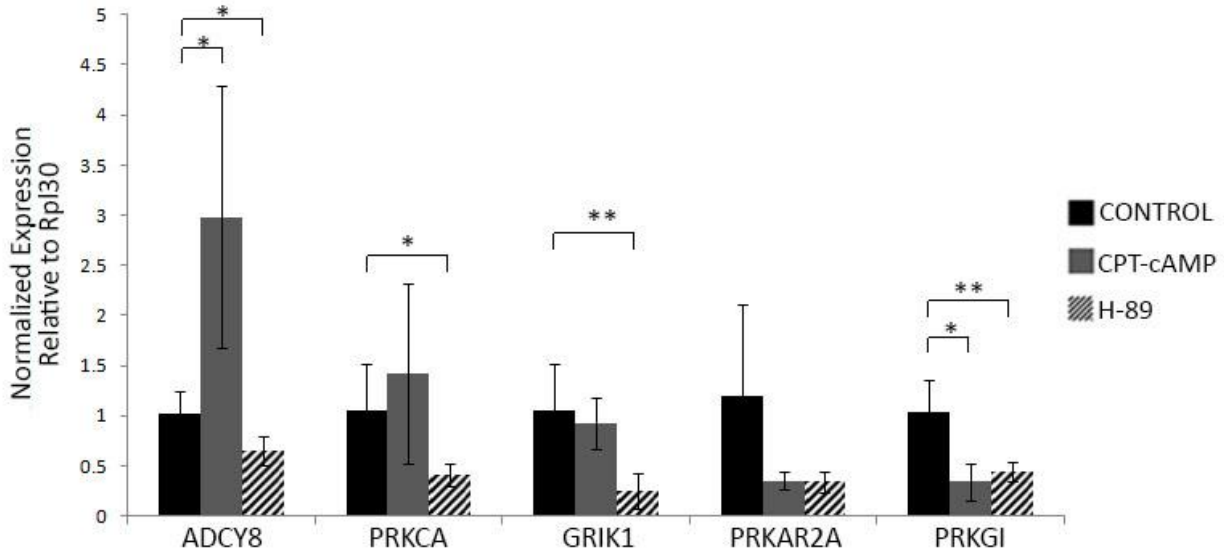
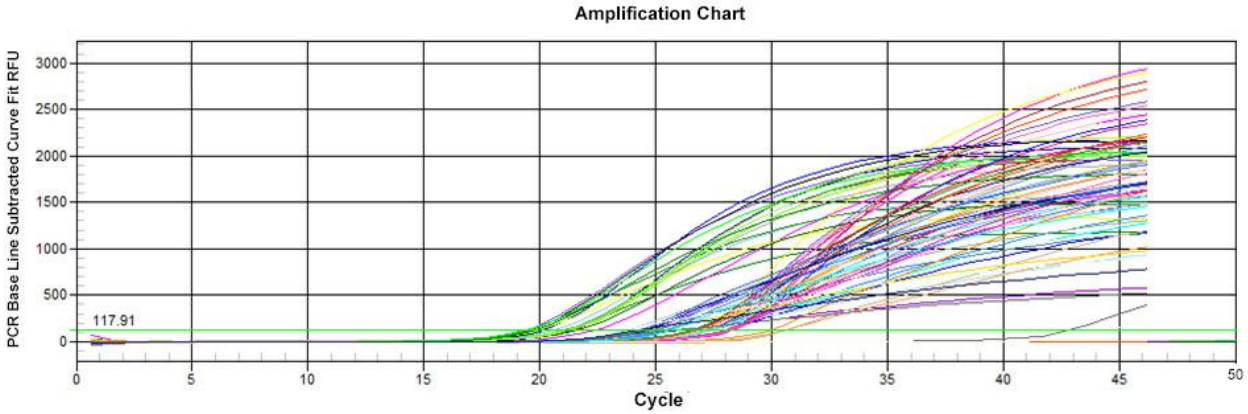
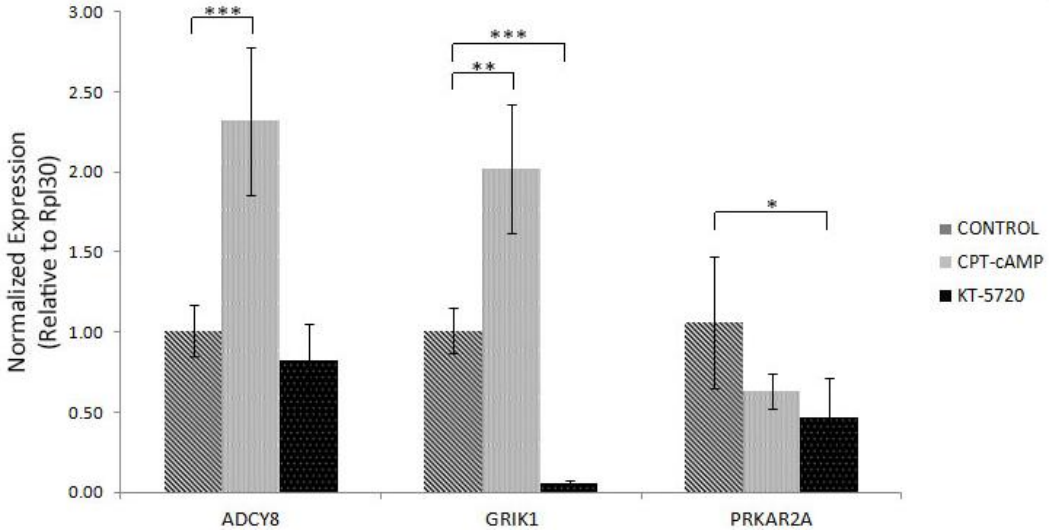


Figure 3. qPCR analysis of cDNA from Experiment 2. A) Representative qPCR results for experiment showing SYBR Green Fluorescence as a function of cycle number. B) Quantitation of results shown in panel A and other assays for ADCY8, PRKCA, GRIK1, PRKAR2A, PRKG1 genes normalized to Rpl30 transcription levels. *p<0.1, **p<0.05, ***p<0.01.

