UNIVERSITY OF CALIFORNIA SAN DIEGO

Dopaminergic Neuromodulation of the Inducible Transcription Factor NPAS4

A Thesis submitted in partial satisfaction of the requirements

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in

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by

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The Thesis of Lori Mandjikian is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Dedication

In recognition of the completion of this master's thesis; for listening to me endlessly talk about the experiments detailed herein; for fiercely supporting me, reminding me to be proud of my roots, and for igniting my sense of curiosity, this thesis is dedicated to my loving parents, Khatchig and Houri Mandjikian.

Epigraph

“Any man could, if he were so inclined, be the sculptor of his own brain.”

-Santiago Ramón y Cajal, *Advice for a Young Investigator*

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Abstract of the Thesis

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The brain converts fleeting experiences into long lasting changes in circuit connectivity and function. Activity induced immediate early genes(IEGs) are rapidly expressed in response to cellular stimuli. When a mouse is placed in an enriched environment, the inducible transcription factor(ITF) NPAS4 is expressed in hippocampal CA1 pyramidal neurons in response to depolarization and not other types of stimuli (Lin

et al., 2008; Bloodgood et al., 2013). Expression of NPAS4 is further known to mediate inhibitory circuit rewiring onto pyramidal neurons through an increase in somatic inhibition by CCK basket cells and a decrease in dendritic inhibition (Bloodgood et al., 2013; Hartzell et al., 2018). Inhibitory circuit reorganization in CA1 through depolarization-dependent NPAS4 expression is likely to play an important functional role in encoding pyramidal cell place fields (Del Pino et al., 2017; Wilson et al., 2001; Danielson et al., 2016). However, the role of neuromodulatory inputs on NPAS4 expression an animal experiences in vivo is unknown. Neuromodulators such as dopamine, acetylcholine and noradrenaline are known to be released during periods of attention, novelty, and salience which an animal experiences as it travels through an enriched environment (Avery et al., 2017). With bath application of D1 dopamine receptor agonist SKF81297 on mouse acute slices, we show diminished NPAS4 expression in hippocampal CA1 pyramidal neurons. This result describes potential for dopamine to modulate NPAS4 mediated changes in inhibitory circuit connectivity in vivo and place field formation as the animal explores an enriched environment.

Introduction

Animals extract information from their environment and use this information to drive motor programs, make decisions, and predict future events. Salient events the animal experiences can be encoded in the brain for long periods of time, with some memories lasting for the life of an animal. How do fleeting experiences become permanent memory traces? What are the molecular mechanisms that support long lasting changes in neuronal function and circuit connectivity?

Stimulus dependent gene regulation is a fundamental part of growth, development, and maintenance of all biological systems. In response to stimuli, immediate early genes (IEGs) become transiently expressed without requiring de novo protein synthesis and act as a gateway to further downstream genomic response (Sun et al., 2016; Bartel et al., 1989; Pinaud et al., 2001; Bisler et al., 2002). To date, various IEGs have been identified in various organisms, including *c-Fos*, *Zif268* and *Arc*. These IEGs are induced by various trophic factors, are expressed in many cell types, and affect downstream protein expression that alter neural plasticity (Greenberg et al., 1984; Bartell et al., 1989; Richardson et al., 1992; Lyford et al., 1995; Plath et al., 2006; Shepherd et al., 2006; Flavell et al., 2008). As a subcategory of IEGs, inducible transcription factors (ITFs), such as c-Fos and Zif268, regulate programs of gene expression to alter cellular function. In the brain, ITFs are expressed in response to depolarization driven by sensory stimuli, novelty, and learning. These same stimuli lead to the expression of many genes, including those involved in synapse function and plasticity. This has led to the model that novel or salient sensory stimuli trigger the expression of ITFs which regulate genes that change synapse function and connectivity.

The neuronal bHLH-PAS domain transcription factor protein 4 (NPAS4) is expressed as an animal travels in an enriched environment (Bloodgood et al., 2013). However, it is unique in that its expression is specific to neuronal depolarization events in the form of action potentials (APs) or excitatory postsynaptic potentials (EPSPs) and their associated calcium influx, rather than growth factors or neurotrophins that have been used classically to stimulate expression of the earliest studied immediate early genes (Ooe et al., 2004; Bartel et al., 1989; Greenberg et al., 1984; Lin et al., 2008; Brigidi et al., 2019 in press; West et al., 2001). Expression of NPAS4 in hippocampal CA1 neurons is known to mediate inhibitory circuit rewiring onto pyramidal neurons through an increase in somatic inhibition by CCK basket cells and a decrease in dendritic inhibition (Bloodgood et al., 2013; Hartzell et al., 2018). Circuit reorganization in the hippocampus through depolarization-dependent NPAS4 expression is likely to play an important functional role in forming contextual memories in CA3 pyramidal neurons and encoding place fields in CA1 pyramidal neurons (Sun et al., 2016).

