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Inhibiting Rho-kinase promotes goal-directed decision-making and blocks habitual responding for cocaine

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Inhibiting Rho-kinase promotes goal-directed decision-making and blocks habitual responding for cocaine

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Abstract

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By Andrew Swanson

The ability to select actions based upon a desired outcome is critical for survival. While a number of brain regions are involved in these processes, the prelimbic prefrontal cortex is necessary for associating actions with their consequences, enabling goal-directed decision-making. However, the relationship between deep-layer dendritic spines and outcome-based decision-making remains opaque. We provide evidence, using a Rho-kinase inhibitor, that glucocorticoid receptor-mediated dendritic spine remodeling is causally related to outcome-based decision-making. To better understand how dendritic spines remodel in response to postnatal stressor exposure, we also enumerated dendritic spines during and after chronic stress hormone exposure in hippocampal CA1, deep-layer prefrontal cortex, and the basal amygdala. Corticosteroid exposure modified dendritic spine density in these regions, but with the exception of the orbitofrontal cortex, densities normalized with a recovery period. Using mice with reduced gene dosage of *p190rhogap*, a cytoskeletal regulatory protein localized to dendritic spines, we isolated structural correlates of both behavioral vulnerability (spine elimination) and resilience (spine proliferation) to stress hormone exposure within the orbitofrontal cortex. We also find that the strength of action-outcome conditioning correlates with prelimbic cortical dendritic spine densities, suggesting that new action-outcome learning involves dendritic spine plasticity. We inhibited Rho-kinase, which enhanced action-outcome memory, resulting in goal-directed behavior in mice that would otherwise express stimulus-response habits. Rho-kinase inhibition transiently reduced prelimbic cortical dendritic spine density during a period of memory consolidation, but only when paired with new learning. It also blocked habitual responding for cocaine, an effect that persisted over time, across multiple contexts, and depended upon actin polymerization, suggesting that Rho-kinase inhibition promotes goal-oriented action selection by augmenting the plasticity of prelimbic cortical dendritic spines during the consolidation of new action-outcome memories, and that it has therapeutic potential for cocaine use disorders. Finally, we developed an approach to investigate the structural effects of cocaine on prelimbic cortical dendritic spines *in vivo*. We found that while low-dose cocaine did not alter dendritic spine density, it increased the rate of dendritic spine turnover. Together, these findings provide strong evidence for the importance of deep-layer prelimbic cortical dendritic spine plasticity in outcome-based decision-making.

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Introduction

Actions and habits

Our work presented here focuses on some of the cellular and molecular processes underlying action selection and the development of habits. It has long been understood that humans can learn to associate specific actions with their outcomes. In the early 1980's, Dickinson and colleagues were the first to show that rodents can similarly encode the causal relationship between an action and its outcome (Dickinson, 1980; Adams and Dickinson, 1981). More recent work reveals that some non-mammalian species can do the same (Weir et al., 2002; Taylor, et al., 2012). Such actions are considered goal-directed, and are critical for an animal's ability to survive: selecting actions to ensure optimal outcomes in regards to obtaining food, shelter, mating and care of offspring can all be modeled as action-outcome relationships. In contrast, stimuli can elicit responses, as famously shown by Pavlov's work with dogs (Pavlov, 1927). Stimulus-elicited behaviors are considered habits.

When a human or rodent first engages in a behavior, it is considered goal-directed responding, because they are sensitive to the contingency between the action and its outcome. With repetition, sensitivity to this contingency decreases and can eventually be lost, resulting in responding that is instead governed by stimulus-response relationships (Dickinson, 1980). One can view these two decision-making strategies as opposing poles on a spectrum, with the ability to quantify a behavior as either more goal-directed (increased sensitivity to action-outcome contingencies) or more habitual (decreased sensitivity to action-outcome contingencies, instead relying on stimulus-response relationships) (fig.1).

For context, the classic example is driving to work. When an individual moves or starts a new job and drives to work for the first time, they are actively paying attention to street names, traffic lights, other vehicles, landmarks, and so on. After driving the same route repeatedly, one pays less attention to those things, freeing up attentional

processes to think about other things, daydream, talk on the phone, etc. This is a textbook example of a behavior that starts as goal-directed, and with repetition, becomes habitual. The development of habits with repetition of a behavior is a normal, advantageous adaptive process because it allows for the 'automation' of behaviors, enabling individuals to focus their attention on other things while simultaneously completing the engaged behavior.

However, there are limitations and downsides to this loss of flexible action selection that is concomitant with the formation of habits. To return to the car example, after work one day an individual needs to go to the post office. At the end of the day, as they walk out to the car, they know they need to go to the post office, but once they get in the car, they automatically drive straight home, just like they always do, missing the turn for the post office. Everyone has experienced something like this at least once in their lifetime. Now in this instance, the consequence of automatically engaging in the habitual behavior of driving straight home is insignificant, because it is easy to just turn the car around and go back to the post office. However, we know that habits that form in the context of drugs of abuse, such as cocaine, can have much more severe consequences (Everitt and Robbins, 2016). Further, psychostimulants like cocaine, and a range of other pathological stimuli, bias response strategies to favor habit-based responding (Miles et al., 2003; Schoenbaum and Setlow, 2005; Zapata et al., 2010; Gourley and Taylor, 2016; Hinton et al., 2014; LeBlanc et al., 2013; Corbit et al., 2014; Schmitzer-Torbert et al., 2015; Leong et al., 2016).

While a number of mechanisms have been identified that control and promote the development of action-outcome insensitivity and habitual responding, effectively reversing or breaking habitual responding has proven difficult, particularly in the context of drug addiction and the ruminative thought processes associated with depression. A tremendous amount of research has focused on rodent models of goal-directed and

habitual responding, both behaviors and their underlying biological mechanisms. Our work builds on these existing rodent models to identify a mechanism that can effectively restore goal-directed decision-making after the loss of sensitivity to action-outcome contingencies and concomitant reliance on habitual response strategies. To this end, one needs to consider the neuroanatomy underlying actions and habits, and ultimately the cellular and molecular processes governing these behaviors.

Neuroanatomical basis actions and habits

General neuroanatomy

The central nervous system consists of the brain and the spinal cord, and is composed of two primary cell types: neurons and glia. Neurons are the cells that form electrochemical connections with each other to form networks of neurons that regulate everything from respiration to complex thought. These connections are called synapses, and most often form on dendritic spines (Segal, 2005). Glial cells provide important support functions to the central nervous system, including but not limited to, structure, immune function, and communication. The brain can be divided into regions based upon functional, connective, and developmental criteria. The decision-making strategies discussed here depend upon the cerebral cortex and the striatum (basal ganglia in humans), which are heavily modulated by inputs from the hippocampus, amygdala, thalamus, and other regions (Balleine and O'Doherty, 2010).

Cortical organization

The cerebral cortex consists of four lobes: frontal, parietal, temporal, and occipital. Early work by Korbinian Brodmann created architectonic maps of the cerebral cortex, identifying 52 distinct regions (Brodmann, 1909). The prelimbic cortex, corresponding to Brodmann area 32, is part of the prefrontal cortex which is located in

the frontal lobe. All cortical areas are organized in layers, initially documented by the drawings of Santiago Ramón y Cajal, and while there is some variation between cortical regions and across species, there are canonically six distinct layers (Brodmann, 1909). The organization of these layers reveals that there are specific types of cells present in each layer, and that inputs to cortical regions have layer-specific organization. Layer I is closest to the skull (pial surface), with layer VI being the deepest. Layer I is acellular, primarily consisting of dendrites from deeper layers. Layer II is a granular layer, populated by small cells. Layer III contains pyramidal neurons that project to and receive inputs from other cortical areas. Layer IV is another granular layer, where a majority of thalamic inputs synapse. Layer V is another layer of pyramidal neurons, with these neurons projecting to subcortical areas and receiving subcortical inputs. Layer VI is a highly variable layer of cells and white matter fibers. With the advent of modern genetics, many groups have identified layer-specific genes that regulate cellular organization and function within cortical layers (Molyneaux, 2007).

Cortico-striatal circuits

Over the past few decades, significant progress has been made in identifying the brain regions and neural circuits responsible for action-outcome-based and stimulus-elicited responding. Given that actions and habits are distinguishable from each other, it is no surprise that they are governed by distinct neural circuits. In short, distinct cortico-striatal circuits underlie these decision-making strategies, with the dorsomedial striatum interacting with the prelimbic prefrontal cortex to govern goal-directed responding, and the dorsolateral striatum interacting with a broader set of sensory and motor cortices to drive habitual response strategies (Balleine and O'Doherty, 2010). These 'core' corticostriatal circuits are modulated by a number of other brain regions. Our work

presented here primarily focuses on the prelimbic cortex, so its anatomy will be covered in the most detail.

The initial direct evidence for the dorsomedial striatum being necessary for sensitivity to action-outcome contingencies, and goal-directed responding, came from Yin and colleagues. Lesioning the dorsomedial striatum in rats either before or after training eliminated sensitivity to action-outcome contingencies. Further, infusion of the γ-aminobutyric acid (GABA) agonist muscimol into the dorsomedial striatum, temporarily inactivated those neurons, and replicated the effect of lesions (Yin et al., 2005). Similarly, lesions of the dorsolateral striatum prior to training impaired the development of stimulus-response-based habitual responding, with rats continuing to respond in a goal-directed manner (Yin et al., 2004).

The first evidence identifying the prelimbic cortex as necessary for sensitivity to action-outcome relationships was that lesions of the prelimbic cortex resulted in rats that were insensitive to changes between action-outcome contingencies (Balleine and Dickinson, 1998). A subsequent study provided additional direct evidence that lesions of the prelimbic cortex prior to behavioral training resulted in rats that were insensitive to changes in action-outcome contingencies, instead relying on habit-based decision-making (Corbit and Balleine, 2003). Interestingly, lesions of the prelimbic cortex after training had no effect on responding, with rats remaining goal-directed, suggesting that the prelimbic cortex is necessary for the acquisition, but not expression, of goal-directed responding (Ostlund and Balleine, 2005). Again, this is in contrast to the dorsomedial striatum, which is necessary for both the acquisition and expression of goal-directed responding (Yin et al., 2005).

Rodent and human homology

The initial studies identifying the cortico-striatal circuits governing goal-directed and habitual responding were conducted in rodents, but these brain regions also exist in humans and appear to form similar cortico-striatal circuits. Human studies, combining fMRI imaging with behavioral tasks designed to differentiate between goal-directed and habitual response strategies, showed that the human ventromedial prefrontal cortex, which includes the prelimbic cortex, is active during goal-directed responding (Valentin et al., 2007; Gläscher et al., 2009). A further study provided further evidence for the role of the human ventromedial prefrontal cortex as well as the dorsomedial striatum in goal-directed responding in humans (Tanaka et al., 2008). The lateral striatum (posterior putamen) has also been shown to be involved in the development of habits in humans (Tricomi et al., 2009).

Modulation of the prelimbic cortex

A large number of cortical and subcortical regions send projections to the prelimbic cortex (Condé et al., 1995), but if and how many of these regions contribute to action-outcome sensitivity are not well characterized. A handful of regions have been shown to play an important role in regulating neural function within the prelimbic cortex. This includes other cortical regions, the hippocampus and amygdala, both part of the limbic system, the mediodorsal (MD) nucleus of the thalamus, and monoaminergic systems. Changes in connectivity and activity between these regions can have both immediate and long-lasting effects on action-outcome sensitivity.

Cortical efferent projections to the prelimbic cortex primarily form synapses on layer III pyramidal neurons. Prominent projections arise from the infralimbic and orbitofrontal cortices, both of which are heavily involved in decision-making (Gabbott et al., 2003; Heidbreder and Groenewegen, 2003). The infralimbic cortex is implicated in habit-based decision-making (Killcross and Coutureau, 2003), while the orbitofrontal

cortex plays an important role in determining outcome value (Schoenbaum et al., 2011; Gourley et al., 2016b).

The thalamus is a major relay point for subcortical inputs to the cortex, and nucleus MD is one of the largest thalamic nuclei. Nucleus MD projects directly to the prelimbic cortex, conveying signals arising from a number of subcortical areas (Balleine and O'Doherty, 2010; Mitchell and Chakraborty, 2013). Importantly, the striatum does not have direct efferents to the prelimbic cortex, instead synapsing on nucleus MD neurons. The hippocampus and amygdala both have indirect projections to the prelimbic cortex that route through nucleus MD. Inhibitory inputs from the substantia nigra pars reticula play an important modulatory role in thalamic activity (Alexander and Crutcher, 1990). Both the amygdala and hippocampus also have indirect prelimbic projections that route through nucleus MD. Prelimbic efferents arising from nucleus MD have been best characterized by Kuroda and colleagues (Kuroda et al., 1995a, 1995b, 1996).

The hippocampus, heavily involved in learning and memory as well as spatial processing, sends abundant inputs to the prelimbic cortex (Jay et al., 1989; Condé et al., 1995; Carr DB and Sesack SR, 1996). The exact functional contributions of these direct projections are not yet well-characterized in the context of action-outcome sensitivity, but the dorsal hippocampus is essential for forming associations between actions and outcomes (Corbit and Balleine, 2000). The ventral region of the hippocampus has been shown to encode location-specific context for outcomes (Komorowski et al., 2013).

Basolateral amygdalar inputs to the prelimbic cortex have a predominantly inhibitory effect, caused by activating local inhibitory interneurons (Ishikawa and Nakamura, 2003; Dilgen et al., 2013). An interesting model of amygdala-hippocampal-prelimbic interactions has been proposed: In heightened emotional situations, such as fight or flight scenarios, amygdala inputs to the prelimbic cortex activate local inhibitory neurons, which in turn inhibit hippocampal inputs to pyramidal prelimbic cortical neurons

(Tejeda and O'Donnell, 2014). Given that goal-directed decision-making is a flexible, cognitively demanding task, it makes sense for the brain to have a mechanism to silence the prelimbic cortex in urgent situations to allow for more rapid, stimulus-elicited responding.

Monoaminergic systems also project to and modulate prelimbic cortical neurons. Local serotonin depletion within the medial prefrontal cortex has been shown to reduce behavioral flexibility, impairing goal-directed decision-making (van der Plasse et al., 2007; Homberg, 2012). Dopamine broadly regulates motivation, and within the context of goal-directed decision-making, it is involved in reward modulation and valuation of outcomes (Lex and Hauber, 2009; Haddon and Killcross, 2011). Norepinephrine projections also target the prelimbic cortex (Morrison et al., 1979; Heidbreder and Groenewegen, 2003), and norepinephrine may play a role in modulating dopamine signaling (Doya, 2008).

Prelimbic cortex

The rodent prelimbic cortex, now broadly considered part of the prefrontal cortex due to an expansion of the definition of prefrontal cortex, is located along the medial wall of the rostral prefrontal cortex (fig.2) (Preuss, 1995; Uylings et al., 2003). Adjacent areas include the medial orbital cortex, cingulate cortex, and infralimbic cortex. This arrangement is consistent across mammalian species. Historically, apart from post-mortem architectonic studies, differentiating between the infralimbic and prelimbic cortices was not possible due to both the deep, medial location of these regions, as well as their size relative to surrounding areas. Even with modern methods, it is still common to see the term 'medial prefrontal cortex' in studies, which typically includes both the prelimbic and infralimbic regions, despite these two regions having distinct organization and function. Here, we focus on the role of the prelimbic cortex in sensitivity to action-

outcome relationships and goal-directed responding, but the prelimbic cortex is also involved in visceral motor responses, and is necessary for the expression of acquired fears (Gabbott et al., 2005; Corcoran and Quirk, 2007).

In rats and mice, the medial prefrontal cortex, including the prelimbic cortex, has an acellular layer IV (Gabbott et al., 1997). Thalamic inputs instead largely synapse on layer V pyramidal neurons, as well as some layer III neurons (Kuroda et al., 1995). Layer V also receives monoaminergic inputs. Given the large number of brain regions involved in goal-directed decision-making that send projections to layer V prelimbic cortical neurons, it's likely that these synapses play a particularly critical role in regulating action-outcome sensitivity.

Layer V primarily consists of pyramidal neurons, which have tree-like dendritic arbors. Layer V prelimbic cortical neurons project to a range of subcortical targets, including the striatum, nucleus accumbens, the central and basolateral regions of the amygdala, and thalamic and brainstem nuclei (Vertes, 2004). A single large, apical dendrite projects towards the pial surface, with many basal dendrites projecting into layer VI. As described above, cortical layers serve as a means to compartmentalize and segment afferent inputs. A further means of organization is the localization of a given input to specific dendritic regions on a neuron. While this localization is well characterized in some brain regions and cell types, much less is known about this synaptic localization within the prelimbic cortex.

For thalamic inputs from nucleus MD, ~95% formed on dendritic spines within the prelimbic cortex, with fairly uniform distribution over apical dendritic arbors (Kuroda et al., 1995a, 1995b, 1996). 60% of layer V prelimbic neurons express serotonin receptors, typically co-expressing both serotonin 5HT1A and 5HT2A receptors. Expression is restricted to proximal dendritic spines, with 5HT1A expressed on basal dendrites and 5HT2A receptors expressed on apical dendritic spines (Puig, 2011). Amygdalar inputs to

the prelimbic cortex are perhaps the best characterized in terms of where the synapses are located. Characterization of amygdalar synapses on layer V corticospinal prelimbic cortical neurons found that ~70% of these synapses were located on basal dendrites. Two important caveats to this study are that corticospinal neurons are only 13% of the neurons in layer V, so this synaptic distribution may differ in other subpopulations. Further, they were unable to analyze the most distal apical dendritic arbors, so the 70/30 split between apical and basilar dendrites may be exaggerated. Given that the subcortical inputs described above are all implicated in goal-directed decision-making and predominantly form synapses on dendritic spines, it provides strong support for the perspective that layer V prelimbic cortical dendritic spines play an important role in regulating goal-directed response strategies.

Dendritic spines

Structure and function

Dendritic spines were first depicted in Santiago Ramón y Cajal's drawings (Ramón y Cajal, 1888), and they visually appear as small protrusions studding the dendrites of neurons (fig.3). Due to methodological limitations, further study and understanding of dendritic spines was limited until the development and use of electron microscopy almost 60 years later. One of the earliest reports utilizing electron microscopy to analyze the morphology and structure of neurons from the cerebral cortex reported that "…has revived an interest in the "spines" or "gemmules" that have been described on dendritic trunks following some silver staining techniques. We are quite certain that no fine lateral branching of this type is to be found after good preservation with osmic acid." (Schultz et al., 1957). It was two years later when dendritic spines were first reported by electron microscopy (Gray, 1959). This led to a proliferation of labs

using electron microscopy to study dendritic spines and other fine compartments of neurons and other cell types.

What we now know is that dendritic spines are the primary site of excitatory synapses in the brain and that their structure impacts their function. Depending on the brain region and cell type, an individual neuron can have thousands to hundreds of thousands of dendritic spines. Dendritic spines vary in size, but are typically 0.5 – 2.0 μm in length, with a volume of 0.01 – 0.8 μm^3 (Hering and Sheng, 2001). Dendritic spines typically form a single synapse, and their structure appears designed to create a physical compartment that is segregated from the rest of the dendrite, creating a discrete electrical and chemical environment that can be used to modulate electrochemical signaling. This is most apparent with calcium signaling in dendritic spines (Sabatini et al., 2001; Segal, 2005). The proliferation of molecular, cellular and biochemical methods in the second half of the 20th century has enabled detailed study of the molecules and signaling mechanisms that control dendritic spine structure and function.

The past few decades have seen the development of microscopy approaches that enable real-time imaging of live neurons and dendritic spines, providing key insights into the remarkable structural plasticity of dendritic spines, and how that in turn impacts their function. Current theories on dendritic spines have been shifting from the perception of dendritic spines as static structures to structures that can remodel on the order of seconds (Murakoshi et al., 2011).

Morphology

From the beginning, researchers have given attention to the varying shapes of dendritic spines (Ramón y Cajal, 1893). With both light and electron microscopy, two general “parts” of dendritic spines are observable: a head and a neck. The neck begins

at the dendrite and is generally considered to end where the dendritic spine enlarges, with all material past that considered the head. The stereotypical dendritic spine has a thin neck that acts as the attachment point to the dendrite, contains scaffolding proteins, and acts as a boundary region between the dendrite and the spine head. It's long been hypothesized that the role of the spine neck is not just structural, but in fact plays an important role in spine function and synaptic strength (Crick, 1982). Recent reports are confirming this hypothesis, with evidence that dendritic spine necks remodel in response to long term potentiation, and that neural activity regulates diffusion across spine necks (Bloodgood and Sabatini, 2005; Tønnesen et al., 2014).

Far more is known about the molecular systems regulating dendritic spine head structure and function, and is covered in detail below. However, many studies have found that synaptic strength increases with the size of dendritic spine heads. Larger dendritic spine heads create more space for surface receptors and intracellular structural and signaling proteins (Arellano et al., 2007).

Classification

In 1970, using a light microscopy with the same Golgi staining used by Ramón y Cajal in conjunction with electron microscopy, an attempt was made to functionally group dendritic spines of the rat cerebral cortex based on their overall shape and morphology. Three "forms" of dendritic spines were proposed: "(1) stubby spines which are generally short and thick; (2) mushroom-shaped spines, each of which has a rather thick stalk that expands into a large bulb, and (3) thin spines, each with a slender stalk that expands into a small oval or rounded end-bulb." (Peters and Kaiserman-Abramof, 1970).

Classification of dendritic spines by shape gained renewed popularity in the latter part of the 20th century with Kristen Harris' electron microscopy investigation of hippocampal dendritic spine morphology and classification (Harris et al., 1992). Harris

and colleagues used very similar classification criteria of stubby, thin, and mushroom spines, as well as a fourth group of branched spines, which contain more than 1 spine head. This extremely thorough work tracked morphological changes of dendritic spines to development over time and provided a key link between dendritic spine structure and synaptic function in the context of long-term potentiation.

One model that arose from classifying dendritic spines in this way was that these spine types existed on a temporal continuum: mushroom spines were ‘mature’ spines, thin spines were immature spines that were in the process of developing into mature, mushroom spines, while stubby spines normally did not contain synapses and were in the process of forming or disappearing completely. Numerous studies have documented the relative presence of the three main spine types in the cortex, and while there is some variation between regions, consensus estimates are that mushroom spines make up ~25% of dendritic spines, 65% thin, and 10% stubby/other (Bourne and Harris, 2007).

One issue with the idea that stubby and thin spines are immature and not synapse-containing is that out of numerous studies of cortical dendritic spines, >95% of spines have been found to be part of a synapse, with the remaining 5% representing filopodia and tiny protrusions that do not have defined spine heads (Arellano et al., 2007). The modern model is that dendritic spine morphology, including relations between head and neck size, is important, but that dendritic spine shape exists along a continuous spectrum. Classifying dendritic spines by type is still commonly reported, and we analyze our dendritic spine data sets this way, but more is learned about the relationship dendritic spine dynamics and function, classification by type will likely be reported less and less in the future. For example, *in vivo* live imaging studies of dendritic spines do not always report individual parameters of morphology like length or head size, instead focusing on changes in dendritic spine density and turnover rates (Liston et al., 2013).

Dendritic arbors and dendritic spine density

While the majority of excitatory synapses form on dendritic spines, dendrites are also critical to neuronal structure and function. Dendritic arborization begins during neuronal morphogenesis and a number of genes and proteins have been identified that regulate these processes (Jan and Jan, 2011). Dendrites maintain some structural plasticity and flexibility after development, with changes in dendrite branching and total dendritic material identified as responses to chronic stress, drugs of abuse, and genetic diseases (Kulkarni and Firestein, 2012; Koleske, 2013). Psychostimulants have been shown to increase dendritic branching in the nucleus accumbens, prefrontal cortex, and other cortical areas (Robinson and Kolb, 1997, 1999; Robinson et al., 2001). A more recent report found that cocaine actually decreased apical dendritic branching in layer II/III prelimbic cortical neurons (Radley et al., 2015). The *arg* gene, discussed in more detail below, is a critical gene for maintaining dendritic structure in adulthood (Warren et al., 2012). Neurons in the medial prefrontal cortex exhibit a retraction of their dendritic arbors in response to chronic stress (Liston et al., 2006).

In addition to dendritic arborization, the number and location of dendritic spines on dendrites play an important role in neuronal function. The distance between a synapse from the soma is correlated with signal strength (Magee, 2000). Alterations in dendritic spine density have been implicated in almost every aspect of health and disease (Hering and Sheng, 2001; Segal, 2005). Psychostimulants, in addition to modifying dendritic arborization, modify dendritic spine density in a number of brain regions (Robinson and Kolb, 1997, 1999; Robinson et al., 2001), including the prelimbic cortex (Gourley et al., 2012; Shen et al., 2009; Radley et al., 2015; Gourley and Taylor, 2016). Stress has also been shown to reduce prelimbic cortical dendritic spine density (Radley et al., 2006). Given that the vast majority of dendritic spines house synapses,

and synaptic transmission is largely responsible for generating behavior, identifying molecules and mechanisms that can eventually be used to protect against or reverse abnormal changes to dendritic arbors and dendritic spines is critically important.

Dendritic spines, learning, and memory

With the advent of live cell and *in vivo* imaging of dendritic spines, we have learned that dendritic spines can be both remarkably plastic, undergoing dramatic morphological changes in a matter of seconds, and remarkably stable, persisting for years, if not a lifetime (Bhatt et al., 2009). All of this together strongly suggests that dendritic spines play a key role in learning and memory. Indeed, there is mounting evidence supporting this hypothesis (Lamprecht et al., 2002; Sutton and Schuman, 2006; Yang et al., 2009). A large amount of work has focused on long-term potentiation, a process by which synaptic strength is increased following certain patterns of excitation (Nicoll, 2017). This process involves substantial activation and remodeling of dendritic spine substructures, and often results in an increase in dendritic spine size and the formation of new dendritic spines (Engert and Bonhoeffer, 1999; Yang et al., 2008; Bosch et al., 2014). Similarly, dendritic spine size can shrink as a result of long-term depression (Bosch and Hayashi, 2012).

In part due to the historical focus on long-term potentiation as a key mechanism underlying learning and memory, the default view is that learning and memory requires either or both increases in synaptic strength or the formation of new dendritic spines and synapses, but there is growing evidence that some types of learning actually involve the elimination of existing dendritic spines. It has been hypothesized, and evidence is emerging, that selective elimination of dendritic spines during sleep helps enhance newly formed memories (Maret et al., 2011; Yang and Gan, 2012; Yang et al., 2014a,b). Acquisition of fear conditioning appears to involve dendritic spine elimination in both the

hippocampus and the frontal association cortex (Lai et al., 2012; Sanders et al., 2012). As more studies combine behavioral paradigms with *in vivo* dendritic spine imaging, we should expect more examples of learning and memory being either dependent upon or enhanced by elimination of shrinkage of dendritic spines as well as other mechanisms.

Molecular regulation of dendritic spine structure

Actin cytoskeleton

The actin cytoskeleton regulates the structure of dendritic spines, growth cones, filopodia, and cell motility. Actin exists in both a monomeric (G) and filamentous (F) polymer form (Dominguez and Holmes, 2011). G-actin molecules exist freely within the cytosol, while F-actin is anchored to other F-actin polymers and scaffolding proteins. Like dendritic spines, the early characterization of actin-regulating proteins described the actin cytoskeleton and its regulation as a fairly static system with regulatory proteins having simple, unidirectional effects. Profilin, which binds G-actin monomers, was initially found to inhibit F-actin polymerization (Carlsson et al., 1977), but subsequent studies revealed that it can also facilitate polymerization by forming a profilin-actin complex (Tilney et all., 1983; Pring et al., 1992). Regulation of the actin cytoskeleton is now viewed as a highly complex, with the net result being a dynamic process that depends on a large number of intra- and extra-cellular factors.

F-actin polymers have polarity, with both a pointed and barbed end. Monomers preferentially dissociate from the pointed end, while it is faster to grow the polymer by adding to the barbed end (Dominguez and Holmes, 2011). Actin-capping proteins, like gelsolin, bind the pointed end, preventing depolymerization. Cofilin binds F-actin and cleaves them into two separate filaments, creating an exposed barbed end for new polymerization. Actin filaments are constantly cycling, with the two opposing forces of polymerization and depolymerization opposing each other. These forces are normally

relatively balanced, producing a steady state with no changes to F-actin lengths, but readily respond to intra- and extra-cellular signals, resulting in extremely rapid increases of decreases in F-actin length and stability (Pontrello and Ethel, 2009).

In dendritic spines, individual F-actin filaments are organized into a lattice network that provides the skeleton for the dendritic spine. In the center of a dendritic spine is a stable 'core' of F-actin in the middle of the dendritic spine, surrounded by more dynamic f-actin filaments. The Arp2/3 complex binds F-actin and provides a branch point for new barbed-end growth (Pollard, 2007). This protein complex is a key regulator of F-actin branching and dendritic spine maturation and as such is regulated by a number of major signaling cascades, including Cdc42, PIP2, PAK, and WASP. Gelsolin and cortactin activity can also enhance F-actin branching (Pontrello and Ethel, 2009). Dendritic spine remodeling can be triggered by a wide range of intracellular and extracellular cues, and is regulated by three major pathways: RhoA, Rac, and Cdc42. Each of these pathways acts to decrease cofilin activity. Our work focuses on RhoA signaling, so we focus on that pathway below.

RhoA signaling

RhoA is a GTPase that is bound by GEF and GAP proteins: Rho-GEF is active while Rho-GAP is inactive (Bos et al., 2007). Rho-associated protein kinase (ROCK) is the major substrate of RhoA (fig.4). β1-integrin is a cell surface receptor that has been implicated in synapse function, long-term potentiation, and dendritic spine stability (Chan et al., 2006; Warren et al., 2012). Abl2/Arg kinase is activated by β1-integrin and inhibits ROCK through p190RhoGAP. Deletion of *arg* leads to adolescent-onset impairments in synapse and spine formation (Sfakianos et al., 2007). RhoA activates ROCK, which activates LIM kinase, which inactivates cofilin by phosphorylation, ultimately resulting in reduced F-actin severing (Yang et al., 1998).

We use ROCK inhibition as a tool to disrupt normal actin cycling. Fasudil is a brain-penetrant ROCK inhibitor, and has been shown to enhance learning and memory in the Morris water maze and water radial arm-maze (Huentelman et al., 2009). Fasudil has a known safety profile and is clinically approved for treatment of ischemic stroke in Japan (Suzuki et al., 2007). Fasudil is less specific than some other commercially available ROCK inhibitors, but is the only one known to be brain penetrant and shown to be safe in humans. While fasudil is a select ROCK inhibitor, it has also been shown to inhibit PRK1, PRK2, PKA and MAPKAP proteins, albeit with less potency (Davies et al., 2000).

ROCK inhibition was originally thought to destabilize dendritic spines, since it would result in active cofilin cleaving F-actin filaments. We now know that cleavage of F-actin filaments is only one piece of actin regulation, and that while the initial cleavage reduces the length of an F-actin filament, it also creates a new barbed end available for polymerization. This has resulted in a more complicated model, where the effects of ROCK inhibition on dendritic spine structure depend upon the external milieu of the actin cytoskeleton and the larger environment of the dendritic spine. A large number of studies have been conducted looking at the effects of ROCK inhibition on dendritic spine structure. Depending upon the cell type, inhibitor, concentration and duration of application, both increases (Kang et al., 2009; Swanger et al., 2016) and decreases (Schubert et al., 2006) in dendritic spine density have been reported. ROCK inhibition has also been shown to be capable of enhancing long-term potentiation (Rex et al., 2009). ROCK inhibition paired with glutamate uncaging directly adjacent to dendritic spines, simulating natural activation of the dendritic spine, blocked both short- and long-term changes in dendritic spine volume (Murakoshi et al., 2011). In chapter 3, we observed something similar, where ROCK inhibition in the absence of learning had no

effect on dendritic spines, but ROCK inhibition paired with a learning event led to dendritic spine elimination.

Approaches to identify structural correlates of decision-making strategies

The main theme of our work presented here is attempting to identify links between cellular structure in the form of dendritic spines and decision-making strategies, with the ultimate goal of identifying tools that can be used to facilitate positive outcomes in the context of addiction and depression. To do this, we use behavioral testing methods that allow us to distinguish between goal-directed and habitual decision-making strategies, in conjunction with techniques to detect and quantify dendritic spines. An overview of these approaches is presented here.

Instrumental conditioning

We use mice for all of our experiments. For experiments involving dendritic spine analyses, *thy1*-YFP-H mice were used, which specifically express YFP in layer V cortical neurons, CA1 and CA3 pyramidal neurons, and a subset of neurons in the basolateral amygdala (Feng et al., 2000). To motivate mice to respond, we food restrict mice to ~90% of their starting body weight. This keeps them healthy while allowing us to use food as a reinforcer during training sessions. We train mice to respond for food pellets in operant conditioning chambers equipped with a food magazine and two nose-poke apertures. When a mouse inserts its nose into an aperture it breaks an infrared photobeam which a computer records and can be programmed to deliver a food pellet into the magazine. Over a number of sessions, mice learn to associate responding on the nose-poke apertures with the delivery of a food pellet. The amount of training a mouse receives influences its response strategy. Moderate amounts of training reliably

produces goal-directed responding in wild type mice, while extensive training sees responding shift to habit-based strategies.

Action-outcome contingency degradation

After the desired amount of instrumental conditioning is complete, we use a task called action-outcome contingency degradation to determine whether mice are sensitive to the relationship between an action and its outcome. First, the likelihood that one response will be reinforced is reduced; instead, food pellets associated with that response are provided non-contingently. In a separate session the next day, the other response remains reinforced. This results in one response having an intact action-outcome contingency and the other response a degraded action-outcome contingency. In a subsequent probe test conducted in extinction, both nose-poke apertures are available, and preferential engagement of the response that is likely to be reinforced provides evidence of knowledge of the action-outcome relationship (Dickinson, 1980; Swanson et al., 2013; Zimmermann et al., 2017) (fig.5).

Outcome devaluation

A second approach to determine action-outcome sensitivity is to alter the valence of the outcome after instrumental conditioning. Here, mice are individually placed in a clean, empty cage with free access to the reinforcer for 1 hour. Immediately afterwards, mice are injected with lithium chloride, which induces transient malaise (Quinn et al., 2007). This pairing is repeated to ensure that mice come to associate consumption of the reinforcer with the sickness sensation, effectively devaluing the outcome. Devaluation is confirmed by measuring the amount of reinforcer consumed across sessions. Mice are then placed back in the operant conditioning chambers for a short probe test. Mice that are sensitive to the change in outcome value will decrease

responding, indicating that these mice are using the value of the reinforcer to guide their actions. Mice that are insensitive to the change in outcome value will continue to respond at rates equal to their response rates during instrumental conditioning, indicating a reliance on habitual response strategies.

Dendritic spine imaging

Using *thy*1-YFP-H mice and fluorescence microscopy, we can visualize dendritic spines from limbic and deep-layer cortical regions. Fixing brains and creating 40-50 um thick coronal sections allows easy detection and visualization of prelimbic cortical dendrites and dendritic spines (figs.2,3). Confocal microscopy is used to collect z-stacks of apical dendritic segments, and enables deep imaging into the mounted coronal sections while maintaining good axial resolution.

***In vivo* methodologies**

To image dendritic spines in real time *in vivo,* we continued to use *thy*1-YFP-H mice, with a multiphoton laser for excitation. Multiphoton excitation uses infrared wavelengths to excite fluorophores. A key advantage of infrared wavelengths is that they are longer than visible wavelengths and therefore scatter less, penetrating deeper into the brain. This is coupled with a high numerical aperture water immersion objective that is optimized for infrared wavelengths to resolve dendritic spines within an intact animal.