A)


Amplification Chart
PCR Base Line Subtracted Curve Fit RFU
3000
2500
2000
1500
1000
500
0
117.91
0
5
10
15
20
25
30
35
40
45
50
Cycle

B)


Normalized Expression
(Relative to Rpl30)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
***
***
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*
ADCY8
GRIK1
PRKAR2A
CONTROL
CPT-cAMP
KT-5720

C)

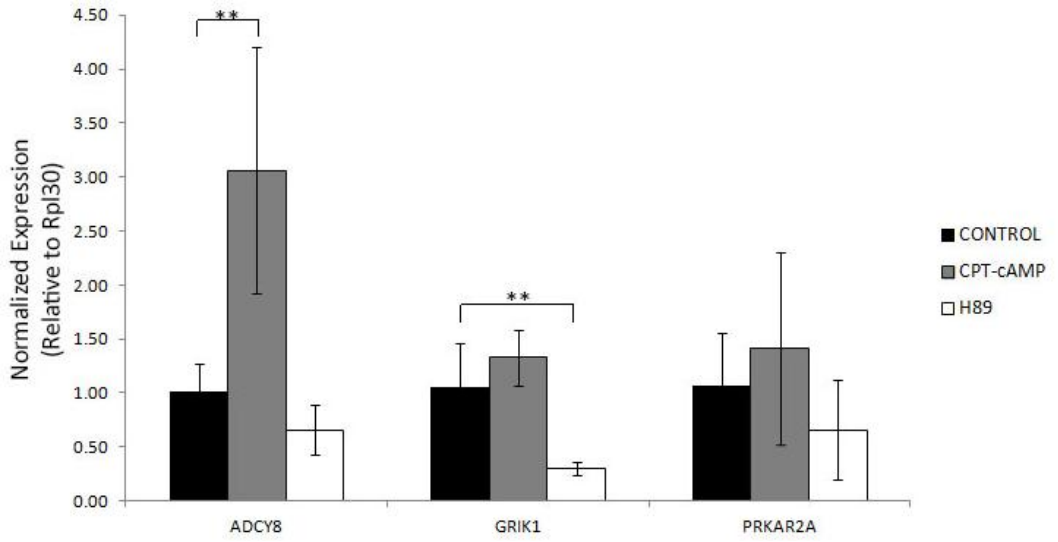
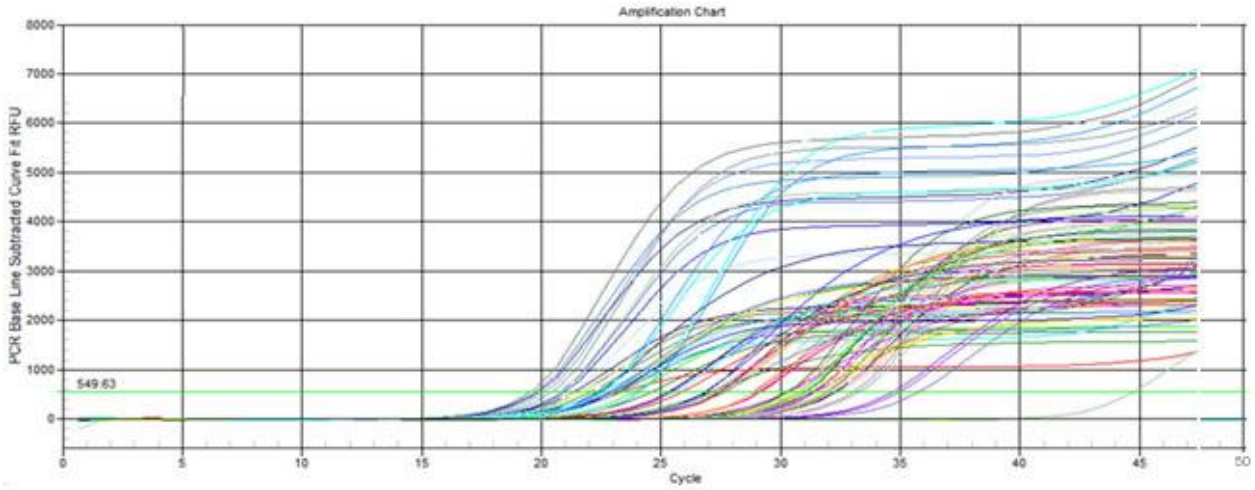

Normalized Expression
(Relative to Rpl30)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
**
**
ADCY8
GRIK1
PRKAR2A
CONTROL
CPT-cAMP
H89

Figure 4. qPCR analysis of cDNA from Experiment 1 and 2, specific to ADCY8, GRIK1, and PRKAR2A. A) Representative qPCR results for experiment showing SYBR Green Fluorescence as a function of cycle number. B) Quantification of results from experiment 1 shown in panel A and other assays for ADCY8, GRIK1, PRKAR2A, genes normalized to Rpl30 transcription levels. C) Quantification of results from experiment 2 shown in panel A and other assays for ADCY8, GRIK1, PRKAR2A, genes normalized to Rpl30 transcription levels. *$p<0.1$, **$p<0.05$, ***$p<0.01$.

