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Pharmacological Enhancement of Adult Hippocampal Neurogenesis Improves Behavioral Pattern Separation in Young and Aged Male Mice

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ABSTRACT

BACKGROUND: Impairments in behavioral pattern separation (BPS)—the ability to distinguish between similar contexts or experiences—contribute to memory interference and overgeneralization seen in many neuropsychiatric conditions, including depression, anxiety, posttraumatic stress disorder, dementia, and age-related cognitive decline. Although BPS relies on the dentate gyrus and is sensitive to changes in adult hippocampal neurogenesis, its significance as a pharmacological target has not been tested.

METHODS: In this study, we applied a human neural stem cell high-throughput screening cascade to identify compounds that increase human neurogenesis. One compound with a favorable profile, RO6871135, was then tested in young and aged mice for effects on BPS and anxiety-related behaviors.

RESULTS: Chronic treatment with RO6871135 (7.5 mg/kg) increased adult hippocampal neurogenesis and improved BPS in a fear discrimination task in both young and aged mice. RO6871135 treatment also lowered innate anxiety-like behavior, which was more apparent in mice exposed to chronic corticosterone. Ablation of adult hippocampal neurogenesis by hippocampal irradiation supported a neurogenesis-dependent mechanism for RO6871135-induced improvements in BPS. To identify possible mechanisms of action, in vitro and in vivo kinase inhibition and chemical proteomics assays were performed. These tests indicated that RO6871135 inhibited CDK8, CDK11, CaMKIIa, CaMKIIb, MAP2K6, and GSK-3β. An analog compound also demonstrated high affinity for CDK8, CaMKIIa, and GSK-3β.

CONCLUSIONS: These studies demonstrate a method for empirical identification and preclinical testing of novel neurogenic compounds that can improve BPS and point to possible novel mechanisms that can be interrogated for the development of new therapies to improve specific endophenotypes such as impaired BPS.

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Pattern separation is the process of separating overlapping sensory information, contexts, and experiences into distinct neural representations. It is believed that this process facilitates the rapid storage of new memories without inducing large amounts of interference (1–3). Computational theories and simulations predicted that this role is performed by the dentate gyrus (DG) (4–7). This function of the DG was later empirically established in rodents (8–14). In mammals, the DG is one of 2 brain regions (with the subventricular zone) that continue to generate new neurons throughout development and adulthood, a phenomenon known as adult hippocampal neurogenesis (AHN) (15,16).

Previous work has shown the importance of AHN for behavioral pattern separation (BPS) in rodents: ablating AHN causes impairments in BPS, whereas enhancing AHN with exercise, enrichment, or genetic manipulation improves BPS (17–25). In addition, transiently silencing immature adult-born granule cells during discrete epochs of a fear discrimination task can disrupt pattern separation, underscoring the role of new neurons in this cognitive task (26). AHN also decreases dramatically with age (18,27–30), as does BPS performance (20,31–33).

In humans, tasks have been developed to test pattern separation, and when studied in conjunction with functional magnetic resonance imaging, they have also been shown to reliably engage the DG and downstream CA3 region (14,34–42). Deficits in BPS may contribute to overgeneralization of negative emotion seen in depression, anxiety, and trauma-related disorders (43–50). BPS also declines with aging in humans (42,51,52), an effect that is even more pronounced in patients with mild cognitive impairment (37,42,53) and further impaired in Alzheimer's disease (53,54).

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Based on these observations, enhancement of AHN is thought to be a promising target for therapeutic development to treat conditions demonstrating BPS deficits, such as depression, anxiety disorders, posttraumatic stress disorder, and age-related cognitive decline as well as dementia (50). In the present study, a high-throughput in vitro screening cascade was used to empirically identify compounds with human neurogenic properties. One family of promising neurogenic molecules from this screen, piperazinones, was chemically optimized and the resulting compound RO6871135 was then tested in vivo. We found that RO6871135 enhanced AHN and improved BPS in a neurogenesis-dependent manner.

METHODS AND MATERIALS

See the Supplement for detailed methods and materials.

High-Throughput Screen for Human Neurogenesis

Human neural stem cells (hNSCs) were derived from human embryonic stem cells according to previously reported procedures (55,56).

Animal Care

All experimental procedures were conducted in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the New York State Psychiatric Institute. Chronic corticosterone experiments were conducted in compliance with protocols approved by another Institutional Animal Care and Use Committee (council directive no. 87–848, October 19, 1987, Ministère de l'agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale). Mice were housed 2 to 5 per cage and maintained on a 12-hour light/dark schedule with access to food and water ad libitum, except when otherwise stated. All data presented are from male mice.

Drug Administration

For behavioral studies, adult male C57BL/6 mice received 7.5 mg/kg of RO6871135 or vehicle daily by oral gavage for 21 days before behavioral testing. On behavioral testing days, animals were gavaged after behavior was completed.

Behavior

Anxiety-related and BPS behavioral tasks were performed as described in Supplemental Methods. For fear discrimination context parameters, see Tables S1 and S2. Hippocampal irradiation (57–59) and chronic corticosterone (60) interventions were performed as described previously.

Binding Analyses

In vitro pharmacological screening for off-target effects was also performed as described (61), tested at Cerep (now Eurofins Pharma Discovery). In vitro kinase screening assays were performed to determine kinase activity inhibition as described (62) via LeadHunter Drug Discovery Services Panels (Eurofins DiscoverX Products, LLC), and dissociation constants (K_d) for compound-kinase interactions were calculated. In situ kinase binding was assayed using the KiNativ platform (ActivX) (63–65). Brain and liver tissue samples were collected from RO6871135- or vehicle-treated mice. Chemical proteomics analysis was conducted in hNSCs using 2 close chemical analogs of RO6871135: 1 neurogenically active and 1 neurogenically inactive compound.

Statistical Analyses

Statistical analyses were performed using the Python packages statsmodels (66) and SciPy (67), R version 4.3.2, and GraphPad Prism (version 9.5.1 for macOS; GraphPad Software, http://www.graphpad.com). For all comparisons, values of $p < .05$ were considered as significant.

