

1 **Title page**

2 **Title:** β -arrestin-dependent ERK signaling positively correlates with reduced anxiety-like and
3 conditioned fear-related behavior in mice.

4

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20 **One sentence summary:** Using pharmacological and genetic strategies, we reveal the importance
21 of non-canonical β -arrestin-mediated G protein-coupled receptor signaling in anxiety-like
22 behaviors.

23

1 **Abstract**

2 Exposure to anxiety- or fear-invoking stimuli initiates a convergence of executive actions
3 orchestrated by multiple proteins and neurotransmitters across the brain. Dozens of G protein-
4 coupled receptors (GPCRs) have been linked to regulation of fear and anxiety. GPCR signaling
5 involves canonical G protein pathways but may also engage downstream kinases and effectors
6 through β -arrestin scaffolds. Here, we investigate whether β -arrestin signaling can regulate
7 anxiety-like and fear-related behavior. Using the δ -opioid receptor (δ OR) as a model GPCR, we
8 found that β -arrestin 2-dependent activation of extracellular signal-regulated kinases (ERK1/2) in
9 the dorsal hippocampus and the amygdala are critical for δ OR agonist-induced anxiolytic-like
10 effects. In contrast, G protein-mediated δ OR signaling was associated with decreased ERK1/2
11 activity and increased fear-related behavior. Our results also indicate unique contributions for β -
12 arrestin isoforms in modulation of anxiety-like and fear-related behavior. Overall, our findings
13 highlight the significance of non-canonical β -arrestin signaling in the regulation of emotions.

1 Introduction

2 Anxiety- and fear-related behaviors are evolutionary adaptive behaviors important for
3 human survival. However, excessive stimulation of the neural circuits that regulate anxiety- and
4 fear-related behaviors can manifest as psychiatric disorders, such as post-traumatic stress disorder
5 and phobias. The expression of anxiety- and fear-related behaviors is regulated by complex
6 integration of both internal and external physiological and sensory cues that influence reflexive
7 behavior, cognitive control, and executive functions. Accordingly, behavioral correlates of anxiety
8 and fear are regulated by a harmonious activity of neurotransmitters and cellular actions across
9 many overlapping and distinct neural circuits (1).

10 With regard to cellular actions underlying (patho-)physiological behavior, G protein-
11 coupled receptors (GPCRs) play an important role in neuronal signaling. GPCRs bind
12 neurotransmitters and initiate intracellular signal transduction pathways which ultimately affect
13 neuronal excitability, neurotransmitter release and synaptic plasticity. GPCRs, including
14 serotonergic (2), dopaminergic (3), adrenergic (4), opioidergic (5) and corticotropin-releasing
15 factor receptors (6) have well-documented roles in the modulation of anxiety and fear and thus are
16 exciting drug targets to treat anxiety disorders.

17 Although drug development at GPCRs has traditionally focused on the canonical G protein
18 pathways, the prior two decades have introduced arrestin-dependent signaling as a new concept of
19 GPCR signal transduction. In particular, the ‘non-visual’ arrestins 2 and 3, referred here as β -
20 arrestin 1 and 2, respectively, have been studied for their ability to drive G-protein-independent
21 signaling (7). The primary role of β -arrestins is to desensitize GPCRs, for example increases β -
22 arrestin 2 expression in mice amygdala, caused by HIV1-tat infection, leads to significant
23 reduction in morphine efficacy in this region (8). Beyond desensitization, β -arrestin 2 may also

1 partake in receptor signaling by scaffolding with various kinases. For example, β -arrestin 2 p38
2 MAP kinase signaling has been linked to the aversive effects of κ -opioid receptor (κ OR) agonists
3 (5, 9), whereas a β -arrestin 2-GSK3 β /AKT scaffold appears to be driving the antipsychotic effects
4 of dopamine D₂ receptors agonists (10, 11). Currently, few studies have investigated how signaling
5 scaffolds involving the β -arrestin isoforms may influence anxiety- and fear-like behavior. It is
6 important to begin to address this gap in our current knowledge of the GPCR modulation of
7 psychiatric behavior, especially since the majority of medications that target GPCRs were
8 developed without consideration of the potential adverse or therapeutic effects of β -arrestin
9 signaling. Yet, it is now possible to develop molecules that preferentially activate or avoid β -
10 arrestin signaling and thus have the potential to treat psychiatric disorders more effectively and
11 with a wider therapeutic window (12, 13).