A)



B)

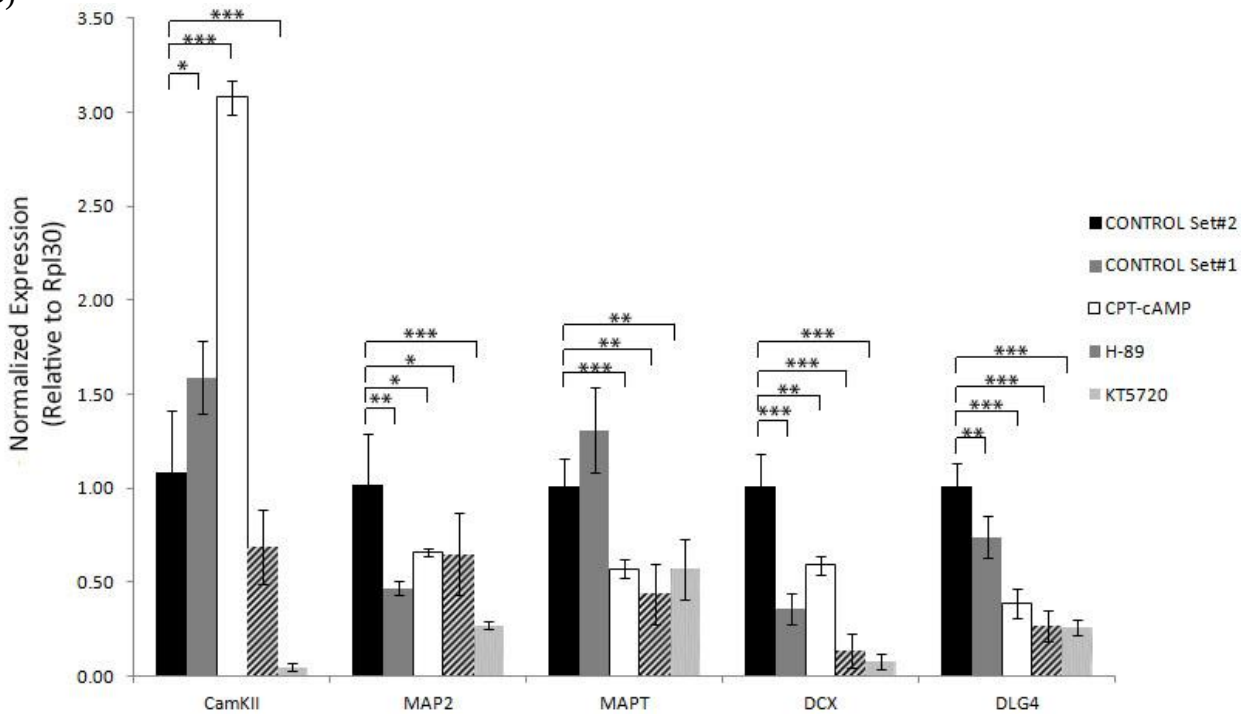
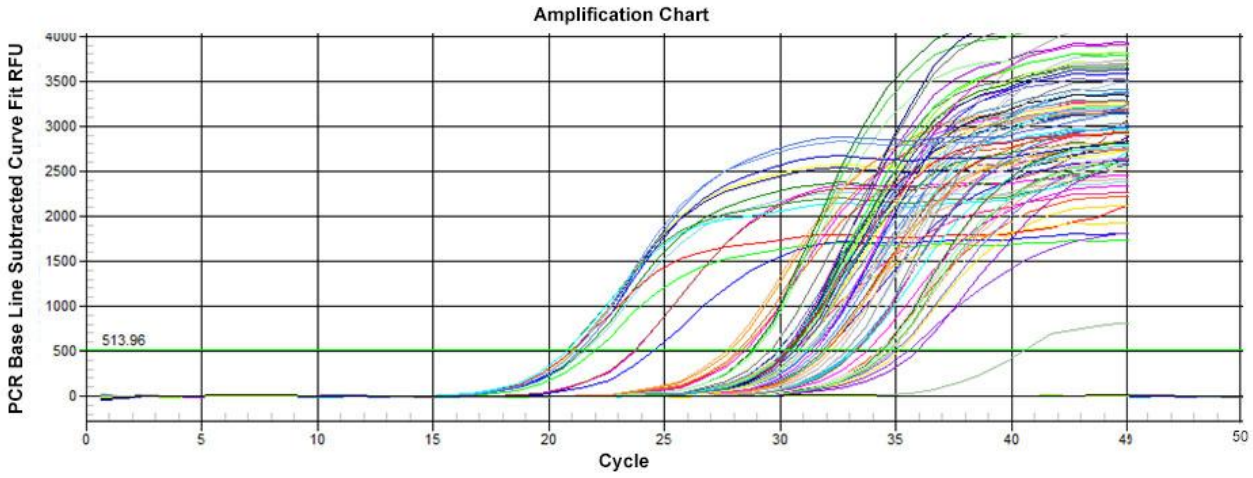


Figure 5. qPCR analysis of cDNA from Experiment 1 and 2, specifically for CamKII, MAP2, MAPT, DCX, DLG4. A) Representative qPCR results for experiment showing SYBR Green Fluorescence as a function of cycle number. B) Quantification of results from experiment 1, 2, and 3 shown in panel A and other assays for CamKII, MAP2, MAPT, DCX, and DLG4 genes normalized to Rpl30 transcription levels. *p<0.1, **p<0.05, ***p<0.01.