RESULTS

In Vitro Neurogenesis Screen

hNSCs were derived from human embryonic stem cells as previously described (55,68) (Figure S1A). hNSCs were exposed to factors known to modulate neurogenesis and then stained for DAPI to quantify cell number and Tuj1, a marker of immature neurons (Figure S1B). As expected, DAPT, which blocks Notch signaling, accelerated differentiation (69), resulting in the upregulation of Tuj1 and reduced proliferation, evidenced by reduced cell number. Consistent with previous in vitro and in vivo findings, Wnt3a promoted both proliferation and differentiation of hNSCs to immature neurons (70,71). Addition of the known mitogen FGF-2 also promoted proliferation of neural progenitor cells while inhibiting neuronal differentiation. Recapitulation of these effects supported the use of hNSCs as a model to screen for novel human neurogenic modulators (Figure 1A). Approximately 1 million compounds were screened, and the results are plotted as a histogram with >10,000 and >3000 hits, 3 and 4 standard deviations from the mean, respectively (Figure S2A). The dose-response curve of a chemically optimized molecule from an original hit, RO6871135 (Figure 1B), had a potency of 26 nM for increasing hNSC cell counts and was essentially inactive on the counter screen in human embryonic stem cell–derived mesenchymal stem cells (Figure S2B). To determine whether RO6871135 was truly neurogenic, high content screening was performed to directly quantify both nuclei and immature neurons (Figure 1C). Dose-response curves of RO6871135 on cell number (Figure S2C) and neurite network (Figure S2D) revealed potency in the range of 20 nM, consistent with potency seen in the previous step of the screen, which used an ATP (adenosine triphosphate) assay to quantify cell number. RO6871135 was profiled in a standard battery of drug development assays at Roche (61), such as hepatic enzyme activity effects and other safety tests, indicating a favorable profile (Table S3). Off-target assays to assess risk of adverse drug reactions (61,72) indicated no pharmacological activity at concentrations relevant to in vitro potencies and in vivo testing (Table S4).

In Vivo Screening for Increased Neurogenesis

RO6871135 showed good pharmacokinetic parameters after single-dose administration in mice (Table S3). After 14 days of oral administration in 129/Sv male mice (Figure 2A, top), there were dose-dependent increases in markers of proliferation (Ki67), survival of adult-born cells (BrdU), and increased numbers of doublecortin (DCX)+ immature neurons (Figure 2).

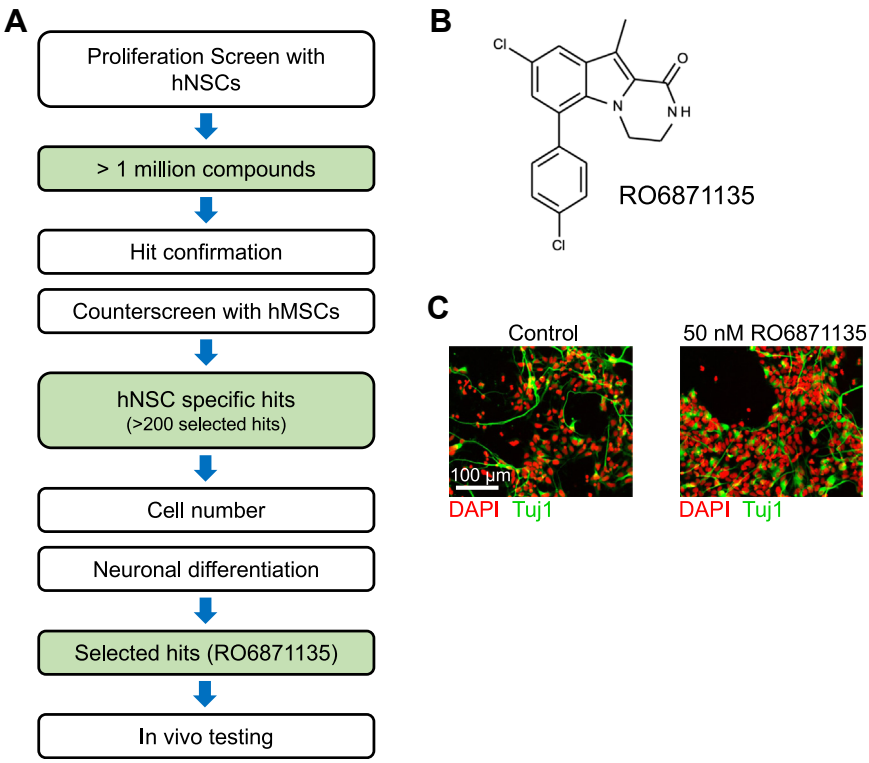


Figure 1. RO6871135 increases in vitro human neurogenesis. **(A)** High-throughput in vitro screening cascade to identify novel neurogenic compounds selected for in vivo testing. **(B)** Molecular structure of RO6871135. **(C)** Representative images of differentiating human embryonic stem cell–derived neural stem cells in the presence or absence of 50 nM of RO6871135 in the media. DAPI in red for cell number, including hNSCs and neural progenitor cells, while Tuj1 staining shown in green reflects initial differentiation or immature neurons. hMSC, human mesenchymal stem cell; hNSC, human neural stem cell.

For behavioral experiments, a longer treatment schedule was used before testing to allow for the accumulation of immature granule cells, which generally require at least 2 weeks to begin integrating into the surrounding circuit (73,74). Of note, these histology studies were conducted in 129/Sv mice, and there are baseline differences in neurogenesis markers and sensitivity to enhancement of neurogenesis from exercise between strains (75,76). C57BL/6 mice were used for all behavioral studies.

Chronic RO6871135 Alters Contextual Fear Discrimination but Not Contextual Fear Conditioning

After >21 days of treatment (Figure 2A), there was no observed difference in freezing between vehicle- and RO6871135-treated groups on the retrieval day after contextual fear conditioning (Figure 2E). There was also negligible freezing in both treatment groups in novel context C on day 3 (Figure 2F). On day 4, when mice were re-exposed to context A that followed the similar context B, both treatment groups exhibited comparable levels of freezing between the 2 contexts (Figure 2D, G). Over subsequent days, freezing levels in the 2 contexts diverged. Repeated-measures analysis of variance of freezing in both treatment groups indicated a significant effect of context and day and a significant context × day interaction. Freezing time in context A versus B was statistically different in vehicle-treated mice starting on day 8 and in RO6871135-treated mice by day 6.

Freezing difference scores were calculated for each mouse ($freezing_A - freezing_B$) and compared between treatment groups with repeated-measures analysis of variance (Figure 2H). There was a significant effect of group, day, and a group × day interaction; days 8 and 10 were significantly different by post hoc testing. We also confirmed that there was increased DCX staining after RO6871135 in behaviorally tested mice (Figure 2I).

