Archival Report

Biological Psychiatry

Orexin 1 Receptor Antagonism in the Basolateral Amygdala Shifts the Balance From Pro- to Antistress Signaling and Behavior

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ABSTRACT

BACKGROUND: Stress produces differential behavioral responses through select molecular modifications to specific neurocircuitry elements. The orexin (Orx) system targets key components of this neurocircuitry in the basolateral amygdala (BLA).

METHODS: We assessed the contribution of intra-BLA Orx_1 receptors (Orx_1Rs) in the expression of stress-induced phenotypes of mice. Using the Stress Alternatives Model, a social stress paradigm that produces two behavioral phenotypes, we characterized the role of intra-BLA Orx_1R using acute pharmacological inhibition (SB-674042) and genetic knockdown (AAV-U6-Orx_1R-shRNA) strategies.

RESULTS: In the BLA, we observed that Orx_1R (*Hcrtr1*) messenger RNA is predominantly expressed in CamKIIα^+ glutamatergic neurons and rarely in GABAergic (gamma-aminobutyric acidergic) cells. While there is a slight overlap in *Hcrtr1* and Orx_2 receptor (*Hcrtr2*) messenger RNA expression in the BLA, we find that these receptors are most often expressed in separate cells. Antagonism of intra-BLA Orx_1R after phenotype formation shifted behavioral expression from stress-sensitive (Stay) to stress-resilient (Escape) responses, an effect that was mimicked by genetic knockdown. Acute inhibition of Orx_1R in the BLA also reduced contextual and cued fear freezing responses in Stay animals. This phenotype-specific behavioral change was accompanied by biased molecular transcription favoring *Hcrtr2* over *Hcrtr1* and *Mapk3* over *Plcb1* cell signaling cascades and enhanced *Bdnf* messenger RNA.

CONCLUSIONS: Functional reorganization of intra-BLA gene expression is produced by antagonism of Orx_1R, which promotes elevated *Hcrtr2*, greater *Mapk3*, and increased *Bdnf* expression. Together, these results provide evidence for a receptor-driven mechanism that balances pro- and antistress responses within the BLA.

https://doi.org/10.1016/j.biopsych.2021.12.019

Stress-induced alterations in neurocircuitry result in divergent behavioral responses. Enhanced stress reactivity (prostress) in rodent models is similar to human affective dysfunction in mood disorders such as depression, fear- and anxiety-related disorders, or posttraumatic stress disorder (1). Current pharmacotherapies for affective disorders have had limited success, and a mechanistic understanding remains elusive.

Balance within key stress circuits may be disrupted during periods of intense or prolonged stress to shift signaling dynamics in pro- or antistress pathways (2–4). Stressful stimuli are interpreted, in part, through converging signals in the basolateral amygdala (BLA), where glutamatergic projection neurons are influenced by distinctive GABAergic (gamma-aminobutyric acidergic) interneurons, to direct behavioral responses (5). In addition, activity in the BLA is modified by hypothalamic orexinergic neurons, which are critical for panic (6,7) and motivation (8,9).

Orexin (Orx) A and Orx_B, neuromodulators derived from a single pre-propeptide, activate two G protein–coupled receptors: Orx_1 receptors (Orx_1Rs) bind Orx_A and Orx_B (half maximal effective concentration = 30 vs. 2500 nM), as do Orx_2Rs (half maximal effective concentration = 38 vs. 36 nM) (10,11). These receptors stimulate G_q proteins, which increase intracellular Ca^{2+} (11) by activating phospholipase C (PLC) pathways (12). The $PLC_{\beta 1}$ isozyme variant is transcribed in the amygdala (13), and its dysfunction is linked to psychopathologies such as depression (14), bipolar disorder (15), addiction (16), and schizophrenia (17,18).

Stimulation of Orx_1R can also activate ERK (extracellular signal–regulated protein kinase). In the amygdala, recruitment of ERKs is important for consolidation, reconsolidation, and extinction of fear memories (19,20). While Orx_1Rs in the BLA are important in regulating fear (21,22), depression (23,24), and anxiety (25), it is unclear how shifts in molecular signaling

SEE COMMENTARY ON PAGE 775

ISSN: 0006-3223

cascades mediate such responses and initiate stress-induced phenotype development.

Using the Stress Alternatives Model (SAM), a behavioral paradigm that separates individuals into social stress–resilient (Escape; as validated by social interaction/preference test) and stress-vulnerable (Stay) (26) populations, we explored how Orx_1R activity in the BLA is involved in the formation of stress-related phenotypes. As a social interaction and avoidance paradigm in which smaller subjects encounter intense attacks from larger novel aggressors over a 4-day period, the SAM produces two separate subsets of animals exhibiting social avoidance or enhanced fear-conditioned responses (27,28). Unlike a traditional social defeat outcome, the SAM provides mice an opportunity to avoid social aggression by exiting the arena through escape tunnels only large enough for the smaller mouse. By the end of the second day of social interaction, test subjects commit to a phenotype: Escape or Stay. These stable phenotypes may be altered through pharmacological manipulations (Escape reduced by anxiogenic drugs, Stay reduced by anxiolytic drugs) administered on the third day of the SAM (28–30). Thus, the SAM is a useful tool for studying the development of stress-induced phenotypes while providing an opportunity to explore physiological and clinically relevant molecular mechanisms.

We investigated if inhibition of intra-BLA Orx_1Rs alters the formation of social stress–induced behavioral phenotypes. We predicted that pharmacological inhibition or genetic knockdown will shift behavioral patterns in vulnerable (Stay) populations toward resilience (Escape). Furthermore, we explored if Orx_1R inhibition affects conditioned fear responses and alters expression of genes responsible for balancing signaling in pro- and antistress neurocircuitries. Together, these results allow us to propose a neurocircuit model that defines the role of intra-BLA Orx_1R signaling in the balance of pro- and antistress states.

METHODS AND MATERIALS

Social Stress and Choice Paradigm

Aggressive social interactions between larger novel CD1 and smaller male C57BL/6NHsd mice dyads in the SAM apparatus (Figure 1) involve four trials, lasting up to 5 minutes each, that allow test animals the opportunity to shorten stressful encounters by making use of size-restricted tunnels at the apical end of the oval open field interaction arena. A tone given during isolation in the SAM apparatus before social interaction permits comparisons between cued and contextual fear conditioning. The escape routes provide a choice, producing two stable phenotypes: active avoidance (Escape) and accepting confrontation (Stay), which may be modified by drug treatment on day 3. The treatment regimen allows for statistical comparisons between groups and within subjects by comparing responses to SAM interactions before and after treatment. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and approved by the University of South Dakota Institutional Animal Care and Use Committee.

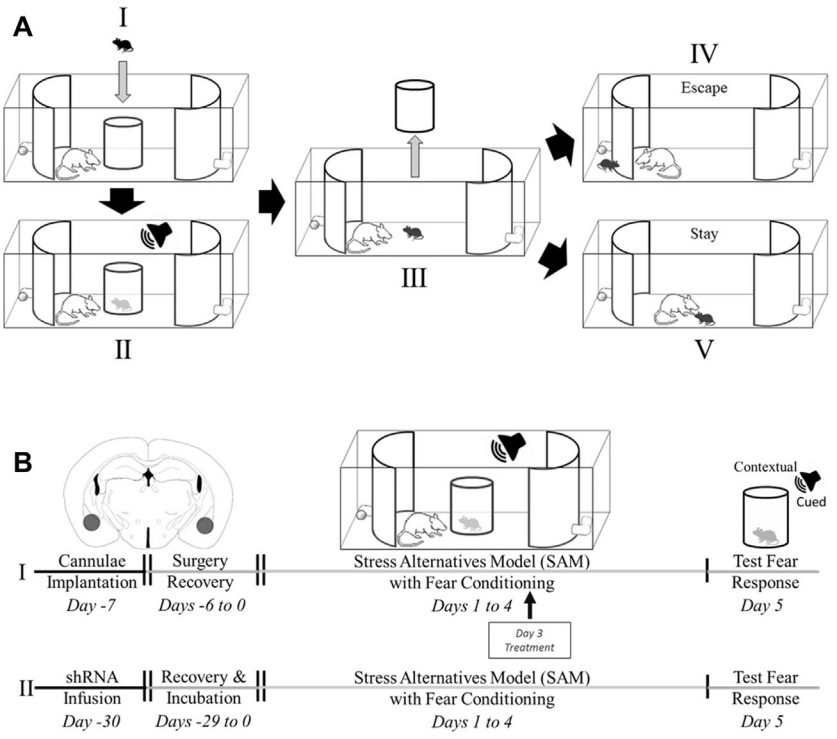

Figure 1. The SAM is used to assess the development of stress-induced phenotypes. **(A)** The SAM is a 4-day behavioral paradigm in which (I) a test mouse is placed into an opaque cylinder, (II) presented a tone, (III) exposed to social aggression, and commits to a phenotype: (IV) Escape or (V) Stay. **(B)** The behavioral timelines for (I) pharmacology and (II) genetic knockdown experiments (mice are the same age at testing) include surgeries targeting the basolateral amygdala, SAM exposure (days 1–4), and the testing of contextual and cued fear responses (day 5). SAM, Stress Alternatives Model; shRNA, short hairpin RNA.