Generally, two *in vivo* methodologies are used to image dendritic spines just below the surface of the skull: a thinned skull approach (Yang et al., 2010) or a cranial window (Holtmaat and Svoboda, 2009). Hybrid approaches that leave a portion of the skull intact while implanting a cover slip have also been developed (Drew et al., 2010). There has been substantial debate in the field about the relative merits of each approach, with the major point of contention concerning the invasiveness of each

approach and how much, or little, damage the necessary surgical steps causes to the brain (Xu et al., 2007; Dorand et al., 2014).

The thinned skull approach involves mechanically thinning a small portion of the skull to the point that it can be imaged through allows for acute imaging over a period of a few hours, although techniques have been developed to allow extend the imageable time frame to a few days (Yang et al., 2013). The cranial window involves complete removal of the skull and permanent implantation of a small coverslip where the skull was removed. This allows long-term imaging over a period of weeks to months, but concerns have been raised about the amount of damage this approach causes to the brain, potentially confounding the results.

In the past few years, *in vivo* multiphoton imaging has begun to be used in awake, behaving mice. Regardless of the relative merits of thinned-skull vs. cranial window approaches, behavioral training typically requires multiple sessions over a period of days, which heavily favors cranial window approaches (Johnson et al., 2016). Although labs are using the thinned-skull technique in awake mice (Yang et al., 2014b), the viability of the thinned-skull approach for imaging over a period of weeks or months remains to be seen. Regardless of which surgical preparation is used, the next major challenge will be to develop apparatus that allow for head-restrained mice to engage in complex behavioral tasks to provide direct evidence linking changes in neural structure with complex behaviors.

Dendritic spine analysis

Quantification of post-mortem dendritic spine images typically concerns several criteria: dendritic spine density, dendritic spine length, head diameter, and if classifying spines by type, neck diameter. As discussed above, changes in any of these parameters have been implicated in some aspect of learning and memory, disease, and psychiatric

pathologies. Historically, detection and analysis was conducted by hand, with individuals manually drawing lines for spine lengths and diameters, and measuring those lines. The advent of modern computers has helped automate this process, but not all analyses can be automated yet. It is no surprise that computers are more consistent at measuring lengths, especially over very small distances (Swanger et al., 2011). However, computers do not do well when image quality, measured by the signal:noise ratio, is poor. Analysis of *in vivo* dendritic spine images is still done manually due to decreased image quality relative to post-mortem samples.

A number of free and commercial software packages exist to quantify dendritic spines. The past decade has seen dramatic increases in processing power and hard drive capacity which has made 3D reconstruction and analysis of z-stacks acquired from fluorescent samples a reality, improving accuracy relative to 2D analysis (Rodriguez et al., 2006). This enables calculation of dendritic spine volume, as well as measuring all possible axes when determining diameters, resulting in a more accurate representation of dendritic spine shape. We use the Imaris software package for semi-automated detection and reconstruction of dendritic spines.

These same measures are available with *in vivo* datasets, but the addition of time as a dimension enables analysis of turnover rates. While post-mortem studies designed to collect samples at different time points can identify changes in dendritic spines as a population over time, *in vivo* imaging allows for tracking individual dendritic spines over time. Aggregate rates of dendritic spine formation and elimination can be measured, and brings us one step closer to directly linking learning and memory to changes at the level of individual dendritic spines (Yang et al., 2014a).

Major points of the dissertation

With this background and methodological foundation in mind, we were broadly interested in the relationship between dendritic spines and decision-making strategies. Our initial findings led us to the hypothesis that ROCK inhibition could serve as a tool to restore goal-directed decision-making after the development of habitual responding. In Chapter 1, we investigate the impact of prolonged corticosterone exposure on prelimbic cortical dendritic spines, and show preliminary evidence that ROCK inhibition can impact decision-making strategies. Chapter 2 details the effects of stress hormone exposure on dendritic spines throughout cortico-limbic regions implicated in decision-making strategies and stress-related mood disorders, and identifies a genetic factor underlying structural resilience of dendritic spines. Chapter 3 examines the relationship between prelimbic cortical dendritic spine dynamics and goal-directed vs. habitual decision-making, and a model for how the ROCK inhibitor fasudil can be used to enhance goal-directed decision-making, including in the context of cocaine-induced habits. Finally, Chapter 4 examines the regulation of prelimbic cortical dendritic spines in real time.

References

Adams CD, Dickinson A (1981). Instrumental responding following reinforcer devaluation. Q J Exp Psychol 33B: 109–121.

Alexander GE, Crutcher MD (1990) Functional architecture of basal ganglia circuits: neural substrates of parallel processing. Trends Neurosci 13:266-271.

Arellano JI, Espinosa A, Fairén A, Yuste R, DeFelipe J (2007) Neuroscience 145:464-469.

Balleine BW, Dickinson A (1998) Goal-directed instrumental action: contingency and incentive learning and their cortical substrates. Neuropharmacology 37:407-419.

Balleine BW, O'Doherty JP (2010) Human and rodent homologies in action control: corticostriatal determinants of goal-directed and habitual action. Neuropsychopharmacology 35:48-69.

Bhatt DH, Zhang S, Gan WB (2009) Dendritic spine dynamics. Annu Rev Physiol 71:261-282.

Bloodgood BL, Sabatini BL (2005) Neuronal activity regulates diffusion across the neck of dendritic spines. Science 310:866-869.

Bos JL, Rehmann H, Wittinghofer A (2007) GEFs and GAPs: Critical Elements in the Control of Small G Proteins. Cell 129:865-877.

Bosch M, Hayashi Y (2012) Structural plasticity of dendritic spines. Curr Opin Neurobiol 22:383–388.

Bosch M, Castro J, Saneyoshi T, Matsuno H, Sur M, Hayashi Y (2014) Structural and molecular remodeling of dendritic spine substructures during long-term potentiation. Neuron 82:444-459.

Bourne J, Harris KM (2007) Do thin spines learn to be mushroom spines that remember? Curr Opin Neurobiol 17:381-386.

Brodmann K (1909) Vergleichende lokalisationslehre der großhirnrinde : in ihren prinzipien dargestellt auf grund des zellenbaues. Leipzig: Barth.

Carlsson L, Nyström LE, Sundkvist I, Markey F, Lindberg U (1977) Actin polymerizability is influenced by profilin, a low molecular weight protein in non-muscle cells. J Mol Biol 115:465–483.

Carr Db, Sesack SR (1996) Hippocampal afferents to the rat prefrontal cortex: synaptic targets and relation to dopamine terminals. J Comp Neurol 369:1-15.

Chan CS, Weeber EJ, Zong L, Fuchs E, Sweatt JD, Davis RL (2006) Beta 1-integrins are required for hippocampal AMPA receptor-dependent synaptic transmission, synaptic plasticity, and working memory. J Neurosci 26:223–232.

Condé F, Maire-Lepoivre E, Audinat E, Crepel F (1995) Afferent connections of the medial frontal cortex of the rat. II. Cortical and subcortical afferents. J Comp Neurol 352:567-593.

Corbit LH, Balleine BW (2000) The role of the hippocampus in instrumental conditioning. J Neurosci 20:4233-4239.

Corbit LH, Balleine BW (2003) The role of prelimbic cortex in instrumental conditioning. Behav Brain Res 146:145-157.

Corbit LH, Chieng BC, Balleine BW (2014) Effects of repeated cocaine exposure on habit learning and reversal by N-Acetylcysteine. Neuropsychopharmacology 39:1893-1901.

Corcoran KA, Quirk GJ (2007) Activity in prelimbic cortex is necessary for the expression of learned, but not innate, fears. J Neurosci 27:840-844.

Crick F (1982) Do dendritic spines twitch? Trends Neurosci 5:44-46.

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

Dickinson A (1980) Contemporary Animal Learning Theory. Cambridge: Cambridge University Press.

Dilgen J, Tejeda HA, O'Donnell P (2013) Amygdala inputs drive feedforward inhibition in the medial prefrontal cortex. J Neurophysiol 110:221-229.

Dominguez R, Holmes KC (2011) Actin structure and function. Annu Rev Biophys 40:169-186.

Dorand RD, Barkauskas DS, Evans TA, Petrosiute A, Huang AY (2014) Comparison of intravital thinned skull and cranial window approaches to study CNS immunobiology in the mouse cortex. Intravital 3:e29728.

Doya K (2008) Modulators of decision making. Nat Neurosci 11:410-416.

Drew PJ, Shih AY, Driscoll JD, Knutsen PM, Blinder P, Davalos D, Akassoglou K, Tsai PS, Kleinfeld D (2010) Chronic optical access through a polished and reinforced thinned skull. Nat Methods 7:981-984.

Engert F, Bonhoeffer T (1999) Dendritic spine changes associated with hippocampal long-term synaptic plasticity. Nature 399:66-70.

Everitt BJ, Robbins TW (2016) Drug Addiction: Updating actions to habits to compulsions ten years on. Annu Rev Psychol 4:23-50.

Feng G, Mellor RH, Bernstein M, Keller-Peck C, Nguyen QT, Wallace M, Nerbonne JM, Lichtman JW, Sanes JR (2000) Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. Neuron 28:41-51.

Gabbott PL, Dickie BG, Vaid RR, Headlam AJ, Bacon SJ (1997) Local-circuit neurones in the medial prefrontal cortex (areas 25, 32 and 24b) in the rat: morphology and quantitative distribution. J Comp Neurol 377:465-499.

Gabbott PL, Warner TA, Jays PR, Bacon SJ (2003) Areal and synaptic interconnectivity of prelimbic (area 32), infralimbic (area 25) and insular cortices in the rat. Brain Res 993:59-71.

Gabbott PL, Warner TA, Jays PR, Salway P, Busby SJ (2005) Prefrontal cortex in the rat: projections to subcortical autonomic, motor, and limbic centers. J Comp Neurol 492:145-177.

Gläscher J, Hampton AN, O'Doherty JP (2009) Determining a role for ventromedial prefrontal cortex in encoding action-based value signals during reward-related decision making. Cereb Cortex 19:483-495.

Gourley SL, Olevska A, Warren MS, Taylor JR, Koleske AJ (2012) Arg kinase regulates prefrontal dendritic spine refinement and cocaine-induced plasticity. J Neurosci 32:2314-2323.

Gourley SL, Taylor JR (2016) Going and stopping: dichotomies in behavioral control by the prefrontal cortex. Nat Neurosci 19:656-664.

Gourley SL, Zimmermann KS, Allen AG, Taylor JR (2016b) The medial orbitofrontal cortex regulates sensitivity to outcome value. J Neurosci 36:4600-4613.

Gray EG (1959) Electron microscopy of synaptic contacts on dendrite spines of the cerebral cortex. Nature 183:1592-1593.

Haddon JE, Killcross S (2011) Rat prefrontal dopamine and cognitive control: Impaired and enhanced conflict performance. Behav Neurosci 3:344-349.

Harris KM, Jensen Fe, Tsao B (1992) Three-dimensional structure of dendritic spines and synapses in rat hippocampus (CA1) at postnatal day 15 and adult ages: implications for the maturation of synaptic physiology and long-term potentiation. J Neurosci 12:2684-2705.

Heidbreder CA, Groenewegen HJ (2003) The medial prefrontal cortex in the rat: evidence for a dorso-ventral distinction based upon functional and anatomical characteristics. Neurosci Behav Rev 27:555-579.

Hering H, Sheng M (2001) Dendritic spines: structure, dynamics and regulation. Nat Rev Neurosci 2:880-888.

Hinton EA, Wheeler MG, Gourley SL (2014) Early-life cocaine interferes with BDNF-mediated behavioral plasticity. Learn Mem 21:253-257.

Holtmaat A, Svoboda K (2009) Experience-dependent structural synaptic plasticity in the mammalian brain. Nat Rev Neurosci 10:647-658.

Homberg JR (2012) Serotonin and decision making processes. Neurosci Biobehav Rev 36:218-236.

Huentelman MJ, Stephan DA, Talboom J, Corneveaux JJ, Reiman DM, Gerber JD, Barnes CA, Alexander GE, Reiman EM, Bimonte-Nelson HA (2009) Peripheral delivery of a ROCK inhibitor improves learning and working memory. Behav Neurosci 123:218-223.

Ishikawa A, Nakamura S (2003) Convergence and interaction of hippocampal and amygdalar projections within the prefrontal cortex in the rat. J Neurosci 23:9987-9985.

Jan YN, Jan LY (2010) Branching out: mechanisms of dendritic arborization. Nat Rev Neurosci 11:316-328.

Jay TM, Glowinski J, Thierry A-M (1989) Selectivity of the hippocampal projection to the prelimbic area of the prefrontal cortex in the rat. Brain Res 505:337-340.

Johnson CM, Peckler H, Tai LH, Wilbrecht L (2016) Rule learning enhances structural plasticity of long-range axons in frontal cortex. Nat Commun 7:10785.

Kang MG, Guo Y, Huganir RL (2009) AMPA receptor and GEF-H1/Lfc complex regulates dendritic spine development through RhoA signaling cascade. Proc Natl Acad Sci U S A 106:3549-3554.

Killcross S, Coutureau E (2003) Coordination of actions and habits in the medial prefrontal cortex of rats. Cereb Cortex 13:400-408.

Koleske AJ (2013) Molecular mechanisms of dendrite stability. Nat Rev Neurosci 14:536-550.

Komorowski RW, Garcia CG, Wilson A, Hattori S, Howard MW, Eichenbaum H (2013) Ventral hippocampal neurons are shaped by experience to represent behaviorally relevant contexts. J Neurosci 33:8079-8087.

Kulkarni VA, Firestein BL (2012) The dendritic tree and brain disorders. Mol Cell Neurosci 50:10-20.

Kuroda M, Murakami K, Kishi K, Price JL (1995a) Thalamocortical synapses between axons from the mediodorsal thalamic nucleus and pyramidal cells in the prelimbic cortex of the rat. J Comp Neurol 356:143-151

Kuroda M, Murakumi K, Shinkai M, Ojima Hisayuki, Kishi K (1995b) Electron microscopic evidence that axon terminals from the mediodorsal thalamic nucleus make direct synaptic contacts with callosal cells in the prelimbic cortex of the rat. Brain Research 677:348-353.

Kuroda M, Murakami K, Igarashi H, Okada A (1996) The convergence of axon terminals from the mediodorsal thalamic nucleus and ventral tegmental area on pyramidal cells in layer V of the rat prelimbic cortex. Eur J Neurosci 8:1340-1349.

Lai CS, Franke TF, Gan WB (2012) Opposite effects of fear conditioning and extinction on dendritic spine remodelling. Nature 483:87-91.

Lamprecht R, Farb CR, LeDoux JE (2002) Fear memory formation involves p190 RhoGAP and ROCK proteins through a GRB2-mediated complex. Neuron 36:727-738.

LeBlanc KH, Maidment NT, Ostlund SB (2013) Repeated cocaine exposure facilitates the expression of incentive motivation and induces habitual control in rats. PLoS One 8:e61355.

Leong KC, Berini CR, Ghee SM, Reichel CM (2016) Extended cocaine-seeking produces a shift from goal-directed to habitual responding in rats. Physiol Behav 165:330-335.

Lex B, Hauber W (2009) The role of dopamine in the prelimbic cortex and the dorsomedial striatum in instrumental conditioning. Cereb Cortex 20:873-883.

Liston C, Miller MM, Goldwater DS, Radley JJ, Rocher AB, Hof PR, Morrison JH, McEwen BS (2006) Stress-induced alterations in prefrontal cortical dendritic morphology predict selective impairments in perceptual attentional set-shifting. J Neurosci 26:7870-7874.

Liston C, Cichon JM, Jeanneteau F, Jia Z, Chao MV, Gan WB (2013) Circadian glucocorticoid oscillations promote learning-dependent synapse formation and maintenance. Nat Neurosci 16:698-705.

Magee JC (2000) Dendritic integration of excitatory synaptic input. Nat Rev Neurosci 1:181-190.

Maret S, Faraguna U, Nelson AB, Cirelli C, Tononi G (2011) Sleep and waking modulate spine turnover in the adolescent mouse cortex. Nat Neurosci 14:1418-1420.

Miles FJ, Everitt BJ, Dickinson A (2003) Oral cocaine seeking by rats: action or habit? Behav Neurosci 117:927-938.

Mitchell AS, Chakraborty S (2013) What does the mediodorsal thalamus do? Front Syst Neurosci 7:37.

Molyneaux BJ, Arlotta P, Menezes JR, Macklis JD (2007) Neuronal subtype specification in the cerebral cortex. Nat Rev Neurosci 8:427-437.

Morrison JH, Molliver ME, Grzanna R, Coyle JT (1979) Noradrenergic innervations patterns in three regions of medial cortex: an immunofluorescence characterization. Brain Res Bull 4:849-857.

Murakoshi H, Wang H, Yasuda R (2011) Local, persistent activation of Rho GTPase during plasticity of single dendritic spines. Nature 472:100-104.

Nicoll RA (2017) A brief history of long-term potentiation. Neuron 93:281-290.

Ostlund SB, Balleine BW (2005) Lesions of medial prefrontal cortex disrupt the acquisition but not the expression of goal-directed learning. J Neurosci 25:7764-7770.

Pavlov IP (1927) Conditioned reflexes: an investigation of the physiological activity of the cerebral cortex (1927) Oxford, England: Oxford Univ. Press Conditioned reflexes: an investigation of the physiological activity of the cerebral cortex.(1927). xv 430 pp.

Paxinos G, Franklin KBJ (2003) The mouse brain in stereotaxic coordinates. Academic; San Diego.

Peters A, Kaiserman-Abramof IR (1970) The small pyramidal neuron of the rat cerebral cortex. The perikaryon, dendrites and spines. Am J Anat 127:321-355.

Pollard T (2007). Regulation of actin filament assembly by Arp2/3 complex and formins. Annu Rev Biophys Biomol Struct 36:451–77.

Pontrello CG, Ethell IM (2009) Accelerators, Brakes, and Gears of Actin Dynamics in Dendritic Spines. Open Neurosci J 3:67-86.

Preuss TM (1995) Do rats have prefrontal cortex? The rose-woolsey-akert program reconsidered. J Cogn Neurosci 7:1-24.

Pring M, Weber A, Bubb MR (1992) Profilin-actin complexes directly elongate actin filaments at the barbed end. Biochemistry 31:1827–36.

Puig, MV (2011) Serotonergic modulation of the prefrontal cortex: from neurons to brain waves. Psychiatric Disorders - Worldwide Advances, Dr. Toru Uehara (Ed.).

Quinn JJ, Hitchcott PK, Umeda EA, Arnold AP, Taylor JR (2007) Sex chromosome complement regulates habit formation. Nat Neurosci 10:1398-1400.

Radley JJ, Rocher AB, Miller M, Janssen WG, Liston C, Hof PR, McEwen BS, Morrison JH (2006) Repeated stress induces dendritic spine loss in the rat medial prefrontal cortex. Cereb Cortex 16:313-320.

Radley JJ, Anderson RM, Cosme CV, Glanz RM, Miller MC, Romig-Martin SA, LaLumiere RT (2015) The Contingency of Cocaine Administration Accounts for Structural and Functional Medial Prefrontal Deficits and Increased Adrenocortical Activation. J Neurosci 35:11897-11910.

Ramón y Cajal S. (1888). Estructura de los centros nerviosos de las aves. Rev. Trim. Histol. Norm. Pat. 1, 1–10.

Ramón y Cajal S (1893) Neue darstellung vom histologischen baudes centralnervensystems. Archiv fur Anatomie und Entwickelungs-geschichte. Anatomische Abtheilung des Archives fur Anatome und Physiologie 319-428.

Rex CS, Chen LY, Sharma A, Liu J, Babayan AH, Gall CM, Lynch G (2009) Different Rho GTPase-dependent signaling pathways initiate sequential steps in the consolidation of long-term potentiation. J Cell Biol 186:85-97.

Robinson TE, Kolb B (1997) Persistent structural modifications in nucleus accumbens and prefrontal cortex neurons produced by previous experience with amphetamine. J Neurosci 17:8491-8497.

Robinson TE, Kolb B (1999) Alterations in the morphology of dendrites and dendritic spines in the nucleus accumbens and prefrontal cortex following repeated treatment with amphetamine or cocaine. Eur J Neurosci 11:1598-1604.

Robinson TE, Gorny G, Kolb B (2001) Cocaine self-administration alters the morphology of dendrites and dendritic spines in the nucleus accumbens and neocortex. 39:257-266.

Rodriguez A, Ehlenberger DB, Hof PR, Wearne SL (2006) Rayburst sampling, an algorithm for automated three-dimensional shape analysis from laser scanning microscopy images. Nat Protoc 1:2152-2161.

Sabatini BL, Maravall M, Svoboda K (2001) Ca(2+) signaling in dendritic spines. Curr Opin Neurobiol 11:349-356.

Sanders J, Cowansage K, Baumgärtel K, Mayford M (2012) Elimination of dendritic spines with long-term memory is specific to active circuits. J Neurosci 32:12570-12578.

Schmitzer-Torbert N, Apostolidis S, Amoa R, O'Rear C, Kaster M, Stowers J, Ritz R (2015) Post-training cocaine administration facilitates habit learning and requires the infralimbic cortex and dorsolateral striatum. Neurobiol Learn Mem 118:105-112.

Schoenbaum G, Takahashi Y, Liu TL, McDannald MA (2011) Does the orbitofrontal cortex signal value? Ann N Y Acad Sci 1239:87-89.

Schoenbaum G, Setlow B (2005) Cocaine makes actions insensitive to outcomes but not extinction: implications for altered orbitofrontal-amygdalar function. Cereb Cortex 15:1162-1169.

Schubert V, Da Silva JS, Dotti CG (2006) Localized recruitment and activation of RhoA underlies dendritic spine morphology in a glutamate receptor-dependent manner. J Cell Bio 30:453-467.

Schultz RL, Maynard EA, Pease DC (1957) Electron microscopy of neurons and neuroglia of cerebral cortex and corpus callosum. Am J Anat 100:369-407.

Segal M (2005) Dendritic spines and long-term plasticity. Nat Rev Neurosci 6:277-284.

Sfakianos MK, Eisman A, Gourley SL, Bradley WD, Scheetz AJ, Settleman J, Taylor JR, Greer CA, Williamson A, Koleske AJ (2007) Inhibition of Rho via Arg and p190RhoGAP in the postnatal mouse hippocampus regulates dendritic spine maturation, synapse and dendrite stability, and behavior. J Neurosci 27:10982-10992.

Shapiro LP, Parsons RG, Koleske AJ, Gourley SL (2017) Differential expression of cytoskeletal regulatory factors in the adolescent prefrontal cortex: Implications for cortical development. J Neurosci Res 95:1123-1143.

Shen HW, Toda S, Moussawi K, Bouknight A, Zahm DS, Kalivas PW (2009) Altered dendritic spine plasticity in cocaine-withdrawn rats. J Neurosci 29:2876-2884.

Sutton MA, Schuman EM (2006) Dendritic protein synthesis, synaptic plasticity, and Memory. Cell 127:49-58.

Suzuki Y, Shibuya M, Satoh S, Sugimoto Y, Takakura K (2007) A postmarketing surveillance study of fasudil treatment after aneurysmal subarachnoid hemorrhage. Surg Neurol 68:126-131.

Swanger SA, Yao X, Gross C, Bassell GJ (2011) Automated 4D analysis of dendritic spine morphology: Applications to stimulus-induced spine remodeling and pharmacological rescue in a disease model. Mol Brain 4:38.

Swanger SA, Mattheyses AL, Gentry EG, Herskowitz JH (2016) ROCK1 and ROCK2 inhibition alters dendritic spine morphology in hippocampal neurons. Cell Logist 5:e1133266.

Swanson AM, Shapiro LP, Whyte AJ, Gourley SL (2013) Glucocorticoid receptor regulation of action selection and prefrontal cortical dendritic spines. Commun Integr Biol 6:e26068.

Taylor AH, Miller R, Gray RD (2012) New Caledonian crows reason about hidden causal agents. Proc Natl Acad Sci U S A 109:16389-16391.

Tejeda HA, O'Donnell P (2014) Amygdala inputs to the prefrontal cortex elicit heterosynaptic suppression of hippocampal inputs. J Neurosci 34:14365-14374.

Tilney LG, Bonder EM, Coluccio LM, Mooseker MS (1983) Actin from Thyone sperm assembles on only one end of an actin filament: a behavior regulated by profilin. J Cell Biol 97:112–24.

Tønnesen J, Katona G, Rózsa B, Nägerl UV (2014) Spine neck plasticity regulates compartmentalization of synapses. Nat Neurosci 17:678-685.

Tricomi E, Balleine BW, O'Doherty JP (2009) A specific role for posterior dorsolateral striatum in human habit learning. Eur J Neurosci 29:2225-2232.

Valentin VV, Dickinson A, O'Doherty JP (2007) Determining the neural substrates of goal-directed learning in the human brain. J Neurosci 27:4019-4026.

Van der Plasse G, La Fors SSBM, Meerkerk DTJ, Joosten RNJMA, Uylings HBM, Feenstra MGP (2007) Medial prefrontal serotonin in the rat is involved in goal-directed behavior when affect guides decision making. Psychopharmacology 194:435-449.

Vertes RP (2004) Differential projections of the infralimbic and prelimbic cortex in the rat. Synapse 51:32-58.

Warren MS, Bradley WD, Gourley SL, Lin YC, Simpson MA, Reichardt LF, Greer CA, Taylor JR, Koleske AJ (2012) Integrin β1 signals through Arg to regulate postnatal dendritic arborization, synapse density, and behavior. J Neurosci 32:2824-2834.

Weir AA, Chappell J, Kacelnik A (2002) Shaping of hooks in New Caledonian crows. Science 298:981.

Uylings HB, Groenewegen HJ, Kolb B (2003) Do rats have a prefrontal cortex? Behav Brain Res 146:3-17.

Xu HT, Pan F, Yang G, Gan WB (2007) Choice of cranial window type for in vivo imaging affects dendritic spine turnover in the cortex. Nat Neurosci 10:549-551.

Yang G, Pan F, Gan WB (2009) Stably maintained dendritic spines are associated with lifelong memories. Nature 462:920-924.

Yang G, Pan F, Parkhurst CN, Grutzendler J, Gan WB (2010) Thinned-skull cranial window technique for long-term imaging of the cortex in live mice. Nat Protoc 5:201-208.

Yang G, Gan WB (2012) Sleep contributes to dendritic spine formation and elimination in the developing mouse somatosensory cortex. Dev Neurobiol 72:1391-1398.

Yang G, Pan F, Chang PC, Gooden F, Gan WB (2013) Transcranial two-photon imaging of synaptic structures in the cortex of awake head-restrained mice. Methods Mol Biol 1010:35-43.

Yang G, Lai CS, Cichon J, Ma L, Li W, Gan WB (2014a) Sleep promotes branch-specific formation of dendritic spines after learning. Science 344:1173-1178.

Yang G, Pan F, Chang PC, Gooden F, Gan WB (2014b) Transcranial two-photon imaging of synaptic structures in the cortex of awake head-restrained mice. Methods Mol Biol 1010:35-43.

Yang N, Higuchi O, Ohashi K, Nagata K, Wada A, Kangawa K, Nishida E, Mizuno K (1998) Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. Nature 393:809-812.

Yang Y, Wang XB, Frerking M, Zhou Q (2008) Spine expansion and stabilization associated with long-term potentiation. J Neurosci 28:5740-5751.

Yin HH, Knowlton BJ, Balleine BW (2004) Lesions of dorsolateral striatum preserve outcome expectancy but disrupt habit formation in instrumental learning. Eur J Neurosci 19:181-189.

Yin HH, Ostlund SB, Knowlton BJ, Balleine BW (2005) The role of the dorsomedial striatum in instrumental conditioning. Eur J Neurosci 22:513-523.

Zapata A, Minney VL, Shippenberg TS (2010) Shift from goal-directed to habitual cocaine seeking after prolonged experience in rats. J Neurosci 30:15457-15463.

Zimmermann KS, Yamin JA, Rainnie DG, Ressler KJ, Gourley SL (2017) Connections of the mouse orbitofrontal cortex and regulation of goal-directed action selection by brain-derived neurotrophic factor. Biol Psychiatry 81:366-377.

Figures

Figure 1. Goal-directed vs. habitual decision-making. When an animal is sensitive to the relationship between an action and its outcome, it is termed goal-directed, while an animal insensitive to that relationship instead relies on stimulus-response relationships, resulting habitual response strategies. A number of mechanisms have been identified that facilitate or accelerate the transition from goal-directed to habitual response strategies, but reversing habits has proven difficult. Here, we demonstrate Rho-kinase (ROCK) inhibition can be used as a tool to restore goal-directed decision-making after the development of habitual responding.

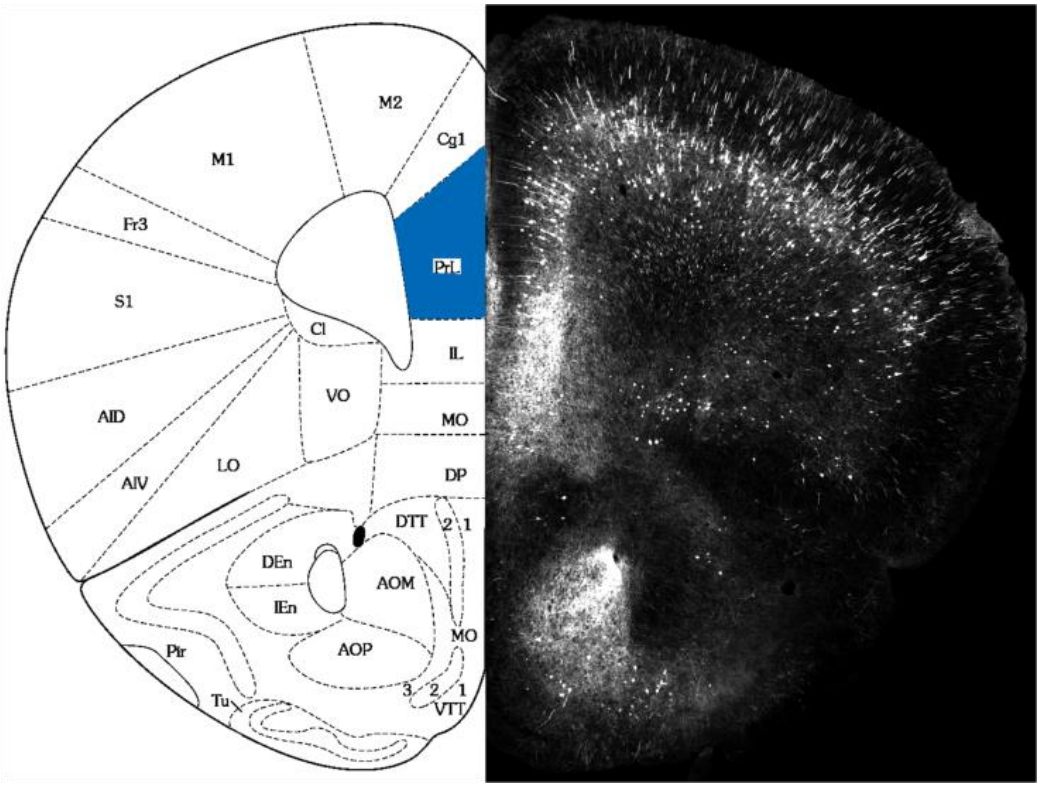

Figure 2. The prelimbic cortex is part of the prefrontal cortex. Left: A coronal plate of the prefrontal cortex, with the prelimbic cortex, located along the medial wall, highlighted in blue (Paxinos and Franklin, 2003). Right: Corresponding fluorescence image of a thy1-YFP coronal section. The cell body layer is located deep in the brain, with prelimbic cortical apical dendrites projecting horizontally towards the midline.

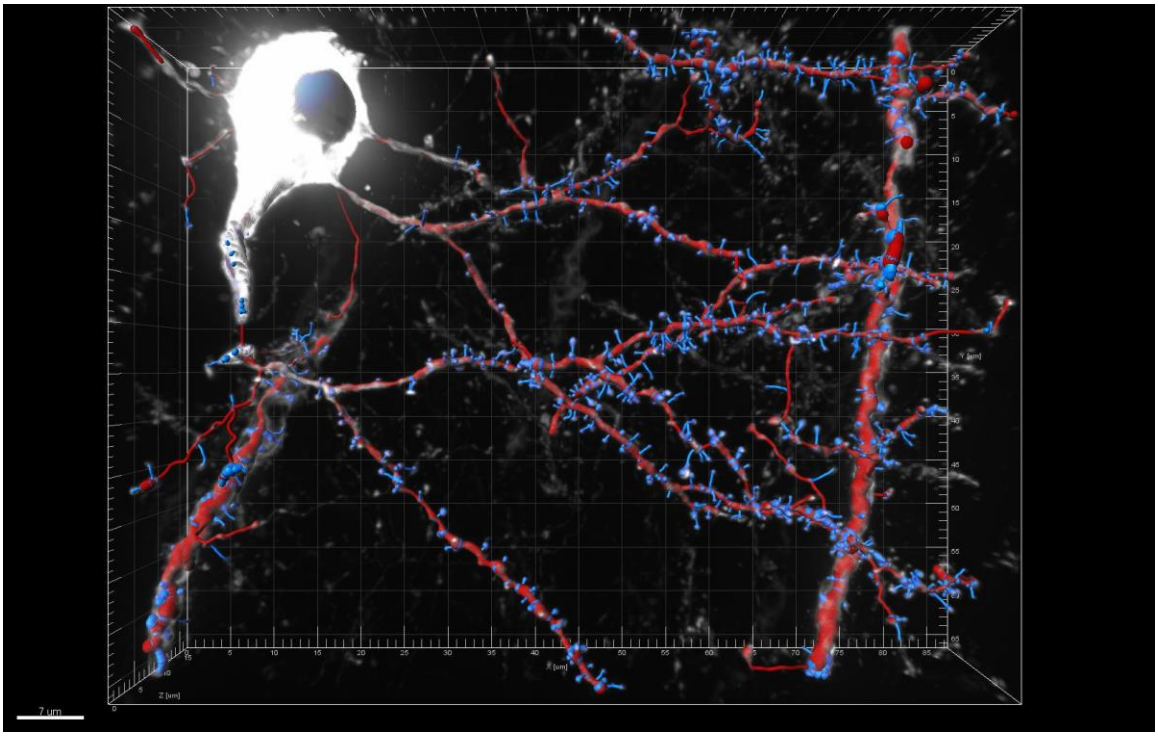

Figure 3. 3-D reconstruction of dendritic spines. Imaris was used to create a 3-D reconstruction of deep-layer cortical neurons. The original fluorescence z-stack is in white. The soma is indicated by the blue circle. Dendrites and axons are in red and dendritic spines are in blue. Scale bar = 7 µm.