A)



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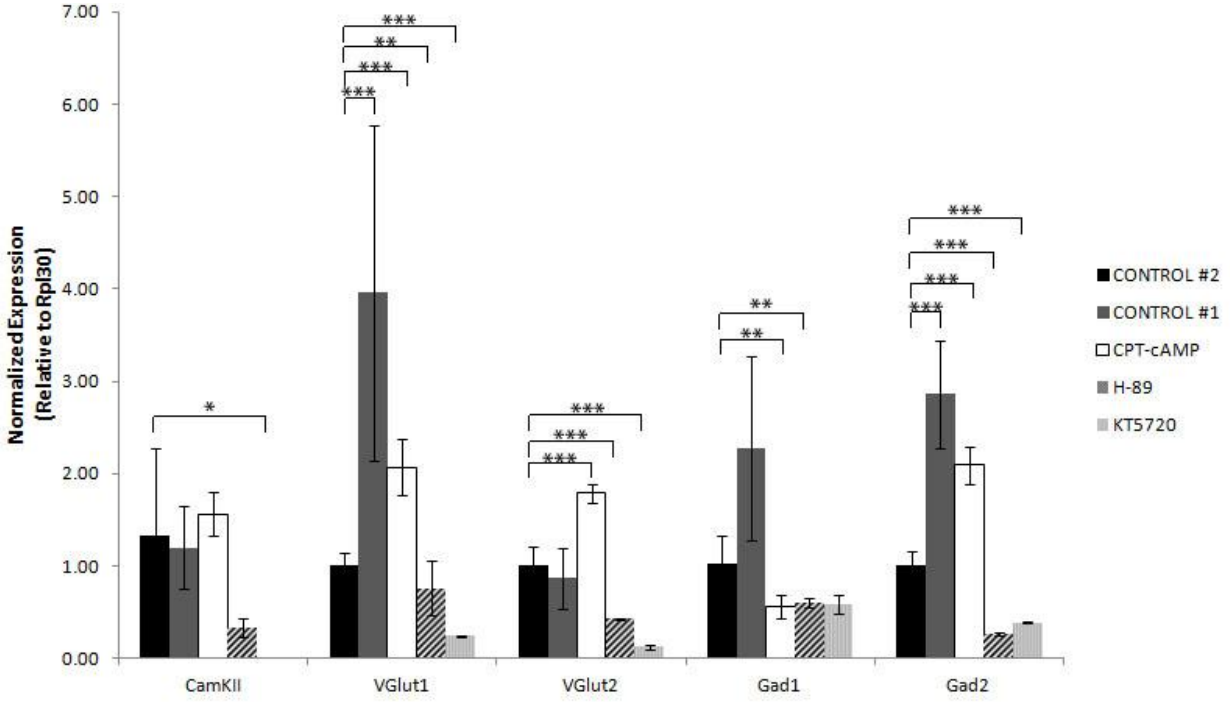

Figure 6. qPCR analysis of cDNA from Experiment 1 and 2, specifically for CamKII, vGlut1, vGlut2, Gad1, and Gad2. A) Representative qPCR results for experiment showing SYBR Green Fluorescence as a function of cycle number. B) Quantification of results from experiment 1, 2, and 3 shown in panel A and other assays for CamKII, VGlut1, VGlut2, Gad1, and Gad2 genes normalized to Rpl30 transcription levels. *p<0.1, **p<0.05, ***p<0.01.

Discussion

Overall, we observed significant gene expression changes when comparing hNPCs treated with CPT-cAMP and the inhibitors H89 and KT-5720. These changes observed in the presence of inhibitors are similar to the reduced expression of ADCY8, GRIK1, PRKG1, and PRKCA observed in SCZD hiPSCs as reported by Brennand et al. (Brennand et al., 2011).

In our experiment we observed that ADCY8 was up-regulated in the presence of CPT-cAMP and down-regulated in the presence of inhibitors. Adenylate cyclase catalyses the formation of cAMP from ATP, allowing cAMP to act as a second messenger. In previous studies it was found that ADCY8 has high expression level in the thalamus and cerebral cortex (Moulder et al., 2008). ADCY8 has an important role in resetting the balance of active to silent synapses after adaptation to strong activity (Moulder et al., 2008); it is also critical for some forms of synaptic plasticity, including long-term potentiation as well as long-term memory formation (Wang and Storm, 2002). The role of ADCY8 in mood disorders has been shown through studies in mice. ADCY8 knockout mice demonstrate lower level of anxiety and greater risk-taking tendencies behavior in comparison to wild-type mice, indicating that ADCY8 involvement in stress responses (Razzoli et al., 2010). Additionally, ADCY8 has been shown to be involved in multiple aspects of addictive behaviors such as cocaine use, alcohol consumption and opiate dependence. The relationship between bipolar and substance use disorders demonstrates that ADCY8 is a potential target for mood disorders (Razzoli et al, 2010). Neurons derived from schizophrenia have a 3 fold decrease expression of ADCY8 (Brennand et al., 2011). In our experiment, we were able to show that ADCY8 expression is altered by the presence of CPT-cAMP and PKA inhibitors. By increasing activation of PKA, we increased expression of ADCY8 by 2-3 fold. By inhibiting PKA with H89 and KT-5720, we showed a slight decrease in

expression of ADCY8. Our results are consistent with a decrease in cAMP activity in schizophrenia neurons. More research involving small interfering RNA (siRNA) as a gene knockout for ADCY8 may help elucidate its role in schizophrenia.

With the presence of H89 and KT-5720, we were also able to mimic glutamate gene expression changes seen in patients with schizophrenia. It was previously reported that GRIK1 showed a fold decrease of 3.9 in schizophrenic neurons relative to the control (Brennand et al., 2011). By decreasing activation of PKA with H89 and KT-5720, we were able to consistently mimic this decrease in expression. In only one set of our experiments, we saw by increasing PKA signaling, we increased expression of GRIK1. Glutamate receptors are excitatory neurotransmitter receptors and are activated in many neurophysiologic processes. It has been previously reported that there is a reduction of the GRIK1 gene in the dorsolateral prefrontal cortex of patients with schizophrenia (Hirata et al., 2012).

However, the presence of H89 and KT-5720 did not mimic the altered expression for genes such as PRKAR2A. While it was previously reported that SCZD neurons show an increased expression of PRKAR2A (Brennand et al., 2011), we observed a slight decrease with treatment with CPT-cAMP and a significant decrease in expression with treatments of KT-5720 (Figure 4B). One possible explanation for the decreased gene expression is that there is an optimal level of PKA phosphorylation of CREB and carbohydrate response-element-binding protein (ChREBP). Phosphorylation of carbohydrate response-element-binding protein (ChREB) by PKA inhibits DNA binding. In contrast to CREB, ChREB is activated by de-phosphorylation (Kawaguchi et al., 2001). It is possible that treatment with CPT-cAMP phosphorylated ChREBP, blocked DNA binding and decreased expression of certain genes such as PRKAR2.