RO6871135 Partially Rescues Lower AHN and BPS Deficits in Aged Mice

We also tested RO6871135 in aged (>18 months) and young mice (Figure 3A). Compared with young mice, there were dramatic decreases in all measures of neurogenesis in vehicle-treated aged mice, in keeping with previous findings (18,27–30). RO6871135 in aged mice significantly increased detectable BrdU and DCX staining (Figure 3B). For the fear discrimination task, we used a nonrandomized order for context presentation (Figure 3C), given that aged mice were expected to have difficulty with the randomized order paradigm (20). Young and aged mice showed comparable levels of freezing in the shock-associated context A after 1 day of exposure, indicating no effect of age on contextual fear conditioning (Figure 3D). There was no significant difference in freezing between contexts A and B in young mice on the first day of exposure to both contexts but significantly higher freezing levels in context B in both vehicle- and RO6871135-treated aged mice (Figure 3D). By the next day, young mice were discriminating between the 2 contexts, while vehicle- and

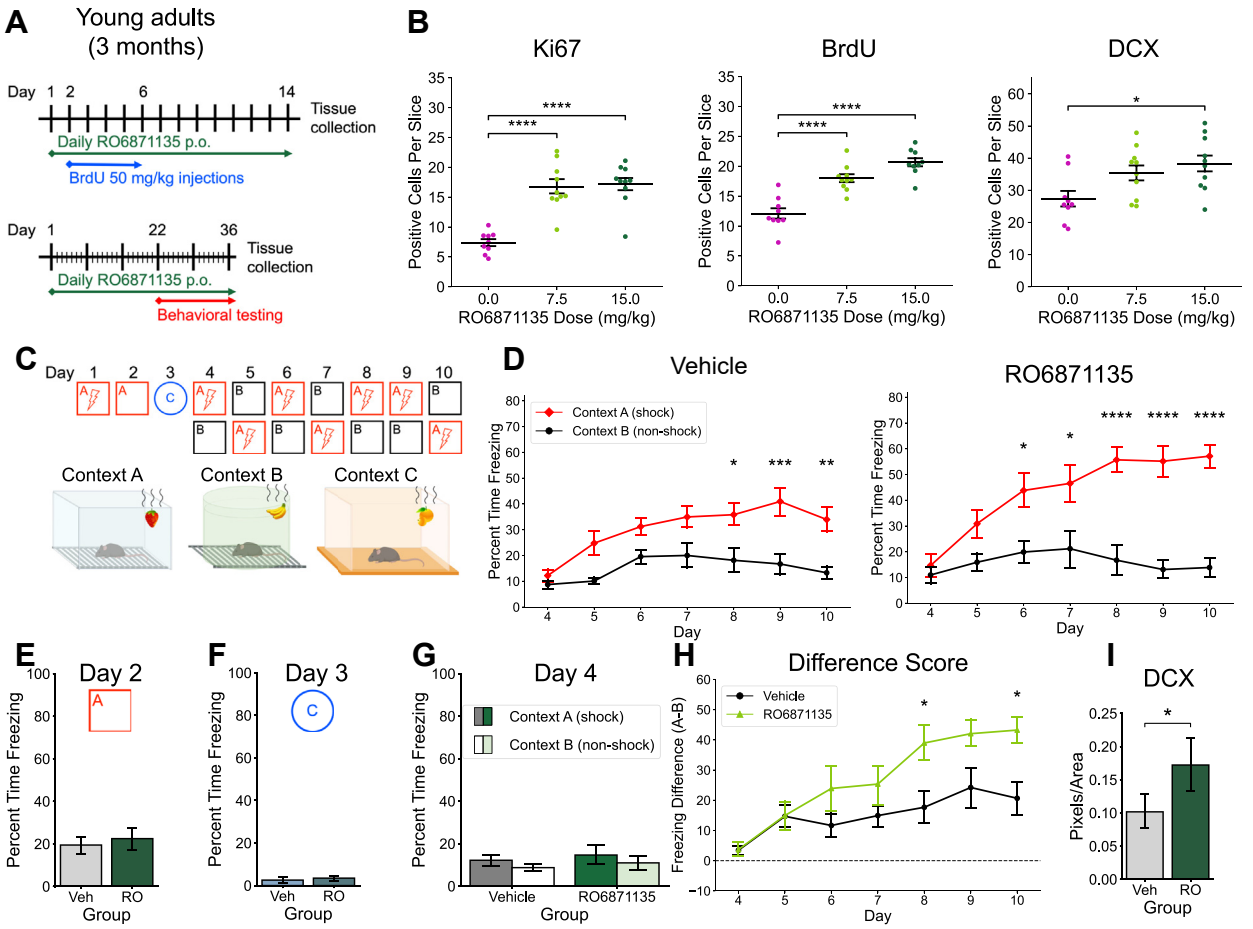


Figure 2. Chronic in vivo administration of RO6871135 increases neurogenesis and improves pattern separation. **(A)** Timelines of experimental design: 14-day administration for histology studies is shown on the top row, and 21-day administration for behavioral testing followed by histology shown on the bottom row. **(B)** Positive cell counts per slice (±SEM) for Ki67, BrdU, and DCX. There was a significant effect of treatment group in all measures (Ki67 $F_{2,26} = 26.64$, $p < .0001$; BrdU $F_{2,25} = 30.60$, $p < .0001$; DCX $F_{2,26} = 4.658$, $p < .05$). Controls ($n = 9$) had lower cell counts of Ki67 ($p < .0001$) and BrdU ($p < .0001$) than mice treated with 7.5 mg/kg of RO6871135 ($n = 10$), and they had lower cell counts of all 3 markers than mice treated with 15 mg/kg of RO6871135 ($n = 10$; Ki67 $p < .0001$; BrdU $p < .0001$; DCX $p < .05$). **(C)** Schematic for contextual fear conditioning and fear discrimination tasks. **(D)** Percent time freezing across days in the fear discrimination task. Vehicle-treated controls ($n = 8$) had a significant context × day interaction ($F_{6,84} = 2.876$, $p < .05$), with significant differences in freezing starting on day 8. RO6871135-treated mice ($n = 8$) also had a significant context × day interaction ($F_{6,84} = 7.899$, $p < .0001$), with significant differences in freezing between contexts by day 6. **(E, F)** Freezing time after single-shock contextual fear conditioning in the same context or a novel context. RO6871135 treatment did not alter expression of contextual fear ($t_{14} = -0.434$, NS) and did not affect generalization of fear to a different, novel context ($t_{14} = -0.490$, NS). **(G)** Freezing time on the first day of exposure to the similar context B at the beginning of the fear discrimination task, showing similar freezing levels to the shock context A in both groups (context $F_{1,28} = 1.157$, NS; group $F_{1,28} = 0.500$, NS; group × context $F_{1,28} = 0.001$, NS). **(H)** Difference score across days, calculated by subtracting the freezing time in context B from freezing time in context A. There was a significant effect of group ($F_{1,14} = 5.948$, $p < .05$) and a significant group × day interaction ($F_{6,84} = 2.256$, $p < .05$). Post hoc testing indicated significant differences on days 8 and 10 ($p < .05$). **(I)** DCX staining in dentate sections from the same mice that underwent behavioral testing. After more than 5 weeks of treatment with 7.5 mg/kg of RO6871135, there was a significant increase in DCX staining ($t_{15} = -2.648$, $p < .05$). Representative images of DCX staining are shown in Figure 4F. $p \geq .05$ is not significant, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$. DCX, doublecortin; NS, not significant; p.o., per oral; RO, RO6871135; veh, vehicle.