Experimental Overview

See the Supplement for further information. The primary treatments for these experiments are inhibition of BLA Orx_1R via the antagonist SB-674042 (0.3 nmol/0.3 μL delivered bilaterally intra-BLA 1 hour prior to interaction on day 3), contrasted with Orx_1R stimulation (accomplished by Orx_A+Orx_2R antagonism), or short hairpin RNA (shRNA) knockdown (bilateral intra-BLA transfection beginning 30 days prior to SAM interaction). Considering the difference in timing of delivery, these treatments were done and analyzed separately with a priori hypotheses. All behavioral measures were performed during the dark cycle when the animals were active and included Escape (use of the apical tunnels), Stay (remaining in the SAM arena with the novel aggressor), time spent attentive to the escape hole, latency to escape (for Escape mice), fear-conditioned freezing (measured in response to the tone [conditioned stimulus (CS)] and context prior to the social interaction unconditioned stimulus [US] and as a conditioned response [CR] on day 5 in the absence of the US), and food intake. Thus, treatment groups included home-cage control animals and intra-BLA SB-674042 (or vehicle, Orx_A, Orx_A+MK-1064, MK-1064) injection of Escape and Stay mice. In addition, transgenic treatment groups included home-cage control animals, intra-BLA AAV-Orx_1R-shRNA injection, and intra-BLA AAV-scramble-shRNA injection. Brains and blood were collected for visual representations of gene expression (using RNAscope) of *Hcrtr1*, *Hcrtr2*, *Calb1*, *CamkIIα*, *Gad1*, and *Pvalb* in the BLA and to measure plasma concentrations of the stress hormone corticosterone (by enzyme-linked immunosorbent assay). Gene expression (using reverse transcriptase quantitative polymerase chain reaction) of *Hcrtr1*, *Hcrtr2*, *Plcb1*, *Mapk1*, *Mapk3*, *Bdnf*, and *Gapdh* (housekeeping gene) was measured in the BLA tissue. All experimental designs and statistical analyses were based on a priori hypotheses, using two-way repeated-measures analysis of variance, two-way analysis of variance, one-way analysis of variance, regression analyses, and *t* test, followed (where appropriate) by post hoc analyses.

RESULTS

OrxR Expression in the BLA

The glutamatergic marker CamKIIα identified the vast majority of BLA neurons (~80%) (Figure S2) and those expressing *Hcrtr1* (31,32) (also in some calbindin-GABAergic neurons) (Figure 2). Few (<20%) BLA *Hcrtr1*-possessing cells express *Gad1* (GABAergic marker) and co-express parvalbumin (~10%) (Figure 2G–K). Our results suggest that *Hcrtr1* is expressed in 10% to 15% of BLA glutamatergic neurons and ~5% of GABA cells (Figure 2K). In BLA cells, messenger RNA (mRNA) for *Hcrtr1* and *Hcrtr2* largely does not overlap, and ~80% of *Hcrtr1*$^+$ cells do not co-express *Hcrtr2* (Figure 2L–O). Specific BLA GABAergic neurons may predominantly localize Orx_2R (Figure 2P) (28).

Motivation for Active Avoidance (Escape)

In the SAM, animals evenly choose one of two stable (27–29,33) behavioral phenotypes, Escape (44.7%) or Stay (55.3%) (Figure 1A and Figure S1B, C) (26,27,29,33,34). Time spent investigating escape routes predicts active avoidance and indicates motivation to escape (28). Time spent attentive to the hole was significantly greater in vehicle-treated Escape mice (Figure 3A), but intra-BLA infusion of the Orx_1R antagonist (Escape: Figure 3B, C; Stay: Figure 3B, D) or AAV-U6-Orx_1R-shRNA (Figure 3E) increases attention to the escape route. Furthermore, receptor activation with Orx_A reduced time that Escape mice spent investigating the escape route (Figure S3).

Avoidance (Escape)

Upon intra-BLA injections of an Orx_1R antagonist on SAM day 3, a substantial number of Stay mice exhibited Escape behavior (Figure 4A), with a 30% shift that day and a significant increase the day after (day 4 = 70% increase). Intra-BLA activation of both Orx receptors with Orx_A or biased activation of Orx_1R (Orx_A+Orx_2R antagonist) blocked Escape behavior in a small, but not statistically significant, proportion of mice on days 3 and 4 (Figure S4), exhibiting deviation from stable phenotype behavioral patterns.

Because knockdown reduced Orx_1R expression prior to stressful interactions, we did not expect a dramatic change in behavior over the course of SAM trials, but AAV-U6-Orx_1R-shRNA yielded incrementally (although not significantly) more escape on the last 2 days (Figure 4B). By day 4, 72.7% of AAV-U6-Orx_1R-shRNA–treated mice displayed Escape compared with 54.5% of the scramble control mice.

Escape mice spent significantly less time in the SAM arena with the CD1 mouse on days 2 to 4 (26,27,29,33,35); therefore, escape latency was reduced (Figure 4C). Stay mice remained for the entire 5-minute period unless treated with Orx_1R antagonist, significantly reducing time spent with aggressive CD1 mice on day 4 (Figure 4D, F). Inhibition of Orx_1R did not influence escape latency in Escape animals (Figure 4D). Neither of the Orx_1R manipulations, antagonist or knockdown treatments, influenced arousal/locomotion (Figure S5) but did result in small but significant decreases in food intake and body weight (Figure S6).

Cued and Contextual Fear Conditioning

Cued fear responses significantly enhanced freezing in both Escape and Stay phenotypes, and Stay mice displayed heightened freezing behavior to context (opaque cylinder divider) as well (Figure 5A, B). Although inhibition of intra-BLA Orx_1R did not affect the fear freezing profile in Escape mice, antagonist-treated Stay mice exhibited significantly reduced contextual and cued fear responses (Figure 5B and Figure S7; Table S1). Similar to mice of the Escape phenotype, knockdown of BLA Orx_1R did not affect conditioned freezing behavior (Figure S8). Activation of intra-BLA Orx receptors with Orx_A did not change the fear freezing profile in Escape or Stay mice compared with vehicle (Figure 5B and Figure S7; Table S1). However, biased stimulation of Orx_1R in the BLA with a combination of Orx_A+Orx_2R antagonist eliminated the CR in Escape, but not Stay, mice (Figure 5B and Figure S7; Table S1). Furthermore, acute inhibition of Orx_2R in the BLA eliminated the cued freezing response in Escape mice and significantly reduced freezing during the post-tone period (Figure 5B and Figure S7; Table S1). Stay mice treated with an

