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Glucocorticoid receptor regulation of action selection and prefrontal cortical dendritic spines

Andrew M Swanson[1,2,3], Lauren P Shapiro[1,2,4], Alonzo J Whyte[1,2], Shannon L Gourley[1,2,3]*

[1]Department of Pediatrics; Emory School of Medicine; Atlanta, GA USA; [2]Yerkes National Primate Research Center; Emory University; Atlanta, GA USA; [3]Graduate Program in Neuroscience; Emory University; Atlanta, GA USA [4]Molecular and Systems Pharmacology; Emory University; Atlanta, GA USA

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We recently 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.; *Proceedings of the National Academy of Sciences*, 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 simultaneously with 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 provide evidence, using a Rho-kinase inhibitor, that GR-mediated dendritic spine remodeling is causally related to complex decision-making. Finally, we conclude this report with evidence that a history of early-life (adolescent) GR blockade, unlike acute blockade in adulthood, enhances subsequent outcome-based 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, but a history of GR blockade may, under some circumstances, also have beneficial consequences.

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.[1,2] 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 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 recently reported 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.[3] Chronic ligand binding can desensitize GRs,[4] so this pattern suggested to us that desensitization of GRs confers vulnerability to the development of stimulus-response habits.[3] 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,[5–8] and this shorter phenotype is suggestive of a greater density of immature "stubby" spines and increased dendritic spine turnover.[9–11] 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.[3] 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. In this Article Addenda, we delve deeper into the complex relationship between deep-layer prelimbic cortical

*Correspondence to: Shannon L Gourley; Email: shannon.l.gourley@emory.edu
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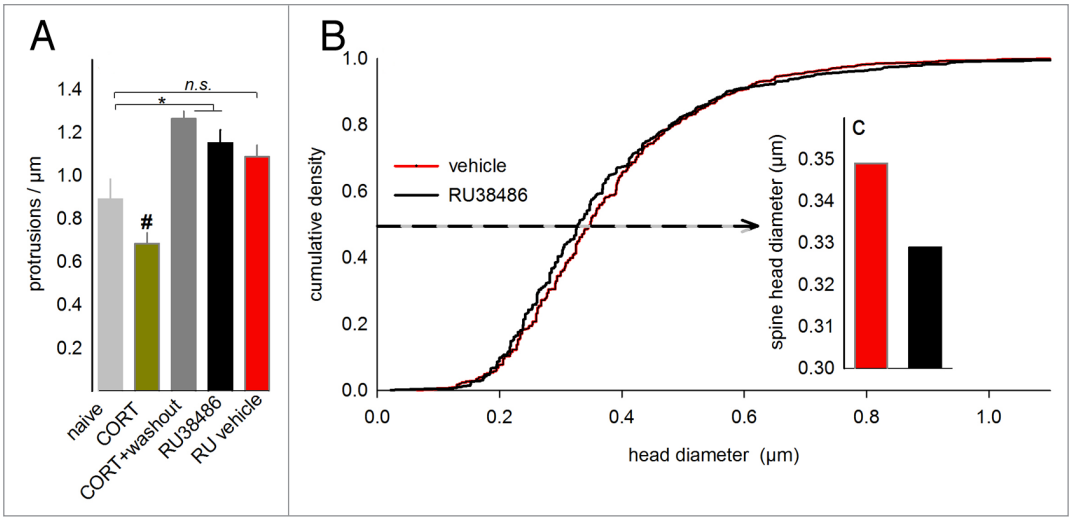

Figure 1. Corticosterone exposure and GR blockade modify prelimbic cortical dendritic spines. (**A**) Dendritic spines were counted (from left to right) in naïve control mice, mice exposed to exogenous corticosterone in the drinking water for 3 weeks, exogenous corticosterone + a 3-week washout period, 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 h 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 ≤ 0.05, #p = 0.08 vs. naive and 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.