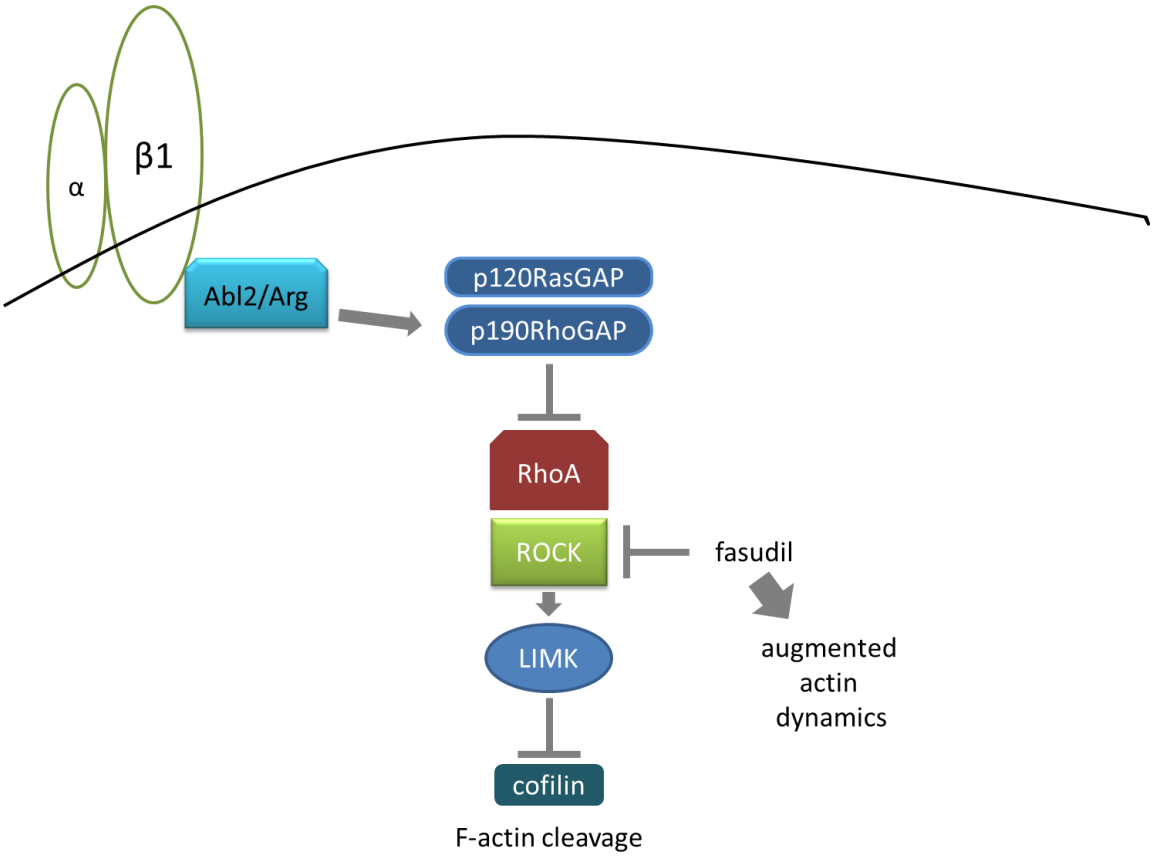

Figure 4. Rho-ROCK signaling. β1-integrin is a transmembrane receptor that forms a dimer with an α subunit. β1-integrin signaling can activate Abl2/Arg, which activates p190RhoGAP, inhibiting RhoA. RhoA phosphorylates LIM kinase, which phosphorylates cofilin, preventing cofilin from cleaving F-actin filaments. Inhibition of ROCK by fasudil leads to dephosphorylation of cofilin, enabling cofilin to cleave F-actin filaments. As such, fasudil can be used as a tool to alter actin dynamics, promoting remodeling of dendritic spine structure (adapted from Shapiro et al., 2017).

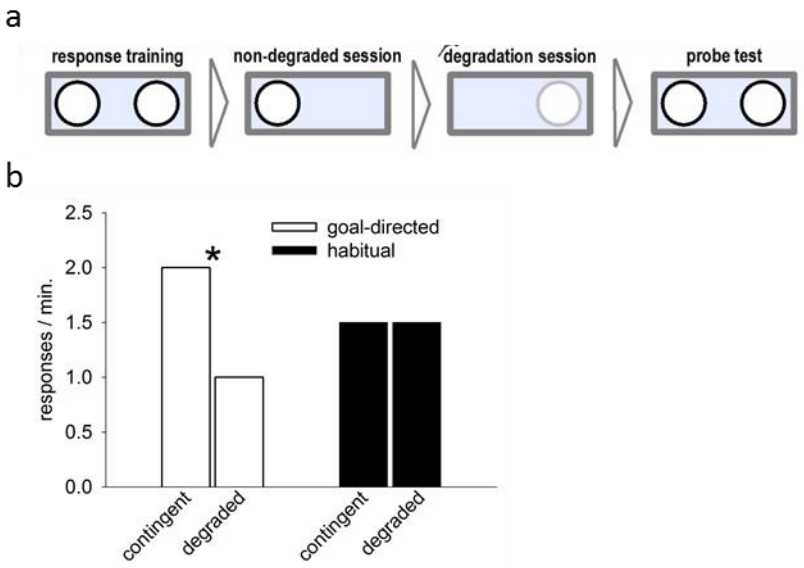

Figure 5. Instrumental conditioning and action-outcome contingency degradation can be used to determine action-outcome sensitivity in mice. (a) Action-outcome contingency degradation outline: Mice are trained to respond on two nose-poke apertures for food pellets. Following training, one nose-poke recess is available, and responding is reinforced. In another session, the opposite nose-poke recess is available, but responding is not reinforced; instead, food pellets are delivered at a rate matched to the reinforcement rate from the previous session, "degrading" the action-outcome contingency. During a probe test, mice have access to both nose-poke apertures. Preferential engagement of the response that is most likely to be reinforced is considered goal-directed, while engaging both nose-poke responses non-selectively is considered a failure in action-outcome conditioning and a deferral to habit-based behavior. (b) Sample results from the probe test, with one group showing a significant preference for the contingent response relative to the degraded response (goal-directed), and the other group engaging each response indiscriminately (habitual).

Chapter 1: Glucocorticoid receptor regulation of action selection and prefrontal cortical dendritic spines

Adapted from

Glucocorticoid receptor regulation of action selection and prefrontal cortical dendritic spines.

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Abstract

Early in my dissertation research, my laboratory group reported that prolonged exposure to the glucocorticoid receptor (GR) ligand corticosterone impairs decision-making that is dependent on the predictive relationship between an action and its outcome (Gourley et al., 2012). Additionally, acute GR blockade, when paired with action-outcome conditioning, also blocks new learning. We then showed that dendritic spines in the prelimbic prefrontal cortex remodeled under both conditions. Nonetheless, the relationship between deep-layer dendritic spines and outcome-based decision-making remains opaque.

We report here that a *history* of prolonged corticosterone exposure increases dendritic spine density in deep-layer prelimbic cortex. When spines are imaged *during* corticosteroid exposure (*i.e.*, without a washout period), dendritic spine densities are, however, reduced. Thus, the morphological response of deep-layer prelimbic cortical neurons to prolonged corticosteroid exposure may be quite dynamic, with spine elimination during a period of chronic exposure and spine proliferation during a subsequent washout period. We also provide preliminary evidence, using a Rho-kinase inhibitor, that GR-mediated dendritic spine remodeling is causally related to complex decision-making. Together, our findings suggest that physiological levels of GR binding enable an organism to learn about the predictive relationship between an action and its outcome. These findings lay the groundwork for further investigation into: the effects of stress hormone exposure on dendritic spines throughout cortico-limbic regions implicated in stress-related mood disorders (Chapter 2); the relationship between prelimbic cortical dendritic spine dynamics and complex decision-making (Chapter 3); and the regulation of prelimbic cortical dendritic spines in real time (Chapter 4).

Body text

Current evidence indicates that both humans and rodents can learn to associate specific actions with their outcomes; such actions are considered goal-directed, while habits are by contrast automated and stimulus-dependent (Yin et al., 2008; Balleine and O'Doherty, 2010). While the development of stimulus-response habits can be behaviorally advantageous, habits are also considered a fundamental etiological factor in several psychopathologies including obsessive-compulsive disorder. Moreover, rodent models of habit formation might have utility in the context of modeling substance abuse and unremitting ruminative thought processes in depression – this is because stimulus-elicited decision-making results in habitual response patterns that, like ruminative thought processes in depression, are resistant to change. Thus, isolating the neuroanatomy and neurobiology of habit formation has the potential for broad impact.

We have shown that both prolonged exposure to the glucocorticoid receptor (GR) ligand corticosterone *and* GR blockade impairs an animal's ability to make decisions based on the predictive relationship between an action and its outcome, resulting in a reliance on familiar, stimulus-response habits (Gourley et al., 2012). Chronic ligand binding can desensitize GRs (*e.g.*, Tasker and Herman, 2011), so this pattern suggested to us that desensitization of GRs confers vulnerability to the development of stimulus-response habits (Gourley et al., 2012). In addition, we showed that dendritic spines in deep-layer prelimbic prefrontal cortex had shortened. This is notable because deep-layer prefrontal cortex is reciprocally connected with downstream structures associated with action-outcome decision-making (Balleine and Dickinson, 1998; Corbit and Balleine, 2003; Killcross and Coutureau, 2003; Tran-Tu-Yen et al., 2009), and this shorter phenotype is suggestive of a greater density of immature "stubby" spines and increased dendritic spine turnover (Peters and Kaiserman-Abramof, 1970; Bhatt et al., 2009; Bourne and Harris, 2011). Thus, at first blush, this pattern may seem to suggest that

diminished GR binding remodels dendritic spines, and that structural remodeling confers maladaptive decision-making. However, the shortened dendritic spine phenotype was shared between multiple experimental conditions, including some that did not obviously impact decision-making strategies (Gourley et al., 2012). Hence, the relationship between deep-layer prelimbic cortical dendritic spine morphology and goal-directed action selection remains unclear.

GR occupation regulates dendritic spine density in deep-layer prelimbic cortex

Dynamic properties of spines, including spine density, shape, turnover, and motility are critical components of functional neural circuits. Here, we delve deeper into the complex relationship between deep-layer prelimbic cortical dendritic spines and outcome-based decision-making. We present new data on this topic and discuss our findings in the context of prior work in the field.

In these analyses, we first used the Yellow Fluorescent Protein (YFP)-expressing tissues collected for our prior report and enumerated dendritic spines on deep-layer excitatory neurons (Feng et al., 2000; Gourley et al., 2012). We compared several conditions: mice with an acute injection of the GR antagonist RU38486 (40 mg/kg, *i.p.*) and then euthanized 24 hours later; the corresponding vehicle-injected mice; mice exposed to the stress hormone corticosterone in the drinking water (25 µg/ml) for 3 weeks followed by a 3-week washout period; mice exposed to corticosterone for 3 weeks and euthanized *without* a washout period; and un-injected control mice. In this exploratory study, n=3 mice/group, and each dendrite was considered an independent sample. Unambiguous dendrites were scored, with lengths ranging from 7-117 µm; the average length was 27 µm. For further methodological details regarding tissue processing and imaging, we refer the reader to our prior report (Gourley et al., 2012).

When spines were quantified, we found that an acute injection of RU38486 increased dendritic spine density in deep-layer prelimbic prefrontal cortex, as did a *history* of prolonged exposure to exogenous corticosterone [$F(4,83)=15.6, p<0.001$] (fig.1a). When spines were imaged *without* a washout period (*i.e.*, when corticosterone was still in the drinking water), dendritic spine densities were *lower* than in other groups, in agreement with a prior report (Liu and Aghajanian, 2009). These findings together suggest that prolonged corticosterone exposure initially eliminates dendritic spines in deep-layer prelimbic cortex, but that spine density "rebounds" with a recovery period, and spines then ultimately over-proliferate. A somewhat similar profile has been observed in the adjacent deep-layer infralimbic cortex (dendritic spine elimination followed by recovery) (Gourley et al., 2013a), but notably not in layer II/III where spines appear to be more resilient to corticosteroid exposure (Cerqueira et al., 2007).

In mice administered an injection of the vehicle for RU38486, dendritic spine density was *qualitatively* higher than in the un-injected control group (fig.1a). Although this difference was statistically non-significant, this general pattern is in agreement with evidence that acute injection stress, unlike *chronic* stressor exposure, results in dendritic spine proliferation in the medial prefrontal cortex (Seib and Wellman, 2003). Moreover, acute exposure to both ethanol and dimethyl sulfoxide, common vehicles for RU38486, stimulate rapid, acute corticosterone secretion even at low concentrations (Allen and Allen, 1975; Olgilvie et al., 1997).

A notable aspect regarding RU38486-exposed mice pertains to the diameter of the dendritic spine head. When we compared spine head diameters, diameters were smaller overall in RU38486-exposed mice relative to vehicle-treated mice (fig.1b,c). This may be significant because smaller dendritic spine heads are less likely to contain synapses (though synapse density was not evaluated here). Whether the increase in

spine density in this group is a compensatory response to maintain overall synapse density also remains unclear.

Regulation of complex decision-making by the GR antagonist RU38486 and Rho-kinase inhibition

We previously reported that acute injection of the GR antagonist RU38486 (40 mg/kg, *i.p.*), when paired with action-outcome contingency degradation, impairs a mouse's ability to subsequently make decisions based on the predictive relationship between a response and its outcome (Gourley et al., 2012). Here, we tested the hypothesis that blocking the dendritic spine remodeling effects of RU38486 would rescue decision-making strategies. As in our prior report, we again trained adult naïve male C57BL/6 mice in standard Med-Associates operant conditioning chambers to respond on two distinct nose poke apertures for food reinforcement. We used a continuous reinforcement schedule and trained mice until responding was stable and side preferences were eliminated (5-7 70-min. sessions). We then 'degraded' the action-outcome relationship associated with one of the apertures by providing food reinforcement non-contingently for 25 min. and at a rate yoked to each animal's own reinforcement rate from the previous session (Hammond, 1980; adapted from Gourley et al., 2012). During this contingency degradation training session, the opposite nose poke was occluded, and an injection of RU38486 immediately followed in order to selectively manipulate the consolidation — rather than acquisition — of new action-outcome associative conditioning. Prior to this session, mice had also received a 25-min. training session during which only the opposite aperture was available, and responding was reinforced without a limit on the number of reinforcers delivered.

During a 10-min. probe test the following day, both apertures were again available. In this case, goal-directed outcome-based decision-making is reflected by

preferential responding on the ‘non-degraded’ aperture, while habits are reflected by equivalent responding, despite action-outcome contingency degradation.

We replicated our prior findings, showing that an injection of the GR antagonist RU38486 immediately after action-outcome contingency degradation — *i.e.*, during the presumptive consolidation phase of new action-outcome learning — in otherwise adult naïve mice *blocks* sensitivity to action-outcome contingency degradation [aperture x group $F(2,19)=3.5, p=0.05$] (fig.2a,b). In this case, we used a 4-fold *lower* dose of RU38486 (10 mg/kg, *i.p.*) than in our prior report (Gourley et al., 2012), providing further evidence that GR receptor binding is a potent regulator of decision-making strategies.

From the perspective of dendritic spine remodeling, aberrant RU38486-mediated spine proliferation could adversely impact new prelimbic cortical-dependent learning. Based on this perspective, we co-administered fasudil (10 mg/kg, *i.p.*), a Rho-kinase inhibitor, in conjunction with RU38486 (10 mg/kg, *i.p.*) in a separate group of mice. Why fasudil? Rho-kinase (also called ROCKII) is a substrate of a master cytoskeletal regulator RhoA GTPase (Rho). RhoA serves as a molecular switch, transitioning between an inactive GDP-bound state and an active GTP-bound state in which RhoA is targeted to cellular membranes. There, RhoA orchestrates the formation of stress fibers and focal adhesions necessary to reorganize cellular membranes through Rho-kinase. Thus, Rho-kinase inhibitors stabilize neural structure or allow for activity-dependent neuronal remodeling, depending on extracellular stimuli (*e.g.*, Murakoshi et al., 2011). We hypothesized that in this context fasudil might have protective benefits. Indeed, fasudil administration in conjunction with RU38486 rescued sensitivity to action-outcome contingency conditioning and preserved decision-making (fig.2b). Thus, we argue that RU38486-mediated remodeling of prelimbic cortical dendritic spines confers vulnerability to the formation of stimulus-response habits; by extension, aberrantly elevated dendritic spine densities may be associated with stimulus-response habits.

Discussion

To summarize, blocking GRs during the consolidation of action-outcome conditioning impairs new learning regarding the predictive relationship between an action (a nose poke) and its outcome (a food pellet). Acute GR blockade also increases dendritic spine density in deep-layer prelimbic cortex. By contrast, a history of GR blockade during adolescence *promotes* subsequent decision-making based on the predictive relationship between a response and its outcome.

Interestingly, prolonged exposure to the GR ligand corticosterone has dynamic structural consequences, eliminating prelimbic cortical dendritic spines initially, but then with a washout period, spine densities are modestly *increased* relative to un-injected control mice. Notably, layer III prelimbic cortical arbors respond somewhat similarly to stressor exposure: With prolonged exposure, arbors are simplified, but with a recovery period, arbors regain their original complexity (Bloss et al., 2010). Interestingly, with advanced age, arbors become less plastic and less able to recover (Bloss et al., 2010), highlighting the possibility that corticosteroid-mediated dendritic spine elimination and the magnitude of "recovery" depend heavily on age.

It is important to note that while the brain region of interest here was the prelimbic cortex, lesion studies in rodents implicate the prelimbic, infralimbic, *and* orbitofrontal prefrontal cortices in action selection. For example, the prelimbic cortex is thought to promote goal-directed decision-making while the infralimbic cortex by contrast supports habit formation (Balleine and Dickinson, 1998; Corbit and Balleine, 2003; Killcross and Coutureau, 2003; Tran-Tu-Yen et al., 2009). And like the prelimbic cortex, the orbitofrontal cortex is implicated in learning the relationship between an instrumental response and its outcome (Gourley et al., 2010; Gourley et al., 2013b), as well as the predictive relationship between a stimulus and an outcome (Ostlund and Balleine, 2007).

We have previously reported that deep-layer infralimbic cortical dendritic spines are eliminated in response to prolonged corticosterone exposure, but densities recover to control levels after a 7-9 day washout period (Gourley et al., 2013a). Deep-layer orbitofrontal cortical dendritic spine densities are also reduced with prolonged corticosterone exposure, but densities *fail to recover within the same time window* (Gourley et al., 2013a). Combined with prelimbic cortical densities that were initially decreased and then *elevated* with a history of corticosterone exposure here, it would appear that deep-layer infralimbic cortex is considerably more resilient to corticosterone than the neighboring prelimbic and orbitofrontal cortices. By extension, the reliance of mice with a history of prolonged corticosterone exposure on familiar stimulus-response habits rather than outcome-based goal-directed response strategies (Gourley et al., 2012) may reflect the preferential engagement of intact infralimbic cortex-mediated response strategies, rather than response strategies that rely on compromised prelimbic and orbitofrontal cortices.

Of course, additional caveats remain: For example, our findings do not shed light onto potential molecular mechanisms of GR-mediated prefrontal cortical dendritic spine remodeling such as Fragile X Mental Retardation Protein and cofilin (Jafari et al., 2012), integrin family receptors (Morsink et al., 2006), p190RhoGAP (Gourley et al., 2013a), *etc.* However, we did find that administering a Rho-kinase inhibitor with RU38486 had protective behavioral effects, with mice maintaining sensitivity to action-outcome relationships relative to mice administered RU38486 alone. Rho-kinase is upstream of cofilin, so it plays an important role in dendritic spine remodeling (*e.g.*, Murakoshi et al., 2011), and given that it was administered during the presumptive consolidation of new memory, it may have “beneficial” effects relating to the consolidation of action-outcome learning. This hypothesis is explored in more detail in Chapter 3.

Further, the sample sizes used in these Addenda for dendritic spine enumeration are small (*n*=3 mice/group), with each dendrite rather than each mouse serving as an independent sample, potentially amplifying relatively minor effects. Even with these provisions, we report these findings with the hope that they contribute to emerging models of stress responsiveness that accommodate multiple cell types in multiple brain regions (*e.g.*, Holmes and Wellman, 2009; Bangasser and Shors, 2010; Riedemann et al., 2010; Duman and Aghajanian, 2012).

Works Cited

Allen JP, Allen CF (1975) The effect of dimethyl sulfoxide on hypothalamic-pituitary-adrenal functions in the rat. Ann NY Acad Sci 243:325-336.

Balleine BW, Dickinson A (1998) The role of incentive learning in instrumental outcome revaluation by sensory-specific satiety. Animal Learning and Behavior 26:46-59.

Balleine BW, O'Doherty JP (2010) Human and rodent homologies in action control: corticostriatal determinants of goal-directed and habitual action. Neuropsychopharmacology 35:48-69.

Bangasser DA, Shors TJ (2010) Critical brain circuits at the intersections between stress and learning. Neurosci Biobehav Rev 34:1223-1233.

Bhatt DH, Zhang S, Gan WB (2009) Dendritic spine dynamics. Annu Rev Physiol 71:261-82.

Bloss EB, Janssen WG, McEwen BS, Morrison JH (2010) Interactive effects of stress and aging on structural plasticity in the prefrontal cortex. J Neurosci 30:6726-6731.

Bourne JN, Harris KM (2011) Coordination of size and number of excitatory and inhibitory synapses results in a balanced structural plasticity along mature hippocampal CA1 dendrites during LTP. Hippocampus 21:354-373.

Cerqueira JJ, Taipa R, Uylings HB, Almeida OF, Sousa N (2007) Specific configuration of dendritic degeneration in pyramidal neurons of the medial prefrontal cortex induced by differing corticosteroid regimens. Cereb Cortex 17:1998-1006.

Corbit LH, Balleine BW (2003) The role of the prelimbic cortex in instrumental conditioning. Behav Brain Res 146:145-157.

Duman RS, Aghajanian GK (2012) Synaptic dysfunction in depression: Potential therapeutic targets. Science 338:68-72.

Feng G, Mellor RH, Bernstein M, Keller-Peck C, Nguyen QT, Wallace M, Nerbonne JM, Lichtman JW, Sanes JR (2000) Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. Neuron 28:41-51.

Gourley SL, Lee AS, Howell JL, Pittenger C, Taylor JR (2010) Dissociable regulation of instrumental action within the mouse prefrontal cortex. Eur J Neurosci 32:1726-1734.

Gourley SL, Olevska A, Warren MS, Taylor, JR, Koleske AJ (2012) Arg kinase regulates prefrontal dendritic spine refinement and cocaine-induced plasticity. J Neurosci 32:2314-2323.

Gourley SL, Swanson AM, Jacobs AM, Howell JL, Mo M, DiLeone RJ, Koleske AJ, Taylor JR (2012) Action control is mediated by prefrontal BDNF and glucocorticoids. Proc Natl Acad Sci U S A 109:20714-20719.

Gourley SL, Swanson AM, Koleske AJ (2013a) Corticosteroid-induced neural remodeling predicts behavioral vulnerability and resilience. J Neurosci 33:3107-3112.

Gourley SL, Olevska A, Zimmermann KS, Ressler KJ, DiLeone RJ, Taylor JR (2013b) The orbitofrontal cortex regulates outcome-based decision-making via the lateral striatum. Eur J Neurosci 38:2382-2388.

Grutzendler J, Kasthuri N, Gan WB (2002) Long-term dendritic spine stability in the adult cortex. Nature 420:812-816.

Hammond LJ (1980) The effect of contingency upon the appetitive conditioning of free-operant behavior. J Exp Analysis Behav 34:297-304.

Holmes A, Wellman CL (2009) Stress-induced prefrontal reorganization and executive dysfunction in rodents. Neurosci Biobehav Rev 33:773-783.

Jafari M, Seese RR, Babayan AH, Gall CM, Lauterborn JC (2012) Glucocorticoid receptors are localized to dendritic spine and influence local actin signaling. Mol Neurobiol 46:304-315.

Killcross S, Coutureau E (2003) Coordination of actions and habits in the medial prefrontal cortex. Cereb Cortex 13:400-408.

Liston C, Gan WB (2011) Glucocorticoids are critical regulators of dendritic spine development and plasticity in vivo. Proc Natl Acad Sci 108:16074-16079.

Liu J, Dietz K, DeLoyht JM, Xiomara P, Kelkar D, Kaur J, Vialou V, Lobo MK, Dietz DM, Nestler EJ, Dupree J, Casaccia P (2012) Impaired adult myelination in the prefrontal cortex of socially isolated mice. Nat Neurosci 15:1621-1623.

Liu RJ, Aghajanian GK (2009) Stress blunts serotonin- and hypocretin-evoked EPSCs in prefrontal cortex: role of corticosterone-mediated apical dendritic atrophy. Proc Natl Acad Sci U S A 105:359-364.

Makinodan M, Rosen KM, Ito S, Corfas G (2012) A critical period for social experience-dependent oligodendrocyte maturation and myelination. Science 337:1357-1360.

Morsink MC, Steenbergen PJ, Vos JB, Karst H, Joëls M, De Kloet ER, Datson NA (2006) Acute activation of hippocampal glucocorticoid receptors results in different waves of gene expression throughout time. J Neuroendocrinol 18:239-252.

Murakoshi H, Wang H, Yasuda R (2011) Local, persistent activation of Rho GTPase during plasticity of single dendritic spines. Nature 472:100-104.

Niwa M, Jaaro-Peled H, Tankou S, Seshadri S, Hikida T, Matsumoto Y, Cascella NG, Kano S, Ozaki N, Nabeshima T, Sawa A (2013) Adolescent stress-induced epigenetic control of dopaminergic neurons via glucocorticoids. Science 339:335-339.

Olgilvie K, See S, Rivier C (1997) Effect of three different modes of alcohol administration on the activity of the rat hypothalamic-pituitary-adrenal axis. Alcohol Clin Exp Res 21:467-476.

Ostlund SB, Balleine BW (2007) Orbitofrontal cortex mediates outcome encoding in Pavlovian but not instrumental conditioning. J Neurosci 27:4819-4825.

Peters A, Kaiserman-Abramof IR (1970) The small pyramidal neuron of the rat cerebral cotex. The perikaryon, dendrites and spines. Am J Anat 127:321-355.

Rakic P, Bourgeois J-P, Goldman-Rakic PS (1994) Synaptic development of the cerebral cortex: Implications for learning, memory, and mental illness. In: The Self-Organizing Brain. 102:227-243.

Riedemann T, Patchev A, Cho K, Almeida OFX (2010) Corticosteroids: way upstream. Molecular Brain 3:2.

Seib LM, Wellman CL (2003) Daily injections alter spine density in rat medial prefrontal cortex. Neurosci Lett 337:29-32.

Spear LP (2000) The adolescent brain and age-related behavioral manifestations. Neurosci Biobehav Rev 24:417-463.

Tasker JG, Herman JP (2011) Mechanisms of rapid glucocorticoid feedback inhibition of the hypothalamic-pituitary-adrenal axis. Stress 14:398-406.

Tran-Tu-Yen DA, Marchand AR, Pape JR, Di Scala G, Coutureau E (2009) Transient role of the rat prelimbic cortex in goal-directed behaviour. Eur J Neurosci 30:464-471.

Van Eden CG, Uylings HB (1985) Postnatal volumetric development of the prefrontal cortex in the rat. J Comp Neurol 241:268-274.

Yin HH, Ostlund SB, Balleine BW (2008) Reward-guided learning beyond dopamine in the nucleus accumbens: the integrative functions of cortico-basal ganglia networks. Eur J Neurosci 28:1437-1448.

Figures

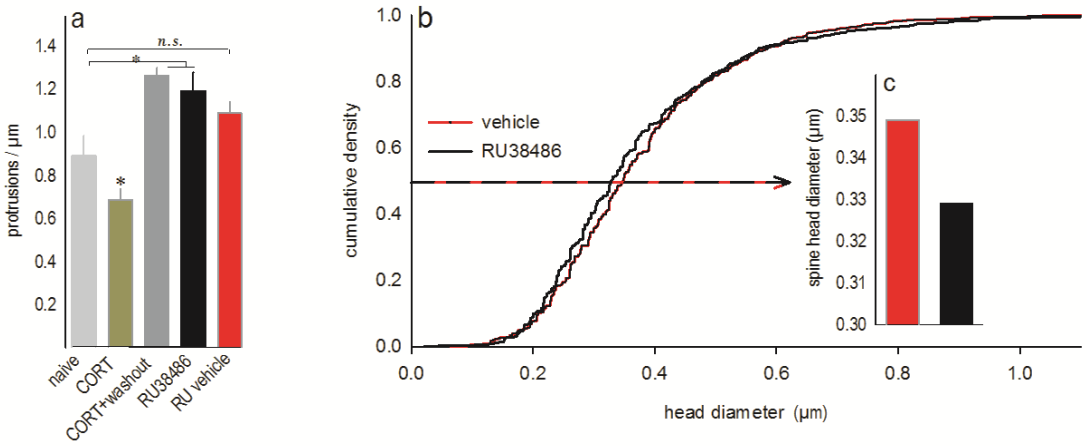

Figure 1. Corticosterone exposure and GR blockade modify prelimbic cortical dendritic spines. (a) Dendritic spines were counted (from left to right) in naïve control mice, mice exposed to exogenous corticosterone in the drinking water for 3 weeks, exogenous corticosterone + a 3-week washout period, acute RU38486 (40 mg/kg, *i.p.*), and the DMSO-based vehicle for RU38486. Chronic corticosterone decreased deep-layer prelimbic cortical dendritic spine density, but a washout period resulted in dendritic spine over-production relative to control mice. Acute RU38486 administered 24 hours prior to euthanasia resulted in the same profile, while acute injection of the RU38486 vehicle resulted in spine densities that differed from neither naïve control nor RU38486-exposed mice. Bars represent means + SEMs, *$p \leq 0.05$, #$p=0.08$ *vs.* naive. $p<0.001$ *vs.* all other groups. (b) We additionally measured spine head diameters in a large population of RU38486-exposed *vs.* vehicle-injected spines ($n=729$ and 1386, respectively). In this case, RU38486 decreased spine head diameter (K-S test, $p<0.001$). (c) At the 50th percentile, control spine heads were nearly 0.35 µm in diameter, while

RU38486-exposed mice had smaller head diameters, less than 0.33 μm in diameter. “CORT” refers to corticosterone.

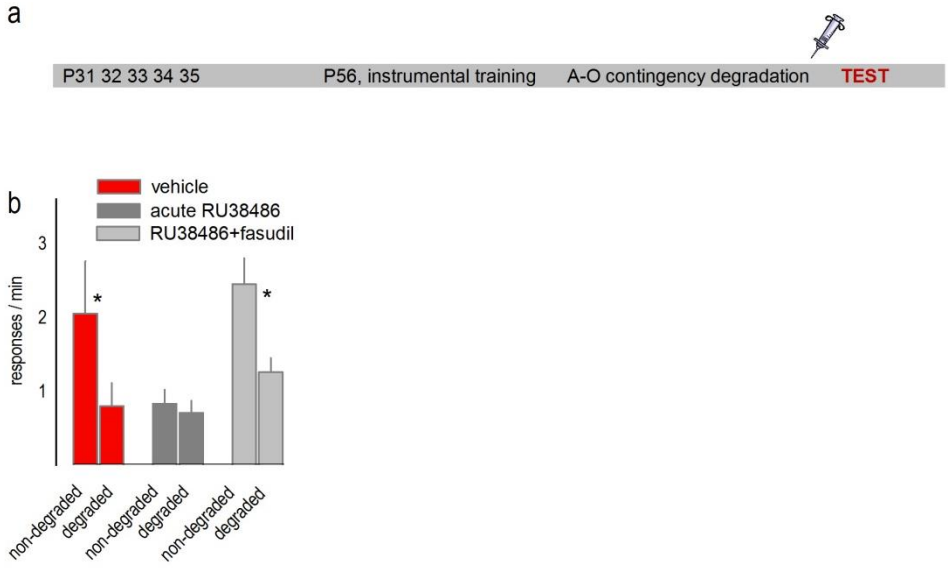

Figure 2. GR blockade regulates outcome-based decision-making. (a) Mice were injected with RU38486 or vehicle immediately following action-outcome contingency degradation training. (b) In a probe test, RU38486 blocked outcome-based decision-making, in that RU38486-treated mice failed to differentiate between the ‘non-degraded’ and ‘degraded’ response. Concomitant injection of the Rho-kinase inhibitor fasudil blocked the behavioral effects of RU38486, suggesting that GR-mediated dendritic spine remodeling is causally related to decision-making strategies. This hypothesis is explored in greater detail in Chapter 3. Bars =means + SEMs, *p=0.05. “A-O” refers to action-outcome.

Chapter 2: Corticosteroid-induced neural remodeling predicts behavioral vulnerability and resilience

Adapted from

Corticosteroid-induced neural remodeling predicts behavioral vulnerability and resilience

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Abstract

Neurons in distinct brain regions remodel in response to postnatal stressor exposure, and structural plasticity may underlie stress-related modifications in behavioral outcomes. Given the persistence of stress-related diseases such as depression, a critical next step in identifying the contributions of neural structure to psychopathology will be to identify brain circuits and cell types that fail to recover from stressor exposure. To this end, we enumerated dendritic spines during and after chronic stress hormone exposure in hippocampal CA1, deep-layer prefrontal cortex, and the basal amygdala. Corticosteroid exposure modified dendritic spine density in these regions, but with the exception of the orbitofrontal cortex, densities normalized with a recovery period. Using mice with reduced gene dosage of *p190rhogap,* a cytoskeletal regulatory protein localized to dendritic spines, we isolated structural correlates of both behavioral vulnerability (spine elimination) and resilience (spine proliferation) within the orbitofrontal cortex. Our findings provide novel empirical support for the perspective that stress-related structural reorganization of certain neuron populations is persistent, despite a "recovery" period from stressor exposure, and that these modifications lay a structural foundation for stressor vulnerability — or resiliency — across the lifespan.

Introduction

Stress hormones, particularly corticosterone (CORT), regulate postnatal dendritic and dendritic spine morphology in distinct brain regions including the prefrontal cortex, hippocampus, and amygdala. Structural remodeling in response to chronic exposure is thought to contribute to aspects of stress-related psychiatric disease. For example, stress-related prefrontal cortical dendritic reorganization predicts impairments in attentional function in rodents (Liston et al., 2006), and reductions in hippocampal volume correlate with the *lifetime duration* of depression in humans (Sheline et al., 1999). Landmark investigations that characterized the consequences of chronic stressor exposure on pyramidal neurons within prefrontal-hippocampal-amygdala circuits largely focused on immediate, rather than persistent, consequences (Woolley et al., 1990; Sousa et al., 2000; Wellman 2001; Vyas et al., 2002). A comprehensive characterization of structural modifications that persist beyond the period of exposure represents a critical next step in understanding mechanisms of lifetime vulnerability and resilience to stress-related psychiatric disease.

Here we focus on cortico-hippocampal-amygdalar structural reorganization in response to prolonged exposure to the major stress hormone CORT. We aimed to identify structural modifications that failed to recover with a washout period, with the hypothesis that these modifications would provide a structural foundation for the development *and persistence* of anhedonic-like behaviors, a hallmark symptom of depression that is thought to involve these circuits (Der-Avakian and Markou, 2012).

Structural remodeling in the central nervous system is orchestrated by Rho family GTPases including RhoA (Rho), Rac1, and Cdc42, which coordinate the actin cytoskeletal rearrangements that are required for spinogenesis or spine elimination. RhoA regulates actin polymerization and actomyosin contractility—for example,

expression of constitutively active RhoA leads to dendritic spine loss (Tashiro et al., 2000), and RhoA activation during the late postnatal period occludes synapse and spine formation (Sfakianos et al., 2007). RhoA is inhibited endogenously by p190RhoGAP, which is activated by integrin receptor binding to extracellular matrix proteins and localized to dendritic spines (Arthur et al., 2000; Lamprecht et al., 2002; Moresco et al., 2007). Thus, we also used mice with reduced gene-dosage of *p190rhogap* as a model of *structural* vulnerability to further isolate correlates of *behavioral* vulnerability to stress hormone exposure.

Results

A history of corticosteroid exposure modifies dendritic spines and behavior

We first investigated dendritic spine density on basal CA1 arbors. Regions of interest for this and subsequent investigations are indicated (fig.1a). In CA1, CORT reduced spine density [no interaction $F<1$, main effect of CORT $F(2,17)=4.8, p<0.05$] (fig.1b), and spine densities normalized with a 1-week washout period.