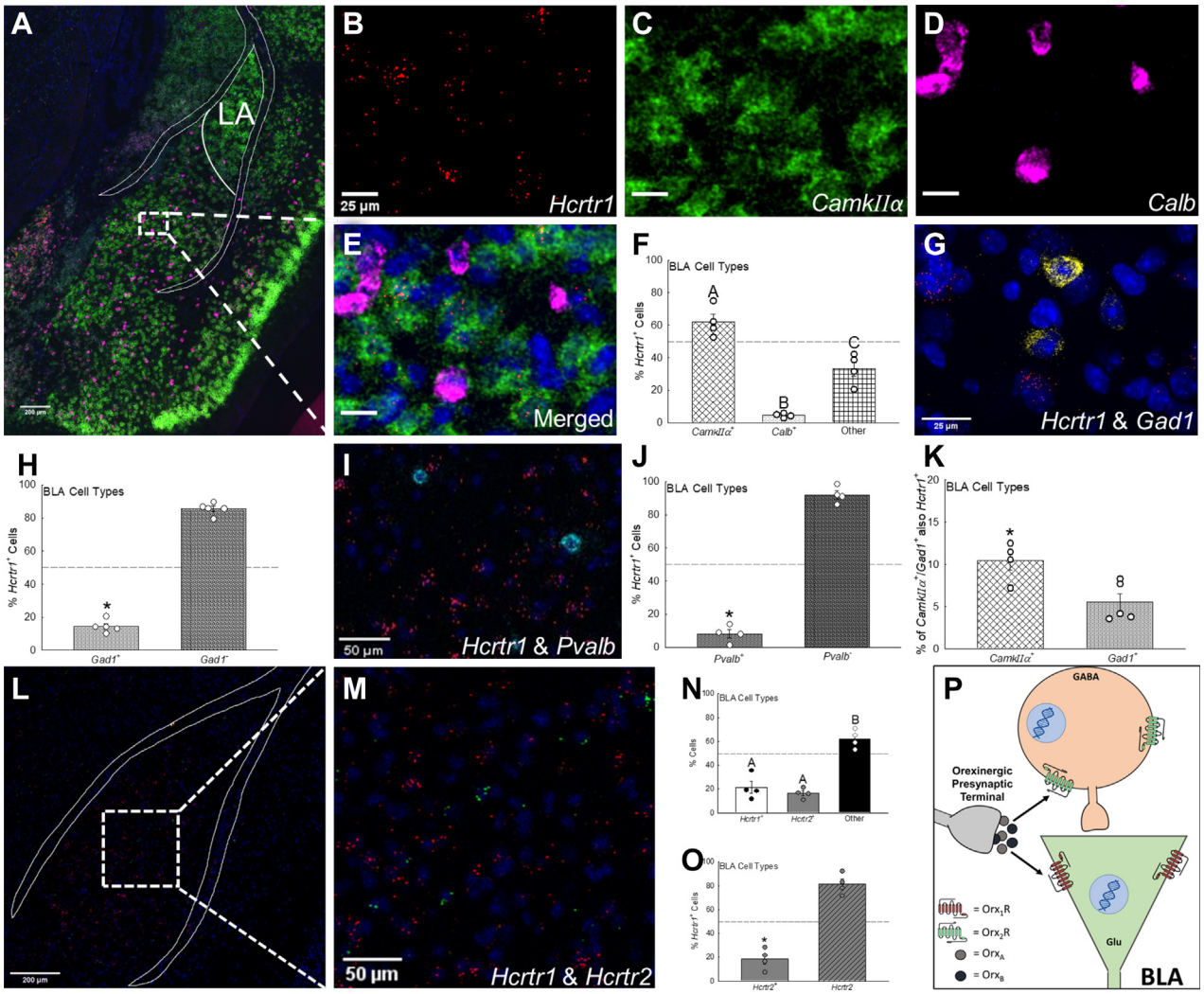

Figure 2. In the untreated BLA, Orx_1Rs are expressed predominantly in glutamatergic neurons and are rarely co-expressed with Orx_2Rs. **(A)** Imaged sections containing BLA cells stained with probes targeting mRNA of **(B)** *Hcrtr1* (red), **(C)** *CamkIIα* (green), and **(D)** *Calb* (magenta) revealed when **(E)** merged (with DAPI) that **(F)** Orx_1R^+ cells mostly co-express the glutamatergic cell marker *CamkIIα* ($n = 4$, $F_{2,9} = 54.4$, $p < .001$; $CamkII\alpha^+$ vs. $Calb^+$: $t_6 = 10.4$, $p < .001$; $CamkII\alpha^+$ vs. other: $t_6 = 5.2$, $p < .001$; $Calb^+$ vs. other: $t_6 = 5.2$, $p < .001$; bars are statistically different from one another as illustrated with unique letters, e.g., A is significantly different from B and C; $p < .001$). **(G)** Expression of *Hcrtr1* (red) and GAD_{67} (*Gad1*) mRNA (yellow) infrequently overlap with **(H)** most $Hcrtr1^+$ cells being absent of the GABAergic marker ($n = 5$, $t_8 = 29.5$, $^*p < .001$). **(I)** While a subset of BLA GABAergic neurons produce the calcium-binding protein parvalbumin ($Pvalb^+$), **(J)** $Hcrtr1^+$ (red) cells are mostly absent of *Pvalb* expression (light blue) with **(K)** <10% being both $Hcrtr1^+$ and $Pvalb^+$ ($n = 4$, $t_6 = 23.1$, $^*p < .001$). **(K)** Furthermore, more BLA glutamatergic ($CamkII\alpha^+$) neurons (compared with GABAergic → $GAD1^+$) also express *Hcrtr1* ($n = 9$, $t_7 = 3.2$, $^*p \leq .015$). **(L)** Images of BLA cells with fluorescent markers labeling **(M)** *Hcrtr1* mRNA (red) and *Hcrtr2* mRNA (green) demonstrate that **(N)** most BLA cells express neither *Hcrtr1* nor *Hcrtr2* ($n = 4$, $F_{2,9} = 42.1$, $p < .001$; $Hcrtr1^+$ vs. other, $t_6 = 7.5$, $p < .001$; $Hcrtr2^+$ vs. other, $t_6 = 8.4$, $p < .001$; bars are statistically different from one another as illustrated with unique letters, e.g., A is significantly different from B). **(O)** Most $Hcrtr1^+$ cells in the BLA do not express *Hcrtr2* ($n = 4$, $t_6 = 10.1$, $^*p < .001$), as depicted in **(P)** showing Orx_1R on glutamatergic neurons. BLA, basolateral amygdala; GABA, gamma-aminobutyric acidergic neuron; Glu, glutamatergic neuron; LA, lateral amygdala; mRNA, messenger RNA; Orx_1R, orexin 1 receptor; Orx_2R, orexin 2 receptor.

Orx_2R antagonist displayed no statistical differences in the levels of contextual and cued freezing (Figure 5B and Figure S7; Table S1).

Corticosterone Concentrations

Social stress in SAM interactions increases corticosterone concentrations in both Escape and Stay animals (27,28,33), although Stay mice have higher levels of corticosterone than Escape mice. Inhibition of BLA Orx_1R decreased Stay corticosterone concentrations compared with vehicle-treated Stay animals and did not differ significantly from nonstressed mice (Figure 5C). Treatments with Orx_A or the combination of Orx_A and an Orx_2R antagonist did not change corticosterone levels relative to vehicle-treated control mice; however, the differences between Escape and Stay were eliminated and levels