It is also possible that the iPS cells from our experiment and those reported by Brennand et al., at the time of treatment with inhibitors, were at different stages of differentiation.

Aside from the genes involved in PKA signaling, we found some changes in the expression of general neuronal markers as well. Treatment with CPT-cAMP, H89, and KT-5720 altered the expression of DLG4 in both sets of experiments. The DLG4 encodes the Psd95 protein that organizes signaling in the post-synaptic membrane and thus plays a crucial role in neurotransmission. This is consistent with previous studies of SCZD neurons that also showed a decrease in expression of DLG4 (Brennand et al., 2011).

While we found some consistencies in changes in gene expression from our results to those previously reported, we also found some discrepancies. Neurons derived from patients with schizophrenia did not show significant changes in VGlut1 gene expression (Brennand et al., 2011). In our experiment, we found that CPT-cAMP up-regulated expression of both VGlut1 and VGlut2 while H89 and KT-5720 down-regulated its expression. Since VGlut1 is rate limiting for the transport of glutamate into the neurotransmitter synaptic vesicle our results are consistent with cAMP enhancing glutamatergic neurotransmission.

By altering cAMP signaling in our experiment, we were able to compare the gene expression changes found after perturbing PKA activity to the changes reported in neurons derived from patients with SCZD. While we were able to mimic changes in ADCY8 and GRIK1, we were not able to mimic changes for PRKAR2A and PRKCA. Ultimately our results were only partially supportive of our hypothesis, but they suggest changes in PKA signaling may be associated with changes in gene expression in SCZD.

It should also be noted that there were significant differences in gene expression for neuronal markers between the two sets of controls in our experiment. This suggests a possibility of inherent differences in the cell culture between the two sets of experiments. The morphology of cells varied in number of cell bodies and length of neuronal process as shown in Figure 1. It is known that neurons mature with time in culture. Perhaps there were fewer neuronal progenitor cells present in the third experiment relative to the first two experiments. These differences in cell culture could have led to the non-significant gene expression changes observed in the presence of CPT-cAMP and H89.

Aside from the differences between the two sets of controls, we were also limited in our sample size. We had only two complete sets of samples from our three experiments. In addition, the iPS cells in our experiment were derived from only one patient. There is known variability in iPS cell lines derived from various patients and between multiple iPS cell lines from a single individual (Vitale et al., 2012). Differences among cell lines may arise in endogenous pluripotency genes, transgene, and protein expression. There are many potential sources of variability in the production and selection of representative iPS cell clones that may affect the ability to identify disease-associated differences in patient derived iPS cells. There is also an inherent variability relating to fibroblasts from which they are derived (e.g. karyotype, disease, genetic background), as well as variability imposed by reprogramming factors (Vitale et al., 2012). For our project, it would have been ideal to have a large number of iPSC lines that are relatively homogenous and representative of the disease, as well as a similar number from health controls to compare the altered gene expression seen in schizophrenia. In addition to our own project, we were limited in our inferences as well; the study that reported gene expression changes observed in SCZD

neurons were only derived from four patients (Brennand et al., 2011). It should also be noted that results from Brennand et al. have yet to be repeated.

Ideally, it would be optimal to repeat the experiment to see if the variance could be reduced between the two controls. We would hope to observe identical changes in treatments with CPT-cAMP between the two sets of experiments. It would also be ideal to have similar gene expression changes observed in the presence of both inhibitors (H89 and KT-5720) even though these two inhibitors differ in their efficacy and specificity (Davies et al., 2000). KT-5720 was previously found to inhibit many protein kinases, some of which are inhibited far more potently than PKA (PHK is inhibited 300-fold more potently than PKA). H89 is a more selective inhibitor, however it too inhibits at least two or more protein kinases with similar potency for PKA (MSK1, S6K1, and ROCK-11 were inhibited with potency similar to or greater than that for PKA). In terms of their efficacy in inhibiting PKA, KT-5720 has an IC_{50} value of 3.3 μM while H89 has an IC_{50} value of 135 nM. At a concentration of 10 μM, H89 reduced the activity of PKA to 2±1% of the control; this is in contrast to KT-5720, which reduced the activity to 39±5% of control (ATP concentration was 0.1 mM in all assays) (Davies et al., 2000). These inherent differences in the PKA inhibitors could have lead to the differences in gene expression in response to KT-5720 and H89.

While in this project we pharmacologically activated and deactivated PKA, it would be ideal to also perform a molecular genetic increase and reduction of cAMP dependent kinase protein, with the expectation that it will alter protein phosphorylation by PKA. Similar to the CPT-cAMP treatment, the expression vector with the catalytic (Cα) subunit of protein kinase A should increase PKA activation. This expression vector for the catalytic subunit will alter CREB-mediated gene expression even in the absence of CPT-cAMP (Uhler and Abou-Chebl, 1991).