RO6871135-treated aged mice had nearly identical freezing levels in contexts A and B (Figure 3E). Vehicle-treated aged mice failed to discriminate between contexts by day 9, while RO6871135-treated aged mice successfully discriminated between contexts after day 5 (Figure 3E). With additional days of exposure, aged vehicle-treated mice did eventually discriminate (Figure S3). We did not observe an effect of RO6871135 in young-adult mice using this behavioral paradigm, given that vehicle-treated young mice already discriminated by the second day of exposure to both contexts (Figure S4).

RO6871135 Effects on Anxiety-Related Behavioral Tests

In the novelty suppressed feeding (NSF) test, which measures approach-avoidance behavior by latency to feed in a novel arena, RO6871135 significantly decreased latency in both young (Figure 3F) and aged (Figure 3G) mice. Comparing NSF

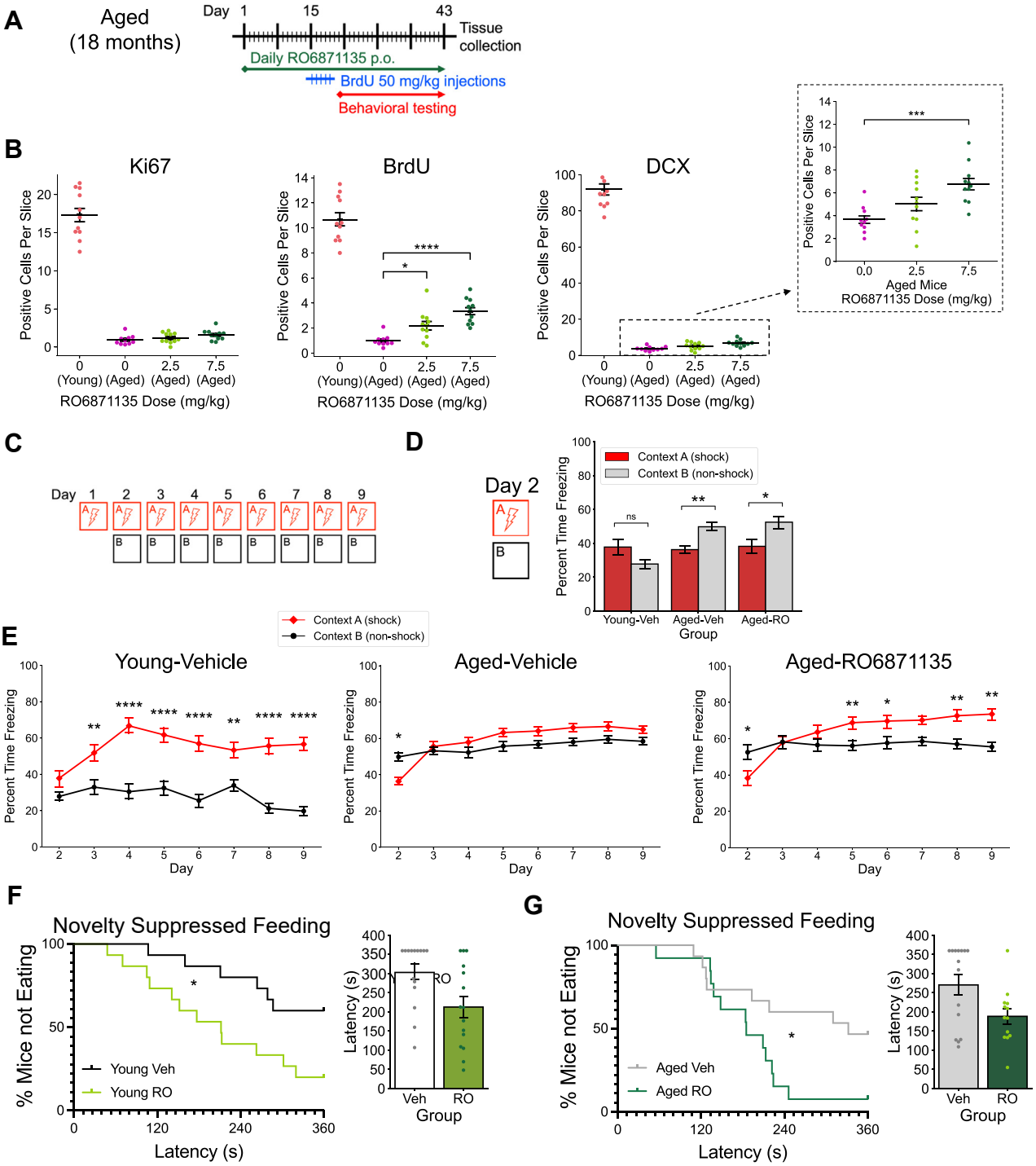


Figure 3. Aged mice have much lower measures of adult hippocampal neurogenesis. They also have a reduced fear discrimination, which is partially rescued with treatment by RO6871135. **(A)** Timeline of experimental design. **(B)** Positive cell counts per slice (±SEM) for Ki67, BrdU, and DCX. Measures from vehicle-treated young mice (n = 11) are shown for reference compared with vehicle-treated aged mice (n = 10). Among aged mice, RO6871135 (n = 10/group) significantly increases counts of cells positive for BrdU ($F_{2,29}$ = 15.99, $p < .0001$) and DCX ($F_{2,29}$ = 8.745, $p < .005$) but does not restore them to the level of young mice. **(C)** Schematic for nonrandomized fear discrimination task (n = 15 young; 24/group aged). **(D)** Freezing time after single-shock contextual fear conditioning in the same context A 1 day after foot shock or on first exposure to the similar context B (context $F_{1,128}$ = 3.672, NS; group $F_{2,128}$ = 6.091, $p <$ .005; group × context $F_{2,128}$ = 6.754, $p < .005$). There was no significant effect of context in the young mice and elevated freezing in the similar context B in both groups of aged mice (vehicle $p < .005$, RO6871135 $p < .05$). **(E)** Percent time freezing across days in the fear discrimination task in young and aged mice.

latency between vehicle-treated young and aged mice, there was no difference between the age groups (Figure S5).