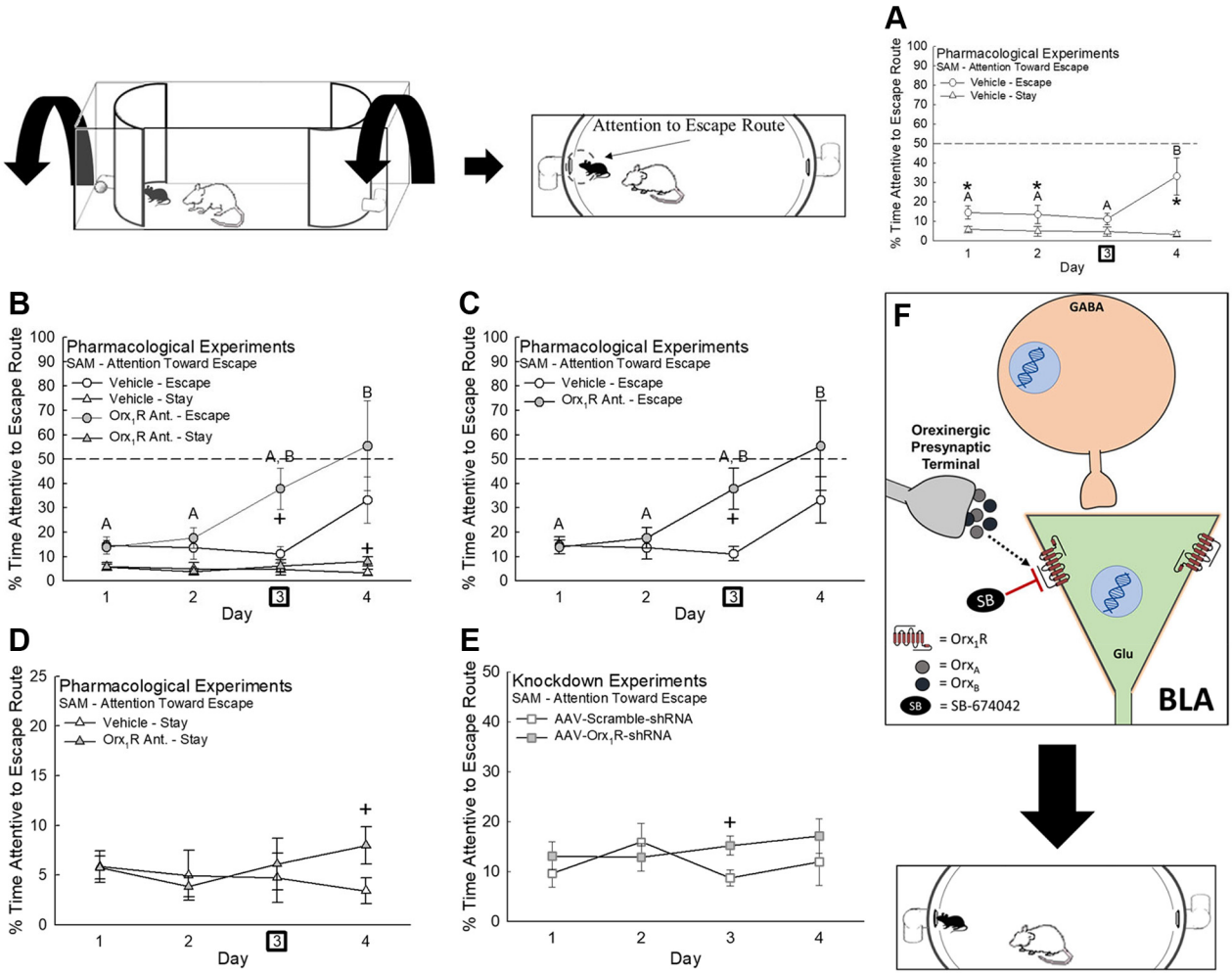

Figure 3. Motivation toward Escape behavior is effected through inhibition of intra-BLA Orx$_1$Rs. **(A)** Escape mice, as compared with those expressing the Stay phenotype, spend a greater percentage of time investigating the SAM escape routes (n = 19; phenotype effect: $F_{1,51}$ = 16.4, $p < .001$; Escape vs. Stay: day 1, t_{17} = 2.6, $^{*}p \leq .018$; day 2, t_{17} = 2.5, $^{*}p \leq .017$; day 4, t_{17} = 4.2, $^{*}p < .001$). **(B)** While Escape mice, in general, explore the escape routes more often, **(C)** inhibition of intra-BLA Orx$_1$R promotes even more attention toward the escape tunnels (n = 34; treatment effect: $F_{1,30}$ = 7.7, $p \leq .019$; day 3 vehicle escape vs. Orx$_1$R Ant. Escape, t_{10} = 2.5, $^{+}p \leq .018$). **(D)** Antagonism of intra-BLA Orx$_1$R only slightly stimulates escape route exploration in Stay mice (day 4 vehicle × Orx$_1$R Ant., t_{20} = 2.1, $^{+}p \leq .05$). **(E)** Knockdown of intra-BLA Orx$_1$R temporarily and minimally increases attention toward escape on day 3 of the SAM (n = 22; day 3 scramble vs. AAV-Orx$_1$R-shRNA, t_{20} = 2.4, $^{+}p \leq .024$). **(F)** Illustration demonstrating that inhibition of intra-BLA Orx$_1$Rs predominantly on glutamatergic neurons promotes attention toward the escape route in the SAM arena. In pharmacological experiments, drug treatment is administered on day 3 as designated by the bold square. Note that the data plotted in panels **(A)**, **(C)**, and **(D)** are the same as those graphed in panels **(B)**; we have separated out these individual comparisons for the sake of clarity. Ant., antagonist; BLA, basolateral amygdala; GABA, gamma-aminobutyric acidergic neuron; Glu, glutamatergic neuron; Orx$_1$R, orexin 1 receptor; Orx$_A$, orexin A; Orx$_B$, orexin B; SAM, Stress Alternatives Model.

were elevated compared with Orx$_1$R antagonist–treated mice (Figure 5C). Inhibition of BLA Orx$_1$R not only reduces social fear responses but also reverses social stress responsiveness.

Antagonism of Intra-BLA OrxR Recruits Alternative Signaling

Although *Hcrtr1* expression was unaltered following vehicle treatment, Orx$_1$R antagonism reduced intra-BLA *Hcrtr1* in Escape mice compared with nonstressed cage control mice (Figure 6A) and simultaneously elevated *Hcrtr2* expression in Stay mice compared with Escape and vehicle-treated Stay mice (Figure 6B; Table S2). In vehicle control animals, *Hcrtr2* expression was higher in Escape mice than both Stay and Orx$_1$R antagonist–treated Escape mice (Figure 6B; Table S2). A reduction in *Hcrtr1* gene expression after Orx$_2$R antagonism was observed, but only in Stay animals relative to vehicle (Figure 6A; Table S2). Expression of *Hcrtr2* in the BLA was reduced in both Escape and Stay phenotypes after blocking Orx$_2$R, contrasting with Orx$_1$R antagonism, which enhanced *Hcrtr2* mRNA levels in Stay mice (Figure 6B; Table S2).

Transcription of BLA PLC$_{\beta 1}$ (*Plcb1*) mRNA (13) is important for Orx$_1$R signaling (36). We predicted that Orx$_1$R antagonist

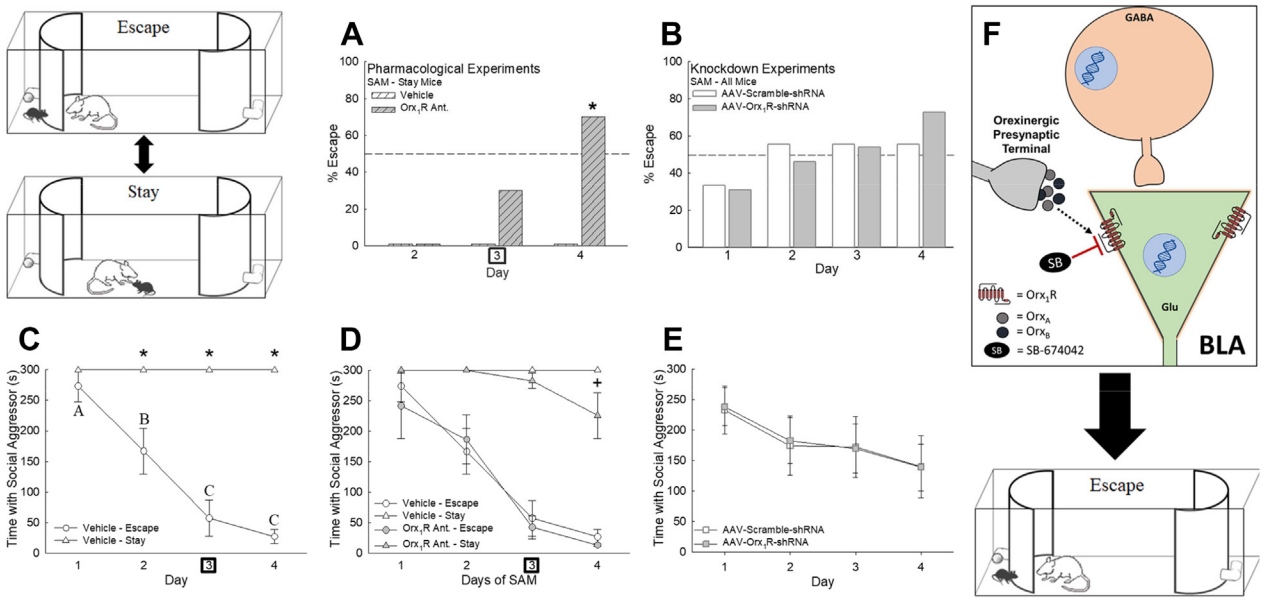

Figure 4. Intra-BLA Orx_1Rs mediate stress-related behavioral phenotype development. **(A)** Infusion of an Orx_1R Ant. (SB-674042) into the BLA promotes escape behavior in Stay mice (n = 22; day 4, χ^2: F_1 = 9.3, $^*p < .001$). **(B)** Knockdown of Orx_1R (AAV-Orx_1R-shRNA) upsets normal day 2 phenotype commitment behavior (as observed with AAV-Scramble-shRNA controls), inducing more escape behavior on days 3 and 4 (n = 22). **(C)** Escape animals learn to efficiently use the escape route to avoid social aggression (escape latency = time with social aggressor) over the course of 4 days while Stay mice remain with the aggressor (n = 19; phenotype effect: $F_{1,45} = 175.3$, $p < .001$; time effect: $F_{3,45} = 26.1$, $p < .001$; interaction effect: $F_{3,45} = 26.1$, $p < .001$; Escape vs. Stay: day 2, $t_{17} = 5.8$, $^*p < .001$; day 3, $t_{17} = 10.6$, $^*p < .001$; day 4, $t_{17} = 11.9$, $^*p < .001$; within–Escape phenotype comparison, $F_{3,18} = 17.8$, $p < .001$, day 1 vs. day 3, $t_6 = 5.7$, $p < .001$; day 1 vs. day 4, $t_6 = 6.5$, $p < .001$; day 2 vs. day 3, $t_6 = 2.9$, $p \leq .009$; day 2 vs. day 4, $t_6 = 3.7$, $p \leq .002$; $p < .05$ for days marked with unique lettering, e.g., A is different from B and C). **(D)** Antagonizing intra-BLA Orx_1Rs promotes aggressor avoidance in Stay mice (n = 34; time effect: $F_{3,54} = 2.9$, $p \leq .043$; interaction effect: $F_{3,54} = 2.9$, $p \leq .043$; day 4 vehicle Stay vs. Orx_1R Ant. Stay, $t_{20} = 3.4$, $^+p < .001$) but has no effect on those animals exhibiting the Escape phenotype. **(E)** Knockdown of intra-BLA Orx_1R does not affect the overall latency of aggressor avoidance (n = 22). Overall, **(F)** inhibition of Orx_1R in the BLA appears to prompt escape behavior. In pharmacological experiments, drug treatment is administered on day 3 as designated by the bold square. Ant., antagonist; BLA, basolateral amygdala; GABA, gamma-aminobutyric acidergic neuron; Glu, glutamatergic neuron; Orx_1R, orexin 1 receptor.

might limit *Plcb1* expression levels (Figure 6C). Escape mice in both vehicle and Orx_1R antagonist groups expressed lower amounts of *Plcb1* than Stay and cage control animals (Figure 6C). Furthermore, greater *Plcb1* followed intra-BLA Orx_2R inhibition compared with vehicle-treated Escape mice (Figure 6C; Table S2).