Previous electrophysiology work in our lab has identified a robust protocol for NPAS4 induction and opened the door to investigating the threshold of NPAS4 induction. When induced in acute slice with antidromic one second 100Hz alveus stimulus, CA1 neurons display AP-mediated somatic expression of NPAS4. This occurred in response to calcium influx via L-type calcium channels (LTCCs), and did not occur with a 3.5 minute 0.5Hz stimulus. Furthermore, when CA1 local afferents were extracellularly stimulated to evoke EPSPs, dendritic expression of protein occurred in specifically in stratum radiatum through NMDA-dependent calcium influx (Bridigi et al., 2019 in press). Furthermore in vivo, NPAS4 expression induced by sensory stimuli in

the enriched environment paradigm led to ~10% of CA1 pyramidal cells exhibitting NPAS4 protein (Hartzell et al 2018). These CA1 pyramidal cells are also characterized as place cells in which subsets of cells increase firing rates when an animal is in a particular location in its environment (O'Keefe et al., 1971; Eichenbaum et al.,1999). As place cells, many CA1 neurons in the enriched environment paradigm are activated, however few display induction of NPAS4 protein. This implies a possible threshold for NPAS4 induction. In identifying the electrophysiological threshold for induction, we also ask if it is possible that neuromodulator release during novel environment discovery onto coincidentally firing CA1 pyramidal neurons modifies this threshold.

The mammalian neuromodulatory system originates from a pool of neurons located in four main regions that project to almost all areas in the brain. The Raphe Nucleus (RN), Basal Forebrain(BF), Locus Coeruleus(LC), and Ventral Tegmental Area(VTA) send serotonergic, cholinergic, adrenergic, and dopaminergic projections respectively, to areas such as the prefrontal cortex, striatum, anterior cingulate cortex, and hippocampus (Avery et al., 2017). The RN, BF, LC, and VTA release various neuromodulators onto the hippocampus when the animal experiences environmental novelty and complexity (Milan et al., 2003; Sarter et al., 2001; Vankov et al., 1995; Avery et al., 2017).When an animal travels through an enriched environment, it undergoes salient experiences with novel cage toys. Observing the modulation of dopamine on NPAS4 expression is particularly interesting due to previous reports of dopamine release in response to salient events from the VTA onto dorsal CA1 where D1 receptors are present (Bromberg-Martin et al., 2010; McNamara et al., 2014; Puighermanal et al., 2016).

We have observed the in vivo induction of NPAS4 through a mouse's interaction with an enriched environment and determined the depolarization events required ex vivo (Bloodgood et al., 2013; Brigidi et al., 2019 in press). However, the role of neuromodulator release onto these hippocampal CA1 neurons is currently unknown. To address this, we begin by investigating the role of dopamine in modulating NPAS4 expression during coincident depolarization in mouse acute slices. It is observed that bath application of a D1 receptor agonist SKF81297, with coincident stimulus via AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) with $GABA_A$ antagonist Picrotoxin, diminishes NPAS4 expression in mouse hippocampal CA1 neurons.

Materials and Methods

Mouse Husbandry

Animal procedures and protocols were performed in accordance with the University of California San Diego Institutional Animal Care and Use Committee. The mouse lines used were wild type (WT C57/BL6 or C57/CD1) ages P21-P28 and were housed in standard conditions (vivarium home cage). Animals were allowed sufficient time for acclimation to lab environment when first removed from vivarium to avoid confounding expression. Experiments utilized both male and female mice and data was pooled together.

Acute Slices

Animals were anesthetized by inhalation of isoflurane and sacrificed by decapitation. The brain was removed quickly and placed into cold choline-aCSF consisting of (in mM): 110 choline-Cl, 25 $NaHCO_3$, 1.25 Na_2HPO4, 2.5 KCl, 7 $MgCl_2$, 25 glucose, 0.5 $CaCl_2$, 11.6 ascorbic acid, 3.1 pyruvic acid and equilibrated with 95% O_2 / 5% CO_2. Blocking cuts were made and tissue was sliced in 300µm transverse sections with a LeicaVT1000s vibratome (Leica Instruments) and transferred to a recovery chamber containing aCSF consisting of (in mM): 127 NaCl, 25 $NaHCO_3$, 1.25 Na_2HPO_4, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 glucose, saturated with 95% O_2 / 5% CO_2, containing 250 µM kynurenic acid (KYN) and 200 nM TTX. Slices were incubated at 31°C for 30 min and then maintained at room temperature (RT) for the duration of experiment up to 2 hours.