We next expanded our survey to other cortico-limbic structures: CORT reduced infralimbic cortex (ILc) spine density as expected (Liu and Aghajanian, 2008), but densities recovered with a washout period [interaction $F(2,19)=4.2, p<0.05$] (fig.1c). In the basal amygdala, CORT elevated spine density, but again, densities normalized with a 1-week washout period [interaction $F(2,17)=4.3, p<0.05$] (fig.1d). In the orbital prefrontal cortex, however, densities declined and *failed to recover* with a washout period [interaction $F(2,18)=4.3, p<0.05$] (fig.1e).

Corticosteroid exposure thus has discrete long-term structural consequences. To evaluate *behavioral* consequences, we measured animals' sucrose consumption in a model of anhedonia one week after CORT washout. Prior CORT exposure reduced sucrose consumption, reflecting a persistent anhedonic-like phenotype [$t(10)=3, p<0.05$] (fig.1f).

To further isolate the relationship between persistent dendritic spine modifications and long-term behavioral consequences of CORT exposure, we generated YFP-expressing mice with reduced gene dosage of the dendrite stability factor *p190rhogap,* exposed them to a subthreshold dose of CORT, and evaluated behavioral and structural outcomes. Throughout, dendritic spine densities and behavioral outcomes were not affected by *p190rhogap* deficiency, but genotype determined the response to corticosterone: Control mice were behaviorally unaffected by the subthreshold dose, but p190RhoGAP-deficient mice were vulnerable, developing an anhedonic-like insensitivity

to the sucrose solution (fig.2a); in parallel, *p190rhogap*+/- spines were eliminated [genotype x CORT interactions $p<0.04$] (fig.2b,c).

Notably, oPFC spines proliferated in *p190rhogap*+/+ mice exposed to subthreshold CORT (fig.2b,c). Also, subthreshold CORT-exposed *p190rhogap*+/+ mice had a larger population of small-headed spines, potentially reflecting new immature protrusions [K-S $ps<0.001$] (fig.2d,e) (Bourne and Harris, 2011). By contrast, neither densities nor head diameters were affected by subthreshold CORT in CA1 [no interaction $F<1$] (fig.2f) [head diameter K-S $ps>0.002$, not shown].

Discussion

The ability of neurons to integrate into networks and regulate behavior is determined by the size, shape, and density of dendrites and dendritic spines, the postsynaptic components of most excitatory synapses in the brain. Spines are remarkably plastic—for example, hippocampal CA3 spines remodel in response to postnatal stressor or corticosteroid exposure (Woolley et al., 1990; Tata and Anderson, 2010). These modifications may play a role in stress-related mood disorders involving cortico-amygdalo-hippocampal circuits (*e.g.*, depression), but identification of structural modifications that—like stress-related mood disorders—persist beyond the period of stressor exposure remains incomplete. We used transgenic mice expressing *thy1*-derived YFP to isolate and reconstruct hippocampal CA1, basal amygdalar, and deep-layer prefrontal cortical dendritic spines. Among the cell populations sampled, all remodeled in response to prolonged corticosteroid exposure, but only oPFC dendritic spines failed to recover with a corticosteroid washout period.

Hippocampal networks and stress-related structural reorganization

Hippocampal CA3 neurons are exquisitely sensitive to stressor exposure (Tata and Anderson, 2009), and certain aspects of stress-related impairment in hippocampal-dependent learning and memory may reflect CA3 remodeling (McEwen et al., 2012; Conrad et al., 1999). The CA1 neuronal response to stressors is poorly characterized by comparison. This is likely because CA1 neurons are regarded as more resilient than their CA3 counterparts, based at least in part on investigations using large bolus CORT doses (33-40 mg/kg) that occlude normal circadian CORT cycling (Woolley et al., 1990; Sousa et al., 2000; Morales-Medina et al., 2009). By contrast, the oral CORT protocol utilized here mimics CORT secretion during restraint stress and leaves circadian cycling intact (Gourley et al., 2008). Oral CORT resulted in dendritic spine elimination 60-90 µm

from the cell body. This region corresponds to the CA1-subiculum intersection targeted by glutamatergic projections from the basal amygdala (Pitkanen et al., 2000), raising the possibility that prolonged hyperexcitability of amygdala projections after stressor exposure (Correll et al., 2005) may be a presynaptic mechanism that results in hippocampal reorganization.

Prefrontal cortical dendritic spines reorganize in response to corticosteroid exposure

CORT eliminated dendritic spines in layer V ILc, consistent with previous reports from layers II/III (Radley et al., 2008) and V (Liu and Aghajanian, 2008). Remarkably little is known, however, regarding stress-related structural modifications in the oPFC, with the exception of one report of dendritic arbor *elaboration* after chronic restraint stress (Liston et al., 2006). By contrast, we found dendritic spine *elimination*. How might we reconcile this apparent contradiction? One possibility is that oPFC dendritic growth serves as a compensatory response to spine elimination, since a long, sparsely-populated dendrite could house as many spines as a short, densely populated dendrite (Bourne and Harris, 2011). In this case, dendritic elaboration would preserve total spine number. The stressor protocol utilized by Liston *et al.* impaired rats' attentional function, but spared reward-related reversal learning, canonically associated with oPFC structural integrity. By contrast, other stressor protocols impair reversal learning (Cerqueira et al., 2007; Lapiz-Bluhm et al., 2009), thus dendritic elaboration observed by Liston *et al.* may reflect a *protective* response to stressor exposure that preserved behavioral function.

This interpretation implies a high degree of spine instability in response to stress hormone exposure, consistent with reports of diminished oPFC *Brain-derived neurotrophic factor (Bdnf)* after CORT (Gourley et al., 2009a) and evidence that postnatal cortical BDNF deficiency destabilizes dendritic spines (Woronowicz et al.,

2010). BDNF is among a constellation of proteins that stabilize cortical neural structure during postnatal development, and p190RhoGAP is another such regulator: Through interactions with the Abl-related gene, it localizes to cellular membranes, inhibits Rho, and attenuates actomyosin contractility (Bradley et al., 2006). In the absence of these critical intracellular interactions, synapses are eliminated and spine heads fail to mature during late postnatal development, corresponding to adolescence in humans (Sfakianos et al., 2007). Adolescence is an established period of vulnerability to the development of treatment-resistant depression, and stressor interference with structural remodeling during this period is thought to play an etiologic role (Thapar et al., 2012), hence we used *p190rhogap*+/- mice as a model of corticosteroid vulnerability. Naïve *p190rhogap*+/- mice did not display anhedonic-like behavior, but they developed anhedonic-like sucrose neglect after prolonged exposure to a subthreshold concentration of CORT that did not impact behavioral outcomes in *p190rhogap*+/+ littermates. In concert, oPFC spines were eliminated, suggesting that elimination confers vulnerability to depression symptomatology, particularly in orbital networks implicated in reward sensitivity (see for further discussion Lapiz-Bluhm et al., 2009; Gourley et al., 2009b; Der-Avakian and Markou, 2012).

p190rhogap+/+ mice exposed to subthreshold CORT had higher oPFC, though not hippocampal, spine densities than all other groups. This pattern suggests that p190RhoGAP-mediated RhoA inhibition, in response to corticosteroid exposure, subserves stressor resilience. These results contribute to an emerging perspective largely from the drug addiction field that dendritic spine reorganization in response to pathological stimuli may in some circumstances have adaptive consequences. For example, pharmacological blockade of cocaine-induced dendritic spine reorganization in the oPFC and nucleus accumbens *increases*, rather than occludes, sensitivity to subsequent cocaine exposures (Toda et al., 2006; Gourley et al., 2012).

p190RhoGAP brakes actomyosin contractility in multiple biological contexts. In neural systems, it also coordinates behaviorally adaptive outcomes—learning about novel environments or aversive stimuli (Sfakianos et al., 2007; Lamprecht et al., 2002), mitigating vulnerability to stress hormone exposure or drugs of abuse (fig.3; Gourley et al., 2012). There are no current pharmacological agents that amplify p190RhoGAP activity, but our experiments add to mounting evidence supporting a shift towards therapeutic approaches to stress-related mood disorders that impact cytoskeletal outcomes. These include agents that directly target the actin cytoskeleton, or those that act indirectly—for example, ketamine, an NMDA receptor antagonist, has rapid antidepressant-like properties that are in part attributable to dendritic spine proliferation in deep-layer prefrontal cortex (Li et al., 2010). Our current findings suggest these structural modifications promote depression recovery through stressor resilience.

Methods

Subjects: Male mice were 5-7 weeks old at the start of the experiments. Wild type (wt) C57BL/6 mice were purchased from Charles River Laboratories (Kingston, NY), and mutant mice expressed *thy1*-derived YFP (Feng et al., 2000) to enable dendritic spine imaging. Our final experiment utilized YFP-expressing p190RhoGAP-deficient mice (*p190rhogap*+/-), which have at least a 32-40% reduction in p190RhoGAP protein expression (Brouns et al., 2000), and YFP+*p190rhogap*+/-littermates, All were bred on a C57BL/6 background, maintained on a 12-hour light cycle (0700 on), and provided food and water *ad libitum* unless otherwise indicated. Procedures were Emory IACUC-approved.

CORT exposure: CORT (4-pregnen-11β-21-DIOL-3-20-DIONE-21-hemisuccinate; Steraloids, Inc., Newport, RI) was dissolved in water and administered for 20 days (25 µg/ml free-base, translating to an average dose of 4.97 mg/kg/day). This protocol recapitulates blood CORT levels in mice exposed to chronic restraint stress (Gourley et al., 2008). Mice were euthanized at 20 days or 20 days + a 1-week washout period.

In a final experiment using both wt and *p190rhograp*+/- mice, 10 µg/ml was used as a subthreshold CORT concentration. This dose is described in text as a "subthreshold CORT."

Dendritic spine imaging and enumeration: As described (Gourley et al., 2012), fresh YFP-expressing brains were submerged in 4% paraformaldehyde for 48 hours, then transferred to 30% w/v sucrose, followed by sectioning into 40 µm-thick sections on a microtome held at -15°C. Unobstructed dendritic segments running parallel to the surface of the section were imaged on a spinning disk confocal (VisiTech International, Sunderland, UK) on a Leica microscope. Z-stacks were taken with a 100x 1.4NA

objective using a 0.1 µm step size, sampling above and below the dendrite. After imaging, we confirmed at 10X that the image was collected from the intended subregions.

Collapsed z-stacks were analyzed using NIH ImageJ: Each protrusion ≤4 µm was considered a spine (Peters and Kaiserman-Abramof, 1970). Individual planes were evaluated to detect protrusions perpendicular to the z-stack. Bifurcated spines were considered singular units. To generate density values, spine number for each segment was normalized to the length of the segment. Four-6 independent segments from secondary and tertiary dendritic branches within 50-150 µm of the soma were collected. Each animal contributed a single density value (its average) to statistical analyses. Due to the relatively stellate appearance of amygdalar and oPFC neurons, apical *vs*. basal branches were not distinguished (Liston et al., 2006; Kolb et al., 2008). A single blinded rater scored spines.

Semi-automated dendritic spine reconstruction: To evaluate both spine density and head diameter in YFP-expressing *p190rhogap*+/- samples, 3D reconstructions were accomplished with the FilamentTracer module of Imaris (Bitplane AG, Zurich, Switzerland) as described (Swanger et al., 2011). Here, a dendritic segment ~25 µm in length sampled from the oPFC (as above) or basal CA1 (60-90 µm from the soma) was drawn using the autodepth function. FilamentTracer processing algorithms centered the segment and determined dendrite diameter. The autodepth function drew dendritic spines along the dendrite. Each spine was then reconstructed in 3D using the FilamentTracer algorithm. A single blinded individual processed all images.

Behavioral modeling: Here we utilized a model of anhedonia: 1% (w/v) sucrose replaced regular drinking water for 2 days starting 2 days after CORT exposure. Then,

animals were habituated to water restriction by removing the water bottle for 19 hours. Next, mice were again water-restricted overnight, and each mouse was allowed 1-hour access to the sucrose solution in its home cage while cagemates were housed in a clean cage in a quiet room. Liquid consumption was recorded. This approach allows us to evaluate sucrose consumption in individual mice while still maintaining standard laboratory housing in groups of 2-5 (Gourley et al., 2008,2009a); the average water restriction period for each cage was 16 hours. The test was repeated the following day with water to confirm that fluid consumption did not differ between groups.

In the first experiment (fig.1), mice were wild type (control *vs.* CORT, *n*=6/group), in the second (fig.2), YFP-expressing *p190rhogap*+/- and YFP-expressing littermate controls exposed to CORT or CORT-naïve (4 groups, *n*s=7-14/group depending on litter composition). These mice were euthanized after test for dendritic spine imaging.

Statistics: Sucrose consumption and morphometric measures were analyzed by 1- or 2-factor ANOVA as appropriate, with repeated measures when morphometric values were analyzed as a function of distance from the soma. Post-hoc comparisons were made using Tukey's comparisons, and when significant, results are indicated graphically. When two groups were compared, 2-tailed *t*-tests were used. To highlight how genotype determined dendritic spine sensitivity to corticosteroid exposure, percent change from baseline (the mean value of CORT-naïve mice of the same genotype) was calculated and compared to 0 (no change) by location *t*-test. $p<0.05$ was considered significant, and values >2 standard deviations outside of the mean were excluded.

Spine head diameters were analyzed by Kolmogorov-Smirnov (K-S) comparisons. Because of the high degree of statistical power generated by K-S tests, $p<0.001$ was considered significant.

References

Arthur WT, Petch LA, Burridge K (2000) Integrin engagement suppresses RhoA activity via a c-Src-dependent mechanism. Curr Biol 10:719-722.

Bourne JN, Harris KM (2011) Coordination of size and number of excitatory and inhibitory synapses results in a balanced structural plasticity along mature hippocampal CA1 dendrites during LTP. Hippocampus 21:354-373.

Bradley WD, Hernandez SE, Settleman J, Koleske AK (2006) Integrin signaling through Arg activates p190RhoGAP by promoting its binding to p120RasGAP and recruitment to the membrane. Mol Biol Cell 17:4827-4836.

Brouns MR, Matheson SF, Hu KQ, Delalle I, Caviness VS, Silver J, Bronson RT, Settleman J (2000) The adhesion signaling molecule p190 RhoGAP is required for morphogenetic processes in neural development. Development 127:4891-4903.

Conrad CD, LeDoux JE, Magarinos AM, McEwen BS (1999) Repeated restraint stress facilitates fear conditioning independently of causing hippocampal CA3 dendritic atrophy. Behav Neurosci 113:902-913.

Correll CM, Rosenkranz JA, Grace AA (2005) Chronic cold stress alters prefrontal cortical modulation of amygdala neuronal activity in rats. Biol Psychiatry 58:382-391.

Cerqueira JJ, Mailliet F, Almeida OFX, Jay TM, Sousa N (2007) The prefrontal cortex as a key target of the maladaptive response to stress. J Neurosci 27:2781-2787.

Der-Avakian A, Markou A (2012) The neurobiology of anhedonia and other reward-related deficits. Trends Neurosci 35:68-77.

Feng G, Mellor RH, Bernstein M, Keller-Peck C, Nguyen QT, Wallace M, Nerbonne JM, Lichtman JW, Sanes JR (2000) Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. Neuron 28:41-51.

Fuchs RA, Eaddy JL, Su ZI, Bell GH (2007) Interactions of the basolateral amygdala with the dorsal hippocampus and dorsomedial prefrontal cortex regulate drug context-induced reinstatement of cocaine-seeking in rats. Eur J Neurosci 26:487-498.

Gourley SL, Kiraly DD, Howell JL, Olausson P, Taylor JR (2008) Acute hippocampal BDNF restores motivational and forced swim performance after corticosterone. Biol Psychiatry 64:884-890.

Gourley SL, Kedves AT, Olausson P, Taylor JR (2009a) A history of corticosterone regulates fear extinction and cortical NR2B, GluR2/3, and BDNF. Neuropsychopharmacology 34:707-716.

Gourley SL, Koleske AJ, Taylor JR (2009b) Loss of dendrite stabilization by the Abl-related gene (Arg) kinase regulates behavioral flexibility and sensitivity to cocaine. Proc Natl Acad Sci 106:16859-16864.

Gourley SL, Olevska A, Warren MS, Taylor JR, Koleske AJ (2012) Arg kinase regulates prefrontal dendritic spine refinement and cocaine-induced plasticity. J Neurosci 32:2314-2323.

Kolb B, Cioe J, Comeau W (2008) Contrasting effects of motor and visual spatial learning tasks on dendritic arborization and spine density in rats. Neurobiol Learn Mem 90:295-300.

Lamprecht R, Farb CR, LeDoux JE (2002) Fear memory formation involves p190 RhoGAP and ROCK proteins through a GRB2-mediated complex. Neuron 36:727-738.

Lapiz-Bluhm MD, Soto-Piña AE, Hensler JG, Morilak DA (2009) Chronic intermittent cold stress and serotonin depletion induce deficits of reversal learning in an attentional set-shifting test in rats. Psychopharmacology 202:329-341.

Liston C, Miller MM, Goldwater DS, Radley JJ, Rocher AB, Hof PR, Morrison JH, McEwen BS (2006) Stress-induced alterations in prefrontal cortical dendritic morphology predict selective impairments in perceptual attentional set-shifting. J Neurosci 26:2870-7874.

Li N, Lee B, Liu RJ, Banasr M, Dwyer JM, Iwata M, Li XY, Aghajanian G, Duman RS (2010) mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. Science 329:959-964.

Liu RJ, Aghajanian GK (2008) Stress blunts serotonin- and hypocretin-evoked EPSCs in prefrontal cortex: Role of corticosterone-mediated apical dendritic atrophy. Proc Natl Acad Sci 105:359-364.

McEwen BS, Eiland L, Hunter RG, Miller MM (2012) Stress and anxiety: Structural plasticity and epigenetic regulation as a consequence of stress. Neuropharmacology 62:3-12.

Morales-Medina JC, Sanchez F, Flores G, Dumont Y, Quirion R (2009) Morphological reorganization after repeated corticosterone administration in the hippocampus, nucleus accumbens and amygdala in the rat. J Chem Neuroanat 38:266-272.

Peters A, Kaiserman-Abramof IR (1970) The small pyramidal neuron of the rat cerebral cortex. The perikaryon, dendrites and spines. Am J Anat 127:321-355.

Pitkänen A, Pikkarainen M, Nurminen N, Ylinen A (2000) Reciprocal connections between the amygdala and the hippocampal formation, perirhinal cortex, and postrhinal cortex in rat. A review. Ann N Y Acad Sci 911:369-391.

Radley JJ, Rocher AB, Rodriguez A, Ehlenberger DB, Dammann M, McEwen BS, Morrison JH, Wearne SL, Hof PR (2008) Repeated stress alters dendritic spine morphology in the rat medial prefrontal cortex. J Comp Neurol 507:1141-1150.

Sfakianos MK, Eisman A, Gourley SL, Bradley WD, Scheetz AJ, Settleman J, Taylor JR, Greer CA, Williamson A, Koleske AJ (2007) Inhibition of Rho via Arg and p190RhoGAP in the postnatal mouse hippocampus regulates dendritic spine maturation, synapse and dendrite stability, and behavior. J Neurosci 27:10982-10992.

Sheline YI, Sanghavi M, Mintun MA, Gado MH (1999) Depression duration but not age predicts hippocampal volume loss in medically healthy women with recurrent major depression. J Neurosci 19:5034-5043.

Sousa N, Lukoyanov NV, Madeira MD, Almeida OFX, Paul-Barbosa MM (2000) Reorganization of the morphology of hippocampal neurites and synapses after stress-induced damage correlates with behavioral improvement. Neuroscience 97:253-266.

Swanger SA, Yao X, Gross C, Bassell GJ (2011) Automated 4D analysis of dendritic spine morphology: Applications to stimulus-induced spine remodeling and pharmacological rescue in a disease model. Mol Brain 4:38.

Tashiro A, Minden A, Yuste R (2000) Regulation of dendritic spine morphology by the Rho family of small GTPases: Antagonist roles of Rac and Rho. Cereb Cortex 10:927-938.

Thapar A, Collishaw S, Pine DS, Thapar AK (2012) Depression in adolescence. Lancet 379:1056-1067.

Toda S, Shen HW, Peters J, Cagle S, Kalivas PW (2006) Cocaine increases actin cycling: effects in the reinstatement mode of drug seeking. J Neurosci 26:1579-1587.

Vyas A, Mitra R, Shankaranarayana Rao BS, Chattarji S (2002) Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. J Neurosci. 2002 Aug 1;22(15):6810-8.

Wellman CL (2001) Dendritic reorganization in pyramidal neurons in medial prefrontal cortex after chronic corticosterone administration. J Neurobiol 49:245-253.

Woolley CS, Gould E, McEwen BS (1990) Exposure to excess glucocorticoids alters dendritic morphology of adult hippocampal pyramidal neurons. Brain Res 225-231.

Woronowicz A, Cawley NX, Chang SY, Koshimizu H, Phillips AW, Xiong ZG, Loh YP (2010) Carboxypeptidase E knockout mice exhibit abnormal dendritic arborization and spine morphology in central nervous system neurons. J Neurosci Res 88:64-72.

Figures

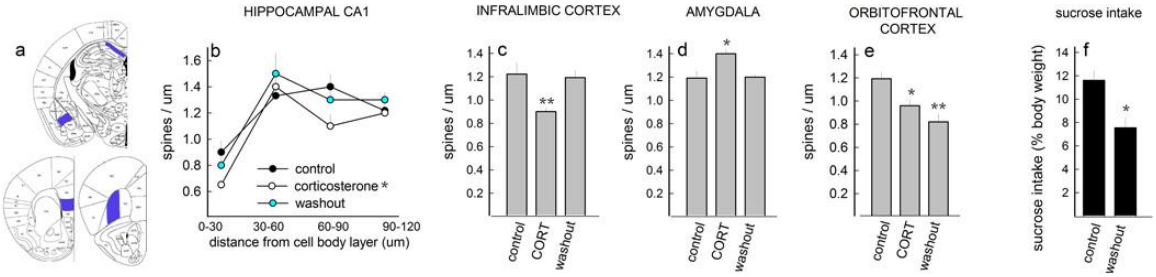

Figure 1. Chronic corticosteroid exposure has regionally-selective effects on dendritic spine density. (a) Sampling sites are highlighted. (b) Dendritic spines on basal CA1 dendritic arbors were analyzed as a function of distance from the cell body—a main effect of group indicated that corticosteroid exposure decreased spine density, but density was restored with a washout period. (c) Prolonged corticosteroid exposure also decreased density on pyramidal neurons in deep-layer ILc, but density recovered with a washout period. (d) Density on pyramidal neurons within the basal amygdala *increased* after CORT, but again, recovered with a washout period. (e) oPFC dendritic spines were eliminated, however here, densities failed to recover. (f) Prior CORT exposure also reduced sucrose consumption in a model of anhedonia, despite a washout period. Symbols/bars=means (+SEM), *p<0.05,**p<0.001 *vs.* control.

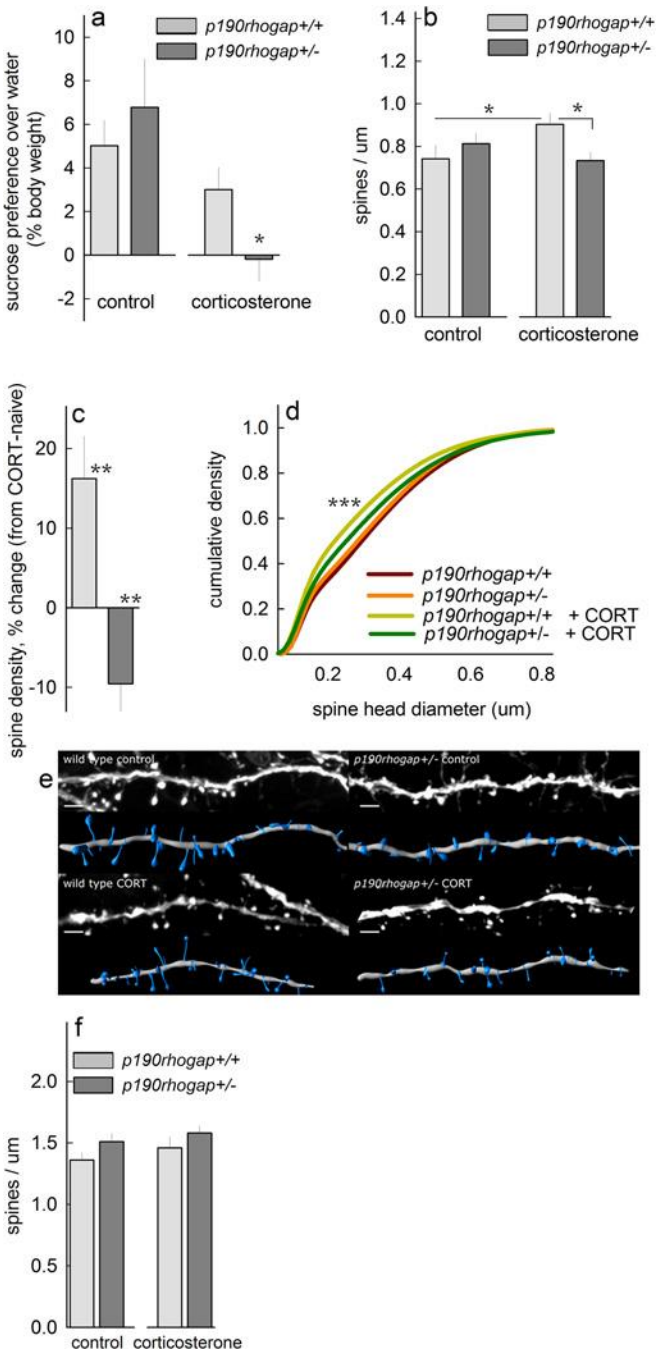

Figure 2. p190RhoGAP determines the cellular response to CORT exposure. (a) In a sucrose preference model of anhedonia, *p190rhogap*+/+ mice were resilient to subthreshold CORT, showing intact sucrose preference over water. By contrast, *p190rhogap*+/- mice developed anhedonic-like sucrose neglect. (b) CORT-exposed *p190rhogap*+/- mice had fewer oPFC spines relative to CORT-naïve *p190rhogap*+/-

counterparts; by contrast, CORT-exposed *p190rhogap*+/+ mice had increased spines relative to CORT-naïve counterparts. (c) Expressed as % change, subthreshold CORT increased spine density by >15% in *p190rhogap*+/+ mice but eliminated approximately 10% of spines in p190RhoGAP-deficient mice. (d) Resilient *p190rhogap*+/+ mice also had an over-representation of spines with small heads. (e) Representative oPFC spines with reconstructions. (f) Subthreshold CORT did not impact CA1 dendritic spine density; head sizes were also unchanged (not shown). Bars=group means (+SEM). **p*<0.05 as indicated; ***p*≤0.005 *vs.* no change (0); ****p*<0.001 *vs.* drug-free *p190rhogap*+/+. Scale bar=3 µm.

Chapter 3: Inhibiting Rho-kinase promotes goal-directed decision-making and blocks habitual responding for cocaine

Adapted from

Inhibiting Rho-kinase promotes goal-directed decision-making and blocks habitual responding for cocaine.

Swanson AM, DePoy LM, Gourley SL

In review

Abstract

The prelimbic prefrontal cortex is necessary for associating actions with their consequences, enabling goal-directed decision-making. We find that the strength of action-outcome conditioning correlates with prelimbic cortical dendritic spine densities, suggesting that new action-outcome learning involves dendritic spine plasticity. To test this, we inhibited the cytoskeletal regulatory factor Rho-kinase, which can otherwise brake structural plasticity. The inhibitor fasudil enhanced action-outcome memory, resulting in goal-directed behavior in mice that would otherwise express stimulus-response habits. Fasudil transiently reduced prelimbic cortical dendritic spine density during a period of memory consolidation, but only when paired with new learning. Fasudil also blocked habitual responding for cocaine, an effect that persisted over time, across multiple contexts, and depended upon actin polymerization. We suggest that Rho-kinase inhibition promotes goal-oriented action selection by augmenting the plasticity of prelimbic cortical dendritic spines during the consolidation of new action-outcome memories, and that it has therapeutic potential for cocaine use disorders.

Introduction

The ability to select actions based upon a desired outcome is critical for survival. Such actions are considered 'goal-directed,' meaning their performance is sensitive to changes in the relationship between the action and its outcome. With repetition, goal-directed behaviors can shift from being outcome-sensitive to being 'habitual,' which is defined by *insensitivity* to changes in the action-outcome relationship. Moreover, a range of pathological stimuli, including cocaine, also bias response strategies to favor habit-based responding (Miles et al., 2003; Schoenbaum and Setlow, 2005; Zapata et al., 2010; Gourley and Taylor, 2016; Hinton et al., 2014; LeBlanc et al., 2013; Corbit et al., 2014; Schmitzer-Torbert et al., 2015; Leong et al., 2016). Although the anatomical connections that organize goal-directed decision-making on the one hand, and habit-based behaviors on the other, are becoming clearer (Hart et al., 2014), underlying cellular and molecular mechanisms are less well-understood. Further, *restoring* goal-directed action selection after habits have formed has proven particularly challenging. This is important because although the development of habits can be a normal, adaptive process, habit-based stimulus-elicited reward seeking that occurs at the expense of engaging in goal-directed response strategies is thought to play an etiological role in the development and maintenance of drug addiction (Everitt and Robbins, 2016).

Studies using lesions indicate that the prelimbic prefrontal cortex is necessary for goal-directed action selection (Balleine and Dickinson, 1998; Corbit and Balleine, 2003; Killcross and Coutureau, 2003), in particular for acquiring and consolidating information regarding the predictive relationship between a behavior and a given outcome (Ostlund and Balleine, 2005). First, we expand on these findings by showing that chemogenetic inactivation of excitatory neurons in the prelimbic cortex similarly impairs an animal's ability to select actions based on their consequences.

Layer V pyramidal neurons receive projections from subcortical areas, such as the thalamus, that are likely involved in developing goal-directed response strategies (Conde et al., 1995; Hart et al., 2014). We thus hypothesized that the structural plasticity of these prelimbic cortical neurons may play an important role in generating goal-directed actions. To test this hypothesis, we inhibited a major substrate of the RhoA GTPase, Rho-kinase (ROCK), which stabilizes filamentous (F)-actin (Olson, 2008). We used the brain-penetrant ROCK inhibitor fasudil, which has been shown to enhance hippocampal- and prefrontal cortical-dependent learning and memory (Huentelman et al., 2009; Couch et al., 2010; Swanson et al., 2013; Zimmermann et al., 2017). Fasudil enriched action-outcome conditioning, resulting in goal-directed response inhibition in mice that would otherwise be expected to express food- or cocaine-seeking habits. We discovered that individual differences in decision-making strategies in mice correlated with dendritic spine densities on layer V prelimbic cortical neurons. Specifically, lower densities were associated with action-outcome-based, goal-directed behavior, with higher densities associated with stimulus-response habits. This correlation raised the possibility that stimulating activity-dependent dendritic spine pruning within the prelimbic cortex might *restore* goal-directed action selection after habits develop. Indeed, ROCK inhibition resulted in a transient pruning of prelimbic cortical dendritic spines, which appears to be necessary to enhance action-outcome conditioning. Taken together, our findings suggest that ROCK inhibition may be an effective tool for promoting goal-directed decision-making in a number of therapeutic contexts, including cocaine use and abuse.

Results

Inducible inactivation of the prelimbic prefrontal cortex impairs the ability of mice to select actions based on their consequences

Throughout several of these experiments, we utilized an action-outcome contingency degradation procedure (fig.1a). In this task, modestly food-restricted mice are trained to generate 2 food-reinforced nose-poke responses, then the likelihood that one response will be reinforced is reduced; instead, food pellets associated with that response are provided non-contingently. In a separate session, the other response remains reinforced. In a subsequent probe test conducted in extinction, both nose-poke apertures are available, and preferential engagement of the response that is likely to be reinforced provides evidence of knowledge of the action-outcome relationship (Dickinson, 1980).

We first delivered viral vectors expressing either CaMKII-driven GFP or G_i-coupled Designer Receptors Exclusively Activated by Designer Drugs (DREADDs (Urban and Roth, 2015)) to the prelimbic cortex (fig.1b). Following recovery, all mice acquired the nose-poke responses, with no differences between groups [no interaction $F<1$, main effect of session $F(6,102)=14.2, p<0.05$] (fig.1c). The DREADD ligand clozapine N-oxide was then paired with action-outcome contingency degradation in all mice. Control GFP-expressing mice subsequently preferentially generated the response most likely to be reinforced in a goal-directed fashion as expected, while G_i-DREADDs-expressing mice responded non-selectively, deferring to a habit-based strategy [interaction $F(1,17)=4.4, p=0.05$] (fig.1d). Thus, chemogenetic silencing of the prelimbic prefrontal cortex blocked action-outcome conditioning.

Prelimbic cortical dendritic spine density predicts reward-related decision-making strategies in mice

Our findings suggest that neuroplasticity in the prelimbic cortex is important for learning about, and updating changes in, action-outcome relationships. Multiple types of learning and memory are thought to require activity-dependent dendritic spine proliferation on the one hand, or dendritic spine elimination, which can also be activity-dependent, on the other (Segal, 2005). To dissect whether dendritic spine dynamics influence action-outcome conditioning, we next manipulated the cytoskeletal regulatory factor ROCK, a major substrate of the RhoA GTPase, since ROCK inhibition can enhance activity-dependent structural remodeling (Schubert et al., 2006; Murakoshi et al., 2011).

In this experiment, mice were given extensive response training using escalating random interval (RI) schedules of reinforcement to induce habit-based responding, which is by definition insensitive to the predictive relationship between a response and an outcome (Balleine and O'Doherty, 2010) (fig.2a). Groups (to be vehicle *vs.* to be fasudil) were designated by matching response rates during training [no interaction $F(15,450)=1.2, p>0.05$, main effect of session $F(15,450)=109.0, p<0.05$, no effect of group $F<1$] (fig.2b).

Immediately following instrumental contingency degradation, we injected mice with either vehicle or the brain-penetrant ROCK inhibitor fasudil. During the subsequent probe test, the vehicle group showed no response preferences, *i.e.*, habit-based responding. In contrast, the fasudil group preferentially generated the response that was likely to be reinforced, a goal-directed strategy [interaction $F(1,30)=4.6, p<0.05$] (fig.2c). Comparing response preference ratios revealed that the fasudil group responded well above chance levels in its preference for the behavior that was most closely associated with reinforcement, while vehicle-treated mice generated non-selective, habitual response strategies, at a ratio of 1 [Mann-Whitney $U=53, p<0.05$] (fig.2d). Although we

focus in this report on male mice, we also found that fasudil had the same effects in gonadally-intact female mice [Mann-Whitney $U=3, p<0.08$] (fig.2e).

Habit-based insensitivity to action-outcome contingencies is often accompanied by insensitivity to outcome value (Balleine and O'Doherty, 2010). To assess whether ROCK inhibition could also enhance value-based responding (and thus, block habits), the male mice in these experiments were trained for 2 additional sessions to reinstate responding and ensure comparable responding between previously vehicle- and fasudil-treated mice. Next, mice were individually placed in an empty cage and allowed free access to the reinforcer pellets used in instrumental conditioning experiments for 1 hour. Immediately following, mice were injected with lithium chloride (LiCl), inducing transient malaise and conditioned taste aversion (CTA), as evidenced by reduced food consumption across 2 sessions [no interaction $F<1$, main effect of session $F(1,21)=16.4, p<0.05$, no effect of group $F(1,21)=2.1, p>0.05$] (fig.2f). When returned to the conditioning chambers drug-free, mice previously treated with vehicle did not reduce responding relative to the last day of training, despite reinforcer devaluation; in other words, control mice responded habitually, consistent with their response strategies following instrumental contingency degradation. By contrast, mice previously treated with fasudil reduced responding, evidence that these mice used the value of the reinforcer to guide their behavior [interaction $F(1,21)=4.8, p<0.05$] (fig.2g).