Alternative molecular pathways recruited during G_q activation are driven by ERK genes (*Mapk1* and *Mapk3*). In Stay mice, Orx_1R antagonism resulted in a significant increase in *Mapk3* expression (*Mapk1* mRNA was unaffected) (Figure S9) compared with similarly treated Escape, vehicle-treated Stay, and nonstressed cage control mice (Figure 6D; Table S2). Inhibition of intra-BLA Orx_2R did not alter *Mapk3* gene expression (Figure 6D; Table S2).

The transcription of BDNF (*Bdnf* [brain-derived neurotrophic factor]) is tied to neuroplasticity (37,38) and behavioral changes such as extinction of fear memories (39), so we predicted that an increase in *Bdnf* might be associated with intra-BLA Orx_1R inhibition (Figure 6E). As hypothesized, intra-BLA Orx_1R antagonism resulted in elevated *Bdnf* in Stay mice compared with Escape mice and vehicle-treated Stay mice (Figure 6E and Table S2). Finally, Orx_2R antagonist treatment enhanced *Bdnf* expression in Escape mice while diminishing transcription in Stay animals, an effect that is phenotypically opposite to that observed after Orx_1R inhibition (Figure 6E; Table S2). Because Stay mice treated with an Orx_1R antagonist experienced shifts from stress-vulnerable to stress-resilient behavioral responses, the alterations in gene expression reported here (Figure 6F, G) may be implicit in this behavioral plasticity.

Molecular Restructuring Is Related to Fear Responsiveness

Expression levels of *Hcrtr2*, but not *Hcrtr1*, in both vehicle-treated and Orx_1R antagonist–treated mice are negatively correlated with cued freezing (Figure 7A, B). Relative expression levels of *Plcb1* were positively correlated with cued freezing behavior in vehicle-treated mice (Figure 7C); however, this relationship is not observed after intra-BLA Orx_1R inhibition (Figure 7D). Contextual freezing behavior was associated with *Mapk3* expression in only vehicle-treated mice (Figure S10I). By contrast, intra-BLA antagonism of Orx_1R cued freezing behavior was negatively correlated to *Mapk3* expression (Figure 7F), but not in vehicle-treated mice (Figure 7E). The lack of gene expression correlations with cued fear freezing when phenotypes were assessed independently (Figure S11) indicates that behavioral and transcriptional relationships exist within collective operational adaptations that link behavioral change to molecular modification. No

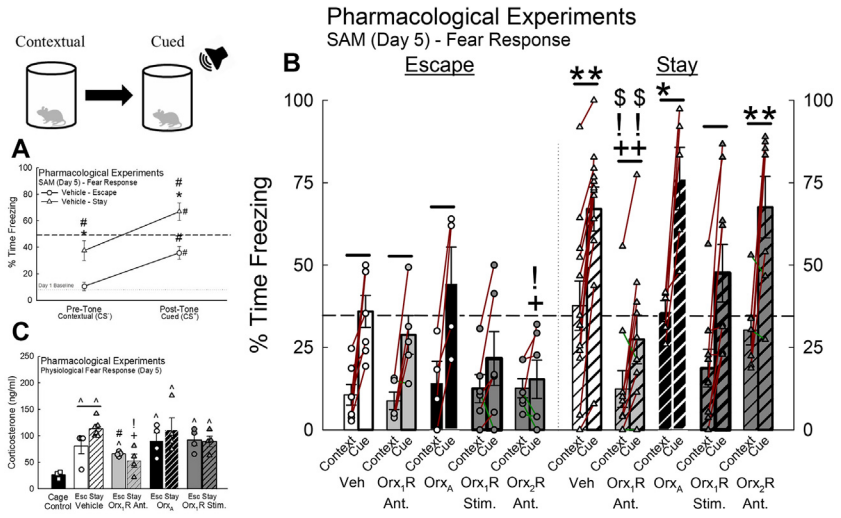

Figure 5. Inhibition of Orx$_1$Rs in the BLA reduced contextual/cued fear responses and stress hormone concentrations. **(A)** Although both Escape and Stay phenotypes learn to associate a cue (tone, CS$^+$) with social aggression (phenotype effect: $F_{1,17} = 7.6$, $p \leq .013$; CS effect: $F_{1,17} = 47.7$, $p < .001$; Escape CS$^-$ vs. CS$^+$, $t_6 = 3.9$, $^{\#}p \leq .008$; Stay CS$^-$ vs. CS$^+$, $t_{11} = 5.7$, $^{\#}p < .001$), Stay mice exhibit heightened freezing behavior to both context (CS$^-$; $t_{17} = 2.8$, $^*p \leq .011$) and tone (CS$^+$; $t_{17} = 2.3$, $^*p \leq .033$). Baseline measurements of freezing are represented by a dotted line. **(B)** Antagonism of intra-BLA Orx$_1$Rs reduces conditioned fear responses in Stay animals while Orx$_2$R inhibition diminishes fear freezing in Escape mice ($n = 71$; * represents significant differences compared with Escape mice in the same treatment group; + signifies significance compared with vehicle-treated animals in the same phenotype group; ! identifies significant differences compared with Orx$_A$-treated mice; $ denotes significant differences compared with Orx$_2$R Ant.-treated animals). See Figure S7 for specific a priori hypotheses comparisons. **(C)** Mice exposed to social stress produce elevated levels of stress hormone ($n = 39$, $F_{2,12} = 24.3$, $p < .001$; cage control vs. vehicle Escape, $t_5 = 3.1$, $^{\wedge}p \leq .028$; cage control vs. vehicle Stay, $t_9 = 9.9$, $^{\wedge}p < .001$); however, Stay animals have the highest concentration (vehicle Escape vs. Stay, $t_{10} = 2.6$, $p \leq .025$). Inhibition of intra-BLA Orx$_1$R reduces corticosterone levels in Stay mice (vehicle Stay vs. Orx$_1$R Ant. Stay, $t_{10} = 5.1$, $^{+}p < .001$; Orx$_1$R Ant. Stay vs. Orx$_A$ Stay, $t_6 = 3.3$, $^{!}p \leq .002$). Ant., antagonist; BLA, basolateral amygdala; CS, conditioned stimulus; Esc, escape; Orx$_1$R, orexin 1 receptor; Orx$_2$R, orexin 2 receptor; Orx$_A$, orexin A; SAM, Stress Alternatives Model; Stim., stimulation; Veh, vehicle.

relationships between gene expression and conditioned fear freezing were observed for any of the tested cell signaling markers after Orx$_2$R antagonism (not Orx$_1$R antagonism) except for *Bdnf*, in which a significant negative correlation was revealed (Figure S12E). Together, these results suggest a functional connection between Orx$_1$R antagonist–induced shifts in gene expression and fear-related behaviors.

Potential Molecular Mechanism Behind Intra-BLA Orx$_1$R Antagonism

To help generate a theoretical mechanism to explain the physiological basis surrounding the observed behavioral and phenotypic shifts resulting from intra-BLA inhibition of Orx$_1$R, we explored transcriptional relationships in systems that exhibited similar regression patterns (Figure 8). With antagonism of Orx$_1$R, there is a strongly positive relationship between *Hcrtr2* and *Mapk3* expression (Figure 8A). This association does not exist after vehicle or Orx$_2$R antagonist treatment (Figure S13). While there are no observed relationships between *Bdnf* and *Hcrtr2* expression levels (Figure 8B and Figure S13), *Bdnf* expression is positively correlated to *Mapk3* expression in animals treated with an Orx$_1$R antagonist (Figure 8C). Notably, no relationships exist between *Hcrtr1* expression and the other genes of interest (Figure S13). These data allowed us to predict a working model to explain how BLA Orx$_1$Rs may function to establish behavioral patterns consistent with stress-induced phenotype development (Figure 9).

DISCUSSION

Antagonism of Orx$_1$Rs in the BLA can reverse or diminish expression of stress-related behavior. Our results suggest that BLA Orx$_1$Rs play a central role in stress responsiveness (40,41) and related behavioral, physiological, and molecular outcomes that are important components of affective disorders (42,43), such as anxiety (7), depression, and posttraumatic stress disorder. Acute inhibition of intra-BLA Orx$_1$Rs promotes Escape over Stay responses and limits freezing during fear conditioning in a phenotype-dependent way. Furthermore, inhibition of Orx$_1$Rs alters gene expression associated with critical signaling cascades. Following intra-BLA Orx$_1$R antagonism, transcription for receptors and intracellular signaling becomes biased toward Orx$_2$R (*Hcrtr2*) over Orx$_1$R (*Hcrtr1*) and ERK$_1$ (*Mapk3*) over PLC$_{\beta 1}$ (*Plcb1*) pathways. Even when BLA Orx$_1$Rs are inhibited, native Orx$_A$ and Orx$_B$ will bind Orx$_2$Rs. The relationship of these behavioral and molecular changes to enhanced expression of *Hcrtr2* mRNA, potentially in BLA neurons that do not contain Orx$_1$R (Figure 2L–O), suggests receptor-mediated mechanisms that balance pro- and anti-stress responses in BLA microcircuits.