Assay

A novel multi-chamber oxygenated container was created for simultaneous benchtop bath application of various agonists (Nimodipine Tocris Cat#0600; R,S AMPA Hydrobromide Tocris Cat#0169; Picrotoxin Tocris Cat#1128; SKF81297 Tocris Cat#1447). In order to place various brain slices from the same animal into different chambers containing controls and agonists in a resource and time efficient manner, each compartment in a clear egg tray holder(Totally Kitchen®) was used as a chamber. Tubes to facilitate oxygenation were clipped onto sides of tray and a cell strainer was placed in each compartment. Using a blunt glass pipette, slices were successfully transferred from recovery bath to each oxygenated chamber containing the floating cell strainer with minimal transfer of solution from the recovery bath to the experimental chamber. After slices were exposed to agonists for five minutes, they were then transferred to their respective aCSF only chamber for one hour to allow for a five minute exposure to the agonist only and allow for ample NPAS4 expression to occur. After expression is ensured in the aCSF only chambers, slices are transferred to a 24 well plate for fixation.

Immunohistochemistry

To fix tissue, slices were placed in 4% PFA/PBS for 2hr at 4°C with gentle agitation in 24 well plates for appropriate divisions for each condition. Then, slices were washed 3x in cold PBS, 5min per wash. For permeabilization and blocking, slices were incubated in 10% Normal goat serum / 0.25% Triton X100 / PBS at 4°C overnight with gentle agitation. For primary antibody incubation, primary antibody mix was created with 1% goat serum / 0.25% Triton X100 / PBS/ primary antibodies used at 1:1000 (Rabbit

monoclonal anti-NPAS4 antibody, guinea pig polyclonal anti-NeuN antibody or chicken polyclonal anti-NeuN antibody). The permeabilization and blocking solution was removed, replaced with primary antibody mix, and incubated for 48 hour at 4°C with gentle agitation. For secondary antibody incubation, the primary antibody mix was removed and slices were washed 3x with cold 1x PBS for 10 minutes per wash at 4°C with gentle agitation. After the third wash, PBS was replaced with antibody mix as described above but with diluted secondary antibodies and incubated overnight at 4°C with gentle agitation (goat anti-rabbit 568, goat anti-guinea pig 647 or goat anti-rabbit 488). For mounting and coverslipping, secondary antibody mix was removed and slices were washed 3x with cold PBS 10 min per wash. After the third wash, tissue sections were mounted carefully on glass slide with blunt glass pipette for transferring and thin paintbrush for positioning. Slices are then mounted with Fluoromount-G with DAPI and carefully coverslipped. Slides were left overnight in dark RT for dryng. To seal coverslip and slide together, clear polish was used around the perimeter of the coverslip. Slides were then stored at 4°C in dark for imaging.

Confocal Imaging

Slices were imaged focusing on hippocampus CA1 using a Nikon A1 confocal microscope (objective 20x / 0.75; UC San Diego School of Medicine Nikon Imaging Center). Top and bottom of each 300µm slice is set and the very middle z plane is objectively determined by the microscope imaging program for final acquisition. Identical acquisition parameters were utilized for slices within a single experiment, set by adjusting with the positive and negative controls for obtaining full dynamic range of fluorescence.

Image Analysis and Fluorescence Quantification

Confocal images for each experiment were accessed through ImageJ. NPAS4 and NeuN channel were separated and saved as tiff files. A region of interest (ROI) was subjectively drawn around stratum pyramidale in CA1. Using a matlab code, average NPAS4 fluorescence values within the NeuN positive cells. Average fluorescence within the ROI but outside of the NeuN mask was used to objectively threshold out fluorescence caused by background signal and non-specific binding.

Results

The post-slicing recovery chamber does not show high levels of slice-induced expression.

Immediate early gene expression has been previously shown to be induced by various inputs, including stressful mechanostimulation caused by axonal shearing, disturbance of long range neural connections, and direct neuronal damage during cutting. Therefore, slice-induced expression is observed closely to avoid a potentially confounding variable. Upon previous experimental consensus in Bloodgood Lab, TTX and kynurenic acid is used to recover slices as a way to block voltage gated sodium channel and AMPA/NMDA/Kainate receptor blocker to avoid excitotoxic cell death. Upon direct fixation after thirty minutes of recovery, average fluorescence values (unitless) are calculated to be 28.55±4.66, n=19 slices (average±s.e.m.) as also indicated in Figure 2C. Post-slicing low expression allows for further pharmacological investigation of NPAS4 expression by bath application to be easily attributable to the drug applied, and not confounded by slice-induced expression.

Low NPAS4 expression occurs in L-type calcium channel blocker and unstimulated slices.

After slices are recovered in the recovery bath containing TTX and kynurenic acid (Figure 2B), the slice is transferred to a bath containing L-type calcium channel blocker (LTCC), nimodipine with aCSF only. LTCC phosphorylation and subsequent calcium influx is shown to be necessary for depolarization dependent expression of many immediate early genes, including NPAS4(Lin et al., 2008). Slices placed in aCSF

and nimodipine only (labeled “saCSF” or silenced aCSF Figure 2B) demonstrated comparable expression levels to slices moved from the recovery chamber to a chamber containing only unstimulated aCSF (aCSF: avg. 28.63±3.09, n=25; saCSF: avg. 28.15±4.17, n=17). This comparison confirms that the slice transfer from the recovery bath to the unstimulated aCSF does not increase NPAS4 expression and does not act as a confounding variable.