In the open field test (OFT), mice treated with RO6871135 showed increased locomotor activity compared with vehicle-treated mice. Aged mice exhibited less locomotion in the OFT, with no significant age × treatment interaction (Figure S6A). There was no main effect of RO6871135 on exploration of the center zone. Aged mice had higher percent distance in the center zone, and we did not observe any significant age × treatment interaction center distance (Figure S6B).

Irradiation Blocks RO6871135 Effects on Contextual Fear Discrimination

To investigate whether immature granule cells were required for the effects of RO6871135 on pattern separation, we used a well-established method of bilateral X-irradiation to permanently ablate AHN across the whole DG (57–59), followed by 2 months of recovery from inflammatory effects of irradiation and then treatment with RO6871135 and fear discrimination testing (Figure 4A). There was no effect of drug treatment on contextual fear conditioning (Figure S7). Vehicle-treated irradiated mice did not discriminate between contexts A and B until day 10 (Figure 4B). Freezing in irradiated RO6871135 mice showed no significant effect of context and no context × day interaction (Figure 4C). The difference scores of freezing in A and B showed no significant effect of the treatment group and no group × day interaction (Figure 4D). Irradiation did not block the effect of RO6871135 in NSF, and drug-treated irradiated mice exhibited decreased latency to feed compared with vehicle-treated irradiated controls (Figure 4E). Ablation of AHN in irradiated mice was confirmed with qualitative histological assessment (Figure 4F).

RO6871135 Reverses Behavioral Effects of Chronic Corticosterone and Stimulates AHN

We next looked at RO6871135 effects after chronic corticosterone exposure (Figure 5A), a model of chronic stress used for anxiety- and depression-related models. Chronic corticosterone increased innate anxiety-like behavior, as measured by exploration of the center zone, and these effects were reversed in mice that had received RO6871135 (Figure 5B). Corticosterone exposure also increased anxiety-like behavior in the NSF test, with increased latency to feed. Treatment with RO6871135 partially reversed this effect and significantly decreased latency to feed compared with the corticosterone/vehicle group (Figure 5C). RO6871135 increased the number of DCX+ cells in the setting of chronic corticosterone treatment as well (Figure 5D, E). Notably, these experiments were conducted at a different facility from noncorticosterone studies, leading to some differences in control group behavioral measures.

Functional Activity and Binding Profiles of RO6871135

As stated above, a panel of assays to screen for G protein–coupled receptor binding was negative at relevant concentrations (Table S4). To identify putative targets of RO6871135, a series of binding assays were performed. In vitro binding against a panel of 96 kinases with the KINOMEscan panel (Table S5) revealed significant functional inhibitory activity for CDK11. Although not included in the initial inhibition assay, CDK8 was added for the calculation of K_d values. Inhibitory activity for CDK8 and CDK11, but no other kinases, was seen at submicromolar concentrations (Table S6).

After the biochemical assays above, we tested for activity in murine brain tissue. In situ kinase profiling was performed using KiNativ for brain tissue from RO6871135-treated mice (Tables S7 and S8). Liver tissue from the same animals was used for comparison (Table S8). Based on previous validation (63), >35% inhibition was considered significant. RO6871135 caused >50% inhibition for CDK8 and CDK11, as well as for CaMKIIa, CaMKIIb, and MAP2K6 in the brain.

A chemical proteomics study was conducted to define the potential targets of RO6871135 in hNSCs (Figure S8). The enriched proteins on the active RO6871135 analog versus the inactive analog are highlighted in Figure S9. CaMKIIa is, among the statistically significant differences, the most enriched protein on the active versus inactive analogs. Seven kinases (GSK-3α, GSK-3β, MAPK1, MAPK3, CaMKIIg, CSNK1A1, and CDK8) exhibited more binding to the active RO6871135 analog than the inactive one.

Top hits from in vitro (KINOMEscan), in situ (KiNativ), and chemical proteomics assays are summarized in Table 1. CDK8 activity was found in all 3 assays. CDK11 and GSK-3β were among the top candidates in 2 of 3 assays. CaMKIIa, which was not directly tested in the KINOMEscan assay, was strongly positive in the other 2 assays. CaMKIIb and MAP2K6 were also not directly tested in the KINOMEscan assay but were strongly positive in the in situ assay.

DISCUSSION

We demonstrated that chronic treatment with RO6871135 was sufficient to enhance AHN and improve BPS in both young and aged male mice and that BPS effects are neurogenesis dependent. This candidate compound was identified from a screening cascade testing for neurogenic effects on hNSC in vitro, which we have also described here. Immature adult-born granule cells from AHN have been shown to play a role in encoding contextual information (26,59,77) and in supporting distinct neural patterns for different contexts (78). The present findings demonstrate a pharmacological method for recapitulating improvements in BPS seen with other means of increasing AHN (20,25).

Vehicle-treated young mice had a significant context × day interaction ($F_{7,196} = 4.598$, $p < .0001$), with significantly higher freezing in the shock context by the second day of exposure to both contexts. Vehicle-treated aged mice also had a significant context × day interaction ($F_{7,378} = 8.994$, $p < .0001$), but, beyond elevated freezing in context B on the first day, did not demonstrate differential freezing across the subsequent 7 days. Aged mice after treatment with RO6871135 had a significant context × day interaction ($F_{7,322} = 9.860$, $p < .0001$), again with elevated freezing in context B on day 2, but with significantly higher freezing in the shock context on days 5, 6, 8, and 9 of the experiment. **(F)** Latency to feed in the novelty suppressed feeding tests in young and aged **(G)** mice ($n = 14–15$/group/age), represented as a survival curve on the left and as the latency measures on the right. Log-rank (Mantel-Cox) test: young $\chi^2 = 5.680$, $p < .05$; aged $\chi^2 = 4.854$, $p < .05$. There was no significant effect of RO6871135 on latency to feed in the home cage in either age group (data not shown). $p \geq .05$ is ns, *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$. DCX, doublecortin; ns/NS, not significant; p.o., per oral; RO, RO6871135; veh, vehicle.