Together, these findings indicate that the ROCK inhibitor fasudil enhances action-outcome conditioning, blocking habits in favor of goal-directed response strategies. Also, fasudil induces sustained sensitivity to action-outcome contingencies, evidenced by reduced responding when a reinforcer loses value.

One cohort of mice tested in the instrumental contingency degradation experiment in fig.2a-d expressed *thy1*-driven YFP and was euthanized 72 hours after injection (fig.2h). Prelimbic cortical dendritic spine densities did not differ [$t(8)=-$

0.88,p>0.05] (fig.2i, inset), nor did dendritic spine length, head diameter, or volume (Supplemental Table 1). However, densities correlated with decision-making strategies, such that lower densities were associated with selecting actions that were likely to be reinforced in a goal-directed fashion [r^2=0.725,p<0.05].

Here, fasudil was administered immediately following action-outcome contingency degradation, with the hypothesis that ROCK inhibition could enhance the consolidation of action-outcome learning and memory. This hypothesis predicts that fasudil treatment that is unpaired from a learning opportunity should have no behavioral effects. To test this, we trained separate mice to respond for food reinforcement (fig.S1a). Next, vehicle or fasudil was delivered, but injections were delayed 4 or 18 hours after action-outcome contingency degradation. In a subsequent probe test, no mice showed a preference for the response that was likely to be reinforced (fig.S2c). Thus, ROCK inhibition appears to enhance the consolidation of action-outcome conditioning during a <4-hour time window, facilitating subsequent goal-directed action selection.

To determine whether the effects of fasudil were attributable to enhancing extinction conditioning, these mice were given 3 days of extinction training, with vehicle or fasudil administered concurrent with each session. All mice extinguished responding, and fasudil had no within- or between-sessions effects (fig.S1d).

We next determined whether the behavioral effects of ROCK inhibition were, at least in part, attributable to actions specifically in the prelimbic prefrontal cortex (fig.2j,k). Intact mice were trained to nose-poke as above, and groups (to be vehicle *vs.* to be fasudil) were designated by matching response rates [no interaction F<1, main effect of session F(6,126)=48.8,p<0.05, no effect of group F(2,21)=1.7,p>0.05] (fig.2l). Here, the training period was shorter because the stress of intracranial surgery would be expected to bias responding towards habits (Schwabe et al., 2013). Immediately following

instrumental contingency degradation training, vehicle or fasudil was infused into either the prelimbic or anterior cingulate cortex as a control. Vehicle groups did not differ and were combined. Subsequently, vehicle-infused mice generated habit-based response patterns (no preference for the response most likely to be reinforced). Prelimbic cortical-targeted fasudil infusions blocked habit-based responding, inducing a significant preference for the response most likely to be reinforced. Meanwhile, anterior cingulate-targeted infusions had no effects [interaction $F(2,21)=2.4, p<0.05$] (fig.2m). These findings suggest that inhibiting ROCK within the prelimbic cortex facilitates goal-directed response selection.

ROCK is endogenously suppressed in the prefrontal cortex and hippocampus by Abl2/Arg kinase, such that Arg ablation disinhibits RhoA GTPase-ROCK interactions (Sfakianos et al., 2007) and blocks dendritic spine plasticity in response to external stimuli such as cocaine (Gourley et al., 2012). We obtained *Arg* knockout mice (*arg-/-*) and found that *Arg* deficiency induced habit-based behaviors (fig.S2), consistent with the *disinhibition* of ROCK. In other words, loss of the endogenous ROCK inhibitor Arg produced the *opposite* behavioral effect relative to the ROCK inhibitor fasudil.

ROCK blockade transiently remodels dendritic spines in a conditioning-dependent manner

Our findings suggest that mice that use action-outcome relationships to guide decision-making strategies have modestly fewer dendritic spines on deep-layer excitatory prelimbic cortical neurons. This could imply that activity-dependent dendritic spine elimination is associated with action-outcome associative learning. This raises the possibility that any gross structural effects of ROCK inhibition within the prelimbic prefrontal cortex will be most apparent during the memory consolidation period immediately following action-outcome conditioning. To test this hypothesis, we again

trained mice to respond for food reinforcement, followed by instrumental contingency degradation training and fasudil treatment (fig.3a). Groups were designated by matching mice based on response rates during training [no interaction $F<1$, main effect of session $F(15,225)=111.8, p<0.05$, no effect of group $F<1$] (fig.3b), and brains were collected 1 hour after injection. Fasudil caused a 9% reduction in prelimbic cortical dendritic spine density [$t(15)=-2.6, p<0.05$] (fig.3c,d). Dendritic spine length, head diameter, and volume did not differ between groups (Supplemental Table 1).

Is fasudil simply "damaging" neurons? One marker of structural damage is dendritic blebbing, causing the dendrite to enlarge (*e.g.*, see Hasbani et al., 2001), however fasudil did not alter dendrite diameter [$t(15)=-0.98, p>0.05$] (fig.3e). We also quantified dendritic spines in the anterior cingulate cortex, where fasudil infusion had no effects on decision-making in our prior experiments, and found no differences in dendritic spine density [$t(15)=-0.21, p>0.05$] (fig.3f), spine length, head diameter, or volume (not shown).

We next repeated the same training protocol [no interaction $F<1$, main effect of session $F(15,195)=119.8, p<0.05$, no effect of group $F<1$] (fig.3g,h), but fasudil was administered 1 day after contingency degradation training, with brains again collected 1 hour after injection. In this case, we found no differences in dendritic spine density [$t(13)=-0.83, p>0.05$] (fig.3i,j), spine length, head diameter, or volume (Supplemental Table 1). Together, these findings suggest that ROCK inhibition eliminates prelimbic cortical dendritic spines in an experience-dependent manner.

ROCK inhibition blocks habitual responding *for cocaine*

We report that inhibiting ROCK using fasudil enhances sensitivity to action-outcome conditioning, blocking habit-based responding. These experiments were conducted using food reinforcement. To build on this finding, we next assessed whether

fasudil can block drug (cocaine)-seeking habits as well (fig.4a). Here, mice were trained to respond on a single operandum for a liquid solution containing cocaine and sucrose that was consumed orally (adapted from Miles et al., 2003). We used an extended training schedule as in the systemic fasudil experiments above (*i.e.*, a training protocol that would be expected to bias response strategies towards habits), with groups determined by matching mice based on response rates during training [no interaction $F(16,496)=1.2, p>0.05$, main effect of session, $F(16,496)=38.5, p<0.005$, no effect of group $F<1$] (fig.4b). Next, the cocaine solution was paired in a separate context with LiCl, inducing CTA, as indicated by reduced consumption over 3 conditioning sessions [no interaction $F<1$, main effect of session $F(2,62)=23.0, p<0.05$, no effect of group $F<1$] (fig.4c). Next, mice were briefly returned to the operant conditioning chambers, providing them with an opportunity to update the association between the cocaine-reinforced response and the now-devalued cocaine reinforcer (Garcia, 1989; Balleine and Dickinson, 1994). Immediately after this session, mice received an *i.p.* injection of vehicle or fasudil. During a probe test the next day, the vehicle-treated group continued to respond at a rate equivalent to the last day of training, indicating a habit-based failure to respond based on outcome value. By contrast, fasudil reduced responding, indicating goal-directed, value-based decision-making [interaction $F(1,31)=4.3, p<0.05$] (fig.4d). A post-probe consumption test revealed no differences between groups [Mann-Whitney $U=121, p>0.05$] and further confirmed that both groups acquired the CTA (fig.4c).

Given that systemic fasudil treatment had persistent effects in food-reinforced experiments (again, fig.2f,g), we next assessed whether fasudil could persistently mitigate cocaine-seeking behavior as well. We also aimed to confirm that the effects of fasudil in the present experiment could be attributed to enhanced sensitivity to the reduced value of cocaine, as opposed to the sucrose included in the cocaine-sucrose solution. To accomplish these goals, a cohort of the mice tested in fig.4a-d was

surgically implanted with indwelling jugular catheters for intravenous cocaine self-administration. After recovery, mice were trained in a different room, in different operant conditioning chambers, to respond for intravenous cocaine delivery. Mice acquired the cocaine-reinforced response [main effect of session $F(6,96)=7.0, p<0.05$], however mice with a history of fasudil treatment generated lower cocaine-reinforced response rates [main effect of group $F(1,16)=6.1, p<0.05$] (fig.4e). Responding on the inactive nose-pokes (*i.e.*, responses that did not result in reinforcement) was also reduced by fasudil, but unlike with cocaine-reinforced responses, only during the first session [interaction $F(6,96)=4.0, p<0.05$]. Further, the fasudil group required more than twice as many sessions to ingest 20 mg/kg in a single session [Mann-Whitney $U=16.5, p<0.05$] (fig.4e, inset). This outcome indicates that ROCK inhibition reduces cocaine seeking in an *i.v.* self-administration model, and also that fasudil enhanced sensitivity to devaluation of the cocaine reinforcer, and not simply the sucrose that was part of the orally-ingested solution.

To address the potential concern that, rather than promoting sensitivity to action-outcome relationships, ROCK inhibition could be having a generalized inhibitory effect on the acquisition of new responses, a cohort of the mice generated in Fig.4b-d were not subjected to catheter implantation, but were instead subsequently trained in different chambers to respond on a different nose-poke aperture for sucrose pellets, a novel reinforcer that had not been devalued (fig.S3a). Groups did not differ in the acquisition of this new sucrose-reinforced response (fig.S3b). Thus, the effects of fasudil were selective to the devalued reinforcer.

In a separate cohort of mice generated to replicate our finding that fasudil enhanced sensitivity to *food-reinforced* action-outcome contingency degradation (again, fig.2a-d), we next implanted indwelling jugular catheters for cocaine self-administration (fig.S3c). This allowed us to assess whether a history of fasudil treatment reduced

cocaine-seeking behavior in general, or whether fasudil specifically interfered with cocaine seeking by enhancing behavioral sensitivity to cocaine devaluation. Here, a history of fasudil resulted in less responding for cocaine on day 1, likely because nose-poking had recently generated no reinforcement, but this difference was quickly lost (fig.S3d). Thus, durable cocaine avoidance results from pairing ROCK inhibition with the devaluation of cocaine.

Inhibiting F-actin polymerization blocks fasudil's effects on action selection

The primary effect of fasudil, through ROCK inhibition, is to increase actin turnover, which is presumably the mechanism by which fasudil eliminates prelimbic cortical dendritic spines and promotes goal-directed decision-making. To confirm this perspective, we sought to disrupt actin turnover with latrunculin A, which blocks F-actin polymerization. In the presence of latrunculin A, fasudil should be ineffective. We again trained mice to respond for an oral cocaine-sucrose solution, followed by LiCl-induced CTA, and then a "reminder session" paired with a systemic injection of vehicle or fasudil. Vehicle or latrunculin A was infused bilaterally into the prelimbic prefrontal cortex (fig.5a,b). Subsequently, mice were given a probe test and finally, a post-probe consumption test to confirm that all mice acquired the CTA.

Groups were matched based on response rates during acquisition [no interaction $F<1$, main effect of session $F(16,448)=5.8, p<0.05$, no effect of injection by infusion $F<1$] (fig.5c). Over the course of 3 pairings of LiCl with the cocaine-sucrose solution, all mice reduced consumption [no interaction $F<1$, main effect of session $F(2,56)=94.1, p<0.05$, no effect of injection by infusion $F(1,28)=1.3, p>0.25$] (fig.5d). When response rates during the probe test were compared against the last day of training, vehicle-vehicle mice were insensitive to the change in outcome value, as expected, while fasudil-vehicle treated mice were sensitive to the change in outcome value and reduced responding in

the probe test, confirming the results from fig.2c. As predicted, local infusion of latrunculin A into the prelimbic prefrontal cortex blocked the effects of fasudil [3-factor interaction $F(1,29)=6.1, p<0.05$] (fig.5e). Surprisingly, mice that received a systemic injection of saline and locally-infused latrunculin A were also sensitive to the change in outcome value and reduced responding. A post-probe consumption test confirmed that all mice acquired the CTA [no interaction $F<1$, no effect of injection $F<1$, no effect of infusion $F<1$] (fig.5d). These findings support the idea that fasudil promotes goal-directed action selection by eliminating prelimbic cortical dendritic spines in an actin turnover-dependent manner.

Discussion

Here we trained mice to generate food- or cocaine-reinforced operant responses, then decreased the likelihood that responding would be reinforced. Response inhibition is thought to reflect new knowledge regarding action-outcome relationships, while a failure to modify response strategies reflects a deferral to familiar, habit-based behaviors (Dickinson, 1980). We found that chemogenetic inactivation of excitatory pyramidal neurons in the prelimbic cortex reduced behavioral sensitivity to action-outcome contingencies, as with prelimbic cortical lesions (Balleine and Dickinson, 1998; Corbit and Balleine, 2003). Systemic and local prelimbic cortical administration of the ROCK inhibitor fasudil enhanced action-outcome conditioning, blocking both food- and cocaine-reinforced habits in an actin-turnover dependent manner. Further, lower dendritic spine densities on deep-layer prelimbic cortical neurons were associated with successful action-outcome conditioning, reminiscent of evidence that other forms of learning and memory involve dendritic spine pruning (Lai et al., 2012; Sanders et al., 2012). ROCK inhibition also transiently reduced prelimbic cortical dendritic spine densities during the consolidation of new action-outcome learning. These findings suggest that endogenous ROCK supports habit-based behaviors by inhibiting structural plasticity within the prelimbic cortex. Meanwhile, inhibiting ROCK can interfere with habit-based behaviors, potentially including in the context of problematic drug-seeking habits.

Cortico-striatal-limbic circuits balance actions and habits

One proposed model for the manner in which the prelimbic cortex coordinates goal-directed action is via interactions with the dorsomedial striatum and basolateral amygdala. Thalamic relays and direct basolateral amygdala inputs innervate deep-layer prelimbic cortical neurons (Kuroda et al., 1996; Gabbott et al., 2012), so we reasoned that dendritic spines receiving these inputs could be involved in action-outcome

conditioning. Supporting this perspective, individual differences in dendritic spine densities on layer V prelimbic cortical neurons correlated with response selection strategies: Lower spine densities were associated with goal-directed responding, and higher densities with habit-based behavior, in multiple cohorts of mice.

Could reducing prelimbic cortical dendritic spine densities enhance sensitivity to action-outcome relationships? To test this possibility, we used the ROCK inhibitor fasudil. ROCK is a major substrate of the RhoA GTPase that phosphorylates LIM-kinase, which then phosphorylates cofilin. Cofilin typically cleaves F-actin, but phosphorylation inactivates it (Olson, 2008). Thus, ROCK inhibition can facilitate activity-dependent plasticity of dendritic spines, including dendritic spine pruning (Schubert et al., 2006; Murakoshi et al., 2011). Fasudil is brain-penetrant, enhances learning and memory in other contexts (Huentelman et al., 2009; Couch et al., 2010; Swanson et al., 2013; Zimmermann et al., 2017), and is clinically approved for therapeutic use in Japan, with a positive safety profile (Suzuki et al., 2007).

Mice were first trained to respond for food reinforcers such that they would be expected to develop habits by virtue of extensive response training. We then administered fasudil immediately following the modification of a familiar action-outcome contingency, during the potential consolidation of new knowledge (*i.e.*, that a familiar behavior is no longer likely to be reinforced). Subsequently, fasudil-treated mice inhibited responding, while control mice persisted in responding, a habit-based strategy. In other words, fasudil enhanced action-outcome conditioning, blocking habit-based behavior. Prelimbic cortical fasudil infusions had the same effects, while infusions into the anterior cingulate cortex were without consequence. This may be because the anterior cingulate cortex is preferentially involved in effort-based decision-making (Walton et al., 2003). Delaying fasudil treatment 4 or 18 hours following training also had no effect, suggesting that fasudil enhances the consolidation, in particular, of action-outcome conditioning.

As predicted, fasudil also reduced dendritic spine densities on prelimbic cortical neurons, sparing neurons in the anterior cingulate cortex. The consolidation of new memory is often assumed to involve the *formation* of new dendritic spines, but growing evidence indicates that dendritic spine pruning is also important (Lai et al., 2012; Sanders et al., 2012), potentially serving to enhance signal:noise during key learning opportunities. Although fasudil reduced dendritic spine density by only 9%, this value exceeds baseline dendritic spine turnover rates in the nearby motor and barrel cortices (Liston and Gan, 2011; Wilbrecht et al., 2010). Further, when fasudil was administered 1 day after conditioning, *i.e.,* after potential memory consolidation, no changes were detected. Together, these findings suggest that ROCK inhibition prunes prelimbic cortical dendritic spines in an activity-dependent manner, facilitating subsequent goal-directed response choice.

ROCK blockade inhibits habit-based cocaine seeking

We next determined whether fasudil could block habitual responding for cocaine. We endeavored to decrease the value of cocaine, with the hypothesis that fasudil would enhance behavioral sensitivity to devaluation. Devaluing cocaine has proven particularly challenging in the field, so we utilized an oral delivery approach previously applied to rats (Miles et al., 2003) and mice (DePoy et al., 2016). In this case, mice responded for a cocaine-sucrose solution, which was later paired with LiCl, inducing transient malaise and thus decreasing its value. Fasudil-treated mice subsequently reduced responding when returned to the conditioning chambers, while control mice failed to modify their behavioral patterns, responding habitually. Thus, fasudil reduced habit-based drug seeking. Orally-delivered cocaine readily penetrates the brain (Pan and Hedaya, 1998), but one concern with this experimental design is that responding in fasudil-treated mice could have decreased because the value of the ingested sucrose, but not necessarily

the cocaine, diminished. To address this possibility, we implanted indwelling jugular catheters, allowing mice to self-administer intravenous-delivered cocaine. Fasudil-treated mice responded less, confirming that fasudil mitigated cocaine, and not simply sucrose, seeking.

Our experimental design can be envisioned as paralleling a common scenario in cocaine use disorders: 1) Mice self-administer cocaine in operant conditioning chambers, while humans use cocaine in their daily lives. 2) As a consequence of the devaluation procedure, mice come to associate cocaine with an adverse outcome (malaise), while humans may seek treatment due to the negative consequences of cocaine abuse. 3) Despite adverse consequences, vehicle-treated mice continue to respond habitually for cocaine, while fasudil-treated mice reduce responding. After treatment in humans, 40-60% of patients relapse (McLellan et al., 2000). Pairing ROCK inhibitors with cognitive behavioral therapy in humans could be an effective pharmacological adjunct to reduce the rate of relapse. Indeed, pharmacological regulators of actin cytoskeleton dynamics may have broad potential in treating drug use disorders. It is widely acknowledged that cocaine modifies dendrite and dendritic spine structure in multiple brain regions (reviewed Depoy and Gourley, 2015). Cocaine also appears to alter actin function (Toda et al., 2006, Shen et al., 2009; Esparza et al., 2012). Additionally, integrins, extracellular matrix protein receptors, and their ligands and intracellular substrates such as p190RhoGAP and Abl2/Arg, regulate the cocaine-induced reinstatement of cocaine seeking in rats and cocaine-induced locomotor sensitization and cognitive impairments in mice (Gourley et al., 2009; Wiggins et al., 2011; Gourley et al., 2012; Warren et al., 2012; Smith et al., 2016; Spencer et al., 2016). Further, infusion of latrunculin A, which inhibits F-actin polymerization, or myosin IIB inhibition, into the basolateral amygdala can block methamphetamine-related memories (Young et al., 2014; Young et al., 2016).

The effects of ROCK inhibition are dependent upon F-actin turnover

We show that the ROCK inhibitor fasudil eliminates prelimbic cortical dendritic spines when paired with action-outcome conditioning, and that dendritic spine density correlates with subsequent response selection strategies, which we interpret as a remnant of activity-dependent spine pruning. To confirm that cellular structural plasticity is *necessary* for fasudil to enhance goal-directed action selection, we co-administered fasudil with latrunculin A, blocking F-actin polymerization. As expected, control mice responded for cocaine even following its devaluation, while fasudil reduced cocaine seeking. However, simultaneous latrunculin A infusion blocked this effect, supporting the perspective that fasudil acts via F-actin turnover in the prelimbic cortex. Interestingly, latrunculin A *alone* also reduced cocaine-seeking behavior. This could be due to spread into the ventrally-situated infralimbic cortex, which is necessary for the expression of habits (Coutureau and Killcross, 2003). This outcome would suggest that structural plasticity in the infralimbic cortex is involved in habit-based behavior, a model that has also been posited for the dorsolateral striatum (Gourley et al., 2013). Further experiments could explicitly test this hypothesis.

Conclusions

During his tenure, former NIMH director Tom Insel repeatedly called for the development of statin-like compounds (such as ROCK inhibitors) to treat psychiatric disorders (*e.g.,* Insel, 2010). Given its favorable safety profile and our evidence that it can mitigate cocaine seeking, fasudil is a strong candidate. An ongoing challenge in the field is understanding at a deeper, mechanistic level how goal-directed and habitual behaviors are balanced. During the acquisition of a new task, deep-layer prelimbic cortical neurons generate high firing rates that then decrease with repetition and habit

formation (Smith and Graybiel, 2013). This gradual “quieting” could explain why extensive training produces insensitivity to changes in action-outcome relationships and outcome value. By extension, the re-engagement of goal-directed response strategies after habits have formed may require re-activation of these neurons. Our findings suggest that optimizing signal:noise via selective dendritic spine pruning could facilitate this process.

Methods

Subjects: Mice were C57BL/6 wildtype from Jackson Labs, transgenic mice expressing *thy1*-YFP-H (Feng et al., 2000) and fully back-crossed onto a C57BL/6 background, or the offspring of *arg*+/- x *arg*+/- crosses (Koleske et al., 1998). *arg* mutant mice were maintained on a mixed 129Sv/J x C57BL/6 background with genotypes confirmed by PCR. Behavioral training began on postnatal day (P) ~42 throughout. Mice were male unless otherwise noted. All procedures were approved by the Emory or Yale institutional animal care and use committees, as appropriate.

Intracranial surgery: Mice were anesthetized with a 100 mg/kg ketamine / 1 mg/kg xylazine mixture and placed in a stereotaxic frame. With infusion needles centered at bregma, a hole was drilled in the skull corresponding to +2.0 AP, +/-0.1 ML, -2.8 DV.

For viral vector experiments, AAV5-CaMKII-HA-hM4D(Gi)-IRES-mCitrine or AAV5-CaMKII-GFP (UNC Viral Vector Core) was infused bilaterally (0.5 µl/hemisphere) over 5 min, with the needle left in place for an additional 5 min. Mice were sutured and allowed to recover for 3 weeks, allowing time for viral vector expression.

For fasudil infusions, 360 µM fasudil (Arita et al., 2009) or sterile saline (0.1 µl/hemisphere) was infused over a period of 1 minute. Latrunculin A or sterile saline (0.15 µl/hemisphere) was delivered in a concentration of 5 µg/µl over 2 min (adapted from Toda et al., 2010; Gourley et al., 2012), 30 min after systemic injection of saline or fasudil. Needles were left in place for 2 additional minutes following infusion, and mice were sutured and allowed to recover for 3 days.

Histology: Mice were euthanized by rapid decapitation and brains were submerged in 4% paraformaldehyde for 48 hours and then transferred to 30% w/v sucrose. 50 µm-thick sections were prepared on a microtome held at -15°C±1. Sections were mounted

and infusion terminals were documented following examination under magnification. In the case of DREADDs experiments, GFP or mCitrine were imaged.

Instrumental response training: Mice were food restricted to 87-90% of their original body weight. Med-Associates operant conditioning chambers equipped with 2 distinct nose-poke recesses and a food magazine were used. Mice were trained to respond on both recesses for food reinforcement (20 mg grain-based pellets; Bioserv), with 30 pellets associated with each response (60 pellets/session). All mice were trained for 5-6 sessions using an FR1 schedule, then 2 sessions at RI30. For extended training – *i.e.*, to induce habit behavior in intact control mice – 9 RI60 sessions followed. Sessions terminated when all pellets had been delivered or at 135 minutes.

Cocaine-reinforced experiments utilized the same operant conditioning chambers, but with a 75 µg/ml cocaine (Sigma) + 10% w/v sucrose solution delivered into the magazine by a motorized dipper that held 100 µl. In this case, a single response (nose-poke) was reinforced, and up to 30 reinforcers were delivered/session. The average daily dose of cocaine following the response acquisition phase was 12.5 mg/kg.

Action-outcome contingency degradation: Methods were as described previously (Swanson et al., 2013; Zimmermann et al., 2017; DePoy et al., 2016; Gourley and Taylor, 2016). Briefly, on one day, one of the two nose-poke recesses was occluded, and pellet delivery was contingent upon responses on the remaining available nose-poke recess. A variable ratio 2 (VR2) schedule of reinforcement was used for 25 min. During another session, only the opposite nose-poke recess was available. In this case, pellets were delivered independently of animals' interactions with this nose-poke recess for 25 min, thus violating, or "degrading," the predictive relationship between responding on that particular nose-poke recess and pellet delivery. The pellet delivery rate was

matched to each animal's mean reinforcement rate from the previous session (also as in Barker et al., 2013). Thus responding on one nose-poke recess became significantly less predictive of pellet delivery than the other.

The following day, both nose-poke recesses were available for 10 or 15 min during a probe test; responding was not reinforced. Preferential engagement of the highly-reinforced response is considered "goal-directed," while non-selective responding reflects habit-based behavior (Balleine and O'Doherty, 2010).

Outcome devaluation: After instrumental response training, mice were placed in a clean empty cage and allowed 1 hour of free access to the reinforcer pellets or oral cocaine-sucrose solution used in instrumental conditioning experiments. Immediately following, mice were injected with lithium chloride (LiCl) (0.15 M, 4ml/100g body weight, *i.p.* Quinn et al., 2007), inducing temporary malaise and CTA. This pairing was repeated 2 or 3 times, as indicated in the figures. The amount of food or liquid consumed was measured after each pairing.

For mice trained to respond for a cocaine-sucrose solution, a "reminder session" consisted of mice being placed in the operant conditioning chambers for 10 min, providing mice with an opportunity to update the association between the operant response and the now-devalued reinforcer (Garcia, 1989; Balleine and Dickinson, 1994). Saline or fasudil was paired with this session, as indicated in the figure timelines.

For all mice, a probe test measured whether mice responded for the now-devalued reinforcer. Following the probe test, mice were given a "post-probe consumption test," consisting of one hour of free access to the reinforcer, to confirm the acquisition of CTA. One mouse each in the initial oral cocaine (fig.4) and latrunculin A experiments (fig.5) did not reduce consumption, as measured between the first pairing and the post-probe consumption test, and were excluded.

Intravenous catheter implantation: Mice were anaesthetized with a 100 mg/kg ketamine / 1 mg/kg xylazine mixture, and the dorsal and ventral sides were shaved and disinfected. The right jugular vein was exposed by blunt dissection, and a sterile Silastic catheter was placed as described (Thomsen and Caine, 2005) and then exteriorized posterior to the scapulae. The entrance and exit wounds were sutured, and mice were housed individually. During the 5-7 day recovery period, catheter patency was ensured by infusing mice daily with 0.05 ml heparinized saline. Subsequently, catheter patency was tested weekly using a 0.03 ml ketamine challenge (15 mg/ml). If mice were insensitive to ketamine at any point, defined by a failure to lose muscle tone within 10 seconds of infusion, they were excluded.

Intravenous cocaine self-administration: Following catheter implantation, cocaine self-administration was tested in contextually distinct conditioning chambers relative to the chambers in which oral cocaine and food-reinforced test sessions had been conducted. Mice were tested daily, during which a single nose-poke response on the center of 3 nose-poke recesses was reinforced with an infusion of cocaine (20 µl; 1.25 mg/ml) delivered through a catheter connected to a swivel holding armored polyethylene tubing. Delivery culminated in extinction of the house light and a 20-sec timeout. Sessions ended when mice self-administered 30 infusions or at 120 min. Mice were considered to have acquired the cocaine-reinforced response when mice self-administered $\geq$20 infusions (~20 mg/kg) (Butkovich et al., 2015).

Extinction training: Mice were placed in the conditioning chambers for 2 consecutive 45 minute sessions (1/day) conducted in 3 x 15-min bins with no reinforcement delivered.

Clozapine N-oxide (CNO) delivery: For DREADDs experiments, all mice, regardless of viral vector, received 1 mg/kg CNO (2% DMSO in saline, *i.p.*; Sigma) 1 hour prior to the "degradation" training session. Mice were tested in the probe test in the absence of further injection.

Systemic fasudil delivery: A 10 mg/kg dose of fasudil (Swanson et al., 2013), dissolved in sterile saline, was administered *i.p.* (1 ml/100 g) at the time points indicated in the experimental outlines.

Dendritic spine imaging and analysis: *thy1*-YFP-expressing mice were euthanized by rapid decapitation, and brains were submerged in 4% paraformaldehyde for 48 hours and then transferred to 30% w/v sucrose. 50 µm-thick sections were prepared on a microtome held at -15°C±1. Images were acquired on a Leica DM5500B microscope equipped with a spinning disk confocal (VisiTech International) and a Hamamatsu Orca R2 camera using a 100x 1.4 NA objective. Z-stacks of dendritic segments were acquired using a 0.1 µm step size. 8 dendrites were acquired bilaterally from 8 independent neurons per animal. The location of the imaged segments within target regions was confirmed by zooming out to a low magnification. Care was taken to select second-order or higher apical dendrites of a known distance from the soma. Dendritic spines were sampled dendritic spines 50-150 µm from soma. A single blinded user generated all images.

Mice were euthanized at various time points, as indicated in the experimental outlines. For example, to assess the rapid effects of fasudil on dendritic spine density and morphology, trained mice were euthanized 1 hour after injection. This timing was selected to give fasudil time to enter the brain and impact dendritic spines while the brain

was presumed to be consolidating new information regarding action-outcome contingencies.

Dendritic spine reconstruction: The FilamentTracer module of Imaris (Bitplane AG) was used as described (Swanger et al., 2011): A dendritic segment 15-25 µm in length was drawn with the autodepth function. Dendritic spine head location was manually indicated, and FilamentTracer processing algorithms were used to calculate morphological parameters. A single blinded individual quantified all dendritic spines within an experiment, with inter-rater reliability ensured between two individuals.

Statistics: Response rates were compared by ANOVA with group, or group and session, as factors, with repeated measures as appropriate. Following significant interactions, Tukey's posthoc comparisons were used. Response preference ratios were calculated by dividing non-degraded / degraded response rates and comparing group means by Mann-Whitney U tests. Food/cocaine ingestion was compared by ANOVA or t-test as appropriate. For dendritic spine densities, each mouse contributed a single value for comparison by ANOVA or t-test as appropriate. Correlations were analyzed by linear regression. Two mice in the local fasudil infusion experiment (fig.2) and 2 mice in the local latrunculin A experiment (fig.5) generated response rates greater than 2 standard deviations above the mean and were excluded. In the case of non-normal distributions, square root or arcsin transformations were used as appropriate (for ANOVAs), or Mann-Whitney U tests (in place of t-tests) were applied. $p<0.05$ was considered significant.

Individual dendritic spine parameters reported in Supplementary Table 1 were compared by Kolmogorov-Smirnov tests. Due to the considerable degree of power generated in these analyses, only $p<0.001$ was considered significant.

References

Allen Institute for Brain Science (2012). Allen Brain Atlas [Internet]. Available from: http://www.brain-map.org.

Arita R, Hata Y, Nakao S, Miura M, Kawahara S, Zandi S, Tayyari F, Shimokawa H, Hafezi-Moghadam A, Ishibashi T (2009) Rho kinase inhibition by fasudil ameliorates diabetes-induced microvascular damage. Diabetes 58:215-226.

Balleine BW, Dickinson A (1994) Role of cholecystokinin in the motivational control of instrumental action in rats. Behav Neurosci 108:590-605.

Balleine BW, Dickinson A (1998) Goal-directed instrumental action: contingency and incentive learning and their cortical substrates. Neuropharmacology 37:407-419.

Balleine BW, O'Doherty JP (2010) Human and rodent homologies in action control: corticostriatal determinants of goal-directed and habitual action. Neuropsychopharmacology 35:48-69.

Barker JM, Torregrossa MM, Taylor JR (2013) Bidirectional modulation of infralimbic dopamine D1 and D2 receptor activity regulates flexible reward seeking. Front Neurosci 7:126.

Butkovich LM, DePoy LM, Allen AG, Shapiro LP, Swanson AM, Gourley SL (2015) Adolescent-onset GABAA α1 silencing regulates reward-related decision making. Eur J Neurosci 42:2114-2112.

Condé F, Maire-Lepoivre E, Audinat E, Crépel F (1995) Afferent connections of the medial frontal cortex of the rat. II. Cortical and subcortical afferents. J Comp Neurol 352:567-593.

Corbit LH, Balleine BW (2003) The role of prelimbic cortex in instrumental conditioning. Behav Brain Res 146:145-157.

Corbit LH, Chieng BC, Balleine BW (2014) Effects of repeated cocaine exposure on habit learning and reversal by N-Acetylcysteine. Neuropsychopharmacology 39:1893-1901.

Couch BA, DeMarco GJ, Gourley SL, Koleske AJ (2010) Increased dendrite branching in AbetaPP/PS1 mice and elongation of dendrite arbors by fasudil administration. J Alzheimers Dis 20:1003-1008.

Coutureau E, Killcross S (2003) Inactivation of the infralimbic prefrontal cortex reinstates goal-directed responding in overtrained rats. Behav Brain Res 146:167-174

DePoy LM, Allen AG, Gourley SL (2016) Adolescent cocaine self-administration induces habit behavior in adulthood: sex differences and structural consequences. Transl Psychiatry 6:e875.

DePoy LM, Gourley SL (2015) Synaptic cytoskeletal plasticity in the prefrontal cortex following psychostimulant exposure. Traffic 16:919-940.

Dickinson A (1980) Contemporary Animal Learning Theory. Cambridge: Cambridge University Press.

Esparza MA, Bollati F, Garcia-Keller C, Virgolini MB, Lopez LM, Brusco A, Shen HW, Kalivas PW, Cancela LM (2012) Stress-induced sensitization to cocaine: actin cytoskeleton remodeling within mesocorticolimbic nuclei. Eur J Neurosci 36:3103-3117.

Everitt BJ, Robbins TW (2016) Drug Addiction: Updating actions to habits to compulsions ten years on. Annu Rev Psychol 4:23-50.

Feng G, Mellor RH, Bernstein M, Keller-Peck C, Nguyen QT, Wallace M, Nerbonne JM, Lichtman JW, Sanes JR (2000) Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. Neuron 28:41-51.

Gabbott P, Warner T-A, Brown J, Salway P, Gabbott T, Busby S (2012) Amygdala afferents monosynaptically innvervate corticospinal neurons in rat medial prefrontal cortex. J Comp Neurol 520:2440-2458.

Garcia J (1989) Aversion, Avoidance and Anxiety: Perspectives on Aversively Motivated Behavior. Hillsdale, NJ: Lawrence Erlbaum.

Gourley SL, Koleske AJ, Taylor JR (2009) Loss of dendrite stabilization by the Abl-related gene (Arg) kinase regulates behavioral flexibility and sensitivity to cocaine. Proc Natl Acad Sci U S A 106:16859-16854.