Robust synaptically-induced stimulus is established with bath application of AMPA and Picrotoxin to display high NPAS4 expression.

After slices were recovered in the recovery bath, some were randomly selected for stimulation to observe possibilities for a pharmacologically induced high level of expression. We used 10uM AMPA to stimulate AMPA receptors on pyramidal neurons with 50uM Picrotoxin to inhibit firing of inhibitory interneurons in the hippocampus from dampening the excitatory effect of AMPA, as interneurons also express AMPA receptors. After five minutes of exposure to this stimulation, slices were placed in a chamber containing aCSF only for one hour to allow for maximal NPAS4 induction to occur (Lin et al., 2008; Brigidi et al., 2019 in press). As shown in Figure 2B, AMPA with Picrotoxin resulted in a significant increase in NPAS4 expression in CA1 pyramidal neurons measured at avg. 102.25±15.39, n=12, **p<0.01, one-way ANOVA, Tukey HSD test.

Synaptic stimulus with coincident D1 activation results in diminished NPAS4 expression.

As NPAS4 expression is induced in vivo when an animal is exposed to an enriched environment (Bloodgood et al., 2013), it has also been shown that expression occurs ex vivo in acute slice when an extracellular stratum radiatum 100Hz stimulus is applied (Brigidi et al., 2019 in press). With these behavioral and physiological methods of induction as possibilities, we were interested in investigating the possibility that dopamine would affect induction of NPAS4 protein, as the dopaminergic neurons in the ventral tegmental area also become activated when an animal experiences novelty in vivo and dopaminergic neurons from the VTA innervate the hippocampus(Avery et al., 2017). Hippocampal pyramidal neurons express D1 receptors and therefore have the capacity to be modulated by dopamine (Huang et al., 1999, Neve et al., 2004). As a D1/D5 agonist, 10uM SKF81297 was added to bath for acute slice exposure for five minutes (Varela et al., 2009). This was done with coincident depolarization by 10uM AMPA and 50uM Picrotoxin, as would occur in the scenario that NPAS4 expression occurring in the enriched environment as described above would be modulated. With the most representative image shown in hown in Figure 2B, Average fluorescence for bath application of 10uM SKF81297 with 10uM AMPA and 50uM Picrotoxin was avg. 24.94±3.49, n=9. This fluorescence value is considerably lower than the AMPA with Picrotoxin alone, as indicated above. Therefore, when the D1 agonist SKF81927 is applied with coincident stimulation, NPAS4 induction decreases.

Figure1A.