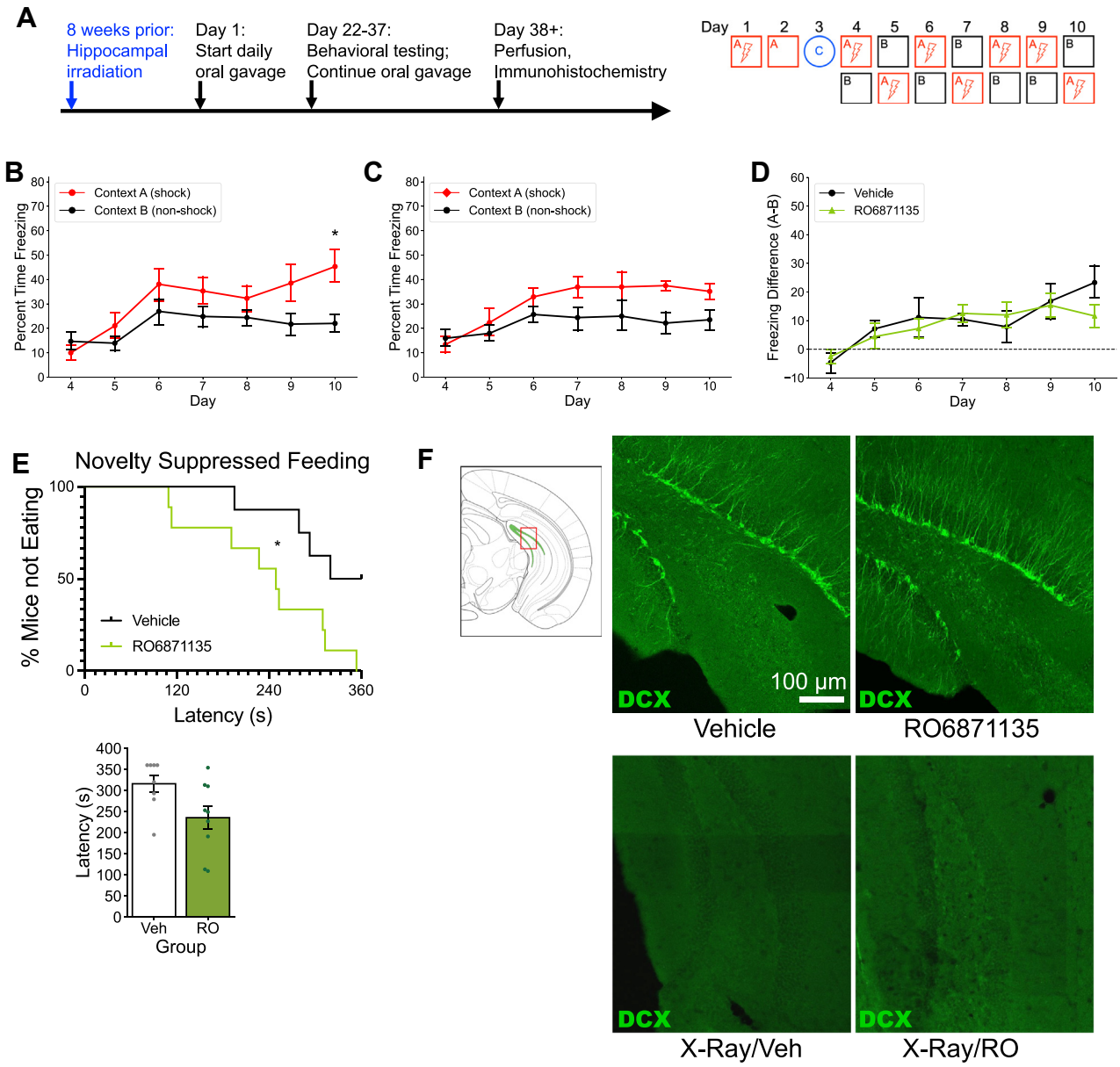


Figure 4. Irradiation blocks the effect of RO6871135 on behavioral pattern separation, but not on novelty suppressed feeding latency to feed. **(A)** Timeline of focal irradiation followed by 8 weeks of recovery and then initiation of daily dosing of RO6871135 and behavioral testing, as conducted with nonirradiated mice. **(B)** Vehicle-treated mice with chronic ablation of adult hippocampal neurogenesis showed a significant context × day interaction, with a significant difference in freezing on day 10 ($n = 9$). **(C)** Irradiated mice ($n = 9$) that received RO6871135 ($n = 9$) had no significant effect of context ($F_{1,16} = 3.333$) and no context × day interaction ($F_{6,96} = 1.889$). **(D)** The difference scores had a significant effect of day ($F_{6,90} = 6.607$, $p < .0001$), but no significant effect of drug group ($F_{1,15} = 0.1623$, NS) and no group × day interaction. **(E)** Latency to feed in the novelty suppressed feeding test shows that decreased latency in the RO6871135-treated group remains even after irradiation ($n = 8$–9/group). (Top) Survival curve log-rank (Mantel-Cox) test: $\chi^2 = 6.034$, $p < .05$. (Bottom) Latency measures of individual mice in the novelty suppressed feeding. There was no significant effect of RO6871135 on latency to feed in the home cage (data not shown). **(F)** Top left panel shows an atlas image of the dentate gyrus and approximate field of view for microscope images (red rectangle). Following panels show representative images of doublecortin staining in nonirradiated and irradiated mice treated with vehicle of RO6871135. Lack of staining in irradiated mice confirms ablation of adult hippocampal neurogenesis and lack of immature neurons observable by DCX staining. One example image from the ventral hippocampus is shown here, but irradiation was applied to the whole hippocampus. Quantification of DCX staining from nonirradiated mice is shown in Figure 2I. *$p < .05$. DCX, doublecortin; NS, not significant; RO, RO6871135; veh, vehicle.

RO6871135 is not currently being developed as a clinical molecule due to evidence of proliferation in the liver and other peripheral organs of mice and rats in vivo, as measured by Ki67 staining (data not shown). While there were no detrimental effects of RO6871135 over the time courses examined, including in 18-month-old mice, chronic carcinogenicity