12 Here, we describe our efforts to elucidate the roles of β -arrestin isoforms in mediating
13 GPCR signaling in relation to the modulation of anxiety and fear-like behavior. We chose to utilize
14 the δ -opioid receptor (δ OR) as a model GPCR for multiple reasons. Previous studies have shown
15 that the δ OR selective agonist SNC80 is an efficacious recruiter of β -arrestin 1 and 2 proteins (14-
16 16), and has anxiolytic-like (17, 18) and fear-reducing effects (17, 19). Moreover, the δ OR-
17 selective agonist TAN67, which is a poor β -arrestin 2 recruiter does not reduce anxiety-like
18 behavior in naïve mice (20), providing support for our hypothesis that β -arrestin 2 signaling is
19 correlated with and reduced anxiety-like behavior.

20 Mitogen activated protein kinases (MAPKs) have been implicated with mood disorders
21 and can scaffold with β -arrestin (21, 22). Studies have suggested that MAPK signaling, specifically
22 ERK1/2, in the hippocampus and the basolateral amygdala is required for the acquisition and
23 extinction of fear memory (23, 24). Therefore, we further hypothesize that β -arrestin-dependent

1 MAPK signaling may contribute to anxiety-like and fear-related behavior. To test our hypotheses,
2 we assessed the degree to which β -arrestin isoforms and MAPK activation were involved in δ OR
3 agonist-mediated modulation of unconditioned anxiety-related behavior and cued-induced fear-
4 related behavior. Our results suggest that ERK1/2 activity is differentially modulated by G protein
5 and β -arrestin signaling and is correlated with anxiety-like and fear-related responses in C57BL/6
6 mice. We noted that different β -arrestin isoforms were involved in the activation of ERK1/2 across
7 various brain regions, including the striatum, hippocampus and amygdala.

8

9

Results

10 **Involvement of β -arrestin 2 in the modulation of anxiety-like behavior** In 2016, Astra Zeneca
11 revealed that their novel δ OR-selective agonist AZD2327 (**Fig. 1a**) was capable of reducing
12 anxiety-like behavior in mice (25). While AZD2327 is not commercially available it is structurally
13 similar to SNC80, a commercially available δ OR-selective agonist (**Fig. 1a**). SNC80 is a known
14 super-recruiter of β -arrestin 2 (14) (**Fig. 1b**) and similar to AZD2327 exhibits anxiolytic-like
15 effects in rodents (17, 20). These previous findings led us to hypothesize that β -arrestin 2 may be
16 required for the anxiolytic effects of SNC80 and AZD2327. Using two models of anxiety-like
17 behavior, the elevated plus maze (EPM) test and dark-light box transition test (**Fig. 1c**), we
18 measured the behavioral effects of SNC80 in β -arrestin 2 KO mice at a dose known to produce
19 anxiolytic-like effects in wild-type (WT) mice (20). As expected, systemic administration of
20 SNC80 (20 mg/kg, s.c.) significantly increased the time WT mice spent in the open arm of the
21 elevated plus maze (**Fig. 1d**; see **Table S1** for two-way ANOVA and post-hoc multiple comparison)
22 and the light chamber of dark-light transition box (**Fig. 1e**; see **Table S1** for two-way ANOVA
23 and post-hoc multiple comparison). As we predicted, the anxiolytic effects of SNC80 were

1 attenuated in β -arrestin 2 KO mice (**Fig. 1d and e**; see **Table S1** for two-way ANOVA and post-
2 hoc multiple comparison). Although the total movement in the elevated plus maze was slightly
3 lower in β -arrestin 2 KO mice than WT mice, no drug effects were observed in both genotypes
4 (**Fig. 1f**; see **Table S1** for two-way ANOVA and post-hoc multiple comparison). Likewise, no
5 statistical difference in total transition was observed in the dark light transition box test (**Fig. 1g**
6 see **Table S1** for two-way ANOVA and post-hoc multiple comparison); however, as previously
7 described, SNC80 produced hyperlocomotive behavior in mice ((14), **Fig. S1**, see **Table S1** for
8 detailed statistics).

9
10 **The β -arrestin recruiting δ OR agonist SNC80 strongly activates ERK1/2 in vitro and in vivo**