Aggressive social interactions in SAM produced two behavioral phenotypes that represent risk assessment and choice: Escape and Stay. These phenotypes, similar to those exposed to social defeat paradigms (44,45), exhibit resilience (tightly linked to Escape) and susceptibility (highly correlated with Stay) in the social interaction/preference test (28). However, unlike traditional social defeat, SAM-separated phenotypes are expressed early in the behavioral paradigm, providing insight into the development and progression of stress-induced behavior and pathophysiology. Anxiolytic drugs (such as CRF$_1$ receptor antagonist antalarmin and the Orx$_2$R agonist [Ala^{11}, d-Leu^{15}]–Orx$_B$) promote escape, while anxiogenic drugs (such as the α_2 antagonist yohimbine and the Orx$_2$R antagonist MK-1064) delay and/or block escape behavior (28,29). Surprisingly, neither the Orx$_1$R antagonist (Figure 4D) nor knockdown (Figure 4E) influenced escape latency, although it is reduced by anxiolytic factors such as exercise, neuropeptide S, and antalarmin and increased by anxiogenic factors such as yohimbine (29). We posit that enhanced escape on day 4, following BLA Orx$_1$R inhibition (on

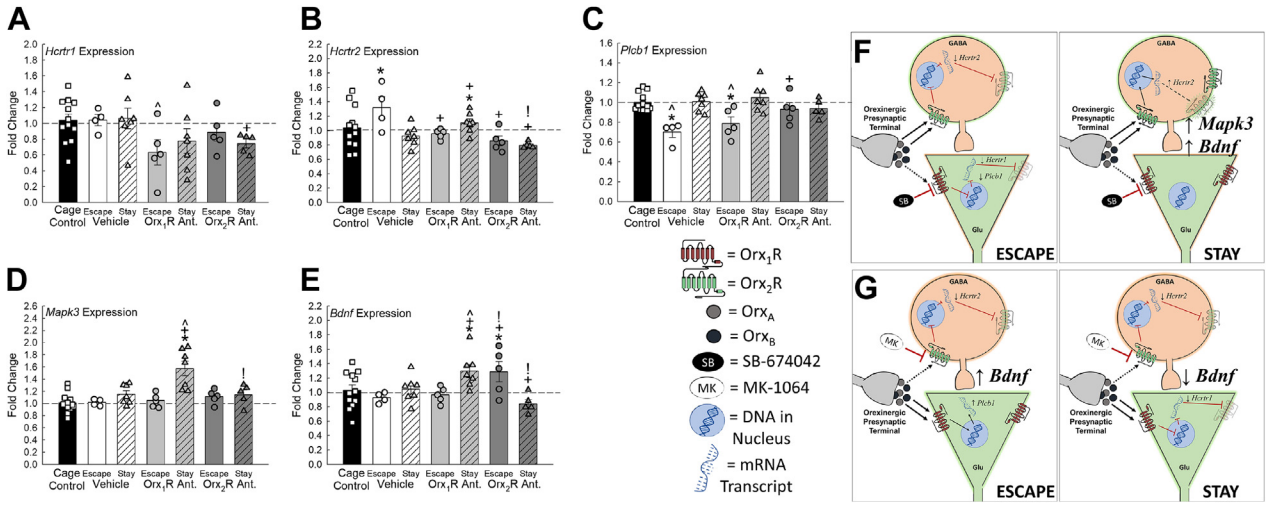

Figure 6. Transcriptional changes (relative to home-cage naïve controls) in BLA after Orx$_1$R or Orx$_2$R antagonism shifts signaling profile. **(A)** Antagonism of Orx$_1$R in the BLA reduces *Hcrtr1* expression (n = 45; treatment effect: $F_{2,27} = 3.5$, $p \leq .043$), but only significantly so in animals expressing the Escape phenotype (cage control vs. Orx$_1$R Ant. Escape, $t_{11} = 2.2$, $^{\wedge}p \leq .050$), whereas infusion of an Orx$_2$R Ant. in the BLA reduces *Hcrtr1* expression in Stay mice compared with vehicle animals of the same phenotype ($t_{10} = 2.2$, $^{+}p \leq .044$). **(B)** While Escape mice (treatment effect: $F_{2,27} = 9.8$, $p < .001$; interaction effect: $F_{2,27} = 8.6$, $p < .001$) treated with vehicle express higher *Hcrtr2* levels than Stay mice ($t_9 = 3.0$; $^{*}p \leq .016$) and Orx$_1$R or Orx$_2$R Ant.-treated Escape animals (vehicle vs. Orx$_1$R Ant., $t_7 = 2.6$, $^{+}p \leq .035$; vehicle vs. Orx$_2$R Ant.: $t_7 = 4.5$, $^{+}p < .001$; Orx$_1$R Ant. vs. Orx$_2$R Ant.: $t_8 = 3.5$, $^{!}p < .001$), Orx$_1$R antagonism results in elevated levels (Escape vs. Stay, $t_{10} = 2.2$, $^{*}p \leq .05$; vehicle vs. Orx$_1$R Ant., $t_{12} = 2.4$, $^{+}p \leq .034$) while Orx$_2$R inhibition leads to a reduction (vehicle vs. Orx$_2$R Ant.: $t_{10} = 3.5$, $^{+}p \leq .002$; Orx$_1$R Ant. vs. Orx$_2$R Ant.: $t_{10} = 4.7$, $^{!}p < .001$) of *Hcrtr2* in Stay mice. **(C)** A reduction of *Plcb1* (phenotype effect: $F_{1,27} = 19.1$, $p < .001$; interaction effect: $F_{2,27} = 4.3$, $p \leq .023$) that is found in Escape mice under control conditions (cage control vs. vehicle Escape, $t_{10} = 5.1$, $^{\wedge}p < .001$; Escape vs. Stay, $t_9 = 5.0$, $^{*}p < .001$) and Orx$_1$R antagonism (Escape vs. Stay, $t_{10} = 3.1$, $^{*}p \leq .012$; cage control vs. Orx$_1$R Ant., $t_{11} = 3.3$, $^{\wedge}p \leq .007$) was eliminated with intra-BLA Orx$_2$R antagonism (vehicle vs. Orx$_2$R Ant.: $t_7 = 2.8$, $^{+}p \leq .017$). **(D)** While Stay mice treated with an Orx$_1$R Ant. express higher levels of *Mapk3* (phenotype effect: $F_{1,27} = 11.3$, $p \leq .002$; treatment effect: $F_{2,27} = 4.3$, $p \leq .023$; interaction effect: $F_{2,27} = 5.1$, $p \leq .013$) in the BLA compared with vehicle control animals ($t_{12} = 3.1$, $^{+}p < .001$), administration of an Orx$_2$R Ant. does not induce the same transcriptional response (Orx$_1$R Ant. vs. Orx$_2$R Ant.: $t_{10} = 2.7$, $^{!}p \leq .022$). **(E)** Expression of *Bdnf* in the BLA after treatment (interaction effect: $F_{2,27} = 10.6$, $p < .001$) with an Orx$_2$R Ant. was enhanced in Escape mice (Orx$_2$R Ant. Escape vs. Stay: $t_8 = 2.9$, $^{*}p \leq .019$; vehicle vs. Orx$_2$R Ant.: $t_7 = 2.7$, $^{+}p \leq .013$; Orx$_1$R Ant. vs. Orx$_2$R Ant.: $t_8 = 2.5$, $^{!}p \leq .017$) and reduced in Stay animals (vehicle vs. Orx$_2$R Ant.: $t_{10} = 2.2$, $^{+}p \leq .05$; Orx$_1$R Ant. vs. Orx$_2$R Ant.: $t_{10} = 3.9$, $^{!}p < .001$); a phenotypically opposite effect was observed after Orx$_1$R antagonism (Escape vs. Stay, $t_{10} = 2.8$, $^{*}p \leq .018$; Orx$_1$R Ant. Stay vs. vehicle Stay, $t_{12} = 2.2$, $^{+}p \leq .049$). Transcriptional changes after **(F)** intra-BLA Orx$_1$R antagonism and **(G)** Orx$_2$R inhibition were differentially regulated in a phenotype-dependent fashion. Ant., antagonist; BLA, basolateral amygdala; GABA, gamma-aminobutyric acidergic neurons; Glu, glutamatergic neurons; mRNA, messenger RNA; Orx$_1$R, orexin 1 receptor; Orx$_2$R, orexin 2 receptor; Orx$_A$, orexin A; Orx$_B$, orexin B.

day 3 drug treatment), is a reflection of the shift toward antistress signaling indicated by downregulation in prostress signaling (*Hcrtr1*) and upregulation of antistress systems (*Hcrtr2, Mapk3, Bdnf*). Thus, BLA dual Orx$_1$R/Orx$_2$R inhibition may not promote behavioral change. These stress-induced effects are paired with important learning and motivational components during SAM interactions (27,29,33,35) and in human affective disorders (46).