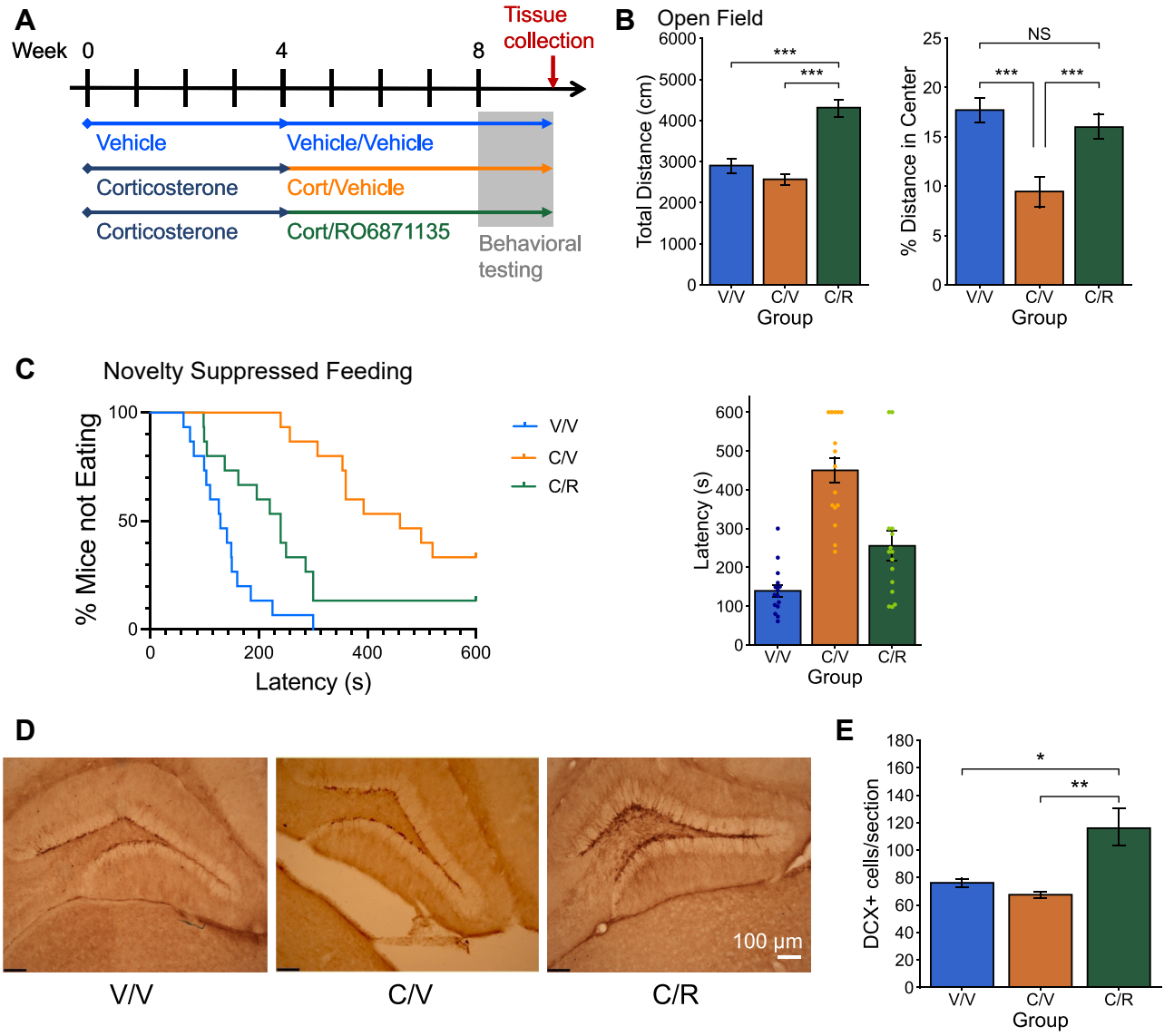


Figure 5. Chronic corticosterone increases anxiety-like behavior, which is reversed by treatment with RO6871135. **(A)** Experimental timeline. Mice are treated with vehicle or corticosterone for 4 weeks before starting daily RO6871135 or vehicle treatment for another 4 weeks while corticosterone or vehicle is continued (n = 15/group). **(B)** There was no significant effect of corticosterone on total distance traveled in an open field, but RO6871135 increased locomotion compared with both the vehicle and corticosterone groups ($F_{2,42}$ = 26.828, $p < .0001$). Stars indicate significant differences in post hoc testing by Tukey's honest significant difference test for multiple comparisons. Chronic corticosterone decreased the percent of distance traveled in the center of the open field arena, and this effect was reversed with RO6871135 treatment ($F_{2,42}$ = 9.724, $p < .001$). Mice that received chronic corticosterone also spent decreased time the center of the open field arena, and this effect was reversed with RO6871135 treatment ($F_{2,42}$ = 8.421, $p < .001$). **(C)** Chronic corticosterone increased latency to feed in the novelty suppressed feeding test, and RO6871135 partially reversed this effect, represented as a survival curve and the latency values per mouse. Log-rank (Mantel-Cox) test: χ^2 = 32.98, $p < .0001$. Bonferroni-corrected α = 0.017 for multiple comparisons, and all 3 treatment groups had significantly different latency values compared with either other group. There was no difference in home cage food consumption relative to body weight between vehicle/vehicle mice and those treated with RO6871135 (data not shown). **(D)** Representative images of DCX staining in mice treated with vehicle vs. corticosterone and vehicle vs. RO6871135. **(E)** Quantification of DCX staining ($F_{2,18}$ = 8.297, $p < .01$). There was no significant change in DCX staining from chronic corticosterone alone, but RO6871135 treatment increased DCX in corticosterone-exposed mice. $p \geq .05$ is not significant, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$. C/R, corticosterone/RO6871135; C/V, corticosterone/vehicle; DCX, doublecortin; V/V, vehicle/vehicle.

studies over longer time courses would be needed to properly derisk the compound. Alternatively, chemical optimization could potentially increase central efficacy. Therefore, we did not further test RO6871135 in female mice to see whether effects generalize, but this should be evaluated with future candidate compounds. In humans, there are known sex differences in psychiatric illness rates (79–81), likely due to an interplay of the biological variable of sex and the psychosocial

Table 1. A Summary of Proteins (Putative Neurogenic Piperazinone Targets) That Showed Significant Binding or Activity Changes in the Presence of RO6871135

Assay	KINOMEscan	KiNativ	Chemoproteomics
CDK11[b]	++	++	–
CDK8[a]	+	++	+
CaMKIIa	Not tested	++	++
CaMKIIb	Not tested	++	–
GSK-3α	–	–	++
GSK-3β[b]	–	+	++
MAP2K6	Not tested	++	–

KINOMEscan results represent an in vitro assay of RO6871135. The KiNativ assay was performed in situ on brain tissue collected from mice treated with RO6871135. Three proteins with high inhibition in the KiNativ assay were not tested in the KINOMEscan assay. + indicates a significant hit for that assay, while ++ denotes more binding or activity relative to the other positive hits. Raw values for individual assays are shown in Figure S9 and Tables S4–S8.

[a]CDK8 was found across all 3 assays.

[b]CDK11 and GSK-3β were identified in 2 assays.

construct of gender (82–85). The current study serves as a proof of concept for high-throughput screening and preclinical testing of neurogenic compounds for BPS effects, which could then be followed by studies to identify the exact mechanisms of action and determine the impact of sex, age, or stress exposure on these effects.