11 Activation of κ OR has been associated with β -arrestin 2-mediated p38 phosphorylation (9). To
12 determine if δ OR agonism similarly stimulates mitogen-activated protein kinases (MAPKs), we
13 measured p38, JNK, and ERK1/2 activation in Chinese Hamster Ovarian cells stably expressing
14 δ OR and β -arrestin 2 (CHO- δ OR- β Arr2) following stimulation with 10 μ M SNC80, a
15 concentration that will fully activate G-protein signaling and induce β -arrestin 2 recruitment (26).
16 We found that SNC80 led to a rapid increase in ERK1/2 phosphorylation within 3 minutes in
17 CHO- δ OR- β Arr2 cells, which lasted until 60 minutes, in agreement with previous δ OR-mediated
18 ERK activation in CHO cells (27). We did not observe strong activation of p38 and JNK by SNC80
19 (**Fig. 2a**). The δ OR mediated ERK1/2 signaling in these cells were not an artifact of the
20 recombinant overexpression of δ OR and β -arrestin 2 in the CHO cells as we observed a similar
21 profile for ERK1/2 activation in NG108-15 neuroblastoma cells endogenously expressing δ OR
22 and β -arrestin (28-30) (**Fig. 2b**). We similarly found ERK1/2 activation in several mouse brain
23 regions, known to express δ ORs, including the dorsal hippocampus, the amygdala and the striatum

1 (31, 32) (**Fig. 2c**). The SNC80-induced ERK1/2 activation in these regions was confirmed and
2 quantified by the Western blot analysis of flash-frozen tissue punches upon collection (**Fig. 2d**).
3 Here, we observed that SNC80 (20 mg/kg, i.p.) significantly increased ERK1/2 phosphorylation
4 at the 10-minute time-point in all tested brain regions except for the ventral hippocampus of WT
5 mice (**Fig. 2e-i**; see **Table S2** for one-way ANOVA and post-hoc multiple comparison), and these
6 activations returned to basal levels 30 minutes after the SNC80 administration. The SNC80-
7 induced ERK1/2 activation was not observed in the dorsal hippocampus and the amygdala of δ OR
8 KO mice (**Fig. S2**), further confirming this unique profile of ERK1/2 activation by SNC80 is
9 mediated by δ OR.

10

11 **β -arrestin 2 is required to activate ERK1/2 signaling in the limbic structures of the brain** To
12 determine if β -arrestin 2 is responsible for the ERK1/2 activation observed in wild-type mice, we
13 quantified ERK1/2 activation across the same hippocampal, striatal and amygdalar regions of β -
14 arrestin 2 KO mice (**Fig. 3a**) upon systemic administration of SNC80 (20 mg/kg, i.p.) (**Fig. 3b**).
15 While SNC80 still strongly activated ERK1/2 in the nucleus accumbens (**Fig. 3c**; see **Table S2** for
16 one-way ANOVA and post-hoc multiple comparison) and revealed an increasing trend of ERK1/2
17 activation in striatum (**Fig. 3b**; see **Table S2** for one-way ANOVA and post-hoc multiple
18 comparison) of the β -arrestin 2 KO mice, we did not observe significant SNC80-induced ERK1/2
19 activation in the dorsal hippocampus, the amygdala, and the dorsal hippocampus of β -arrestin 2
20 KO mice (**Fig. 3e-g**; see **Table S2** for one-way ANOVA and post-hoc multiple comparison).

21

22 **ERK1/2 signaling plays a key role in the SNC80-mediated anxiolytic-like effects** We next
23 assessed if the anxiolytic effects of SNC80 were dependent on ERK1/2 activation. We

1 administered wild-type mice with SL327 (50 mg/kg, s.c.), a MEK1/2 inhibitor that indirectly
2 prevents ERK1/2 activation, (**Fig. 4a**) (33). We found that pre-administration of SL327 ablated
3 the anxiolytic-like effects of SNC80 (20 mg/kg, i.p.) in WT mice (**Fig. 4b**; see **Table S3** for one-
4 way ANOVA and post-hoc multiple comparison). The hippocampus is a brain region associated
5 with anxiety-like behavior in the elevated plus maze (34) and δ OR-agonism in the dorsal
6 hippocampus, and the amygdala is associated with reduced anxiety-like behavior in the open field
7 test (18, 35). These published findings agree with our observation of SNC80-induced β -arrestin 2-
8 dependent ERK1/2 activity specifically in these two brain regions (**Fig. 3e,f**). Therefore, if the β -
9 arrestin-mediated ERK1/2 signaling in these two regions was critical for the anxiolytic-like effects
10 of SNC80, we would expect ERK1/2 activity to be abolished in these regions in the mice with
11 SNC80 (20 mg/kg, i.p.) and SL327 (50 mg/kg, s.c.). Indeed, we found that SL327 effectively
12 decreased SNC80-induced ERK1/2 activity in the dorsal hippocampus and the amygdala (**Fig.**
13 **4c,d**, see **Table S3** for detailed statistics), but not in the dorsal striatum, the nucleus accumbens,
14 and the ventral hippocampus (**Fig. S3**, see **Table S3** for detailed statistics). As an additional
15 approach to investigate the importance of β -arrestin 2 signaling on ERK1/2 signaling and anxiety-
16 like behavior, we utilized a δ OR agonist that is a weaker β -arrestin recruiter than SNC80.
17 Specifically, we utilized ADL5859, which affinity ($K_i = 0.84$ nM) and G-protein potency ($EC_{50} = 20$
18 nM) at δ OR is not significantly different from SNC80 ($K_i = 1.2$ nM, $EC_{50} = 10$ nM, (36)), but has
19 50-fold lower potency ($pEC_{50} = 6.6 \pm 0.1$, **Fig. S4a**) and slightly lower efficacy ($E_{max} = 119\% \pm 5$,
20 relative to Leu-enkephalin **Fig. S4a**) than SNC80 ($pEC_{50} = 8.2 \pm 0.1$, $E_{max} = 142\% \pm 9$, (14)). Systemic
21 administration of ADL5859 (30 mg/kg, p.o.) at a dose that produces robust δ OR-mediated
22 analgesia (37) did not result in ERK1/2 activation in the dorsal hippocampus and the amygdala
23 (**Fig. S4b,c**) nor reduce anxiety-like behavior (**Fig. S4d**), suggesting that ERK1/2 activation in the