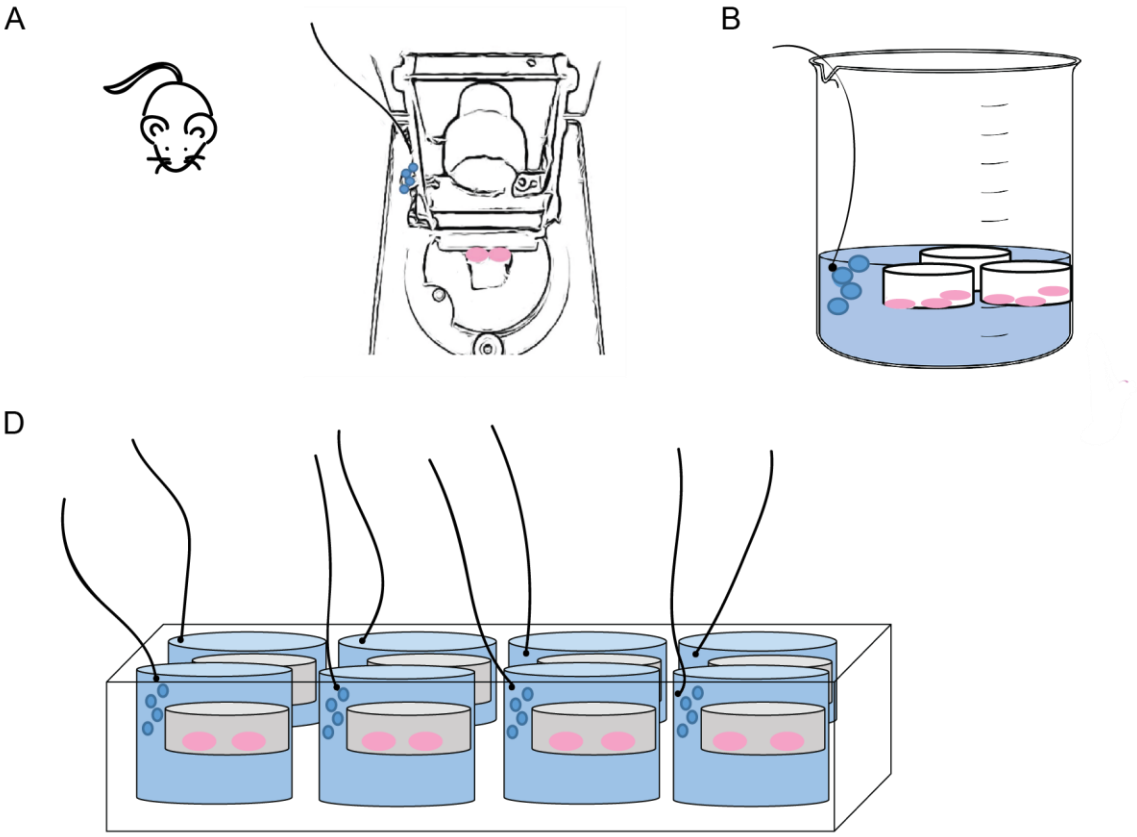

Figure 1A. Mouse acute slices are exposed to agonists in oxygenated chambers.

A. Hippocampal slices from C57/BL6 or C57/CD1 mice (ages P21-P28) are transversely sectioned(300um) using a vibratome, in cold oxygenated choline-acsf.
B. Slices are transferred to a recovery chamber containing oxygenated aCSF with TTX (200nM) and kynurenic acid(250uM) for 30min incubated at 31°C then maintained at room temperature during the duration of the experiment (1-2hrs).
D. Various bubbling chambers containing desired compound is created and used for pharmacological application. For example, a slice is exposed to a desired agonist diluted in aCSF for 5min in any of the the rear four chambers. It is then transferred to its respective front row chamber that contains only bubbling aCSF for 1hr to allow time for NPAS4 expression.

Figure 1B.

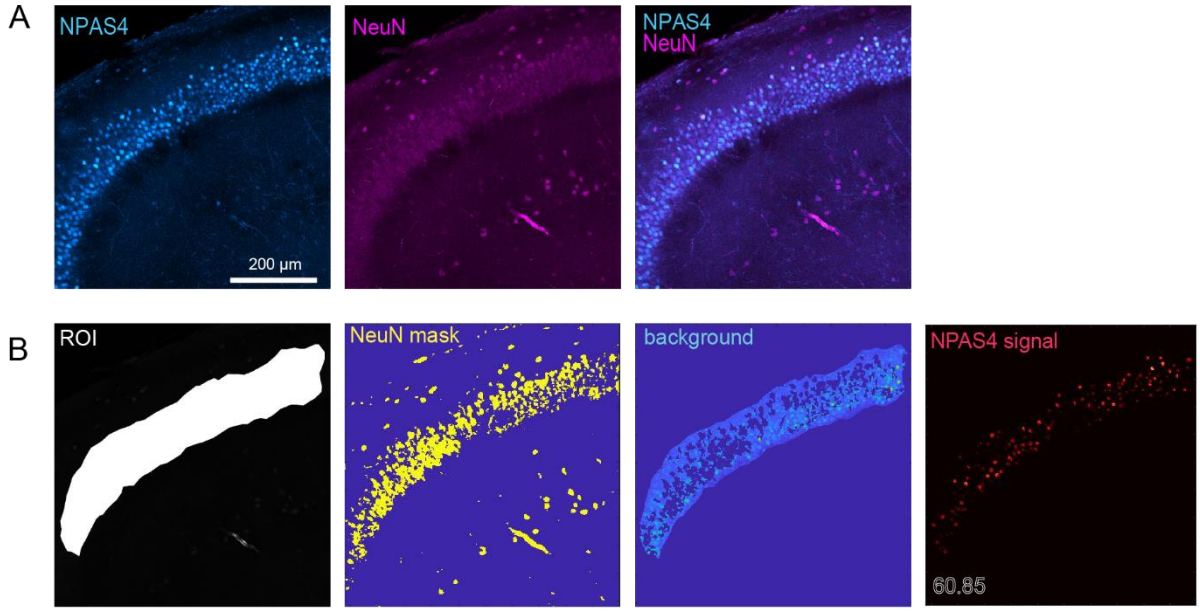

Figure 1B. Quantification of average fluorescence counts workflow.

A. Confocal images of hippocampus CA1 stained with NeuN and NPAS4 by IHC (Nikon A1+, 20x, na 0.75).
B. Fluorescence value was quantified in NeuN positive cells. NeuN channel was utlized as a mask for NPAS4 quantification. ROI was drawn and NPAS4 pixel values within NeuN mask is quantified. Values less than or equal to the average pixel value within the ROI and outside the NeuN mask was disregarded to account for background and non-specific primary antibody binding. Therefore, NPAS4 average fluroscence was quantified, shown here in the above example as 60.85.
C. Average fluorescence quantification formula.