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 our additional analyses, we first used the Green Fluorescent Protein (GFP)-expressing tissues collected for our prior report and enumerated dendritic spines in deep-layer excitatory neurons.[3,12] We compared several conditions: mice with an acute injection of the GR antagonist RU38486 (40 mg/kg, *i.p.*) and then euthanized 24 h 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.[3]

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.[13] 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),[14] but notably not in layer II/III where spines appear to be more resilient to corticosteroid exposure.[15]

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.[16] Moreover, acute exposure to both EtOH and DMSO, common vehicles for RU38486, stimulate rapid, acute corticosterone secretion even at low concentrations.[17,18]

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

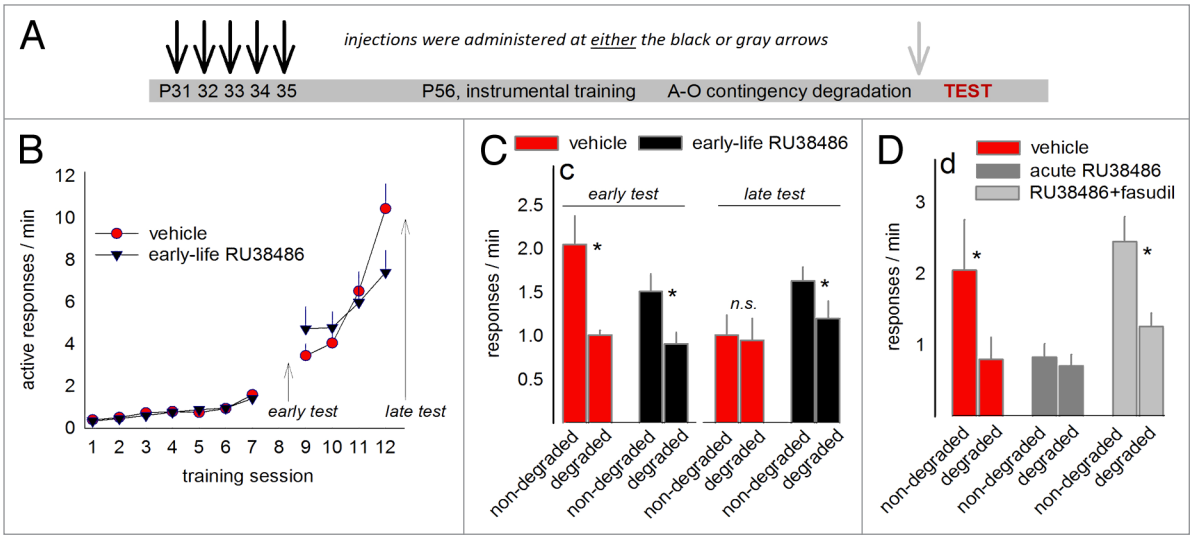

Figure 2. GR blockade regulates outcome-based decision-making. (**A**) Mice were injected with RU38486 or vehicle either in adolescence from P31–35 (black arrows) or immediately following action-outcome contingency degradation training in adulthood (gray arrow). (**B**) Mice exposed to either RU38486 (30 mg/kg, *i.p.*) or an EtOH-based vehicle solution during adolescence were unaffected in their instrumental response acquisition in adulthood. Response rates are shown, with the timing of each action-outcome contingency degradation test indicated by arrows. (**C**) After an initial action-outcome contingency degradation training session, all mice showed sensitivity to action-outcome contingencies, responding preferentially on the 'non-degraded' aperture during a probe test. However, with further training, control mice developed stimulus-response habits, responding equally on both instrumental apertures. By contrast, mice with a history of GR blockade preserved outcome-based decision-making strategies, as indicated by preferential responding on the 'non-degraded' aperture. (**D**) In the case of injection immediately following contingency degradation training, however, 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. Bars and symbols = means + SEMs, *p ≤ 0.05. "A-O" refers to action-outcome.

mouse's ability to subsequently make decisions based on the predictive relationship between a response and its outcome.[3] For the present report, 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 (adapted from 3,19). 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 and D**). In this case, we used a 4-fold *lower* dose of RU38486 (10 mg/kg, *i.p.*) than in our prior report,[3] 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). Rho serves as a molecular switch, transitioning between an inactive GDP-bound state and an active GTP-bound state in which Rho is targeted to cellular membranes. There, Rho 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.[20] 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. 2D**). 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.

Long-Term Effects of Adolescent RU38486 Exposure

Acute GR antagonism blocks action-outcome conditioning (**Fig. 2**; 3), but does a *history* of GR blockade have the same consequences? In a separate group of male C57BL/6 mice, we repeatedly injected mice with RU38486 or the EtOH-based vehicle in which RU38486 is dissolved (in this experiment, 2% EtOH v/v in phosphate-buffered saline) and evaluated long-term consequences (timeline in **Fig. 2A**).