In addition to species-specific anxious behavior and learning, social stress promotes behavioral inhibition, depressed motivation, and depressed behavioral drive in some individuals (47), plus a lower rate of adaptive behavior (48). Behavioral depression reveals two distinctive phenotypes related to stress responsiveness in humans and other animals (45,49,50). In SAM social interactions, Stay animals do less exploration of the escape route (Figure 3A) and show indecisiveness relative to escape (35). Measuring motivation in the SAM is derived from a simple choice process, Escape or Stay (26,27). Antagonism and knockdown of Orx$_1$Rs increases interest in the escape route for both Stay and Escape mice (Figure 3C, D). Thus, BLA Orx$_1$Rs regulate stress-induced motivational behaviors, greatest in Escape mice but marking a dramatic behavioral reversal in Stay mice that typically avoid the escape route (Figure 3B, C). Attention to the escape route happens prior to escape and is thus the first evidence of phenotypic differentiation in the SAM (28,35). Latency to escape and escape behavior also are influenced by motivation, although as previously demonstrated, these behaviors are strongly affected by stress and fearfulness associated with familiarity of the SAM or social interaction (27–29,33,35). Our results, similar to those of others, suggest that Orx activity plays a fundamental role in motivation (8,51) and, in this case, specifically in the BLA for behaviors associated with stress-related motivation and choice.

Understanding the development of choice and motivation in the SAM is enhanced by pairing aversive aggression (US) with a nonthreatening stimulus (tone CS) prior to interaction, promoting potent cued and contextual CR similar to standard fear conditioning approaches that use foot shock as a US (52). While the CRs elicited are similar, e.g., freezing (53), the ethological and ecological relevance of the US to the subject are not. By associating naturally aversive US with a benign stimulus (54), the SAM allows views into development of fear learning as it relates to the etiology of stress-provoked

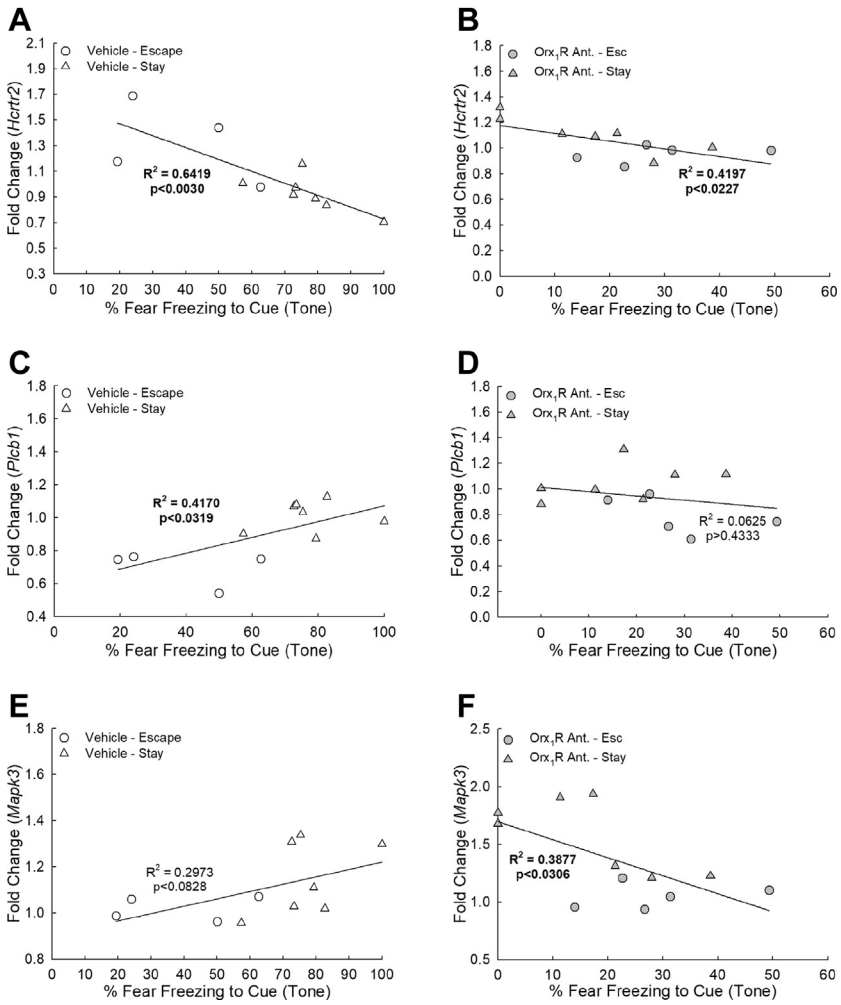

Figure 7. Conditioned fear freezing response is related to gene expression changes (fold change relative to home-cage naïve control mice) resulting from intra–basolateral amygdala Orx$_1$R antagonism. In both **(A)** vehicle-treated (n = 11, $F_{1,9}$ = 16.1, R^2 = 0.6419, $p \leq .003$) and **(B)** Orx$_1$R Ant.-treated (n = 12, $F_{1,10}$ = 7.2, R^2 = 0.4197, $p \leq .023$) animals, a negative correlation exists between *Hcrtr2* expression and cued fear freezing. **(C)** With vehicle treatment, relative *Plcb1* expression is positively associated with cued fear freezing ($F_{1,9}$ = 6.4, R^2 = 0.417, $p \leq$ .0319). **(D)** This relationship is not observed in mice that were administered an Orx$_1$R Ant. ($F_{1,10}$ = 0.7, R^2 = 0.0625, $p \geq .4333$). **(E)** While there is not a significant association between *Mapk3* expression and cued fear freezing after vehicle treatment ($F_{1,9}$ = 3.8, R^2 = 0.2973, $p \geq .0828$), **(F)** a significant negative correlation is observed after Orx$_1$R antagonism ($F_{1,10}$ = 6.3, R^2 = 0.3877, $p \leq .0306$). Ant., antagonist; Esc, escape; Orx$_1$R, orexin 1 receptor.

neurocircuitry changes and demonstrates a connection between stress-induced fear expression and phenotype (Figure 5). While early work suggested that only Stay mice exhibited cued fear learning (27,33), it is now clear that both Stay and Escape mice respond to auditory cues with enhanced freezing compared with pretone freezing, and Stay mice also show contextual (prior to the cue) fear conditioning (Figure 5A).

Fear responses are mediated through Orx$_1$R activity in the amygdala and in the locus coeruleus, which connects to the amygdala (22,55–57). Our results similarly demonstrate that Orx$_1$R, but not Orx$_2$R, inhibition diminishes both contextual and cued conditioned fear freezing in Stay animals (Figure 5B; Table S1). While antagonizing Orx$_1$R reduces fear- and panic-induced freezing (7,56,58), Orx$_2$R antagonism appears to eliminate fear learning in Escape mice, suggesting a phenotype-dependent effect (Figure 5B; Table S1). Although Orx$_2$R antagonism in the BLA reduced cued freezing only in Escape mice, we have previously demonstrated a potential anxiogenic effect of blocking receptor function (25,28). This response may be dependent on brain region because Orx$_2$R activity in the nucleus accumbens shell and prelimbic prefrontal cortex may enhance anxious behavior (59,60). Furthermore, Orx$_2$R antagonism has demonstrated antidepressive capabilities in a clinical setting (61).

Stimulation of intra-BLA Orx$_1$Rs and Orx$_2$Rs using Orx$_A$ in Stay mice produces no reduction in contextual or cued fear conditioning (Figure 5B; Table S1), suggesting that the inhibition of both types of learned fear responses result specifically from Orx$_1$R inhibition in Stay mice. To clarify the roles of Orx$_1$R and Orx$_2$R, we administered Orx$_A$ while concurrently inhibiting Orx$_2$R (MK1064), leaving Orx$_1$R stimulated, and again there was no statistically significant reduction in either type of fear conditioning response (Figure 5B; Table S1). Knockdown of Orx$_1$R did not affect the fear freezing profile (Figure S8). Because knockdown occurred before the introduction of social stress, activity levels of Orx$_1$R after SAM exposure allowed for fear learning (higher freezing after CS), but did not diminish freezing as observed with acute antagonism after stress and phenotype development (Figure 5B).

Molecular gene expression during SAM fear conditioning and phenotype development indicated potential shifts in receptor-linked intracellular signaling cascades (Figure 6).