Figure 2.

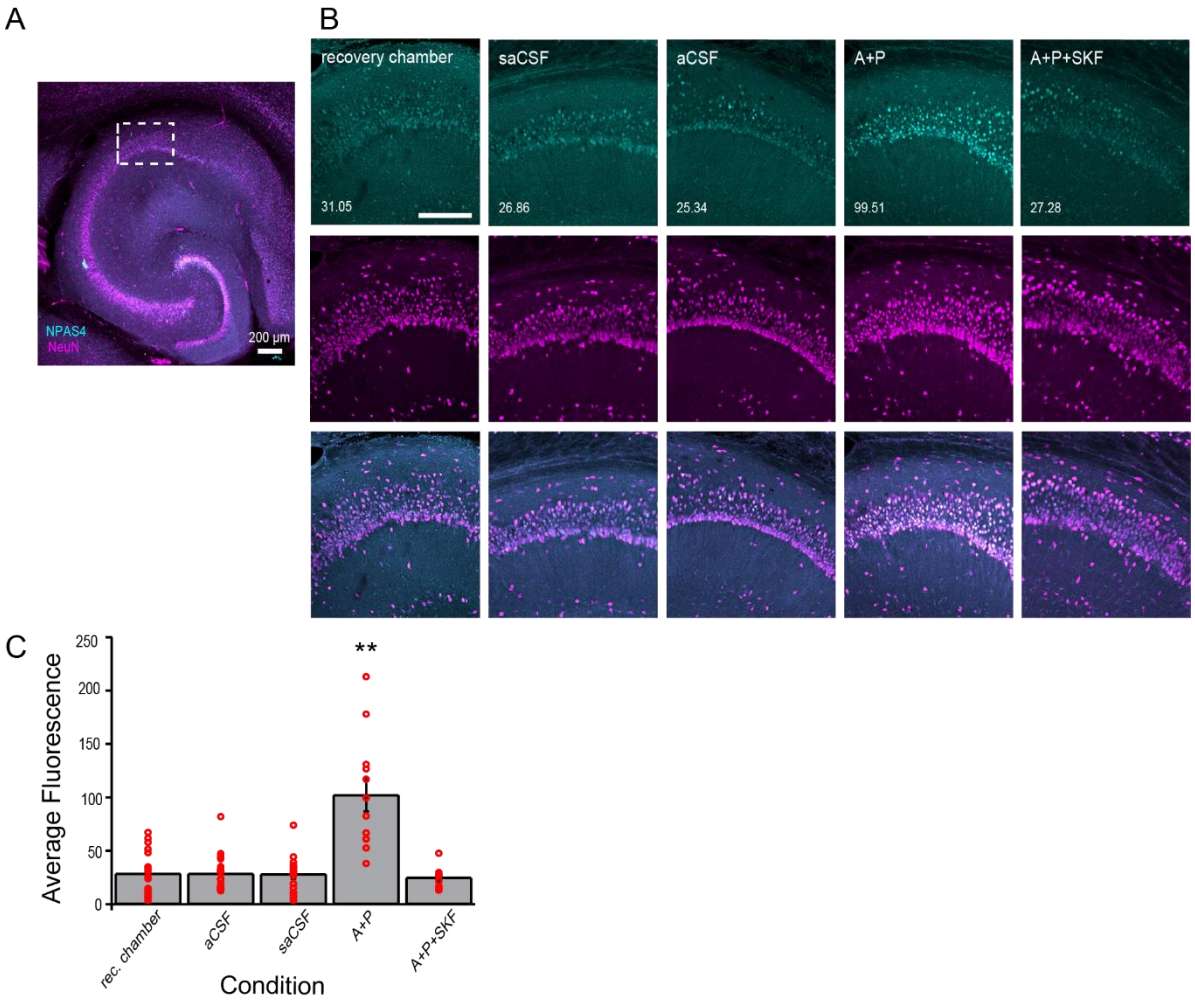

Figure 2. Bath application of D1 agonist SKF81297 diminishes NPAS4 expression in mouse hippocampus CA1.

A. Mouse hippocampus region CA1 (approximate region of interest within dashed box shown hereinafter) immunostained with antibodies recognizing NPAS4 (blue) and NeuN (magenta) and subsequently confocal imaged.
B. The highest NPAS4 expression occurred with bath application of AMPA (10uM) with picrotoxin (50uM) for 5 min. This expression was diminished when SKF81297(10uM) was added. Recovery chamber (aCSF, 200nM TTX, 250uM KYN), saCSF (aCSF, 20uM Nim), and aCSF only chambers showed similar expression. Fluorescence values within region of interest for each image indicated on bottom left of image.
C. Average fluorescence values for each condition. (Recovery chamber: avg. 28.55, sem. 4.66, n=19 slices. aCSF: avg. 28.63, sem. 3.09, n=25. saCSF: avg. 28.15, sem. 4.17, n=17. A+P: avg. 102.25, sem. 15.39, n=12, **p<0.01, one-way ANOVA, Tukey HSD test. A+P+SKF: avg. 24.94, sem. 3.49, n=9.)