Mice were injected for 5 d starting at postnatal day (P) 31, corresponding to early adolescence in rodents.[21] Twenty-one days after the last injection, we trained mice to respond for food reinforcers (**Fig. 2B**) using a continuous reinforcement schedule as above and "degraded" the relationship between one action and its outcome, while the action-outcome contingency associated with the other aperture remained intact. In this case, all mice showed sensitivity to action-outcome contingency degradation, responding preferentially on the non-degraded aperture during a subsequent 10 min. probe test (**Fig. 2C**). We next trained mice using a random interval 30 s schedule of reinforcement since random interval schedules promote stimulus-response habit formation (**Fig. 2B**).[22] After another session of action-outcome contingency degradation, control mice responded equally on both apertures, insensitive to action-outcome contingency degradation (**Fig. 2C**); in other words, control mice developed stimulus-response habits as expected. By contrast, RU38486-pretreated mice maintained goal-directed response strategies and preferentially responded on the 'non-degraded' instrumental response aperture (**Fig. 2C**). In other words, a history of GR blockade preserved decision-making based on the predictive relationship between a response and its outcome. Response rates were analyzed by 2-factor (aperture x RU38486) ANOVA: $F_{(1,29)} = 4.4$, $p = 0.04$.

Discussion

i. Summary.

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.[23] Interestingly, with advanced age, arbors become less plastic and less able to recover,[23] highlighting the possibility that corticosteroid-mediated dendritic spine elimination and the magnitude of "recovery" depend heavily on age.

ii. Multiple prefrontal cortical subregions regulate action selection.

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.[5–8] And like the prelimbic cortex, the orbitofrontal cortex is implicated in learning the relationship between an instrumental response and its outcome,[24,25] as well as the predictive relationship between a stimulus and an outcome.[26]

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 d washout period.[14] Deep-layer orbitofrontal cortical dendritic spine densities are also reduced with prolonged corticosterone exposure, but densities *fail to recover within the same time window*.[14] 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[3] 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, several 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 the Fragile X Mental Retardation Protein and cofilin,[27] integrin family receptors,[28] p190RhoGAP,[14] *etc.* Moreover, 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.[29–32]

iii. GR regulation of neurobehavioral outcomes in adolescence vs. adulthood.

One notable aspect of our report pertains to the age when mice were exposed to RU38486 in cases when it *preceded* instrumental conditioning (**Fig. 2A–C**). Specifically, we administered RU38486 from P31–35, corresponding to early adolescence in rodents.[21] In this case, a history of RU38486 treatment during

adolescence enhanced outcome-based decision-making in adulthood; specifically, these mice were sensitive to changes in action-outcome associative relationships despite extended training that resulted in stimulus-response habit formation in drug-naïve mice.

Why might this be? Adolescence is characterized by rapid spinogenesis and synaptogenesis, followed by a protracted period of spine pruning that results in synaptic elimination; also, cortical neural structure is markedly more plastic in adolescence than in adulthood.[11,34–36] Furthermore, adolescent stressor systems have gained increasing attention as determinants of long-term behavioral and structural outcomes (*e.g.,* 37–39). In rodents, P31 corresponds to the onset of a major period of gross structural refinement and dendritic spine pruning in the prefrontal cortex.[40,41] Recent findings by Liston and Gan indicate that *adolescent-onset dendritic spine formation*, *though not elimination*, is GR-dependent.[35] Thus, subchronic GR blockade during a period predominated by spine elimination might *optimize* programmed spine elimination processes and thereby confer long-term behavioral benefits, as was indeed observed here. Further studies testing such a model are, however, necessary.

iv. Multiple stress systems regulate action selection.

Finally, it is important to note that *multiple* stress response systems, not just GR-mediated systems, regulate habit formation: Studies in humans indicate that acute stressor exposure impairs the *expression* of new action-outcome learning, and this effect can be attributed to dual activation of corticosteroid and noradrenergic systems (42; reviewed 43). When considered in light of our findings here, as well as a prior report focused on stressor-exposed rats,[44] it may be that the *expression* of new action-outcome learning is dependent on dual GR/noradrenergic systems, while the *consolidation* of action-outcome contingencies instead requires physiological levels of GR binding. In this case, both aberrantly high and low binding levels would result in a dependence on familiar, habitual response patterns rather than outcome-based strategies that require new learning and the adjustment of familiar behavioral routines.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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