Gourley SL, Olevska A, Warren MS, Taylor JR, Koleske AJ (2012) Arg kinase regulates prefrontal dendritic spine refinement and cocaine-induced plasticity. J Neurosci 32:2314-2323.

Gourley SL, Olevska A, Gordon J, Taylor JR (2013) Cytoskeletal determinants of stimulus-response habits. J Neurosci 33:11811-11816.

Gourley SL, Taylor JR (2016) Going and stopping: dichotomies in behavioral control by the prefrontal cortex. Nat Neurosci 19:656-664.

Hart G, Leung BK, Balleine BW (2014) Dorsal and ventral streams: the distinct role of striatal subregions in the acquisition and performance of goal-directed actions. Neurobiol Learn Mem 18:104-118.

Hasbani MJ, Schlief ML, Fisher DA, Goldberg MP (2001) Dendritic spines lost during glutamate receptor activation reemerge at original sites of synaptic contact. J Neurosci 21:2393-2403.

Hinton EA, Wheeler MG, Gourley SL (2014) Early-life cocaine interferes with BDNF-mediated behavioral plasticity. Learn Mem 21:253-257.

Huentelman MJ, Stephan DA, Talboom J, Corneveaux JJ, Reiman DM, Gerber JD, Barnes CA, Alexander GE, Reiman EM, Bimonte-Nelson HA (2009) Peripheral delivery of a ROCK inhibitor improves learning and working memory. Behav Neurosci 123:218-223.

Insel TR (2010) Rethinking schizophrenia. Nature 468:187-193.

Killcross S, Coutureau E (2003) Coordination of actions and habits in the medial prefrontal cortex of rats. Cereb Cortex 13:400-408.

Koleske AJ, Gifford AM, Scott ML, Nee M, Bronson RT, Miczek KA, Baltimore D (1998) Essential roles for the Abl and Arg tyrosine kinases in neurulation. Neuron 21:1259-1272.

Kuroda M, Murakami K, Igarashi H, Okada A (1996) The convergence of axon terminals from the mediodorsal thalamic nucleus and ventral tegmental area on pyramidal cells in layer V of the rat prelimbic cortex. Eur J Neurosci 8:1340-1349.

Lai CS, Franke TF, Gan WB (2012) Opposite effects of fear conditioning and extinction on dendritic spine remodelling. Nature 483:87-91.

LeBlanc KH, Maidment NT, Ostlund SB (2013) Repeated cocaine exposure facilitates the expression of incentive motivation and induces habitual control in rats. PLoS One 8:e61355.

Leong KC, Berini CR, Ghee SM, Reichel CM (2016) Extended cocaine-seeking produces a shift from goal-directed to habitual responding in rats. Physiol Behav 165:330-335.

Liston C, Gan WB (2011) Glucocorticoids are critical regulators of dendritic spine development and plasticity in vivo. Proc Natl Acad Sci USA 108:16074-16079.

McLellan AT, Lewis DC, O'Brien CP, Kleber HD (2000) Drug dependence, a chronic medical illness: implications for treatment, insurance, and outcomes evaluation. JAMA 284:1689-1695.

Miles FJ, Everitt BJ, Dickinson A (2003) Oral cocaine seeking by rats: action or habit? Behav Neurosci 117:927-938.

Murakoshi H, Wang H, Yasuda R (2011) Local, persistent activation of Rho GTPases during plasticity of single dendritic spines. Nature 471:100-104.

Olson MF (2008) Applications for ROCK kinase inhibition. Curr Opin Cell Biol 20:242-248.

Ostlund SB, Balleine BW (2005) Lesions of medial prefrontal cortex disrupt the acquisition but not the expression of goal-directed learning. J Neurosci 25:7764-7770.

Pan WJ, Hedaya MA (1998) An animal model for simultaneous pharmacokinetic/pharmacodynamic investigations: application to cocaine. J Pharm Tox Methods 39:1-8.

Quinn JJ, Hitchcott PK, Umeda EA, Arnold AP, Taylor JR (2007) Sex chromosome complement regulates habit formation. Nat Neurosci 10:1398-1400.

Rosen GD, Williams AG, Capra JA, Connolly MT, Cruz B, Lu L, Airey DC, Kulkarni K, Williams RW (2000) The Mouse Brain Library [Internet]. Available from http://www.mbl.org.

Sanders J, Cowansage K, Baumgärtel K, Mayford M (2012) Elimination of dendritic spines with long-term memory is specific to active circuits. J Neurosci 32:12570-12578.

Schmitzer-Torbert N, Apostolidis S, Amoa R, O'Rear C, Kaster M, Stowers J, Ritz R (2015) Post-training cocaine administration facilitates habit learning and requires the infralimbic cortex and dorsolateral striatum. Neurobiol Learn Mem 118:105-112.

Schoenbaum G, Setlow B (2005) Cocaine makes actions insensitive to outcomes but not extinction: implications for altered orbitofrontal-amygdalar function. Cereb Cortex 15:1162-1169.

Schubert V, Da Silva JS, Dotti CG (2006) Localized recruitment and activation of RhoA underlies dendritic spine morphology in a glutamate receptor-dependent manner. J Cell Bio 30:453-467.

Schwabe L (2013) Stress and the engagement of multiple memory systems: integration of animal and human studies. Hippocampus 23:1035-1043.

Segal M (2005) Dendritic spines and long-term plasticity. Nat Rev Neurosci 6:277-284.

Sfakianos MK, Eisman A, Gourley SL, Bradley WD, Scheetz AJ, Settleman J, Taylor JR, Greer CA, Williamson A, Koleske AJ (2007) Inhibition of Rho via Arg and p190RhoGAP

in the postnatal mouse hippocampus regulates dendritic spine maturation, synapse and dendrite stability, and behavior. J Neurosci 27:10982-10992.

Shen HW, Toda S, Moussawi K, Bouknight A, Zahm DS, Kalivas PW (2009) Altered dendritic spine plasticity in cocaine-withdrawn rats. J Neurosci 29:2876-2884.

Smith AC, Scofield MD, Heinsbroek JA, Gipson CD, Neuhofer D, Roberts-Wolfe DJ, Spencer S, Stankeviciute NM, Smith R, Allen NP, Lorang MR, Griffin WC 3rd, Boger HA, Kalivas PW (2017) Accumbens nNOS interneurons regulate cocaine relapse. J Neurosci 37:742-756.

Smith KS and Graybiel AM (2013) A dual operator view of habitual behavior reflecting cortical and striatal dynamics. Neuron 79:361-374.

Spencer S, Garcia-Keller C, Roberts-Wolfe D, Heinsbroek JA, Mulvaney M, Sorrell A, Kalivas PW (2016) Cocaine use reverses striatal plasticity produced during cocaine seeking. Biol Psychiatry Epub ahead of print.

Suzuki Y, Shibuya M, Satoh S, Sugimoto Y, Takakura K (2007) A postmarketing surveillance study of fasudil treatment after aneurysmal subarachnoid hemorrhage. Surg Neurol 68:126-131.

Swanger SA, Yao X, Gross C, Bassell GJ (2011) Automated 4D analysis of dendritic spine morphology: applications to stimulus-induced spine remodeling and pharmacological rescue in a disease model. Mol Brain 4:38.

Swanson AM, Shapiro LP, Whyte AJ, Gourley SL (2013) Glucocorticoid receptor regulation of action selection and prefrontal cortical dendritic spines. Commun Integr Biol 6:e26068.

Thomsen M, Caine SB (2005) Chronic intravenous drug self-administration in rats and mice.Curr Protoc Neurosci 9:20.

Toda S, Shen H, Kalivas PW (2010) Inhibition of actin polymerization prevents cocaine-induced changes in spine morphology in the nucleus accumbens. Neurotox Res 18:410-415.

Toda S, Shen HW, Peters J, Cagle S, Kalivas PW. (2006) Cocaine increases actin cycling: effects in the reinstatement model of drug seeking. J Neurosci 26:1579-1587.

Urban DJ, Roth BL (2015) DREADDs (designer receptors exclusively activated by designer drugs): chemogenetic tools with therapeutic utility. Annu Rev Pharmacol Toxicol 55:399-417.

Walton ME, Bannerman DM, Alterescu K, Rushworth MF (2003) Functional specialization within medial frontal cortex of the anterior cingulate for evaluating effort-related decisions. J Neurosci 23:6475-6579.

Warren MS, Bradley WD, Gourley SL, Lin YC, Simpson MA, Reichardt LF, Greer CA, Taylor JR, Koleske AJ (2012) Integrin β1 signals through Arg to regulate postnatal dendritic arborization, synapse density, and behavior. J Neurosci 32:2824-2834.

Wiggins A, Smith RJ, Shen HW, Kalivas PW (2011) Integrins modulate relapse to cocaine-seeking. J Neurosci 31:16177-16184.

Wilbrecht L, Holtmaat A, Wright N, Fox K, Svoboda K (2010) Structural plasticity underlies experience-dependent functional plasticity of cortical circuits. J Neurosci 30:4927-4932.

Young EJ, Aceti M, Griggs EM, Fuchs RA, Zigmond Z, Rumbaugh G, Miller CA (2014) Selective, retrieval-independent disruption of methamphetamine-associated memory by actin depolymerization. Biol Psychiatry 75:96-104.

Young EJ, Blouin AM, Briggs SB, Sillivan SE, Lin L, Cameron MD, Rumbaugh G, Miller CA (2016) Nonmuscle myosin IIB as a therapeutic target for the prevention of relapse to methamphetamine use. Mol Psychiatry 21:615-623.

Zapata A, Minney VL, Shippenberg TS (2010) Shift from goal-directed to habitual cocaine seeking after prolonged experience in rats. J Neurosci 30:15457-15463.

Zimmermann KS, Yamin JA, Rainnie DG, Ressler KJ, Gourley SL (2017) Connections of the mouse orbitofrontal cortex and regulation of goal-directed action selection by brain-derived neurotrophic factor. Biol Psychiatry 81:366-377.

Figures

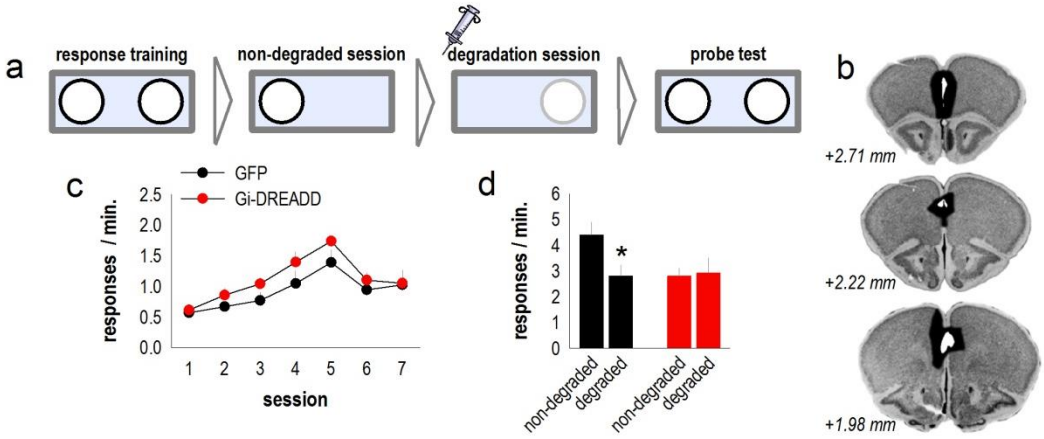

Figure 1. Chemogenetic silencing of the prelimbic prefrontal cortex impairs the ability of mice to select actions based on their consequences. (a) Task outline: Food-restricted mice are placed in operant conditioning chambers and trained to respond on two nose-poke apertures for food pellets. Following training, one nose-poke recess is available, and responding is reinforced. In another session, the opposite nose-poke recess is available, but responding is not reinforced; instead, food pellets are delivered at a rate matched to the reinforcement rate from the previous session, "degrading" the action-outcome contingency. During a probe test, mice have access to both nose-poke apertures. Preferential engagement of the response that is most likely to be reinforced is considered goal-directed, while engaging in both nose-poke responses non-selectively is considered a failure in action-outcome conditioning and a deferral to habit-based behavior. (b) The largest and smallest viral vector spread is represented on images from the Mouse Brain Library (Rosen et al., 2000). (c) All mice acquired the instrumental responses, with no differences between groups. (d) Following instrumental contingency degradation, control GFP-expressing mice (*n*=12) preferentially engaged the response most likely to be reinforced. Gi-DREADDs-expressing mice did not

differentiate between the responses, deferring instead to habit-based responding (*n*=7). Bars and symbols = means + SEMs, *p<0.05.

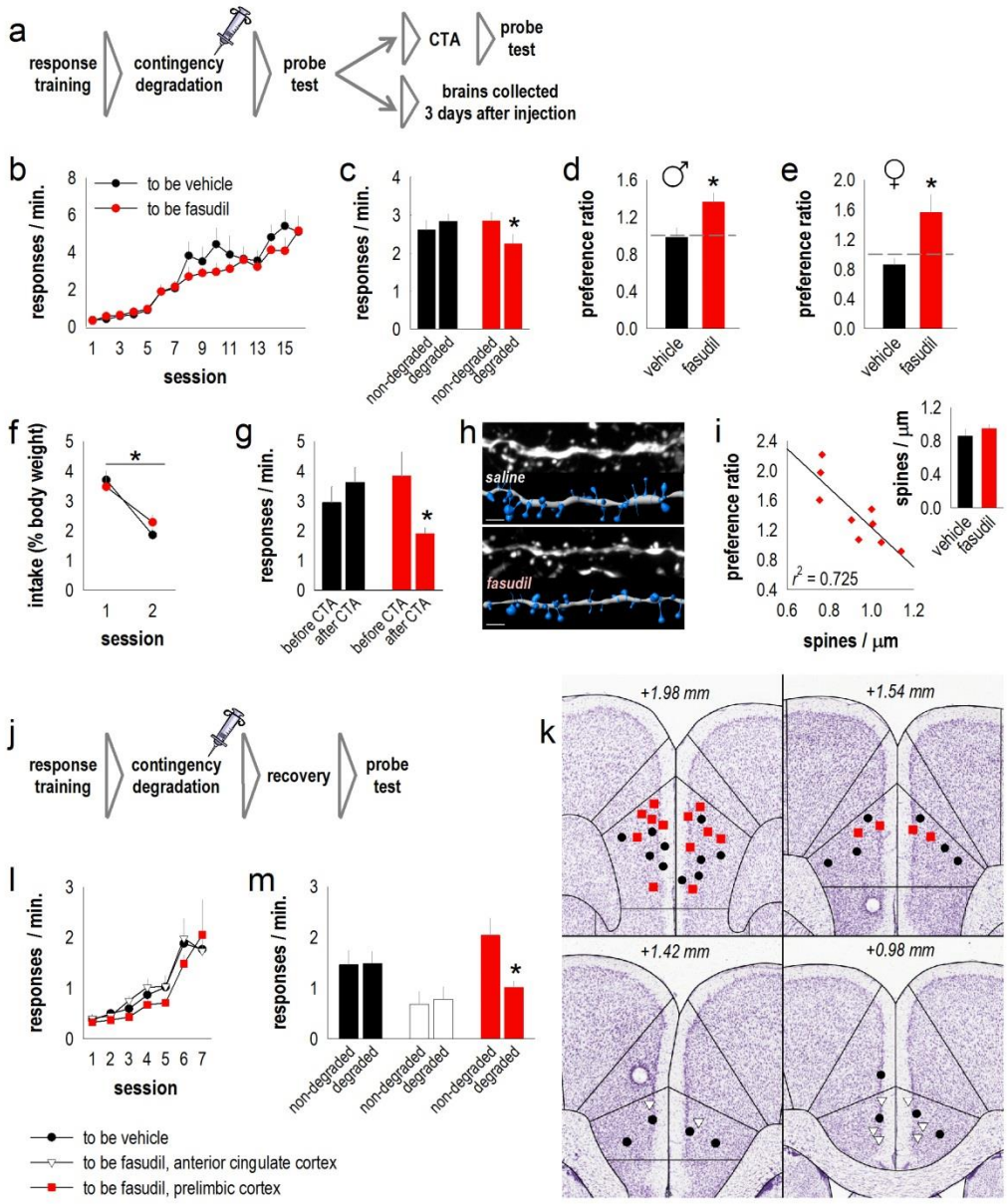

Figure 2. ROCK inhibition enhances goal-directed action selection. (a) Experimental timeline: Mice were trained using escalating random interval (RI) schedules of reinforcement to induce habit-based behavior. Immediately following instrumental contingency degradation, during the presumptive consolidation of new learning, mice were given a systemic injection of either vehicle (*n*=15) or the ROCK

inhibitor fasudil (*n*=17). Following a probe test, brains were either collected or mice were tested further for sensitivity to outcome value using lithium chloride (LiCl)-based conditioned taste aversion (CTA). (b) Groups did not differ during instrumental response acquisition. (c) Following instrumental contingency degradation, vehicle-treated mice did not differentiate between the responses that were likely, *vs.* unlikely, to be reinforced. However, fasudil-treated mice preferentially engaged the response most likely to be reinforced. (d) The same data are represented as preference ratios (non-degraded / degraded contingencies), with values >1 indicating goal-directed responding. Vehicle-treated mice responded at chance levels, while fasudil-treated mice utilized a goal-directed response strategy. (e) Although we focus our report on males, we found that female mice (*n*=7-8/group) were also sensitive to fasudil in the same task. (f) We next induced CTA (*n*=11-12/group), reducing the amount of food consumed over 2 pairings. (g) Despite CTA, vehicle-treated mice failed to reduce responding, consistent with habitual behavior following instrumental contingency degradation (in c). Meanwhile fasudil-treated mice reduced responding, sensitive to the now-reduced value of the reinforcer. (h) Representative prelimbic cortical dendritic segments with corresponding digital reconstructions below. (i) Dendritic spine densities correlated with response selection strategies, although spine densities did not differ between groups (inset). (j) Experimental timeline: Mice were again trained to respond on two nose-poke apertures for food reinforcement. Immediately after instrumental contingency degradation training, mice were anesthetized, and vehicle or fasudil was infused into either the prelimbic or anterior cingulate cortex (*n*=4-12/group). (k) Infusion sites, with coordinates relative to Bregma, are indicated on images from the Allen Brain Atlas (Allen Institute for Brain Science, 2012). (l) All mice acquired the responses, with no differences in responding. (m) In a probe test, vehicle-infused mice failed to differentiate between the responses that were more, *vs.* less, likely to be reinforced (non-degraded *vs.* degraded), deferring

to habit-based behavior. Prelimbic cortical fasudil infusions produced a strong preference for the response most likely to be reinforced, indicating goal-directed responding. Infusions terminating in the anterior cingulate cortex had no effects. Scale bar = 2 µm. Bars and symbols = means + SEMs except in i where diamonds represent individual mice. *p<0.05.

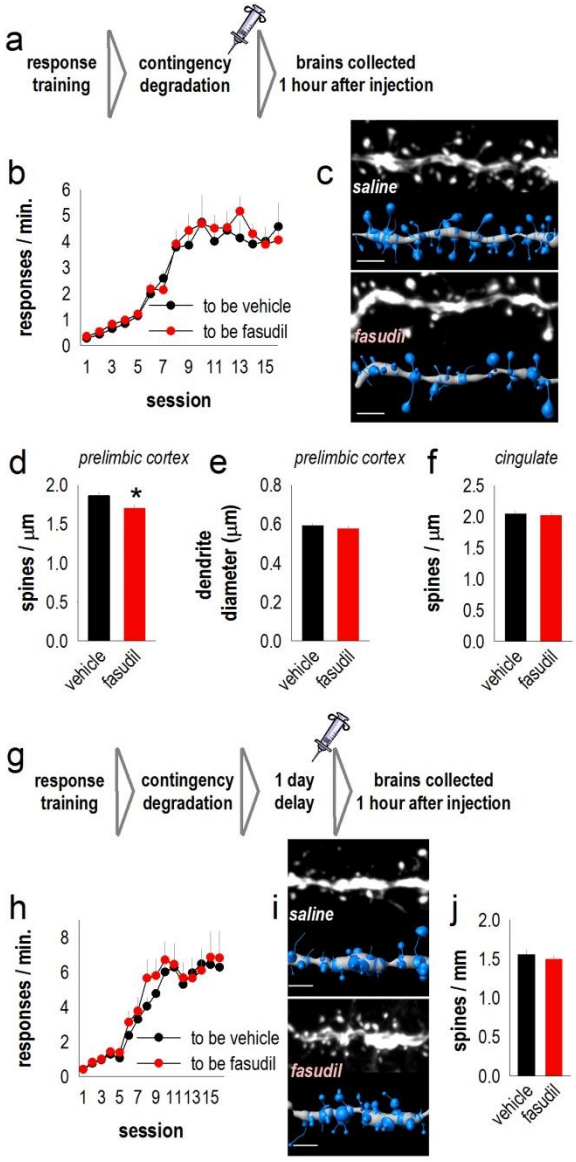

Figure 3. ROCK inhibition remodels prelimbic cortical dendritic spines. (a) Experimental timeline: Mice were given extensive response training to promote habit-based responding. A systemic injection of vehicle or fasudil was administered

immediately following instrumental contingency degradation, with brains collected 1 hour after the injection (*n*=8-9/group). (b) Mice acquired the instrumental responses, with no group differences. (c) Representative prelimbic cortical dendritic segments with corresponding digital reconstructions below. (d) Fasudil reduced prelimbic cortical dendritic spine density. (e) Dendrite diameter did not differ between groups. (f) Further, dendritic spine density in the adjacent anterior cingulate cortex did not differ between groups. (g) Experimental timeline: Another group of mice was trained and tested identically except that injections were administered 1 day after instrumental contingency degradation. Again, brains were collected 1 hour after the injection (*n*=7-8/group). (h) All mice acquired the responses without differences between groups. (i) Representative prelimbic cortical dendritic segments with corresponding digital reconstructions below. (j) A delayed injection of fasudil did not alter prelimbic cortical dendritic spine density, suggesting that fasudil-induced dendritic spine elimination is activity-dependent, *i.e.*, fasudil must be paired with a learning event to induce spine elimination. Scale bar = 2 µm. Bars and symbols = means + SEMs, *p<0.05.

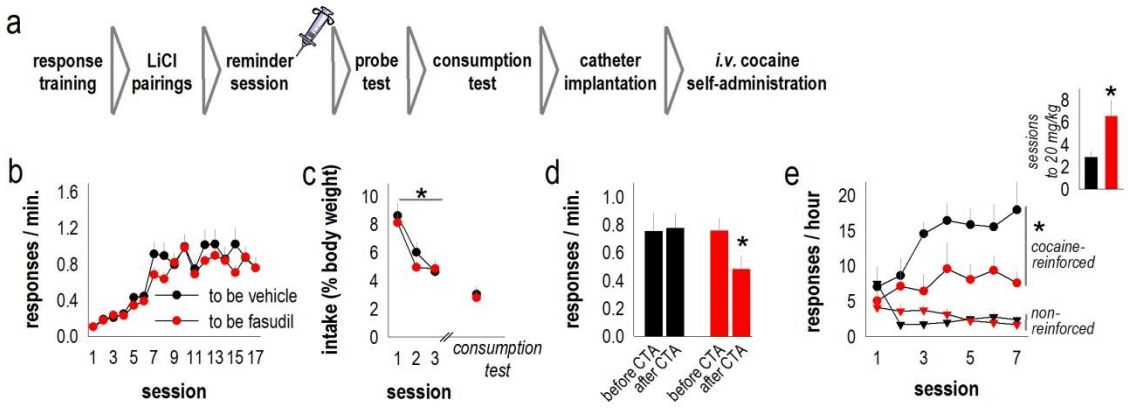

Figure 4. ROCK inhibition blocks habitual responding for cocaine. (a) Experimental timeline: Mice were trained to respond for an orally-ingested cocaine-sucrose solution. Then, mice were subject to LiCl-induced CTA. Mice were then placed in the conditioning chambers for a brief "reminder session," which served as an opportunity for mice to

update the association between the now-devalued outcome and responding. Mice were administered either vehicle or fasudil, followed by a probe test and finally, a post-probe consumption test. These mice were then implanted with indwelling jugular catheters for *i.v.* cocaine self-administration. (b) All mice responded for the cocaine-sucrose solution, without differences between groups (*n*=15-18/group). (c) During CTA, consumption diminished. (d) During a probe test, however, vehicle-treated mice did not reduce responding, despite acquired CTA, indicating insensitivity to the now-reduced value of the cocaine reinforcer. In contrast, fasudil-treated mice reduced responding, indicating sensitivity to outcome value. (e) Mice were trained in contextually-distinct chambers to self-administer *i.v.*-delivered cocaine. While all mice ultimately acquired the response, mice with a prior history of fasudil treatment responded less throughout and (inset) required more sessions to acquire 20 mg/kg. This indicates that fasudil enhanced sensitivity to devaluation of the cocaine reinforcer, and not simply the sucrose that was part of the orally-ingested solution. Bars and symbols = means + SEMs, *p<0.05.

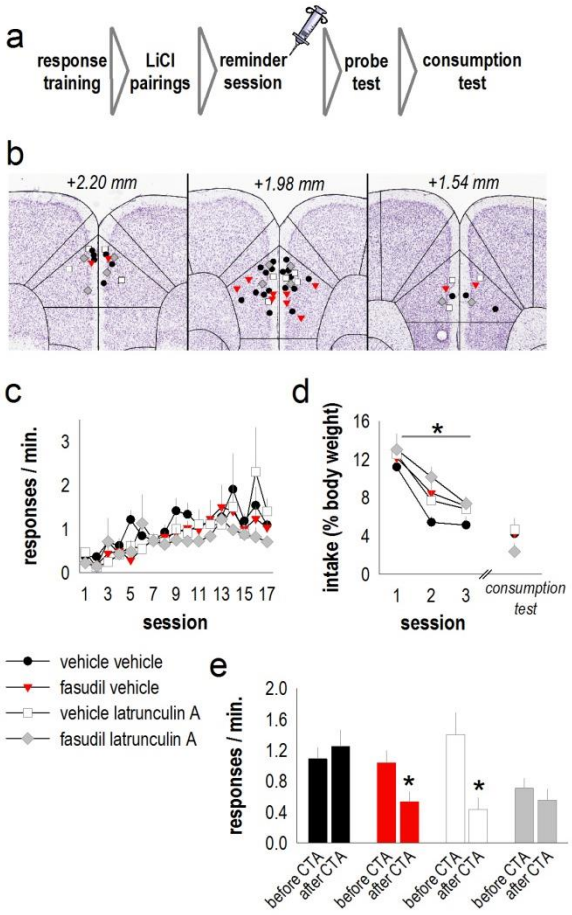

Figure 5. Blocking F-actin polymerization in the prelimbic cortex prevents ROCK inhibition from promoting goal-directed action selection. (a) As in fig.4, mice were extensively trained to respond for an orally-ingested cocaine-sucrose solution, followed by LiCl-mediated CTA and a brief "reminder session" paired with vehicle or fasudil. 30 minutes after this injection, vehicle or latrunculin A was infused into the prelimbic cortex. Probe and post-probe consumption tests followed. (b) Prelimbic cortical infusion sites are indicated on images from the Allen Brain Atlas (Allen Institute for Brain Science, 2012). (c) All mice acquired the cocaine-reinforced response, with no group differences (*n*=4-14/group). (d) CTA reduced cocaine-sucrose consumption. (e) During the probe test, however, vehicle-vehicle treated mice did not reduce responding, despite CTA, indicating a reliance on habitual response strategies. As expected, fasudil-vehicle treated mice reduced responding, showing sensitivity to the now-reduced value of the

reinforcer. This effect was blocked by prelimbic cortical-selective latrunculin A infusion. Bars and symbols = means + SEMs, *p<0.05.

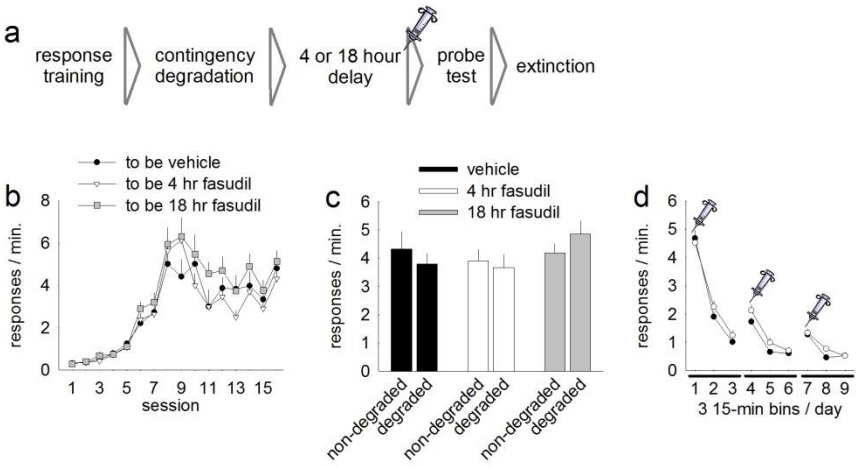

Figure S1. Delayed fasudil injection has no effects, and ROCK inhibition also does not influence response extinction. (a) Experimental timeline: To determine whether fasudil must be paired with an opportunity to learn new outcome-related information in order to enhance action-outcome conditioning, mice underwent identical response training as in main text fig.2, but here, injections were delayed either 4 or 18 hours following instrumental contingency degradation. We also tested the effects of fasudil on extinction conditioning. (b) Groups (to be vehicle *vs.* to be fasudil) were designated by matching response rates. Vehicle groups did not differ and were combined [no interaction F<1, main effect of session F(15,315)=51.9,p<0.05, no effect of group F(2,21)=1.1,p>0.05] (*n*=8/group). (c) In a probe test following delayed injections, no groups responded preferentially, instead relying on habitual response strategies [no interaction F<1, no effect of response F(1,42)=1.5,p>0.05, no effect of group F<1]. (d) Fasudil also did not alter response extinction [no interaction F<1, main effect of session F(8,176)=109.2,p<0.05, no effect of group F<1]. Bars and symbols = means + SEMs. *p<0.05.

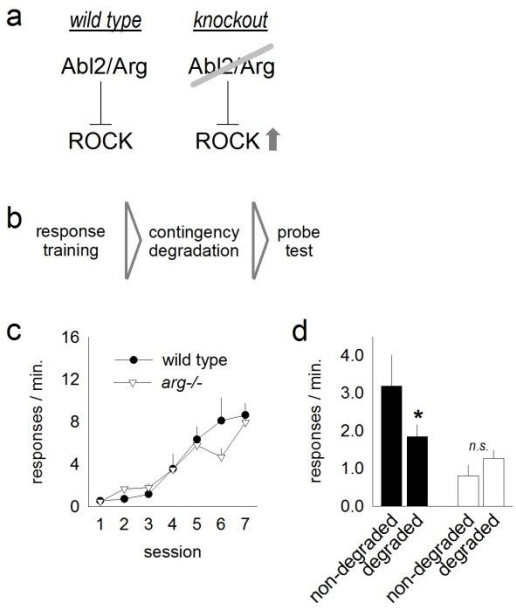

Figure S2. Ablating Arg kinase, an endogenous ROCK inhibitor, induces habit-based behavior. (a) In the brain, ROCK is endogenously suppressed by Abl2/Arg, such that ablating Arg disinhibits ROCK (Sfakianos et al., 2007) and causes failures in dendritic spine motility (Gourley et al., 2009). (b) Experimental timeline: Mice with reduced levels of Arg and their wild type littermates were trained to nose poke for food reinforcers, followed by instrumental contingency degradation and a probe test. (c) *arg*-/- mice did not differ from wild type mice during instrumental response acquisition [no interaction $F<1$, main effect of session $F(6,54)=14.9, p<0.05$, no effect of group $F<1$]. (d) Following instrumental contingency degradation, however, wild type mice exhibited a preference for the reinforced response – *i.e.*, goal-directed responding – while *arg*-/- mice showed no preference for the intact contingency, indicating habits-based responding [interaction $F(1,9)=4.5, p=0.05$] (*n*=5-6/group). Bars = means + SEMs, *$p<0.05$.

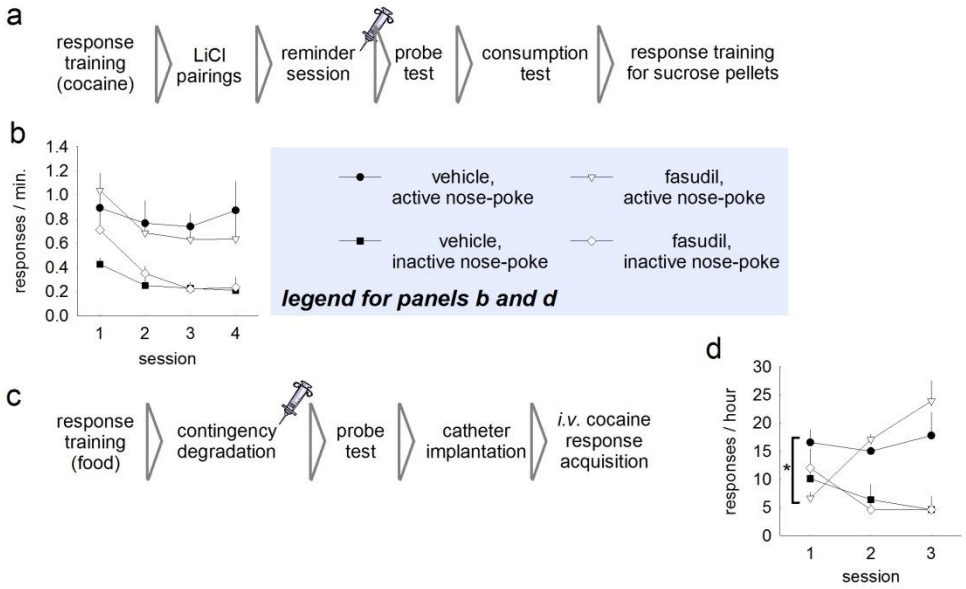

Figure S3. Fasudil selectively enhances sensitivity to action-outcome contingency. (a) Experimental timeline: Mice were trained to respond for an orally-ingested cocaine-sucrose solution, followed by LiCl-induced CTA (see main text fig.4). A brief "reminder session" was paired with vehicle or fasudil, followed by a probe test the following day and finally, a post-probe consumption test. These mice were then placed in distinct conditioning chambers and trained to respond for novel sucrose pellets. (b) Here, response rates did not differ between groups, evidence that fasudil selectively enhanced sensitivity to the reduced value of the cocaine reinforcer (compare to main text fig.4) and did not *generally* reduce response rates [no interaction $F(1,6)=2.4, p>0.05$, main effect of session $F(1,6)=17.1, p<0.05$, no effect of group $F<1$]. (c) Experimental timeline: Another cohort of mice originally generated to replicate our finding that fasudil enhanced sensitivity to action-outcome contingency degradation (see fig.2a-d) was implanted with indwelling jugular catheters. (d) Here, a history of fasudil decreased cocaine-reinforced responding on day 1, likely because operant responding had recently been unpaired from reinforcer delivery, but *unlike* when fasudil was paired with the devaluation of

cocaine, this group difference was quickly lost [interaction $F(2,12)=3.9$, $p<0.05$]. Bars and symbols = means + SEMs, *$p<0.05$.