Reduced PKA activation can be accomplished using two different methods: a mutant regulatory subunit of PKA and a protein kinase inhibitor (PKI). The mutant regulatory subunit of PKA has amino acid mutations in each of the two cAMP binding sites in the RIα protein, so that when the protein is produced, it binds to the catalytic subunit but cannot be activated by cAMP. Protein phosphorylation by the C subunit is therefore blocked in cells, producing the mutant RIα protein (Uhler and Abou-Chebl, 1991). In a similar way, the PKI expression vector will block activation of the C subunit because it binds to the substrate binding site of the C subunit and prevents protein substrates from binding to the C subunit (Huang et al., 2011). These two vectors should produce the same results as the treatment of H89 and KT-5720, namely that neurons derived from normal iPS cells should become more "schizophrenic" in terms of the changes in gene expression for genes such as GRIK1, NRG1, etc. We would transfect each of the 3 vectors in iPSCs using Amaxa electroporation . Selection of the progenitor cells that have taken up the plasmid would be done by using puromycin treatment. All cells that did not incorporate the plasmid would die in the presence of puromycin. These progenitor cells would differentiate into neurons as described previously in the methods section. Next RNA-seq would be performed to map RNA pieces to human chromosome and compare the "SCZD" neurons and control iPSC neurons. However, in performing this molecular genetic reduction, it may be difficult to create the vector. We encountered clonal difficulties when attempting to obtain a plasmid with the UbiC promoter, mutant RIα, and Rpl10acherry-3x tag with an IRES subunit.

In addition to these studies that are performed in vitro, using a mouse model to observe the effect of inactivating the Prkaca (protein kinase, cAMP dependent, catalytic, alpha) gene could help understand the role of PKA in the behaviors associated with schizophrenia. The protein coding region for Prkaca is located on chromosome 8. By using the Cre-LoxP system, expression of

Prkaca can be inactivated only in the cortical neuronal cells. Homologous recombination in ES will be used to create the floxed mouse; the essential exon of the Prkaca gene is flanked by 2 lox P sites located in intronic sequences. Mice with these LoxP sites would be bred with transgenic mice expressing the Cre recombinase (Gavériaux-Ruff and Kieffer, 2007). Cre expression would be controlled by a promoter for deleted expression only in the cortical cells. Cre expression would be controlled by a promoter for expression only in the cortical cells. RNA isolated from the brains of the adult male mice could be reverse transcribed into cDNA. In order to assess behaviors associated with schizophrenia, tests including prepulse inhibition (PPI) (shows deficit to filter out unnecessary information), social interaction, sucrose preference (SP) (assess anhedonia), and locomotor response to d-amphetamine (Distler et al., 2012). By assessing these behaviors in the mice deficient of PKA, the role of PKA in schizophrenia can be better understood in an animal model.

In the field of disease modeling, iPSCs have allowed for investigation of the early human development, etiology, and progression of different diseases (Yung et al., 2013). Research in the development of treatments for diseases should involve disease-specific tissues obtained from patients In studying neurological diseases, it has been difficult to biopsy brain tissue due to its highly invasive procedure and to perform biochemical analysis from autopsy specimens due to rapid post-mortem changes (Ito et al., 2012). The advent of iPSCs has made it possible to recapitulate the disease with the same genomic materials of the patient.

Human iPSCs generated from schizophrenic patients have shown alterations in neuronal phenotypes and gene expression (Brennand et al., 2011). In order to further understand the molecular mechanisms underlying schizophrenia and understand the role of cAMP signaling, we have taken iPSCs, differentiated them into neuronal progenitor cells, and perturbed PKA

signaling in order to model schizophrenia. With the use of iPSCs, we were able to compare the gene expression changes from the control and experimental neuronal progenitor cells. Our findings suggest that changes in PKA signaling may be associated with changes in gene expression in schizophrenia. This further suggests drug treatments altering the expression of some cAMP-regulated genes to normal levels may be more effective in treating schizophrenia.

However, at present, there are a number of technical problems in using iPSCs. These include large clonal variation and the heterogeneity of differentiated neurons. The methods for reprogramming and inducing differentiation have yet to be standardized (Vitale et al., 2012). It is important to reduce the variability between iPS cells derived from individual and multiple patients and determine criteria for selecting the iPS cell lines.

In the future, these patient derived iPSCs have the potential to study disease mechanisms for a variety of neurological disorders and to allow for drug discovery to delay the progression of neurodegenerative diseases. Currently, there have been neurological disease-specific iPS cells used in used in pathogenesis research, drug screening, diagnosis and regenerative medicine (Ito et al., 2012). These iPSCs can help us understand the molecular mechanisms for many diseases including amyotrophic lateral sclerosis (ALS), sporadic Parkinson's disease (PD), Duchenne/Becker muscular dystrophy, and Huntington disease. With further advancement of iPSCs, should be possible to better understand and develop improved drug treatments for a complex diseases such as schizophrenia.

List of Abbreviations

Abbr.	Abbreviation
5-HT	serotonin
5-HT1A	5-hydroxytryptamine (serotonin) receptor 1A, G protein-coupled
5-HT2A	5-hydroxytryptamine (serotonin) receptor 2A, G protein-coupled
5-HT2C	5-hydroxytryptamine (serotonin) receptor 2C, G protein-coupled
5-HT6	5-hydroxytryptamine (serotonin) receptor 6, G protein-coupled
5-HT7	5-hydroxytryptamine (serotonin) receptor 7, G protein-coupled
ADCY8	adenylate cyclase 8 (brain)
Akt/AKT	protein kinase B
ALS	amyotrophic lateral sclerosis
AMP	adenosine monophosphate
ATP	adenosine-5'-triphosphate
BDNF	brain-derived neurotrophic factor
bZIP	basic leucine zipper domain
cAMP	Cyclic adenosine monophosphate
CNS	central nervous system
CPLXII	complexin II
CPT-cAMP	8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate sodium salt
CRE	consensus cAMP response element
CREB	cAMP response element binding protein
CT	computed tomography
D1	dopamine receptor D1
D2	dopamine receptor D2
D3	dopamine receptor D3
D4	dopamine receptor D4
DA	dopamine
ERK	Ras/extracellular signal regulated kinase
ES	embryonic stem
et al.	`et alii' to mean "and others"
FACS	fluorescence activated cell sorted
FGF	fibroblast growth factor
FGFRs	fibroblast growth factor receptors
fMRI	functional magnetic resonance imaging
GABA	γ-Aminobutyric acid
GRIK1	glutamate receptor, ionotropic, kainate 1
GRIN2A	glutamate receptor, ionotropic, N-methyl D-aspartate 2A
GRM7	glutamate receptor, metabotropic 7