Discussion

Developing a Robust Stimulus

First, we show that a robust stimulation to induce high levels of NPAS4 is possible through pharmacological bath application. Not established previously, this pharmacological method of producing consistently high levels of induction using AMPA was brought about by first considering receptors present on CA1 pyramidal neurons and how we would be able to depolarize the cell. It was also important to consider the pathways of NPAS4 induction that are possible in order to design a successful stimulus.

NPAS4 expression has been detailed to occur in response to two depolarization stimulus pathways, as detailed above. One pathway to expression occurs in response to EPSPs, in which a dendritic expression occurs where calcium influx through NMDA receptors drives a fast local expression and protein is trafficked to the soma over time. The second pathway to expression occurs in response to AP spikes in which the cell experiences depolarization above threshold, an influx of calcium through voltage gated L-type calcium channels (Cav1.2), and the induction of NPAS4 in the soma on a longer time scale compared to dendrites due to nuclear transport involved in transcription and translation (Brigidi et al., 2019 in press). Ultimately, the dendritic-EPSP-NMDA and the somatic-AP-LTCC pathways allows the cell to record its history of inputs through EPSPs and outputs through APs.

With these two pathways of expression in mind, we realized it was necessary to bring the cell to receive inputs in the form of EPSPs or push the cell above threshold to fire APs. A bath application of AMPA acts as an agonist specifically for AMPA receptors present on pyramidal neurons, which are ionotropic transmembrane receptors for glutamate that flux primarily sodium ions into the cell, leading to depolarization of the

cell (Purves et al., 2001). Therefore, once enough sodium ions have moved into the cell along their concentration gradient, the cell's resting membrane potential should slowly depolarize above -70mV to the reversal potential of the AMPA receptor, which is close to 0mV—allowing for considerable depolarization inside the neuron (Purves et al 2001). This depolarization would relieve the voltage-dependent magnesium block present in the NMDA receptor and coincident Schaffer Collateral inputs would release glutamate onto NMDA receptors present on CA1 pyramidal cells. This would allow for increased calcium influx through the NMDA receptor, which further depolarizes the cell and facilitates downstream calcium associated cascades.

AMPA receptors are also present on GABAergic inhibitory interneurons in the hippocampus (Leranth et al., 1996), which means that their GABA release onto CA1 pyramidal cells or even CA3 pyramidal cells that synapse onto CA1 through Schaffer Collaterals would be inhibited. Therefore, 50uM Picrotoxin was simultaneously bath applied with AMPA. Picrotoxin noncompetitively binds $GABA_A$ receptors present on inhibitory interneurons present in CA3 and CA1 (Yoon et al., 1993, Johnston et al., 2009).This AMPA stimulus combined with circuit disinhibition by Picrotoxin should depolarize the cell enough to lead to NPAS4 expression. This should also be confirmed in future experiments. Indeed, our immunohistochemistry quantifications demonstrate high levels of NPAS4 expression as demonstrated in Figure 2B. However, we can only speculate the mechanism of depolarization which plays an important role in understanding the effect demonstrated by dopamine agonist.

One possibility is that stimulus with AMPA and Picrotoxin to the hippocampal slice is causing action potentials in CA1 inducing somatic expression through LTCC

opening and subsequent calcium influx. Another possibility is that stimulus with AMPA and Picrotoxin to the hippocampal slice is causing EPSPs in the dendrites of CA1 by direct agonist stimulation and/or supplemented by Schaffer Collateral input from CA3. A Third and likely possibility is that both EPSPs and APs are being evoked with the stimulus paradigm. Protein expression could be occurring in the dendrites and later being trafficked to the soma over time, in line with the time course experiments completed previously in the lab (Brigidi et al., in press). In these sets of experiments, it is not possible to discern the mechanism of induction with AMPA and Picrotoxin, highlights future experimental directions to confirm the membrane potentials with the addition of these stimulating agonists. Understanding the mechanism of induction with our pharmacological stimulus would help explain the diminishing effect exhibited by the application of D1 agonist SKF81297.