Supplemental Table 1: Morphological measurements of prelimbic cortical dendritic spines

Corresponding to Fig.2	spine length (µm)	spine head diameter (µm)	spine volume (µm^3)
Saline	1.44 ± 0.021	0.38 ± 0.010	0.51 ± 0.020
Fasudil	1.37 ± 0.020	0.38 ± 0.009	0.51 ± 0.019
K-S test result	p=0.045	p=0.454	p=0.811
Corresponding to Fig.3			
Saline	1.24 ± 0.013	0.32 ± 0.005	0.36 ± 0.0002
Fasudil	1.28 ± 0.016	0.31 ± 0.005	0.34 ± 0.0002
K-S test result	p=0.012	p=0.538	p=0.323
Delayed saline	1.13 ± 0.014	0.41 ± 0.007	0.53 ± 0.014
Delayed fasudil	1.13 ± 0.013	0.38 ± 0.006	0.48 ± 0.012
K-S test result	p=0.907	p=0.023	p=0.033

Aggregate measurements of prelimbic cortical dendritic spine morphological parameters. Kolmogorov-Smirnov (K-S) tests were used to determine group differences. Values refer to means ± SEMs or p values as indicated.

Chapter 4: *In vivo* imaging of prelimbic cortical dendritic spines

Abstract

Dendritic spines are the primary sites of excitatory synapses in the brain, and growing evidence links their structure and densities with brain function. Neural remodeling and plasticity can be induced by external stimuli, including pathological stimuli like stress and drug exposure. For example, psychostimulants, like cocaine, are potent regulators of dendritic spine plasticity, and they also alter behavior. To develop better treatments for cocaine abuse, it may be critical to gain a better understanding of how cocaine impacts dendritic spines and how that in turn affects behavior. Prior studies have overwhelmingly used post-mortem samples to study cocaine-induced changes in the brain, typically well after cocaine exposure. Here, we focus on the acute behavioral and structural effects of cocaine. First, we show that when cocaine is paired with new learning about the relationship between an action and its outcome, memory consolidation is effectively disrupted. Next, we used *in vivo* multiphoton imaging to investigate the effects of cocaine on dendritic spines in the prelimbic cortex, a region of the brain that is necessary for consolidating action-outcome relationships. We found that while cocaine did not alter dendritic spine density in the first 30 minutes of exposure, cocaine decreased the rate at which dendritic spines were added and increased the rate of spine elimination from the dendrite. While our report focuses on male mice, we also used the same approach to quantify turnover rates in the prelimbic prefrontal cortex of female mice.

Introduction

An abundance of evidence shows that dendritic spine structure changes over time, that dendritic spines can be gained and lost, and that these changes can impact neural function (Yuste and Bonhoeffer, 2001; Bhatt et al., 2009). These processes begin early in development and continue throughout an organism's lifespan (Yuste and Bonhoeffer, 2004). Many studies have shown that external stimuli, such as stressor exposure and psychostimulants, can induce changes in dendritic spine stability and number (*e.g.*, reviewed DePoy and Gourley, 2015). Traditionally, these studies have been conducted using post-mortem samples, which allows for analysis of changes to the population of dendritic spines, but does not allow for tracking of individual spines over time. Growing evidence links dendritic spine structure and turnover to function (Kasai et al., 2003; Holtmaat and Svoboda, 2009), so understanding how external stimuli like stressor or drug exposure change individual spines is critical. It is also clear that dendritic spine structure and turnover can be altered very rapidly, which is often not observed in post-mortem studies since most studies utilize time points spaced multiple hours (to multiple days) apart, if multiple time points are collected at all.

One way of identifying rapid changes over time in individual dendritic spines is to image live samples at multiple time points. In rodents, this can be accomplished *in vivo* within an intact animal (Kerr and Denk, 2008). *In vivo* imaging has the advantage of being able to image dendritic spines in an intact animal with little-to-no disruption to the nervous system. However, the optical requirements for *in vivo* imaging restrict the depth at which dendritic spines can be imaged, specifically within a few hundred microns of the surface. Thus, this approach is limited to imaging brain regions that are very close to the surface of the brain, with deeper areas of the brain requiring more invasive techniques (Ghosh et al., 2011).

We focus here on the prelimbic prefrontal cortex, a portion of which is the most dorsal part of the brain, sitting directly beneath the skull and meninges, which makes a minimally damaging *in vivo* approach a potentially practical option. Generally, two *in vivo* methodologies are used to image dendritic spines just below the surface of the skull: a thinned skull approach (Yang et al., 2010) or a cranial window (Holtmaat et al., 2009). The thinned skull approach involves mechanically thinning a small portion of the skull to the point that it can be imaged through, while the cranial window involves complete removal of the skull and permanent implantation of a small coverslip where the skull was removed. Thinning the skull allows for acute imaging over a period of a few hours, while the cranial window is chronic and enables long-term imaging. We were interested in the immediate changes to prelimbic cortical dendritic spines after exposure to cocaine. Because of this short window of time, we elected to image prelimbic cortical dendritic spines using a thinned skull approach.

Cocaine produces rapid structural effects in several brain regions, including the prelimbic prefrontal cortex, where cocaine is generally believed to cause a proliferation of dendritic spines. We show that an acute injection of cocaine during the consolidation phase of action-outcome conditioning blocks subsequent goal-directed responding. This is in opposition to the effects of Rho-kinase (ROCK) inhibition in the same paradigm, which *enhances* goal-directed responding and *reduces* prelimbic cortical dendritic spine density (see Chapter 3). We hypothesized that cocaine's negative effects on action-outcome conditioning may be associated with rapid effects on prelimbic cortical dendritic spines. We found that an acute injection of cocaine increased the rate of dendritic spine loss, without changing gross density or dendritic spine morphology.

Although we focus our report on male mice, we also investigated dendritic spine turnover rates in the female prefrontal cortex. Together, these two experiments represent the first reported *in vivo* imaging of dendritic spines specifically in the prelimbic

prefrontal cortex, providing important information about how prelimbic cortical dendritic spine structure changes over a short period of time.

Results

Cocaine blocks the consolidation of action-outcome conditioning, occluding goal-directed responding

As in previous chapters, we used an action-outcome contingency degradation procedure to distinguish between goal-directed and habitual responding (fig.1a). Briefly, mice were trained in operant conditioning chambers to respond on 2 nose-poke apertures for food reinforcement. Mice received 5 days of continuous reinforcement training, followed by 2 days of random interval 30-second training. Sessions terminated when 30 reinforcers associated with each aperture were delivered, or at 135 minutes. Groups were matched based on responding during these training sessions [no interaction $F<1$] (fig.1b). For the contingency degradation procedure, one aperture was occluded, with free access to the remaining aperture, and responding was reinforced according to a variable ratio 2 schedule of reinforcement. In the next session, the opposite aperture was available, but responding was not reinforced. Instead, pellets were delivered at a rate matched to the previous session's reinforcement rate, "degrading" the action-outcome relationship associated with that response. Immediately after this session, mice were injected with either vehicle or 10 mg/kg cocaine *i.p.*

In a probe test the next day, mice again had simultaneous access to both apertures and could choose to direct their efforts to the intact or degraded contingency. The vehicle group preferentially generated the response that was most likely to be reinforced, indicating goal-directed action selection; in contrast, the cocaine group responded indiscriminately on each aperture, suggesting a reliance on habitual, stimulus-response strategies [interaction $F(1,22)=4.4, p<0.05$] (fig.1c). This pattern suggests that cocaine interferes with consolidation of action-outcome learning, resulting in a deferral to habit-based behavior.

Structural effects of acute cocaine in the prelimbic prefrontal cortex

Given that the prelimbic prefrontal cortex is necessary for learning about the relationship between an action and its outcome (Balleine and Dickinson, 1998; Corbit and Balleine, 2003; Killcross and Coutureau, 2003; Ostlund and Balleine, 2005), we hypothesized that cocaine's interference with action-outcome learning could be due to changes in prelimbic prefrontal cortical dendritic spines. To explore this hypothesis, we used a thinned skull preparation (see methods section for details) to image dendritic spines in the prelimbic prefrontal cortex in the absence and presence of cocaine.

Anesthetized mice were placed on the multiphoton microscope stage to obtain a field of view that contained distinct, resolvable dendritic segments. Immediately prior to the first imaging time point, an *i.p.* injection of vehicle (saline) was administered to the mouse. Images were collected 0, 15, and 30 minutes afterwards. Next, 10 mg/kg cocaine was administered *i.p.*, again with images collected 0, 15, and 30 minutes afterwards. ~105 dendritic spines/mouse were quantified from dendritic segments within the acquired z-stack (Table 1). Total dendritic length did not differ between conditions [$F<1$] (not shown). Gross dendritic spine density also did not differ between conditions [no drug x time interaction $F(2,16)=2.1, p=0.148$, no effect of time $F<1$, no effect of drug $F(1,8)=5.3, p=0.051$] (fig.2a,b).

While there were no changes in dendritic spine density over time, we were interested in determining whether there were any changes in dendritic spine *turnover* rates. To evaluate turnover rates, we calculated the number of spines gained or lost relative to the total number of spines present at the previous time point in the imaging session (Liston and Gan, 2011). Cocaine blunted the rate at which new spines appeared [no interaction $F<1$, main effect of drug $F(1,8)=12.4, p<0.05$] (fig.2c). When we analyzed spines that disappeared, we did not identify an overall difference between treatments [$F<1$], but by 30 minutes, cocaine caused significantly more dendritic spines to disappear

relative to vehicle [interaction $F(1,8)=34.6, p<0.05$] (fig.2d). We quantified dendritic spine length and head diameter in these same spines, with no differences between treatments at any single time point (Table 1), or over time [spine length: no drug x time interaction $F<1$, no main effect of drug $F(1,488)=1.745, p=0.187$, no main effect of time $F<1$; head diameter: no drug x time interaction $F(2,926)=2.6, p=0.071$, main effect of drug $F(1,463)=6.2, p=0.01$, no effect of time $F(2,926)=1.7, p=0.181$] (Table 1).

To summarize, while total dendritic spine densities, as well as dendritic spine morphologies, were similar between treatments, cocaine accelerated the disappearance of dendritic spines within the prelimbic prefrontal cortex. This could conceivably be a mechanism underlying cocaine's ability to interfere with consolidation of action-outcome learning and memory.

Turnover rates of cortical dendritic spines in female mice

We again used the thinned skull preparation, this time in female mice age postnatal day (P) 49-59. Z-stacks of a field of view containing prelimbic cortical dendritic segments were obtained every 30 minutes for 2 hours, or until image quality degraded to where dendritic spines were no longer resolvable. As above, dendritic spines from each animal were quantified from a number of dendritic segments (Table 2).

Total dendritic spine density did not change over the course of 2 hours [no effect of time $F<1$] (fig.3a,b), nor did the rate of new spine formation [no effect of time $F<1$] (fig.3c), or spine elimination [no effect of time $F<1$] (fig.3d). Dendritic spine length and head diameter were also quantified at each time point (Table 2). Dendritic spine length did not vary over time [$F(4,1000)=1.2, p=0.292$], nor did head diameter [$F(4,988)=2.3, p=0.053$] (Table 2). (A small number of spine head diameter values were lost during processing, resulting in differing degrees of freedom between dendritic spine length and head diameter analyses.) As a comparison, the values generated for females

were compared against the vehicle-treated males represented in fig.2 and Table 1. Dendritic spine densities did not differ between sexes at 0 or 30 minutes [no interaction $F(1,16)=1.4, p=0.259$, no main effect of sex $F(1,16)=1.1, p=0.311$, no main effect of time $F<1$] (fig.3e), nor did the number of spines gained [$t(16)=0.5, p=0.602$] (fig.3f) or spines lost [Mann-Whitney $U=20, p=0.077$] (fig.3g). When we compared dendritic spine length and head diameter between males and females, we found that at both 0 minutes and 30 minutes, length and head diameter were larger in males [K-S $ps<0.001$]. Due to the difficulties encountered when imaging females (discussed below), it is unclear whether these differences are methodological artifacts or meaningful differences. For example, given that the dendritic spines in females were uniformly smaller than in males, a possible explanation is that the dendrites imaged in females were more distal from the soma relative to those imaged in males.

Discussion

We report that cocaine administered immediately after an action-outcome learning event interferes with memory consolidation, such that vehicle-treated mice are able to flexibly modify their behavior to achieve a goal, while mice that received cocaine fail to modify their behavior. Thus, cocaine interferes with goal-directed behavior. Lesion studies indicate that the prelimbic prefrontal cortex is necessary for goal-directed responding (Balleine and Dickinson, 1998; Corbit and Balleine, 2003; Killcross and Coutureau, 2003), specifically for the acquisition and consolidation of the predictive relationships between actions and their outcomes (Ostlund and Balleine, 2005), and that psychostimulants like amphetamine and cocaine are potent regulators of prefrontal cortical dendritic spine structure (*e.g.*, Robinson and Kolb, 1997; Robinson and Kolb, 1999; Crombag et al., 2005). Given this evidence, we hypothesized that cocaine's interference with the consolidation of action-outcome learning and memory could be associated with changes in prelimbic prefrontal dendritic spines. In support of this perspective, we found that the rate of dendritic spine elimination was elevated 30 minutes after cocaine administration. The rate of spine formation was also blunted. Finally, we also quantified dendritic spine turnover rates female mice.

In Chapter 3, we reported that ROCK inhibition *enhanced* the consolidation of action-outcome learning and memory. Specifically, we found that in mice extensively trained such that they developed habit-based behavior that is by definition insensitive to action-outcome contingencies, a ROCK inhibitor restored sensitivity to action-outcome relationships and thereby induced goal-directed responding. This is the opposite of what we found with cocaine in this chapter, where control mice were trained such that they displayed sensitivity to the predictive relationship between actions and outcomes. Meanwhile, cocaine-treated mice responded indiscriminately, or habitually, indicating a failure in action-outcome conditioning.

In Chapter 3, we also found that ROCK inhibition transiently reduced dendritic spine density in the prelimbic cortex, while psychostimulants are historically associated with dendritic spine proliferation. We thus hypothesized that ROCK inhibition and cocaine affect memory consolidation in part by exerting opposing effects on prelimbic cortical dendritic spines, with ROCK inhibition eliminating spines to enhance signal:noise in the prelimbic cortex (see our discussion in Chapter 3), and acute cocaine exposure causing aberrant spine proliferation (Muñoz-Cuevas et al., 2013) to block memory consolidation. Countering our hypothesis, however, 10 mg/kg cocaine – a dose that affected memory consolidation (fig.1) – did not alter gross dendritic spine densities. Further, cocaine increased dendritic spine elimination rates within the prelimbic cortex.

One explanation for this apparent conflict is that ROCK inhibition-mediated spine elimination may be selective to dendritic spines endogenously active during the consolidation of action-outcome learning, while cocaine non-selectively affects dendritic spines. ROCK inhibition-mediated dendritic spine plasticity is indeed reported to be activity-dependent (Murakoshi et al., 2011). Further, we found that the ROCK inhibitor fasudil only eliminated prelimbic cortical dendritic spines when administered during the presumed consolidation phase of action-outcome learning and memory, with no effects on prelimbic cortical dendritic spine density or morphology when administered in the absence of an action-outcome learning event (see Chapter 3). In other words, ROCK inhibition-mediated dendritic spine elimination could be selectively enhancing the signal:noise ratio for prelimbic cortical neurons involved in action-outcome learning. Meanwhile, cocaine-mediated prelimbic cortical dendritic spine elimination could be non-specific, *decreasing* the signal:noise ratio of prelimbic cortical neurons and impairing those neurons' ability to facilitate action-outcome learning. Given that dendritic spines are the primary sites of excitatory synapses in the brain, and these connections are hypothesized to form networks that govern learning and memory, non-specific dendritic

spine loss could conceivably impair the ability of the prelimbic cortex to participate in the memory consolidation process.

A recent report (Radley et al., 2015) indicated that chronic cocaine self-administration results in a loss of prelimbic cortical dendritic spines, countering several prior reports that instead indicated that repeated amphetamine and cocaine exposure induces dendritic spine proliferation (reviewed DePoy and Gourley, 2015). This finding is nonetheless consistent with our own evidence that acute cocaine exposure reduces the rate of dendritic spine proliferation and that it facilitates elimination. As in the Radley report, we utilized fluorescence imaging, which can better identify thin-type spines, relative to Golgi impregnation methods (Shen et al., 2009). This methodological distinction could potentially account for disparate effects relative to prior reports. Considering our findings and those of Radley et al. (2015) together, it may be that the reduced rate of prelimbic cortical dendritic spine formation and accelerated disappearance of existing spines that occurs with acute cocaine administration may ultimately result in a long-term loss of prelimbic cortical dendritic spines with repeated cocaine exposure. A future study could aim to identify whether the acute effects of cocaine exposure reported here indeed culminate in gross dendritic spine loss upon repeated exposure.

Another recent study reported that acute cocaine increases prefrontal dendritic spine density and the rate of new spine formation 2 hours after injection (Muñoz-Cuevas et al., 2013). This study used a higher dose of cocaine (15 mg/kg vs. 10 mg/kg here) and imaged dendrites within the dorsal prefrontal cortex, which would include more regions than just the prelimbic cortex, *e.g.*, the anterior cingulate cortex. It is possible that cocaine has distinct effects on dendritic spines within the prelimbic cortex relative to other dorsal prefrontal regions, or that cocaine has contrasting temporal effects, with

changes in total density not becoming apparent until an hour or more after administration.

Relative to values typically reported in the literature, our dendritic spine densities are lower than those typically reported in the literature. This is likely due to only being able to target very distal dendritic segments, where dendritic spine densities are typically quite low (Duan et al., 2003; Benavides-Piccione et al., 2013). Further, our reported dendritic spine lengths and head diameters are likely inflated. While two stacks were collected at each time point, the close proximity of major blood vessels to the midline region of interest introduces significant motion artifacts even in the best circumstances. To ameliorate this issue, we flattened portions of the z-stack to form a maximum intensity projection, with the trade-off being some 'smearing' or enlarging of a spine's location across multiple slices, resulting in an apparent 'increase' in measured lengths. Another consideration is that our baseline turnover rates are higher than those reported by others (Wilbrecht et al., 2010; Liston and Gan, 2011), which again is likely attributable to difficulty in using this thinned-skull approach to image neurons so close to major blood vessels. Specifically, despite using a z-projection for analysis, motion of the brain would sometimes cause small portions of analyzed dendritic segments to shift completely out of the plane of focus at one time point, and completely reappear in the next time point, artificially inflating turnover rates.

While we were successful in imaging the acute effects of cocaine on prelimbic cortical dendritic spines in males, we had significantly less success using this same approach in female mice. Bleeding from the vessels in and around the thinned area was much more frequent and difficult to stop in females compared to males, both during the skull-thinning procedure and once multiphoton imaging began. This resulted in decreased image quality over successive time points in females and a concomitant reduction in the number of spines detected (16% loss in females relative to a 1% gain in

males over the course of 60 minutes, even despite cocaine injection in males after 30 minutes). Due to the difficulty in avoiding and stopping spontaneous bleeding, the thinned-skull preparations in females were not of the same quality as achieved in the males. Consequently, the reported values for dendritic spines imaged in female mice have more variance relative to the results from male mice. Interestingly, imaging in the secondary motor cortex, which is farther from the midline and is routinely imaged by other laboratory groups, did not increase success rates or image quality (not shown).

In the future, to selectively image prelimbic cortical dendritic spines *in vivo,* a different approach should be taken. One possibility is a chronic cranial window, which involves completely removing a portion of the skull and replacing it with an optical coverslip that is then imaged through. A chronic cranial window approach does not, however, completely avoid the issue of destabilizing the skull and vasculature near the midline and can trigger bleeding below the coverslip. An angled approach (starting above the secondary motor cortex and moving diagonally towards the midline) using a gradient-index (GRIN) lens could enable imaging of ‘deeper’ areas of the prelimbic cortex while avoiding the midline of the brain (Helmchen and Denk, 2005). It is a more invasive approach, since a portion of the secondary motor cortex would be removed and replaced with a GRIN lens assembly, but it could result in a more stable preparation and visible light access to dendritic spines closer to the soma.

Methods

Subjects: C57BL/6 wild type mice from Jackson labs were used for the instrumental conditioning experiment. C57BL/6 mice expressing *thy*1-YFP-H (Feng et al., 2000) and bred on-site were used for *in vivo* imaging. For the instrumental conditioning experiment, male mice were aged P42. For the subsequent *in vivo* imaging cocaine experiment, male mice were either adult (>P60) or ~P45 at the time of imaging. No differences between age groups were observed, so the groups were collapsed. Females were gonadally intact and ranged in age from P49-59. All procedures were approved by the Emory institutional animal care and use committee.

Drugs: 10 mg/kg cocaine (Sigma) was dissolved in sterile saline. For the behavioral experiment, cocaine was administered *i.p.* at the time indicated in fig.1. For the *in vivo* imaging experiments, cocaine was administered when indicated.

Instrumental conditioning: Male mice were food restricted to 90% of their original body weight. Med-associates operant conditioning chambers equipped with 3 distinct nose-poke recesses and a food magazine were used. Mice were trained to respond on the outer 2 recesses for reinforcement (20 mg grain-based pellets; Bioserv), with the center nose poke inactive. Mice were trained for 5 sessions using a continuous reinforcement schedule, followed by 2 sessions of training using a random interval 30-second schedule of reinforcement, each of which terminated after 30 pellets associated with each active aperture were delivered or at 120 minutes. This training protocol reliably produces goal-directed responding in wild type mice (that is, responding that is sensitive to action-outcome contingencies).

Action-outcome contingency degradation was conducted as previously described (Swanson et al., 2013; Zimmermann et al., 2017; DePoy et al., 2016; Gourley and

Taylor, 2016). Briefly, one aperture was occluded, and mice were given a 25-minute session with free access to the remaining aperture; responding was reinforced according to a variable ratio 2 schedule of reinforcement. In the next session, the opposite aperture was available, but responding was not reinforced. Instead, pellets were delivered at a rate matched to the previous session's reinforcement rate, effectively degrading the action-outcome relationship associated with that response. Immediately after this session, mice were administered either saline or 10 mg/kg cocaine *i.p.*

In the final session, both apertures were again available for a 10 minute probe test conducted in extinction. Preferential engagement of the response that remains reinforced (non-degraded condition) is considered goal-directed, while indiscriminate responding on each aperture is considered habit-based (Balleine and O'Doherty, 2010).

Thinned skull preparation: We adapted Gan's thinned skull approach (Pan and Gan, 2008) to image dendrites within the prelimbic prefrontal cortex. Mice were anesthetized with a ketamine/xylazine mixture (100mg/kg / 1 mg/kg). Once mice were sedated, the head was shaved and ocular ointment was placed on the eyes, and mice were then placed in a stereotaxic instrument. Alternating ethanol and betadine washes (3x each) were used to clean the shaved area. A scalpel was used to place a long midline incision in the scalp. Scissors were used to increase the length of the incision if needed, and the two skin flaps were pulled back. Spring scissors were used to cut away the connective tissue between the scalp and the skull, creating a large working area of exposed skull. The skull was dried and scrubbed to remove any remaining connective tissue. At +2.8 mm anterior from Bregma, the prelimbic cortex sits directly below the skull and extends approximately 500 µm laterally from the midline. Once this area was marked, the mouse was removed from the stereotaxic instrument.

We adapted Gan's method to enable imaging of prelimbic cortical dendritic spines by using the natural curvature of the skull to create a thin area to image through. The skin flaps were positioned to create a circular expanse of skull centered around the marked area. Next, cyanoacrylate glue was placed around the interior opening of a double-edged razor blade, which was then carefully centered over the marked area and placed in direct contact with the skull. Light pressure was applied for 5-10 seconds to secure the blade, then small forceps were used to pull the loose skin flaps up through the side opening of the razor blade, forming the beginning of a well that would ultimately help form the meniscus of the sterile saline for the dipping objective used for imaging. The razor blade was then secured to a custom stage adapter for use under a dissecting microscope and on the multiphoton microscope.

Using a hypodermic needle, small amounts of additional glue were added to seal any remaining gaps between the skin, the skull, and the razor blade. This was done to ensure that imaging media added to the thinned skull area would not escape the imaging area due to gravity. The preparation was allowed to dry for a few minutes. Next, sterile saline was placed into the now-formed well to begin to wash away any un-polymerized cyanoacrylate. Sterile Kimwipes were used to absorb up the sterile saline, and this was repeated as necessary until it was possible to clearly see the skull through the saline with the assistance of a stereo microscope.

Along the dorsal midline of the skull run major blood vessels that fill the space 'between' the dorsal portions of the two hemispheres of the brain. The main central vessel, the superior sagittal sinus is >200 µm wide at the dorsal surface, but the ventral portions are thinner (Cook, 2008). Bone surrounds this main vessel, but consists primarily of loose 'plates' that are less stable than other parts of the skull. Smaller vessels project laterally from this major vessel throughout the skull and along the surface of the brain. The dorsal portion of the brain has a roughly triangle-shaped 'gap' that is

where this vessel and the surrounding bone sits. Instead of making a symmetric, round depression in the skull like one can use when imaging more lateral brain regions, we instead thinned the skull directly adjacent to the midline blood vessels and along the curvature of the skull. The goal was to produce a very thin layer of curved skull without disrupting the major vessel or any of its lateral projections.

Due to the adjacency of our thinning to the central vessel, substantial bleeding from the lateral vessels occurred, which was resolved by either pausing, or continuing to thin and effectively crushing the bleeding vessel shut. If we were not in danger of cracking the ventral-most skull layer, removing the saline and letting the bleeding vessel dry in the air helped accelerate clotting. Air drying was not used if the lowest skull layer was unstable, to protect the meninges from drying out.

The initial thinning of the skull was accomplished using a high speed drill and a very small drill bit. A high drill speed was used to reduce the chance of the bit catching a portion of the skull and tearing through the entire skull and into the brain. This phase of the thinning consisted of lightly moving the bit back and forth over a large area of one hemisphere, with care being taken to not get too close to the midline. Once a smooth working surface was generated, slightly more pressure was applied along the curvature of the skull, such that the bit removed more skull bone close to the midline, and less material was removed from the lateral portions of the skull. Again, this was done slowly, with frequent stops. During these breaks, the media was refreshed and a hypodermic needle was used as a probe to examine skull thickness and stability. Once an angle was formed on the skull, the drill was set aside and a fine scalpel or hypodermic needle was used to manually scrape off the remaining skull to as thin as possible. The goal was to remove as much of the skull as possible without cracking it or applying too much pressure and damaging the meninges and brain tissue below the skull. This was visually detectable when the skull was smooth and often shiny in the absence of any media.

Once this point was reached, the stage adapter and mouse were transferred to the multiphoton microscope for imaging.

Multiphoton imaging: Images were acquired on a Leica SP8 system equipped with a Coherent Chameleon Vision II laser tuned to 920 nm. Initial targeting was completed using the confocal scanner and a 488 nm line from an Argon laser. A 25x 0.95 NA HCX IRAPO dipping objective and a HyD detector were used to acquire images. A 4x zoom was used, resulting in a pixel size of 0.065 µm, and a 0.5 µm step size. Laser power was set to as low as possible while still enabling clear image acquisition. For each time point, 2 z-stacks were acquired back to back, to minimize loss of image quality caused by motion artifacts like animal respiration. Respiration and anesthesia level were checked regularly throughout imaging, and additional anesthetic (20 mg/kg / 0.2 mg/kg ketamine/xylazine *i.p.*) was administered if an animal began to respond to a foot pinch.

A challenge of imaging an area near the midline is that bleeding tends to begin spontaneously. One advantage of thinning a V-shaped portion of the skull is that gravity moves any loose blood or red blood cells to the bottom of the V-shape, which helps keep the imaging area clear. However, when necessary, the media was removed, and the preparation rinsed several times and replaced with fresh media to improve image quality. Occasionally, bleeding would occur partway through an experiment that could not be stopped, at which point the experiment was terminated.

After imaging was complete, the multiphoton laser was turned to high power and used to photobleach an area adjacent to the imaged area to allow for post-mortem confirmation of the region of interest.

Histology: Mice were euthanized by rapid decapitation, and brains were submerged in 4% paraformaldehyde for 48 hours and then transferred to 30% w/v sucrose. Brains

were observed under a fluorescent dissecting microscope to confirm that the area imaged was within the prelimbic prefrontal cortex.

Dendritic spine quantification: Prior to analysis, a median filter, which replaced each pixel's value with the median of it and its neighbors values, was applied to all images to reduce noise in the image. A single rater, blinded to treatment condition and time point, then analyzed all dendritic spines in each experiment. Dendritic segments were selected for analysis on the basis of being present in most or all imaged time points, free of overlap from visual obstructions (*i.e.*, other dendritic material) that would impair visual detection of dendritic spines, and had good signal:noise ratio across time points. Once an appropriate dendritic segment was identified, a maximum intensity projection was made for each time point, with care taken to select sections above and below the plane of focus. Next, the dendrite was drawn, followed by drawing each spine's length and head diameter, with a unique identifier attached to each spine. This unique identifier was used to track the morphological parameters of dendritic spines over time. Turnover rates were quantified as a percentage of spines gained or lost relative to the total number of spines present in the first time point of the indicated interval (Liston and Gan, 2011).

Statistics: Instrumental response rates were compared by 2-factor ANOVA, with repeated measures as appropriate. Dendritic spine densities were compared by 1- or 2-factor repeated measure ANOVA. Turnover rates were compared by 2-factor repeated measure ANOVA. Turnover rates were compared between males and females by t-test or Mann-Whitney rank sum test in the case of non-normal distributions. Significant interactions were followed by Tukey's posthoc comparisons. Spine densities and turnover rates were square root transformed for normality if needed.

Dendritic spine length and head diameter were analyzed by the Kolmogorov-Smirnov test, with $p<0.001$ indicating significance. Additionally, a within-subject analysis of dendritic spine length and head diameter was accomplished using a 2-factor repeated measure ANOVA comparing spine parameters across time.

References

Attardo A, Fitzgerald JE, Schnitzer MJ (2015) Impermanence of dendritic spines in live adult CA1 hippocampus. Nature. 523:592-596.

Balleine BW, Dickinson A (1998) Goal-directed instrumental action: contingency and incentive learning and their cortical substrates. Neuropharmacology 37:407-419.

Balleine BW, O'Doherty JP (2010) Human and rodent homologies in action control: corticostriatal determinants of goal-directed and habitual action. Neuropsychopharmacology 35:48-69.

Benavides-Piccione R, Fernaud-Espinosa I, Robles V, Yuste R, DeFelipe J (2013) Age-based comparison of human dendritic spine structure using complete three-dimensional reconstructions. Cereb Cortex 23:1798-1810.

Bhatt DH, Zhang S, Gan WB (2009) Dendritic spine dynamics. Annu Rev Physiol 71:261-282.

Cook MJ (2008) The Anatomy of the Laboratory Mouse. Web: http://www.informatics.jax.org/cookbook/index.shtml

Corbit LH, Balleine BW (2003) The role of prelimbic cortex in instrumental conditioning. Behav Brain Res 146:145-157.

Crombag HS, Gorny G, Li Y, Kolb B, Robinson TE (2005) Opposite effects of amphetamine self-administration experience on dendritic spines in the medial and orbital prefrontal cortex. Cereb Cortex 15:341-348.

DePoy LM, Gourley SL (2015) Synaptic cytoskeletal plasticity in the prefrontal cortex following psychostimulant exposure. Traffic 16:919-940.

DePoy LM, Allen AG, Gourley SL (2016) Adolescent cocaine self-administration induces habit behavior in adulthood: sex differences and structural consequences. Transl Psychiatry 6:e875.

Duan H, Wearne SL, Rocher AB, Macedo A, Morrison JH, Hof PR (2003) Age-related dendritic and spine changes in corticocortically projecting neurons in macaque monkeys. Cereb Cortex 13:950-961.

Feng G, Mellor RH, Bernstein M, Keller-Peck C, Nguyen QT, Wallace M, Nerbonne JM, Lichtmann JW, Sanes JR (2000) Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. Neuron 28:41-51.

Ghosh KK, Burns LD, Cocker ED, Nimmerjahn A, Ziv Y, Gamal AE, Schnitzer MJ (2011) Miniaturized integration of a fluorescence microscope. Nat Methods 8:871-878.

Gourley SL, Taylor JR (2016) Going and stopping: dichotomies in behavioral control by the prefrontal cortex. Nat Neurosci 19:656-664.

Helmchen F, Denk W (2005) Deep tissue two-photon microscopy. Nat Methods 2:932-940.

Holtmaat A, Bonhoffer T, Chow DK, Chuckowree J, De Paola V, Hofer SB, Hübener M, Keck T, Knott G, Lee WC, Mostany R, Mrsic-Flogel TD, Nedivi E, Portera-Cailliau C, Svoboda K, Trachtenberg JT, Wilbrecht L (2009) Long-term, high resolution imaging in the mouse neocortex through a chronic cranial window. Nat Protoc 4:1128-1144.

Holtmaat A, Svoboda K (2009) Experience-dependent structural synaptic plasticity in the mammalian brain. Nat Rev Neurosci 10:647-658.

Kasai H, Matsuzaki M, Noguchi J, Yasumatsu N, Nakahara H (2003) Structure-stability-function relationships of dendritic spines. Trends Neurosci 26:360-368.

Kerr JN, Denk W (2008) Imaging in vivo: watching the brain in action. Nat Rev Neurosci 9:195-205.

Killcross S, Coutureau E (2003) Coordination of actions and habits in the medial prefrontal cortex of rats. Cereb Cortex 13:400-408.

Liston C, Gan WB (2011) Glucocorticoids are critical regulators of dendritic spine development and plasticity in vivo. Proc Natl Acad Sci USA 108:16074-16079.

Muñoz-Cuevas FJ, Athilingam J, Piscopo D, Wilbrecht L (2013) Cocaine-induced structural plasticity in frontal cortex correlates with conditioned place preference. Nat Neurosci 16:1367-1369.

Murakoshi H, Wang H, Yasuda R (2011) Local, persistent activation of Rho GTPase during plasticity of single dendritic spines. Nature 472:100-104.

Ostlund SB, Balleine BW (2005) Lesions of medial prefrontal cortex disrupt the acquisition but not the expression of goal-directed learning. J Neurosci 25:7764-7770.

Pan F, Gan WB (2008) Two-photon imaging of dendritic spine development in the mouse cortex. Dev Neurobiol 68:771-778.

Radley JJ, Anderson RM, Cosme CV, Glanz RM, Miller MC, Romig-Martin SA, LaLumiere RT (2015) The contingency of cocaine administration accounts for structural and functional medial prefrontal deficits and increased adrenocortical activation. J Neurosci 35:11897-11910.

Robinson TE, Kolb B (1997) Persistent structural modifications in nucleus accumbens and prefrontal cortex neurons produced by previous experience with amphetamine. J Neurosci 17:8491-8497.

Robinson TE, Kolb B (1999) Alterations in the morphology of dendrites and dendritic spines in the nucleus accumbens and prefrontal cortex following repeated treatment with amphetamine and cocaine. Eur J Neurosci 11:1598-1604.

Shen HW, Toda S, Moussawi K, Bouknight A, Zahm DS, Kalivas PW (2009) Altered dendritic spine plasticity in cocaine-withdrawn rats. J Neurosci 29:2876-2884.

Swanson AM, Shapiro LP, Whyte AJ, Gourley SL (2013) Glucocorticoid receptor regulation of action selection and prefrontal cortical dendritic spines. Commun Integr Biol 6:e26068.

Wilbrecht L, Holtmaat A, Wright N, Fox K, Svoboda K (2010) Structural plasticity underlies experience-dependent functional plasticity of cortical circuits. J Neurosci 30:4927-4932.

Yang G, Pan F, Parkhurst CN, Grutzendler J, Gan WB (2010) Thinned-skull cranial window technique for long-term imaging of the cortex in live mice. Nat Protoc 5:201-208.

Yuste R, Bonhoeffer T (2001) Morphological changes in dendritic spines associated with long-term synaptic plasticity. Annu Rev Neurosci 24:1071-1089.

Yuste R, Bonhoeffer T (2004) Genesis of dendritic spines: insights from ultrastructural and imaging studies. Nat Rev Neurosci 5:24-34.