H1	histamine H1 receptor
H89	(N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide)
H89	(N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide)
hES	human embryonic stem
hiPSC	human induced pluripotent stem cell
hNPC	Human neuronal progenitor cells
iPSCs	induced pluripotent stem cells
KT-5720	2,3,9,10,11,12-hexahydro-10S-hydroxy-9-methyl-1-oxo-9R,12S-epoxy-1H-diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid, hexyl ester
M1	muscarinic acetylcholine receptor M1
M4	muscarinic acetylcholine receptor M4
MAP2	microtubule associated protein 2
MAPK	mitogen-activated protein kinase
MDD	major depressive disorder
MEF	mouse embryonic fibroblasts
MRI	magnetic resonance imaging
NANOG	Nanog homeobox
NRG1	neuregulin 1
NSCs	neural stem cells
NSFT	novelty suppressed feeding test
PBS	phosphate buffered saline
PD	Parkinson's disease
PET	positron emission tomography
PFC	prefrontal cortex
PI3K	phosphoinositide 3-kinase
PKA	cAMP-dependent protein kinase/protein kinase A
PKB	protein kinase B
PKI	protein kinase inhibitor
PPI	prepulse inhibition
PRKACA	PKA catalytic subunits
PRKCA	protein kinase C, alpha
ROCK	rho-associated protein kinase
SCZD	schizophrenia
siRNA	small interfering RNA
SNAP-25	synaptosomal-associated protein 25
SNRIs	dual serotonin and norepinephrine reuptake inhibitors
SOX2	SRY (sex determining region Y)-box 2
SP	sucrose preference
SRY	sex determining region Y

SSRIs	selective serotonin reuptake inhibitors
STAT3	signal transducer and activator of transcription 3
TCF4	transcription factor 4
TrkB	tyrosine-related kinase B
WNT7A	wingless-type MMTV integration site family, member 7A
α1	alpha-1 adrenergic receptor
α2	alpha-2 adrenergic receptor

References

Arnold, S.E., Lee, V.M., Gur, R. E., Trojanowski, J. Q. (1991) Abnormal expression of two microtubule-associated proteins (MAP2 and MAP5) in specific subfields of the hippocampal formation in schizophrenia. *Proc. Nati. Acad. Sci. USA* **88**, 10850-10854.

Artigas, F. (2012) Serotonin receptors involved in antidepressant effects. *Pharmacology & Therapeutics*. In Press.

Benes, F. (1993) Neurobiological investigations in cingulate cortex of schizophrenic brain. *Schizophr Bull* **19**, 537-49.

Brennand, K.J., Simone, A., Jou, J., Gelboin-Bukhard, C., Tran, N., Sangar, S., Li, Y., Yangling, M., Chen., Yu, D., McCarthy, S., Sebat, J., Gage, F. (2011) Modelling schizophrenia using human induced pluripotent stem cells. *Nature* **473**, 221-227.

Brenner, H.D., Denker, S.J., Goldstein, M.J., Hubbard, J.W., Keegan, D.L., Kruger, G., Kulhanek, F., Liberman, R. P., Malm, U., Midha, K.K. (1990) Defining treatment refractoriness in schizophrenia. *Schizophr Bull* **16**, 551-61.

Chao, J., Nestler, E.J. (2004) Molecular Neurobiology of Drug Addiction. *Annu. Rev. Med.***55**, 113-32.

Chen, B., Wang, X., Wang, Y., Chen, L., Luo, Z. (2012) Brain- Derived Neurotrophic Factor Stimulates Proliferation and Differentiation of Neural Stem Cells, Possible by Triggering the Wnt/β-Catenin Signaling Pathway. *J Neurosci Res* **0**, 000-000.

Davies, S., Reddy, H., Caivano, M., Cohen, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J.* **351**, 95-105.

Distler, M. G., Opal, M. D., Dulawa, S. C., Palmar, A. A. (2012). Assessment of Behaviors Modeling Aspects of Schizophrenia in Csmd1 Mutant Mice. *PLOS One.* **7**. 1-6.

Duman, R.S., (2002) Synaptic plasticity and mood disorder. *Molecular Psychiatry* **7**, S29-S34.

Elsayed, M., Banasr, M., Duric, V., Fournier, N. M., Licznerski, P., Duman, R. S. (2012) Antidepressant Effects of Fibroblast Growth Factor-2 in Behavioral and Cellular Models of Depression. *Biol Psychiatry* **72**, 258-265.

Finkbeiner, S., Tavazoie, S. F., Maloratsky, A., Jacobs, K. M., Harris, K. M., Greenberg, M. E.(1997) CREB: A Major Mediator of Neuronal Neurotrophin Responses. *Neuron* **19**, 1031-47.

Gavériaux-Ruff, C., Kieffer, B.L. (2007) Conditional gene targeting in the mouse nervous system: Insights into brain function and diseases. Pharmacology & Therapeatuics 113, 619-34.

Gnegy, Margaret E. *Basic Neurochemistry*. 8th ed. N.p.: Elsevier Inc., 2012. 293-95.

Guillemot, F., Zimmer, C. (2011) From Cradle to Grave: The Multiple Roles of Fibroblast Growth Factors in Neural Development. *Neuron* **71**, 574-88.

Hirata, Y., Zai, C.C., Souza, R. P., Lieberman, J. A., Meltzer, H. Y., Kennedy, J. L. (2012). Association study of GRIK1 gene polymorphisms in schizophrenia: case-control and family-based studies. *Hum. Psychophamacol Clin Exp.* **27**, 345-51.