1 dorsal hippocampus and the amygdala as well as δ OR-mediated anxiolytic effects require a strong
2 recruitment of β -arrestin 2.
3
4 **Fear-potentiated startle behavior is correlated with ERK1/2 activity but is not mediated by**
5 **β -arrestin 2.** Besides reducing anxiety-like behavior, δ OR activation can also alleviate
6 conditioned fear-related behavior (17, 38). Based on our results and previous studies, we
7 hypothesized that SNC80 may similarly reduce fear-related behavior through a mechanism that
8 involves β -arrestin 2. To measure conditioned fear, we utilized a mouse behavior paradigm of fear-
9 potentiated startle (FPS) (Fig. 5a). Mice groups were counterbalanced, such that no significant
10 differences between startle reflexes were observed between groups (Fig. S5). In WT mice (Fig.
11 5b), we noted that SNC80 (20 mg/kg, i.p.) significantly reduced startle responses to the
12 unconditioned ‘noise’ cue as well as to the conditioned ‘light+noise’ cue (Fig. 5c; see Table S4
13 for two-way ANOVA and post-hoc multiple comparison). The reduction produced by ‘light+noise’
14 is larger than the reduction produced by ‘noise’, and thus these reductions result in a significant
15 reduction in % FPS response (Fig. 5d; p-value is indicated in figure legends). We also measured
16 if SNC80 would alter %FPS at a dose of 10 mg/kg (i.p.), as this dose of SNC80 has been shown
17 to be effective in other studies effect (17, 95), but in our hands we did not observe any significant
18 effects (Fig. S6). To our surprise, we found that SNC80 was equally effective in reducing % FPS
19 responses in β -arrestin 2 KO mice (Fig. 5e,f; see Table S4 for two-way ANOVA and post-hoc
20 multiple comparison; Fig. 5g; p-value is indicated in figure legends). While SNC80 is a very
21 efficacious recruiter of β -arrestin, it still also fully activates G_i protein signaling (14, 26). Thus, we
22 next hypothesized that the observed fear-reducing effects of SNC80 could be mediated through G_i
23 protein signaling. To address this hypothesis, we utilized a δ OR selective agonist, TAN67, which

1 is a very poor recruiter of β -arrestin, and considered G_i protein-biased (14, 26). However, when
2 we administered TAN67 (25 mg/kg, i.p.) to our WT mice (Fig. 5h), TAN67 did not significantly
3 change startle to the ‘noise’ or to the ‘light+noise’ stimuli (Fig. 5i; see Table S4 for two-way
4 ANOVA). However, there was a significant increase in %FPS (Fig. 5j; p-value is indicated in
5 figure legends). The lack of a direct effect of TAN67 on noise-alone startle is in agreement with
6 our previous finding that TAN67 did not change basal anxiety-like behavior in the elevated plus
7 maze and dark-light transition test (20). Our Western blot analysis of ERK1/2 activities in WT
8 mice revealed that TAN67, decreased ERK1/2 phosphorylation in the dorsal striatum, the nucleus
9 accumbens, the dorsal hippocampus, and the amygdala (Fig. 6a-d). In the ventral hippocampus,
10 TAN67 did not alter ERK1/2 activity (Fig. 6e), which was similar to the lack of ERK1/2
11 modulation by SNC80 in this region (Fig. 2i) and may be indicative of low δ OR expression in the
12 ventral region as previously described (39).