Zimmermann KS, Yamin JA, Rainnie DG, Ressler KJ, Gourley SL (2017) Connections of the mouse orbitofrontal cortex and regulation of goal-directed action selection by brain-derived neurotrophic factor. Biol Psychiatry 81:366-377.

Figures

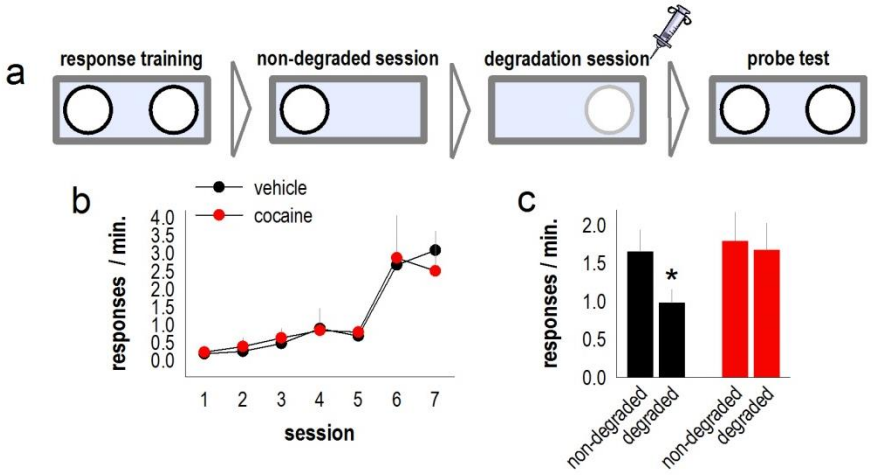

Figure 1: Cocaine interferes with consolidation of action-outcome learning and memory. (a) (Reproduced from Chapter 3) Task outline: Food-restricted mice are placed in operant conditioning chambers and trained to respond on two nose-poke apertures for food pellets. Following training, one nose-poke recess is available, and responding is reinforced. In another session, the opposite nose-poke recess is available, but responding is not reinforced; instead, food pellets are delivered at a rate matched to the reinforcement rate from the previous session, "degrading" the action-outcome contingency. During a probe test, mice have access to both nose-poke apertures. Preferential engagement of the response that is most likely to be reinforced is considered goal-directed, while engaging in both nose-poke responses non-selectively is considered a failure in action-outcome conditioning and a deferral to habit-based behavior. (b) All mice acquired the instrumental responses, with no differences between groups. (c) Following instrumental contingency degradation, vehicle-treated mice (*n*=14) preferentially engaged the response most likely to be reinforced. Mice administered cocaine did not differentiate between the responses, deferring instead to habit-based responding (*n*=10). Bars and symbols = means + SEMs, *p<0.05.

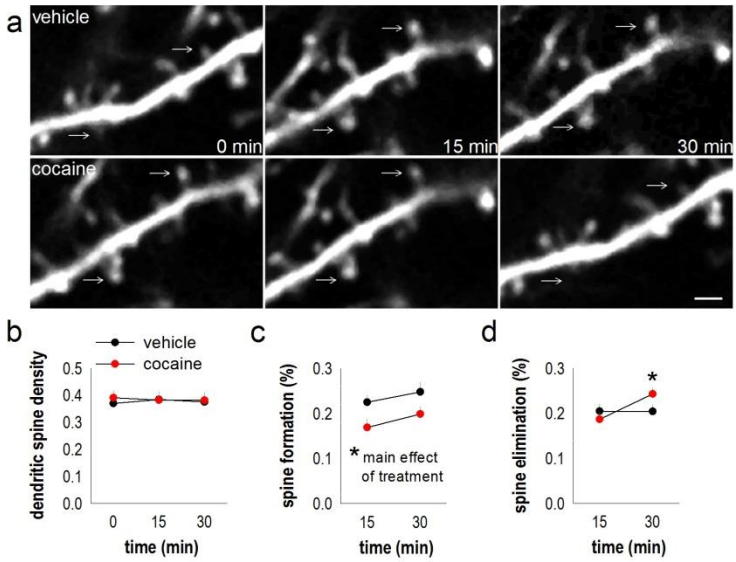

Figure 2: Cocaine decreases the rate of dendritic spine formation and increases the rate of elimination in the prelimbic cortex. (a) Representative prelimbic cortical dendritic segments. Arrows highlight spines that appear or disappear over time. (b) Cocaine did not alter overall dendritic spine density. (c) Cocaine reduced the rate at which new spines were formed, however, with spine formation being defined as the number of new spines relative to the total spines present at the previous time point. (d) By 30 minutes following injection, cocaine had increased the rate of dendritic spine elimination, as determined by number of spines eliminated relative to the total spines present at the previous time point. Scale bar = 2 µm, symbols = means + SEMs, *$p<0.05$.

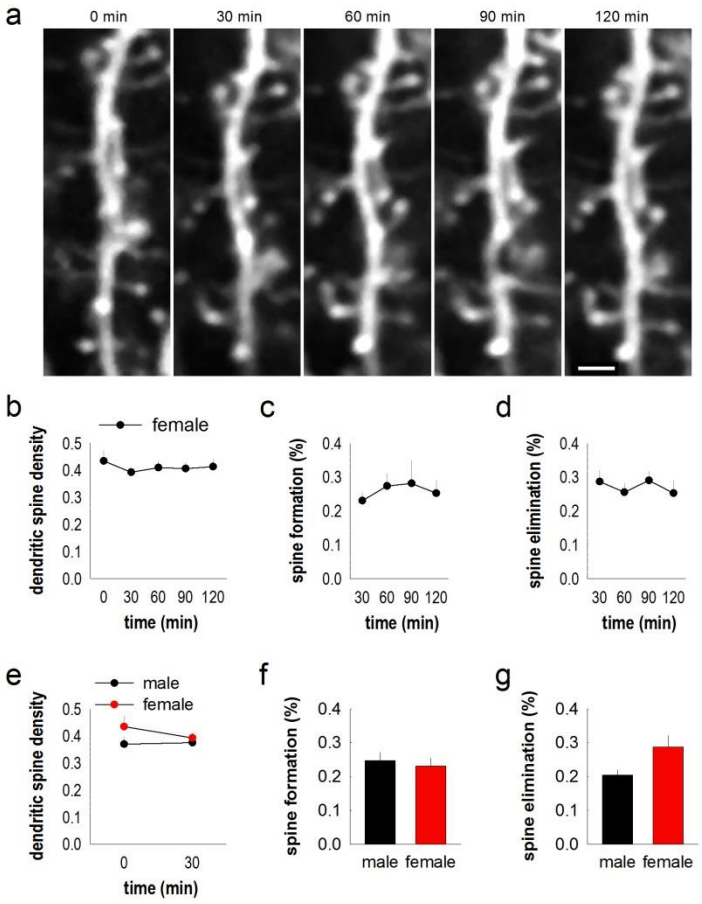

Figure 3: Densities and turnover rates of dendritic spines in the prelimbic prefrontal cortex of female mice. (a) Representative prelimbic cortical dendritic segments. (b) Gross dendritic spine density did not change over time in female mice. (c) The rate at which new spines were formed or (d) eliminated also did not change over time. (e) Dendritic spine densities did not differ between females and males (from fig.2). (f) After 30 minutes of imaging, dendritic spine formation rates did not differ between sexes, (g) nor did dendritic spine elimination rates. Scale bar = 2 µm, symbols and bars = means + SEMs.

Table 1: Morphological parameters of vehicle- and cocaine-treated prelimbic cortical dendritic spines

		time (min)		
		0	**15**	**30**
# of animals		9	9	9
# of spines	vehicle	916	934	955
	cocaine	969	958	929
spine length	vehicle	1.43 ± 0.022	1.45 ± 0.024	1.44 ± 0.024
(mean ± SEM)	cocaine	1.44 ± 0.024	1.45 ± 0.023	1.44 ± 0.023
K-S test p value		0.957	0.954	0.860
head diameter	vehicle	0.859 ± 0.011	0.895 ± 0.012	0.892 ± 0.012
(mean ± SEM)	cocaine	0.899 ± 0.012	0.874 ± 0.011	0.896 ± 0.012
K-S test p value		0.037	0.391	0.838
		0 vs 30 minutes		
Within-treatment comparison	vehicle	0.503		
K-S test p value	cocaine	0.993		
Within-treatment comparison, spine length (mean ± SEM)				
	vehicle	1.55 ± 0.03	1.54 ± 0.03	1.56 ± 0.03
	cocaine	1.56 ± 0.03	1.56 ± 0.03	1.56 ± 0.03
ANOVA	interact	p=0.592	drug p=0.187	time p=0.860
Within-treatment comparison, head diameter (mean ± SEM)	vehicle	0.90 ± 0.02	0.92 ± 0.02	0.94 ± 0.02
	cocaine	0.94 ± 0.02	0.94 ± 0.02	0.94 ± 0.02
ANOVA	interact	p=0.071	drug p=0.013	time p=0.181

Measurements of prelimbic cortical dendritic spines after vehicle and cocaine administration. **Top:** Kolmogorov-Smirnov (K-S) tests were used to determine group differences. **Bottom:** Results were also analyzed by 2-factor repeated measures ANOVA. These within-subject dendritic spine length and head diameter comparisons only included dendritic spines that were present at all time points. Values refer to means ± SEMs or p values as indicated.

Table 2: Morphological parameters of prefrontal cortical dendritic spines in female mice

	time (min)				
	0	**30**	**60**	**90**	**120**
# of animals	9	9	9	7	6
# of spines	1055	980	886	637	551
spine length (mean ± SEM)	1.248 ± 0.017	1.267 ± 0.018	1.278 ± 0.019	1.282 ± 0.022	1.310 ± 0.023
K-S test p value (vs. table 1)	<0.001	<0.001	n/a	n/a	n/a
head diameter (mean ± SEM)	0.761 ± 0.009	0.766 ± 0.008	0.777 ± 0.008	0.801 ± 0.010	0.810 ± 0.010
K-S test p value (vs. table 1)	<0.001	<0.001	n/a	n/a	n/a
Within-subject spine length (mean ± SEM)	1.32 ± 0.04	1.32 ± 0.03	1.37 ± 0.043	1.37 ± 0.04	1.33 ± 0.03
ANOVA	interact	p=0.292			
Within-subject head diameter (mean ± SEM)	0.82 ± 0.02	0.84 ± 0.02	0.86 ± 0.02	0.86 ± 0.02	0.84 ± 0.02
ANOVA	interact	p=0.053			

Measurements of prelimbic cortical dendritic spines in female mice. **Top:** Kolmogorov-Smirnov (K-S) tests were used to compare morphometric measures collected in female mice against those collected from male vehicle-treated mice in Table 1. **Bottom:** Results were also analyzed by repeated measures ANOVA. These within-subjects dendritic spine length and head diameter comparisons only included dendritic spines that were present in all time points. Values refer to means ± SEMs or p values as indicated.

Concluding discussion

Summary

Building on our earlier finding that prolonged exposure to the glucocorticoid receptor (GR) ligand corticosterone impairs action-outcome based decision-making (Gourley et al., 2012), we report here that deep-layer prelimbic cortical dendritic spines exhibit a dynamic response to corticosterone. During extended corticosterone, prelimbic cortical dendritic spine densities are reduced, while after a washout period, dendritic spine densities are increased. To provide a causal link between action-outcome sensitivity and prelimbic cortical dendritic spines, we demonstrate that acute GR blockade impairs sensitivity to action-outcome contingencies, thus blocking goal-directed responding, while pairing GR blockade with the Rho-kinase (ROCK) inhibitor fasudil preserved sensitivity to action-outcome relationships and goal-directed responding. We expanded on these findings by analyzing dendritic spines from several regions involved in goal-directed decision-making both during and after chronic corticosterone exposure. After a washout period, spine densities recovered in some, but not all, regions.

Within the prelimbic cortex, we found that dendritic spine densities on deep-layer neurons correlated with decision-making strategies, with lower densities associated with goal-directed responding, and higher densities associated with habit-based responding. We found that pairing the ROCK inhibitor fasudil with consolidation of an action-outcome learning event promoted goal-directed responding. Fasudil transiently reduced prelimbic cortical dendritic spine density when paired with consolidation, but had no effect on decision-making strategies or dendritic spine density when administered in the absence of consolidation. Fasudil blocked habitual responding for cocaine, suggesting fasudil may have therapeutic potential in the context of cocaine use disorders. Finally, we used *in vivo* imaging to examine normal prelimbic cortical dendritic spine plasticity and how it is impacted by cocaine. Together, our work highlights the importance of deep-layer prelimbic cortical dendritic spines in regulating decision-making strategies, and that

targeting cytoskeletal regulatory systems may have therapeutic potential in treating psychiatric disorders.

Fasudil's mechanism of action

Building on our findings from Chapters 1 and 2 that dendritic spines on deep-layer prelimbic cortical dendritic spines play an important role in regulating action-outcome sensitivity and goal-directed responding, we provide evidence in Chapter 3 that pairing ROCK inhibition with consolidation of outcome-learning restores goal-directed decision-making in mice that otherwise rely on stimulus-dependent response strategies. We focus on the consolidation phase of action-outcome contingency learning because the prelimbic cortex is necessary only for the acquisition, and not expression, of action-outcome learning (Ostlund and Balleine, 2005). In brains collected a few days after this pairing, we detected no differences prelimbic cortical dendritic spine density between vehicle and fasudil groups. However, in brains collected 1 hour after pairing fasudil with an action-outcome learning event, prelimbic cortical dendritic spine density was reduced in the fasudil group. In contrast, fasudil administered in the absence of an action-outcome learning event did not promote goal-directed responding nor did it alter prelimbic cortical dendritic spine densities in brains collected 1 hour after fasudil administration.

To integrate these findings into a working model, a few different situations should be considered (fig.1). First, mice that undergo a moderate amount of instrumental training will reliably exhibit goal-directed responding after undergoing action-outcome contingency degradation. We hypothesize that natural consolidation of an action-outcome learning event is facilitated by structural plasticity and remodeling of prelimbic cortical dendritic spines. Secondly, extensively trained mice lose sensitivity to changes in action-outcome relationships, and will continue to respond habitually after action-

outcome contingency degradation. Here, prelimbic cortical dendritic spines have reduced structural plasticity and do not remodel in response to action-outcome contingency degradation. These mice fail to consolidate that an action-outcome relationship has changed and consquently fail to modify their response strategy. However, if the ROCK inhibitor fasudil is administered in conjunction with the window of consolidation created by action-outcome contingency degradation, this disturbance of the actin cytoskeletal regulatory system acts as an enhancer of the structural plasticity of prelimbic cortical dendritic spines, facilitating consolidation and ultimately restoring goal-directed decision-making. For comparison, we can consider the control situation where mice with extensive training are not exposed to action-outcome contingency degradation and fasudil is instead administered in the absence of any sort of learning or consolidation opportunities. Here, fasudil fails to either alter prelimbic cortical dendritic spine density or restore goal-directed responding.

Cortical dendritic spines are remarkably stable over time, persisting over months and years (Bhatt et al., 2009), and almost all of these dendritic spines are part of a synapse (Arellano et al., 2007). We hypothesize that the amount of ROCK inhibition induced by a single injection of fasudil (10 mg/kg) alone is not enough to meaningfully disrupt the structural stability of dendritic spines. However, this same level of ROCK inhibition, combined with intrinsic structural plasticity induced by a learning event, is cumulative in nature, effectively restoring structural plasticity and enabling consolidation. This model raises two broad categories of questions: what is happening at the broader level of cortico-striatal circuits that determine decision-making strategies, and what the elimination of dendritic spines located on apical branches of deep-layer prelimbic cortical dendritic spines actually does.

The balance between actions and habits

The balance between action-outcome-oriented and stimulus-response-elicited decision-making appears to depend on a delicate balance between their associated neural circuits. The brain regions most closely associated with behavioral sensitivity to action-outcome relationships, and thus promoting goal-directed responding, are the prelimbic cortex and dorsomedial striatum. Meanwhile, stimulus-elicited habits are most closely associated with the dorsolateral striatum and somatosensory cortical areas (Balleine and O'Doherty, 2010). An unresolved question in the field is how the balance between the goal-directed and habitual circuits balance and interact.

Historically, significant attention has been paid to the striatal regions governing decision-making, specifically the dorsomedial and dorsolateral compartments. Growing evidence suggests that during the acquisition of a new task, both areas display high firing rates, with the dorsomedial striatum becoming quiet with repetition and habit formation (Yin et al., 2009; Thorn et al., 2010). This suggests that when these striatal regions are equally active, goal-directed responding takes precedence and that it is only once the dorsomedial striatum becomes quiet that habitual behaviors are engaged. A pattern similar to dorsomedial striatal activity is seen in the prelimbic prefrontal cortex, with neural activity decreasing with progressive amounts of training in a maze task and a concomitant shift to stimulus-response-based decision-making (Smith and Graybiel, 2013). Given that we found fasudil had long-term, persistent effects on action-outcome conditioning that were evident across contexts, one interpretation is that the impact of ROCK inhibition during the consolidation of action-outcome contingencies adjusts the balance between decision-making circuits, such that the neurocircuit governing action-outcome learning is once again as active, or more active, than the "habitual circuit."

Our argument is that effective consolidation of action-outcome learning requires structural plasticity of prelimbic cortical dendritic spines. A simplistic view of this is that when the prelimbic cortex, dorsomedial striatum and the broader "goal-directed circuit"

exhibit high levels of activity, this corresponds to high synaptic activity on prelimbic cortical dendritic spines. Once the “habit circuit” has taken prominence, synaptic activity on these same dendritic spines is reduced to the point that structural plasticity is no longer triggered after a learning opportunity, preventing consolidation. In essence, there is a threshold of synaptic activity that must be reached in order to re-active the prelimbic cortices involvement in action-outcome sensitivity. With ROCK inhibition, rather than increasing the level of synaptic activity above the threshold, the structure of the dendritic spines themselves becomes more plastic, such that the threshold for dendritic spine remodeling itself is reduced, enabling low levels of learning opportunity-related activity to induce structural remodeling. If this is the case, it raises the question of exactly what elimination of prelimbic cortical dendritic spines actually means.

Action-outcome learning and prelimbic cortical dendritic spine elimination

If the model is that elimination of prelimbic cortical dendritic spines, and thus synapses, increases prelimbic cortical function, an obvious possibility is that γ-aminobutyric acidergic (GABA) inhibitory synapses are being eliminated. Our studies focused on deep-layer prelimbic cortical dendritic spines located on secondary apical dendrites 50-100 μm from the soma. In the prelimbic cortex, only a small percentage of GABA synapses form on dendritic spines, with the majority forming on proximal dendritic shafts or the soma (Kuroda et al., 2004). It is possible that the modest number of spines eliminated by ROCK inhibition predominantly form GABAergic synapses. Further, axosomatic and axodendritic synapses also have actin filaments anchoring the post-synaptic side (Fifková and Delay, 1982), so those synapses could also be affected. Indeed, in rats with forebrain ischemia, chronic ROCK inhibition by fasudil was found to restore ischemia-induced impaired LTP function of hippocampal CA3 neurons by modulating $GABA_A$ and $GABA_B$ receptor expression levels (Huang et al., 2014).

Another possibility is that ROCK inhibition, whether by systemic or local administration, is going to affect all synapses of a prelimbic cortical neuron. Therefore, while we report elimination of dendritic spines on secondary apical dendrites 50-100 μm from the soma, the “important” synapses are located elsewhere on the cell. We may only be describing a small piece of the overall mechanism and that there is actually fairly substantial remodeling of deep-layer prelimbic cortical dendritic spines. Future experiments should attempt to identify the specific types of spines being eliminated, both within the region of the dendritic arbor analyzed, as well as elsewhere on the cell. Repeating the behavioral experiments described in Chapter 3 in conjunction with dendritic spine analysis and immunofluorescence labeling of various synaptic markers (GABA, glutamate, dopamine, etc.) would potentially characterize which synapses are being eliminated. This could be coupled that with a broader survey of more dendritic regions to create a relatively clear map both of the normal distribution of synapse type on deep-layer prelimbic cortical neurons but also which ones are involved in fasudil-mediated action-outcome learning.

This leads to the question of whether the elimination of prelimbic cortical dendritic spines by fasudil to promote goal-directed decision-making mimics the endogenous mechanisms of action-outcome learning. One way to answer this question would be turn to the work of Rui Costa who has shown that by using two different schedules of reinforcement during training, one can control for the number of sessions, responses, and reinforcers, and generate one group of mice that exhibit goal-directed responding while the other group relies on habit-based response strategies (Hilário et al., 2007; Gremel and Costa, 2013). One could collect brains from *thy*1-YFP mice shortly after completing contingency degradation, as described in Chapter 3, and analyze prelimbic cortical dendritic spines. If, when comparing the goal-directed vs. habitual groups, we see a similar reduction in prelimbic cortical dendritic spine density in the goal-directed

group, it would suggest that fasudil is simply enhancing endogenous systems. If we do not see a difference between groups, or some other effect on dendritic spines, it would suggest that fasudil is enhancing action-outcome sensitivity by a novel mechanism.

Ultimately, we only analyzed a subset of dendritic spines on layer V prelimbic cortical dendritic spines. In addition to more detailed analysis of other dendritic regions from these same regions, our use of *thy*1-YFP-H mice has generated a library of brains that have fluorescently labeled neurons from many brain regions. With no additional sample preparation steps, these brains could be used to analyze dendritic spines located on brain regions implicated in goal-directed and habitual decision-making. Regions of particular interest include, but are not limited to, the infralimbic and orbitofrontal cortices, hippocampal CA1 and CA3 neurons, and BLA neurons (Balleine and O'Doherty, 2010). Unfortunately, *thy*1-YFP-H is only expressed in excitatory neurons, and there is virtually no expression in striatal neurons. Similar analysis of dorsomedial and dorsolateral striatal dendritic spine involvement would be extremely interesting, but would require repeating the experiments from the beginning with either a different transgenic mouse or another way to fluorescently label striatal neurons. For the same reason, investigating the role of layer II/III prelimbic cortical neurons and dendritic spines would require another way to generate fluorescently labelled neurons.

Correlative vs. causative mechanisms of decision-making strategies

In Chapter 3, we described a correlation between deep-layer prelimbic cortical dendritic spine density and decision-making strategies. Lower dendritic spine densities were associated with goal-directed responding, while higher spine densities were associated with habitual responding. At the time, we used this correlation as support for the hypothesis that if one could reduce prelimbic cortical dendritic spine density, one could shift decision-making strategies away from habits and towards goal-directed

responding. Interestingly, the animals represented in that correlation were a mix of vehicle- and fasudil-treated mice, and no group differences in spine density or morphology were detected. Another interpretation of the correlation is that it instead describes an intrinsic biological set point for susceptibility to habit formation. Even though our *thy*1-YFP mice are on an inbred C57BL/6 background, we still observe individual differences in neuronal morphology, body size, and behavior. It's possible that if one could measure deep-layer prelimbic cortical dendritic spine density ahead of time, it would serve as a predictor for how quickly an individual animal or person would shift to habit-based response strategies, potentially serving as indicator for susceptibility to substance use disorders.

Regardless, we did demonstrate that the ROCK inhibitor fasudil enhanced goal-directed decision-making and also reduced prelimbic cortical dendritic spine density. Ultimately, those findings are still only correlative. Later in chapter 3 we demonstrated that Latrunculin A, which prevents F-actin polymerization (Toda et al., 2010), in conjunction with fasudil paired with action-outcome contingency degradation blocked fasudil's effects on decision-making strategies, providing indirect evidence for a causative link between prelimbic cortical dendritic spine elimination and enhanced goal-directed responding.

What is missing, in part because an appropriate tool may not yet exist, is a way to directly test this correlation. Ideally there would be an experimental approach to selectively shift prelimbic dendritic spine densities before training begins. One could deliver a virus to the prelimbic cortex that would express a gene whose protein would either increase or decrease dendritic spine densities for a prolonged period of time, test the mice for action-outcome sensitivity, and then analyze dendritic spines post-mortem. While we have identified a number of genes that impact dendritic spine density, they invariably also affect other biological systems. One of the most well studied examples is

the *Fmr1* gene. Absence of *Fmr1* in humans results in fragile X syndrome. *Fmr1* knockout results in increased dendritic spine density, but also alters dendritic spine morphology and stability, resulting in impairments in hippocampal-dependent learning and memory and other cognitive tasks (He and Portera-Cailliau, 2013). Using a viral construct that has these or similar 'additional' consequences would confound any conclusions made about causal relationships between dendritic spine density and decision-making strategies. Even if a direct comparison could be made, it still would not rule out the questions raised above regarding other prelimbic cortical layers, brain regions, or whether the dendritic spine elimination caused by fasudil is even the relevant mechanism to be studying.

In vivo imaging of prelimbic cortical dendritic spines

Detailed study of dendritic spine morphology, as well as how it relates to neural function has been occurring for well over 50 years now, and the vast majority of that research has been conducted on post-mortem samples. It is only in the past handful of years that live imaging of dendritic spines within an intact animal has been possible, and while the number is increasing, there are still only a few labs with this capability. However, the methods are becoming established and streamlined, the equipment is more widely available, and the number of researchers proficient in the methodologies is increasing and so it seems as if a transition point is approaching where this approach will become relatively common. This is in part due to the fact that post-mortem samples by definition represent a single point in time, and we now know that dendritic spines are remarkably plastic in their structure and function, necessitating the analysis of dendritic spines over time.

However, a major limitation to the established approaches of either thinning the skull or implanting a chronic cranial window is that the depth of resolvable imaging is at

most 1 mm, and while mouse brains are small, the vast majority of the brain is more than 1mm below the skull. And while there has been significant attention paid to which technique is the least invasive and therefore yields the 'purest' data (Xu et al., 2007; Dorand et al., 2014), it seems that these approaches will largely fall by the wayside in favor of techniques that are capable of imaging much deeper regions of the brain, despite being much more invasive.

While first detailed over a decade ago (Helmchen and Denk, 2005), microlenses, such as gradient-index (GRIN) lenses, can be used in conjunction with traditional *in vivo* multiphoton systems to image dendritic spines in deep tissue (Barretto et al., 2009; Attardo et al., 2015). To outline this approach, it involves surrounding the cylindrical microlens assembly with a biologically inert wrapper and then surgically implanting it into the brain above the area of interest, such that the target area is within the field of view of the microlens. Then a traditional multiphoton laser scanning microscope with a dipping lens is used to image through the microlens assembly and into deep tissue. In terms of invasiveness, this is similar to implanting a cannula, an approach that is already widely used in the fields of behavioral neuroscience.

So while portions of the prelimbic cortex are on the dorsal surface of the brain and are within the depth limitations of traditional *in vivo* multiphoton imaging approaches, its direct adjacency to the superior sagittal sinus makes the thinned-skull technique an extremely problematic approach, as detailed in chapter 4. Implantation of a microlens assembly that is oriented diagonally through the secondary motor cortex towards the prelimbic cortex is much more likely to yield successful live imaging of prelimbic cortical dendritic spines.

Conclusion

The personal, societal, and economic burdens of addiction, depression, and other psychopathologies make it imperative that we develop better treatments for these disorders. Many of these behaviors and pathologies can be modelled as stimulus-response-driven behaviors, so developing a better understanding of how habits overtake actions, and how to reverse that process and “break” habits may have broad therapeutic potential. While our work has focused on enhancing the consolidation of action-outcome learning and memory, it can be viewed more broadly as an attempt to target cytoskeletal regulatory systems as potential treatments for psychopathologies.

Relative to cancer research, where cytoskeletal systems are primary targets for drug development (Fife et al., 2014), little attention is paid to cytoskeletal systems as therapeutic targets. Due to the prominence of the cytoskeleton in cancer drug development, there is a large existing pool of drugs targeting cytoskeletal systems, and many pharmaceutical companies and research institutions have groups dedicated to the screening and development of new cytoskeletal drugs, drugs that could prove useful in treating psychiatric disorders (Rao and Li, 2004). However, the potential of cytoskeletal systems as potential treatment targets in neuroscience is gaining more attention as new findings are published (Rothenfluh and Cowan 2013; DePoy and Gourley, 2015; Young et al., 2015). We report here that the ROCK inhibitor fasudil can be used to block habitual responding for cocaine, but it’s quite likely that drugs targeting different cytoskeletal regulatory proteins would have similar beneficial effects in the treatment of psychiatric disorders.

References

Arellano JI, Espinosa A, Fairén A, Yuste R, DeFelipe J (2007) Neuroscience 145:464-469.

Attardo A, Fitzgerald JE, Schnitzer MJ (2015) Impermanence of dendritic spines in live adult CA1 hippocampus. Nature 523:592-596.

Balleine BW, O'Doherty JP (2010) Human and rodent homologies in action control: corticostriatal determinants of goal-directed and habitual action. Neuropsychopharmacology 35:48-69.

Barretto RP1, Messerschmidt B, Schnitzer MJ (2009) In vivo fluorescence imaging with high-resolution microlenses. Nat Methods 6:511-512.

Bhatt DH, Zhang S, Gan WB (2009) Dendritic spine dynamics. Annu Rev Physiol 71:261-282.

DePoy LM, Gourley SL (2015) Synaptic Cytoskeletal Plasticity in the Prefrontal Cortex Following Psychostimulant Exposure. Traffic 16:919-940.

Dorand RD, Barkauskas DS, Evans TA, Petrosiute A, Huang AY (2014) Comparison of intravital thinned skull and cranial window approaches to study CNS immunobiology in the mouse cortex. Intravital 3:e29728.

Fife CM, McCarroll JA, Kavallaris M (2014) Movers and shakers: cell cytoskeleton in cancer metastasis. Br J Pharmacol 171:5507-5523.

Fifková E, Delay RJ (1982) Cytoplasmic actin in neuronal processes as a possible mediator of synaptic plasticity. J Cell Biol 95:345-350.

Gourley SL, Swanson AM, Jacobs AM, Howell JL, Mo M, DiLeone RJ, Koleske AJ, Taylor JR (2012) Action control is mediated by prefrontal BDNF and glucocorticoids. Proc Natl Acad Sci U S A 109:20714-20719.

Gremel CM1, Costa RM (2013) Orbitofrontal and striatal circuits dynamically encode the shift between goal-directed and habitual actions. Nat Commun 4:2264.

He CX, Portera-Cailliau C (2013) The trouble with spines in fragile X syndrome: density, maturity and plasticity. Neuroscience 251:120-128.

Helmchen F, Denk W (2005) Deep tissue two-photon microscopy. Nat Methods 2:932-940.

Hilário MR1, Clouse E, Yin HH, Costa RM (2007) Endocannabinoid signaling is critical for habit formation. Front Integr Neurosci 1:6.

Huang L, Zhao LB, Yu ZY, He XJ, Ma LP, Li N, Guo LJ, Feng WY (2014) Long-term inhibition of Rho-kinase restores the LTP impaired in chronic forebrain ischemia rats by regulating GABAA and GABAB receptors. Neuroscience 277:383-391.

Kuroda M, Yokofujita J, Oda S, Price JL (2004) Synaptic relationships between axon terminals from the mediodorsal thalamic nucleus and gamma-aminobutyric acidergic cortical cells in the prelimbic cortex of the rat. J Comp Neurol 477:220-234.

McLellan AT, Lewis DC, O'Brien CP, Kleber HD (2000) Drug dependence, a chronic medical illness: implications for treatment, insurance, and outcomes evaluation. JAMA 284:1689-1695.

Murakoshi H, Wang H, Yasuda R (2011) Local, persistent activation of Rho GTPases during plasticity of single dendritic spines. Nature 471:100-104.

Ostlund SB, Balleine BW (2005) Lesions of medial prefrontal cortex disrupt the acquisition but not the expression of goal-directed learning. J Neurosci 25:7764-7770.

Rao J, Li N (2004) Microfilament actin remodeling as a potential target for cancer drug development. Curr Cancer Drug Targets 4:345-54.

Rothenfluh A, Cowan CW (2013) Emerging roles of actin cytoskeleton regulating enzymes in drug addiction: actin or reactin'? Curr Opin Neurobiol 23:507-512.

Schubert V, Da Silva JS, Dotti CG (2006) Localized recruitment and activation of RhoA underlies dendritic spine morphology in a glutamate receptor-dependent manner. J Cell Bio 30:453-467.

Smith KS, Graybiel AM (2013) A dual operator view of habitual behavior reflecting cortical and striatal dynamics. Neuron 79:361-374.

Thorn CA, Atallah H, Howe M, Graybiel AM (2010) Differential dynamics of activity changes in dorsolateral and dorsomedial striatal loops during learning. Neuron 66:781-795.

Toda S, Shen H, Kalivas PW (2010) Inhibition of actin polymerization prevents cocaine-induced changes in spine morphology in the nucleus accumbens. Neurotox Res 18:410-415.

Xu HT, Pan F, Yang G, Gan WB (2007) Choice of cranial window type for in vivo imaging affects dendritic spine turnover in the cortex. Nat Neurosci 10:549-551.

Yin HH, Mulcare SP, Hilário MR, Clouse E, Holloway T, Davis MI, Hansson AC, Lovinger DM, Costa RM (2009) Dynamic reorganization of striatal circuits during the acquisition and consolidation of a skill. Nat Neurosci 12:333-341.

Young EJ, Briggs SB, Miller CA (2015) The Actin Cytoskeleton as a Therapeutic Target for the Prevention of Relapse to Methamphetamine Use. CNS Neurol Disord Drug Targets 14:731-737.

Figures

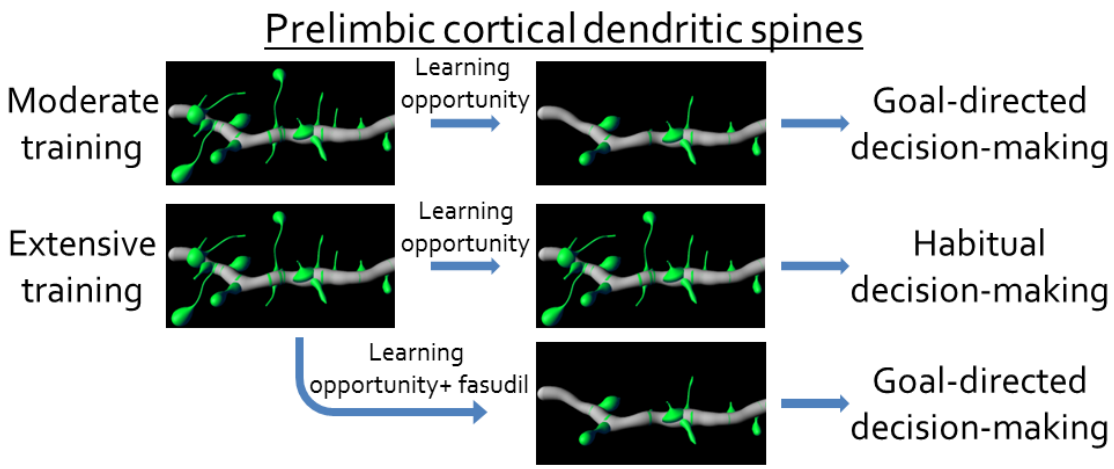

Figure 1. ROCK inhibition facilitates remodeling of prelimbic cortical dendritic spines, promoting goal-directed decision-making. Mice with moderate training undergo action-outcome contingency degradation to update their knowledge of action-outcome relationships, prelimbic cortical dendritic spines plasticity facilitates consolidation of this learning, and the mice exhibit goal-directed responding when tested. In contrast, mice with extensive training are insensitive to action-outcome contingency degradation, do not have structural plasticity of prelimbic dendritic spines, and rely on habitual response strategies when tested. However, intervening with fasudil restores structural plasticity of prelimbic cortical dendritic spines, restoring sensitivity to action-outcome contingencies, and enabling goal-directed responding.