In these studies, much of the context discrimination is driven by increased freezing in the shock context, as mice continue to experience a foot shock on subsequent days of exposure. This increased freezing cannot be attributed to a general increase in immobility from drug treatment, given that RO6871135 treatment actually increased locomotion. In addition, this could not be attributed to a general increase in anxiety-like behavior, given that RO6871135 decreased latency to feed in the NSF test, and while contextual fear conditioning after a single foot shock is unchanged with RO6871135, we demonstrated that the more challenging task of discriminating from a very similar context is where the neurogenic effects are most apparent, consistent with previous findings (25,26). It is possible that RO6871135 acts by enhancing fear learning, driving up freezing in the shock context earlier than vehicle-treated mice, and this is not mutually exclusive with improved BPS, which is believed to support learning and memory (3,86). The fear discrimination task is the sum of 2 possible processes: fear learning in the shock context and safety learning and/or extinction learning in the nonshock context. As observed in other studies (25,87), the level of freezing after initial fear conditioning appears to influence the direction that freezing behavior diverges later in the task. When baseline freezing is low, as in Figure 2D, discrimination is achieved with increased freezing in context A. However, when baseline freezing is higher, as in the aged mice shown in Figure S3, behavioral discrimination is marked by decreased freezing in context B. Although not directly tested in this study, aged mice have not demonstrated an altered response to foot shock compared with young mice (33).

Treatment with RO6871135 after ablation of AHN with targeted hippocampal irradiation failed to improve BPS, but continued to decrease latency to feed in NSF, suggesting that RO6871135 effects on BPS, but not innate anxiety, are neurogenesis dependent. Indeed, the relationship between AHN manipulations and innate anxiety-like behaviors has been less consistent and more apparent with chronic stress in previous studies. While some manipulations require neurogenesis to affect NSF (57,60), others do not (87,88), and some neurogenic manipulations have no effect on NSF at all (25,89). In the OFT, RO6871135 altered innate anxiety-like behavior only in mice exposed to chronic corticosterone, consistent with results from genetically enhanced AHN (25,89,90). Enhanced neurogenesis has been shown to increase resilience to chronic stress (90–94); therefore, the ability of novel neurogenic compounds to enhance resilience to other models of chronic stress should be tested in future studies.

In vivo RO6871135 significantly elevated Ki67, BrdU, and DCX cell counts, and behavior was tested after 3 weeks of treatment, when immature neuron levels would be expected to plateau (95). Given that there were no notable discrepancies in the effects on these 3 neurogenesis markers, the normal neurogenic process does not appear to be altered by RO6871135, as a disproportionate increase in DCX staining would be expected if the compounds were delaying maturation or inducing dematuration.

Pharmacologically induced increases in AHN have been observed with serotonin reuptake inhibitors such as fluoxetine (57,88), tricyclic antidepressants (57,96), monoamine oxidase inhibitors, and norepinephrine reuptake inhibitors (97). RO6871135 showed no direct activity on the serotonin or norepinephrine system at our experimental concentrations and therefore has a novel mechanism of action compared with existing neurogenic medications. However, as with many therapeutics, including many currently available medications, the exact mechanisms of action for RO6871135 are not yet known. Looking at the kinases that were inhibited by RO6871135 in vitro or in situ or those that bound specifically to the neurogenic analog does provide some intriguing targets. The strongest convergence was on cyclin-dependent kinases CDK8 and CDK11, which are both relatively enriched in the hippocampus compared with other brain regions (98). CDKs and CDK inhibitors are instrumental in neural development, regulating cell fate and differentiation (99), and there is evidence that CDK inhibition may be a promising target to upregulate AHN (100). Other CDKs are being targeted by candidate chemotherapeutic agents (101), further highlighting how their role in regulation of cell proliferation and maturation can be exploited for pharmacological manipulation. RO6871135 induced in situ inhibition of CaMKIIa and CaMKIIb, 2 highly abundant proteins in the brain, and the neurogenic analog strongly bound to CaMKIIa as well. Given how ubiquitous CaMKII is, additional studies would be needed to understand how neurogenic compounds interact with it, but CaMKII is known to be crucial for learning and plasticity in mice (102–107) and normal neural development in humans (108–111). MAP2K6, also binding significantly to RO6871135 in this in situ assay, activates mitogen-activated protein kinase p38 (112) within the MAPK/ERK/JNK signaling cascades. Within these cascades, p38 has been shown to have a role in stress response, development, apoptosis, and senescence (113) and may even mediate age-related decline in AHN (114), although there have been some conflicting reports of the

directionality of its effects (114–118). GSK-3β binding was also seen in situ, and this kinase is involved in the Wnt/β-catenin pathway, a regulator of AHN (71,119,120) that may also be a promising target for counteracting neural loss in neurodegenerative disorders (121,122). While the top hits from our activity and binding assays can point to targets for additional neurogenic compounds, clinically effective medications often have multiple targets, and a more efficient method for identifying candidate compounds remains an empirical, high-throughput screen, such as the one described above, followed by additional studies to determine the precise mechanism of action.

Since an initial report of AHN in humans in 1998 (15), techniques for demonstrating evidence of AHN have continued to evolve (29,123–134). While the number of new cells might be quite low in human adults (127), their impact on hippocampal circuitry may proportionally increase when the system is challenged, that is, in the settings of stress, neurodegeneration, or other pathology (135–139). There is also evidence that, although proliferation of adult-born cells decreases with age, maturation time also lengthens, such that the total number of immature neurons in the system is still a significant proportion of cells (140,141). Moreover, AHN in mice may be a useful readout for interventions that affect broader hippocampal functioning in humans, with many neurogenic manipulations in mice (exercise, enrichment, selective serotonin reuptake inhibitors) having therapeutic or resilience-building properties in humans.

In this study, we demonstrated that pharmacologically enhancing AHN is a means for improving BPS. Studies of neural functioning during BPS in humans find that a homologous neural circuit is engaged (34–36,42), and investigations in clinical populations implicate this cognitive process as a promising therapeutic target (14,42–44,48,50–54,142–144). It has even been shown that perceived clinical response to antidepressants is correlated with improvements in BPS performance (145). While medications such as selective serotonin reuptake inhibitors and other treatments for anxiety and depression can increase AHN (57,96,97), they are not effective in some cases (146,147) and also have side effects that are not tolerable to many patients (148–150). Identification of compounds with novel neurogenic mechanisms may provide a means to increase AHN with a higher efficiency and reduced side-effect profiles, ultimately increasing the effectiveness in individuals with insufficient response to existing medications. They may also provide direct and symptomatic treatment for individuals with BPS deficits that work in concert with other types of therapy, including pharmacotherapy, psychotherapy, neural modulation, and/or cognitive rehabilitation. Further translation of neurogenesis as a treatment target should be pursued in clinical trials of neurogenic agents to directly test for improvements in BPS and assess how this correlates with general clinical improvement.

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