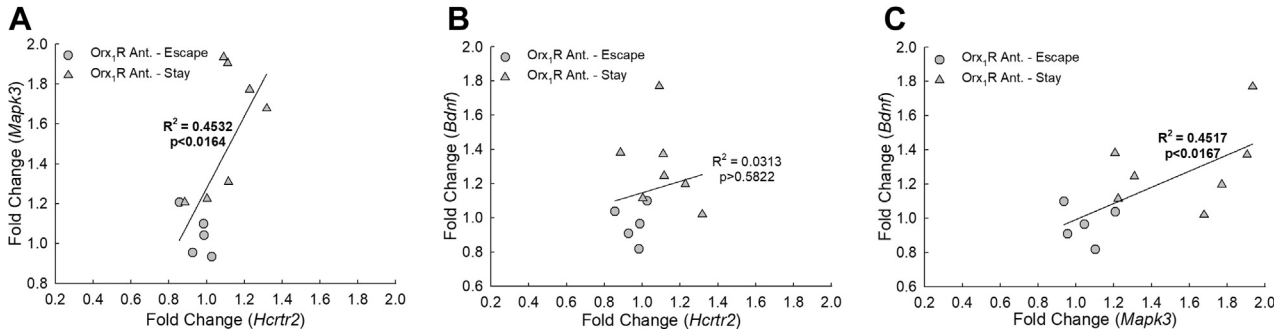

Figure 8. The basolateral amygdala transcriptional changes (relative to home-cage naïve control animals) that result from Orx$_1$R antagonism form relationships that hint at molecular timelines and signaling dynamics. **(A)** While relative gene expression of *Mapk3* is positively correlated to the transcriptional changes of *Hcrtr2* (n = 12, $F_{1,10}$ = 8.3, R^2 = 0.4532, $p \leq .0164$), **(B)** there is no association between *Bdnf* and *Hcrtr2* ($F_{1,10}$ = 0.3, R^2 = 0.0313, $p \geq .5822$). However, **(C)** a positive relationship emerges when comparing *Bdnf* expression to that of *Mapk3* ($F_{1,10}$ = 8.2, R^2 = 0.4517, $p \leq .0167$). Ant., antagonist; Orx$_1$R, orexin 1 receptor.

Acute inhibition of intra-BLA Orx$_1$R, by means of a feedforward rather than feedback mechanism, lowered *Hcrtr1* expression in Escape mice while enhancing *Hcrtr2* mRNA in Stay animals (Figure 6A, B). Antagonism of Orx$_2$R in the BLA did the opposite, reducing *Hcrtr1* mRNA only in Stay mice, and in a similar feedforward way, decreasing *Hcrtr2* expression in both phenotypes (Figure 6A, B). Mice exhibiting escape and reduced fear freezing, expressed lower *Plcb1* compared with the Stay phenotype, an effect unaltered by SB-674042 treatment but reversed by Orx$_2$R antagonism (Figure 6C). However, intra-BLA Orx$_1$R antagonism increased *Mapk3* and *Bdnf* expression in Stay animals only, with Orx$_2$R inhibition having no effect on expression of *Mapk3* and enhancing *Bdnf*, but only in Escape mice, while reducing *Bdnf* in Stay mice (Figure 6D–G; Table S2). These results suggest that social stress disrupts gene expression and potentially alters BLA signaling pathways depending on an individual's stress state. Therefore, pharmacological interventions (such as acute Orx$_1$R antagonism) may functionally amend behavior through signaling adaptations that are phenotype dependent.

Fear conditioning responses appear to be related to specific transcriptional reorganization taking place during/after intra-BLA Orx$_1$R inhibition (Figure 7). In treated animals, negative regressions exist between cued fear freezing behavior and *Hcrtr2* as well as *Mapk3* (62) transcriptional changes (Figure 7B, F). Without treatment (vehicle), cued freezing was positively linked to *Plcb1* gene expression (Figure 7C), an effect not observed with Orx$_1$R antagonism (Figure 7D). These associations provide evidence for potential mechanistic remodeling (Figure 9) in the BLA during periods of stress that is tied to phenotype formation and involves Orx receptor activity. This balancing act between Orx$_1$R and Orx$_2$R creates an influence over BLA microcircuits, which further defines downstream signaling dynamics, in a way that can modify stress-induced behavior (2). Because changes in *Hcrtr2* expression after intra-BLA Orx$_1$R inhibition are positively associated with *Mapk3* but not *Bdnf* transcription levels (Figure 8A, B), it appears that the adjusted bias of Orx$_2$R over Orx$_1$R activity favors ERK$_1$ signaling (Figure 9). Amplification of ERK$_1$, in turn, may lead to enhanced *Bdnf* expression (Figure 8C) and plastic changes within BLA microcircuits (Figure 9) (62,63). These findings highlight a role of intra-BLA Orx$_1$Rs in establishing prostress behavioral states but expose a receptor-driven balance that takes part in the fluid, not static, appearance of phenotype-specific behavior.

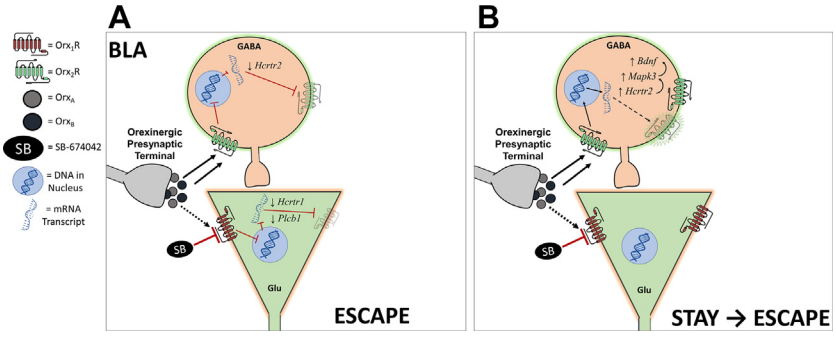

Figure 9. Predicted circuit demonstrates the influence of intra-BLA Orx$_1$R antagonism, during endogenous stimulation through Orx$_A$ and Orx$_B$ release, on microcircuit dynamics in a phenotype-dependent fashion. **(A)** Escape mice treated with an Orx$_1$R Ant. (SB-674042) undergo molecular shifts, including a feedforward reduction of *Hcrtr1* and reduced *Plcb1* transcription, leading to diminished orexin activity on glutamatergic neurons in the BLA. Escape mice also have a feedforward decrease in *Hcrtr2* expression, potentially via (undiagrammed) negative circuit feedback, even while Orx$_2$Rs are stimulated. **(B)** While Orx$_B$ and Orx$_A$ maintain stimulation of some GABAergic neurons through Orx$_2$R, antagonism of some pyramidal neurons via intra-BLA Orx$_1$R inhibition differentially modifies molecular mechanisms in Stay mice through enhancement of Orx$_2$R (*Hcrtr2*), extracellular signal–regulated kinase 1 (*Mapk3*), and *Bdnf* transcription and increased orexin activity in Orx$_2$R-containing neurons (likely GABAergic cells). BLA, basolateral amygdala; GABA, gamma-aminobutyric acidergic neurons; Glu, glutamatergic neurons; mRNA, messenger RNA; Orx$_1$R, orexin 1 receptor; Orx$_2$R, orexin 2 receptor; Orx$_A$, orexin A; Orx$_B$, orexin B.

Conclusions

Modulation of BLA stress-regulatory pathways via Orx_1Rs found predominantly on glutamatergic pyramidal neurons modifies gene expression and behavior. Modulation of prostress BLA microcircuits via Orx_1R inhibition reduces stress-induced behavior. In the process, Orx_1R BLA inhibition modifies gene expression of *Hcrtr2*, which impedes prostress responses. Concurrently, transcription levels for downstream molecular signaling systems associated with Orx receptor signaling are also tilted toward increased ERK_1 (*Mapk3*) rather than $PLC_{\beta1}$ (*Plcb1*) signaling pathways, potentially altering behavior.

ACKNOWLEDGMENTS AND DISCLOSURES

This study was supported by the National Institute of Mental Health of the National Institutes of Health (Grant Nos. R15 MH104485 and R15 MH125306 [to CHS]), the National Institute of Drug Abuse of the National Institutes of Health (Grant No. R25-DA033674), Summer Program for Research in Additions fellowship (to BMJ), the National Science Foundation Research Training Program (USD-N_3 Grant DGE-1633213 [to JDWY and KTK]), a USD Center for Brain and Behavior Research pilot grant, and the Nolop Endowment via the USD Foundation.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health, NSF, the Department of Veterans Affairs, or the United States Government.

We thank Jacob Nordman for comments on this manuscript, J.J. Gale for helping with RNA extraction, Ashley M. Potter for assisting with behavioral analyses, Kelly R. Graber for guiding us on microscope imaging and acquisition, and Raegan Skelton for polymerase chain reaction consultations. We further recognize and commend efforts in the scientific community that stand up against discrimination and social injustices.

The authors report no biomedical financial interests or potential conflicts of interest.

ARTICLE INFORMATION

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Received Jul 29, 2021; revised Dec 18, 2021; accepted Dec 29, 2021.

Supplementary material cited in this article is available online at https://doi.org/10.1016/j.biopsych.2021.12.019.

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