Huang, H.S., Turner, D.L., Thompson, R.C., Uhler, M.D. (2011) Ascl1-induced neuronal differentiation of P19 cells requires expression of a specific inhibitor protein of cyclic AMP-dependent protein kinase. *Journal of Neurochemistry* **120**, 667-83.

Ito, D., Okano, H., Suzuki, N. (2012) Accelerating Progress in Induced Pluripotent Stem Cell Research for Neurological Diseases. *Ann Neurol* **72**, 167-174.

Jibson, M., Tandon, R. (1995) The negative symptoms of schizophrenia. *Dir Psychiatry* **15**, 1-7.

Kawaguchi, T., Takenoshita, M., Kabashima, T., Uyeda, K. (2001) Glucose and cAMP regulate the L-type pyruvate kinase gene by phosphorylation_dephosphorylation of the carbohydrate response element binding protein. *Proc Natl Acad Sci U.S.A.* **98**, 13710-5.

Kelly, C., Sharkey, G., Morrison, J., Allardyce, J., McCreadie, R.G. (2000) Nithsdale Schizophrenia Surveys 20: Cognitive function in a catchment-area-based population of patients with schizophrenia. *British Journal of Psychiatry* **177,** 348-53.

Kerwin, R., Patel, S., Meldrum, B. (1990) Quantitative autographic analysis of glumate binding sites in the hippocampal formation in normal and schizophrenic brain post mortem. *Neuroscience* **39**,25-32.

Lee, H. J., Lim, I.J., Lee, M.C., Kim, S.U. (2010) Human Neural Stem Cells Genetically Modified to Overexpress Brain-Derived Neurotrophic Factor Promote Function Recovery and Neuroprotection in a Mouse Stroke Model. *J Neurosci Res* **88**, 3282-3294.

Levine, M., Ruha, A. (2012) Overdose of Atypical Antipsychotics: Clinical Presentation, Mechanisms of Toxicity and Management. *CNS Drugs* **26**, 601-11.

Moulder, K. L., Jiang, X., Chang, C., Taylor, A. A., Benz, A. M., Conti, A., Muglia, L. J., Mennerick, S. (2008). A specific role for Ca+2- dependent adenylyl cyclases in recovery from adaptive presynaptic silencing. *J Neurosci.* **28**, 5159-68.

Ochoa, S., Usall, J., Cobo, J., Labad, X., Kulkarni, J. (2012) Gender Differences in Schizophrenia and First-Episode Psychosis: Comprehensive Literature Review. *Schizophrenia Research and Treatment* **2012**, 1-9.

Quintana, J., Davidson, T., Kovalik, E., Marder, S.R., Mazziotta, J.C. A compensatory mirror cortical mechanism for facial affect processings in schizophrenia. *Neuropsychopharmacology* **25**, 915-24.

Razzoli, M., Andreoli, M., Michielin, F., Quartra, D., Sokal, D. (2010). Functional Role of Calcium-Stimulated Adenylyl Cyclase 8 in adaptations to psychological stressors in the mouse: implications for mood disorders. *Neuroscience* **170**, 429-40.

Ren, X., Uhler, M. (2008) Microarray transfection analysis of conserved genomic sequences from three immediate early genes. *Genomics* **93**, 159-68.

Reuss, B., von Bohlen und Halbach, O. (2003) Fibroblast growth factors and their receptors in the central nervous system. *Cell Tissue Res* **313**, 139-157.

Shi, Y., Kirwan, P., Smith, J., Robinson, H. P., Livesey, F. (2011). Human cerebral cortex develoment from pluripotent stem cells to functional excitatory synapses. *Nature Neuroscience*, **15**, 477-487.

Stevens, H. E., Jiang, G. Y., Schwartz, M. L., Vaccarino, F. M. (2011) Learning and Memory Depend on Fibroblast Growth Factor Receptor 2 Functioning in Hippocampus. *Biol Psychiatry* **71**, 1090-8.

Stotz-Ingenlath, G. (2000) Epistemological aspects of Eugen Bleuler's conception of schizophrenia in 1911. *Medicine, Health Care and Philosophy* **3.2**, 153-59.

Sullivan, P. F., Kendler, K.S., Neale, M.C. (2003) Schizophrenia as a Complex Trait: Evidence From a Meta-analysis of Twin Studies. *Arch Gen Psychiatry*, **6**, 1187-92.

Tao, X., Finkbeiner, S., Arnold, D.B., Shaywitz, A., Greenberg, M. E. (1998) Ca+2 Influx Regulates BDNF Transcription by a CREB Family Transcription Factor-Dependent Mechanism. *Neuron* **20**, 709-26.

Uhler, M. D., Abou-Chebl, A.(1991) Cellular Concentrations of Protein Kinase A Modulate Prostaglandin and cAMP Induction of Alkaline Phosphatase. *The Journal of Biological Chemistry* **267**, 8658-65.

Vitale, A. M., Matigian, N. A., Ravishankar, S., Bellette, B., Wood, S., Wolvetang, E. J., Macknay-Sim, A. (2012). Variability in the Generation of Induced Pluripotent Stem Cells: Importance for Disease Modeling. *Stem Cells Translational Medicine* **1,** 641-50.

Wang, H., Storm, D. (2002). Calmodulin-Regulated Adenylyl Cyclases: Cross-Talk and Plasticity in the Central Nervous System. *Molecular Pharmacology* **6320**, 463-68.

Wong, A., Van Tol, H. (2003). Schizophrenia: from phenomenology to neurobiology. *Neuroscience and Behavioral Reviews* **27**, 269-306.

World Health Organization. *Mental Health: Schizophrenia*. N.p., n.d. Web. 23 Feb. 2012. <http://www.who.int/mental_health/management/schizophrenia/en/>.

Yung, J. S., Tam, P. K., Ngan, E. S. (2013). Pluripotent stem cell for modeling neurological diseases. *Experimental Cell Research* **319**, 177-184.