Application of Dopamine Agonist SKF81297

To observe dopaminergic neuromodulation of CA1 hippocampal neurons, we bath applied D1-receptor agonist 10uM SKF81297 with coincident stimulation demonstrated by AMPA and Picrotoxin to model neuromodulator release the animal has in vivo. Because we know NPAS4 expression occurs in vivo when an animal traverses an enriched environment, that expression is depolarization dependent, and it is possible that induction is dopamine modulated during the animal's exposure to novelty, we developed a pharmacological CA1 stimulus with coincident dopamine agonist application as an ex vivo model for modulation that occurs in vivo (Bloodgood et al., 2013; Brigidi et al., 2019 in press; Bromberg-Martin et al., 2010). The presence of SKF in the bath diminished expression levels comparable to levels in controls (Figure 2B and

2C). In interpreting the effects of a dopamine agonist SKF, it becomes especially important to understand the mechanism of induction caused by the AMPA and Picrotoxin stimulus originally. It is only possible to speculate and formulate future experiments to understand the mechanism underlying low NPAS4 expression when SKF is in the bath.

In the scenario where the AMPA with Picrotoxin stimulus would cause only EPSPs in the dendrites of the pyramidal neurons, induction of NPAS4 would be NMDA receptor mediated, as described previously. According to Varela and colleagues, SKF alone can have a potentiating and depressive effect in response to EPSPs on the various cells of CA1 in acute slice (Varela et al., 2009). According to Lee and colleagues, bath application of SKF81297 reduces peak and steady state amplitudes when cells are simultaneously stimulated with L-aspartate in cultured HEK293 cells. Furthermore, NR2A subunit-containing NMDA receptors are shown to interact with D1 receptors through the NMDA receptor's carboxyl tail that ultimately reduces the membrane expression of NR2A-containing NMDA receptors during depolarization and simultaneous D1 receptor agonist application in hippocampal cultured neurons (Lee et al., 2002). Moreover, Tran and colleagues show that the blockage of NR2B-containing NMDA receptors in cultured hippocampal neurons did not affect a dendritic NMDA-induced dendritic expression of a CAMKII reported construct, whereas preferential NR2A antagonism diminished protein synthesis in the dendrites (Tran et al., 2007).

Additionally, it has been proposed that NR2A containing NMDA receptors display faster channel kinetics and therefore a faster influx of calcium (Erreger et al., 2005). There is also evidence suggesting NR2A-containing NMDA receptors are found in

synaptic sites (ie. Schaffer Collateral or Perforant Path synapses) whereas NR2B containing receptors are extrasynaptically localized (Stocca et al., 1998, Tovar et al., 1999), which would be especially relevant if stimulus is occurring through CA3 synapses. When examining NMDA-mediated BDNF expression, investigators also detailed a differential modulation of BDNF expression (extrasynaptic mediated expression shuts of synaptic mediated expression), hinting at diverging responses in response to the calcium influx that occurs when the NMDA receptor opens within and outside the synapse (Vanhoutte et al., 2003). Therefore, localization of NR2A subunit containing NMDA receptors in synapses, the requirement of a functional internal NMDA receptor C terminus supports a hypothesis that a diminished effect in NPAS4 expression may be due to NR2A dysfunction due to interactions with the D1 receptor.

In vivo, sources of depolarization will occur through glutamate release via the Schaffer Collaterals or the Perforant Path inputs. This is strictly synaptic release of glutamate-- meaning AMPA, NMDA, and various other receptors with a glutamate ligand will be activated in CA1. It may be hypothesized that EPSP-NMDA mediated dendritic expression of NPAS4 occurs through or is biased towards NR2A containing NMDA receptors. If dopamine is then released coincidentally on these depolarized neurons from projections from the VTA onto CA1, it can be hypothesized that the conformational change that occurs on the N2A subunit, reduces NR2A containing NMDA receptors in the synapse, hinders synaptic EPSPs, diminishes dendritic calcium influx through NMDA receptors and expression of dendritic NPAS4 protein that is not then trafficked to the soma. This ultimately highlights the importance of understanding

the stimulus source, strength, and duration that would occur in the initial depolarization event before the dopamine were to be exposed to the neuron.

In future acute slice experiments, it would be important to first discern membrane potential with AMPA and Picrotoxin stimulus only with whole cell recordings. It would then be confirmed with pharmacological manipulations by antagonizing LTCCs during stimulation, where a presence of somatic protein with the AMPA and Picrotoxin stimulus only would confirm that the protein levels currently observed are expressed strictly through dendritic EPSP-evoked NMDA response. If this is confirmed, an NR2A blocker NVP-AAM077 can be used simultaneously with AMPA and Picrotoxin to see if my results are comparable to when SKF is added. I would then use NR2B blocker ifenprodil in the presence of AMPA and Picrotoxin to confirm that the cell still experiences comparable levels of expression to before blockage. These experiments should then be done with SKF in the bath to confirm NR2A dependence. However, this set of experiments would tie dendritic depolarization to NMDA receptor subunit NR2A, but would not tackle our original interests in understanding modulation of NPAS4 expression via dopamine. To have stimulus specificity, it would be beneficial to determine effects of SKF81297 with electrophysiologically targeted stimulus protocols to drive EPSPs or APs and simultaneously wash in dopamine agonists and quantify expression of NPAS4.

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