13

14 **Potential roles for ERK1/2 and β -arrestin 1 in the modulation of conditioned-fear behavior**

15 Our results suggest that SNC80 reduces conditioned fear through a mechanism that does not
16 involve β -arrestin 2 or G protein signaling. Therefore, we next hypothesized that the effect may be
17 mediated by β -arrestin 1 instead. SNC80 is a potent and efficacious recruiter of β -arrestin
18 (pEC₅₀ = 7.9 ± 0.2, E_{max} = 86% ± 5), particularly compared to TAN-67, which recruits β -arrestin
19 1 with much lower efficacy (pEC₅₀ = 7.7 ± 0.4, E_{max} = 14% ± 2) (Fig. S7). However, SNC80 is
20 known to induce severe seizures in β -arrestin 1 KO mice (16), preventing us from testing the FPS
21 response of SNC80 in this strain. Instead, we measured SNC80-induced ERK1/2 phosphorylation
22 in the β -arrestin 1 KO mice. SNC80 (20 mg/kg, i.p.) was systemically administered at indicated
23 timepoints. In comparison to WT mice (Fig. 2e-i), genetic knockout of β -arrestin 1 prevented

1 SNC80-mediated ERK1/2 phosphorylation in the dorsal striatum and nucleus accumbens (**Fig. 6f-**
2 **g**; see **Table S5** for one-way ANOVA and post-hoc multiple comparison). In the amygdala and
3 dorsal hippocampus of β -arrestin 1 KO mice, SNC80 did increase ERK1/2 phosphorylation (**Fig.**
4 **6h-i**), but in contrast to the response observed in WT mice, the activation was sustained for at least
5 30 minutes. Additionally, we observed ERK1/2 activation in the ventral hippocampus (**Fig. 6j**), a
6 region where we had observed no δ OR agonist-mediated modulation of ERK1/2 in WT mice (**Fig.**
7 **2i** and **Fig. 6e**), suggesting this pattern of ERK1/2 activity may not be δ OR-mediated and is most
8 likely a result of seizure activity.

9

10

Discussion

11 Here, we investigated the hypothesis that β -arrestin can modulate anxiety- and conditioned fear-
12 related behavior through downstream MAPK activation. By utilizing G protein- and β -arrestin-
13 biased δ OR agonists together with β -arrestin-isoform selective knockout mice, we discovered that
14 G protein, β -arrestin 1, and β -arrestin 2 uniquely modulated ERK1/2 activity resulting in
15 differential outcomes in mouse models of anxiety/fear-related behavior. Our results suggest that
16 the reduction in anxiety-like behavior by SNC80 required the presence of β -arrestin 2 as well as
17 activation of ERK1/2. Distinctly in the dorsal hippocampus, we found that ERK1/2 activation was
18 β -arrestin 2-dependent (**Fig. 7a**). We also found that SNC80-induced ERK1/2 activation in the
19 nucleus accumbens and the dorsal striatum required β -arrestin 1, which may be part of the
20 mechanism by which SNC80 decreased FPS (**Fig. 7b**) as well as increase general locomotion (**Fig.**
21 **S1**). Notably, G protein-biased signaling by TAN67 reduced ERK1/2 phosphorylation and was
22 correlated with increased FPS (**Fig. 7c**).

1 *Differential roles for β -arrestin isoforms in neuropsychiatric behavior.* The β -arrestin
2 proteins were discovered in quick succession (40, 41), surprisingly, despite the availability of
3 genetic KO mice for each isoform (42, 43). Studies investigating β -arrestin in the CNS have largely
4 focused on β -arrestin 2 and have generally neglected β -arrestin 1 (44, 45). A potential reason for
5 the preference of studying β -arrestin 2 may be that when the β -arrestin 2 knockout mice were
6 generated their first utilization was to highlight the proteins' involvement with the CNS-mediated
7 adverse effects of μ -opioid receptor agonism (46, 47). Researchers have only recently begun to
8 utilize β -arrestin 1 KO mice to study various neurological disorders. In 2016, Pradhan *et al.* found
9 that different δ OR agonists either preferentially recruited β -arrestin 1 leading to δ OR degradation
10 or recruited β -arrestin 2 causing δ OR resensitization (15). A study in 2017 found that
11 amphetamine-induced hyperlocomotion was amplified in β -arrestin 1 KO mice, but attenuated in
12 β -arrestin 2 KO mice (48), further emphasizing the importance of studying both β -arrestin isoforms.

13 While our study identified that SNC80-induced anxiolytic-like effects were β -arrestin 2-
14 dependent, β -arrestin 2 KO mice still exhibited the fear-reducing effect of SNC80, which could
15 suggest a potential role for β -arrestin 1 in the modulation of fear-related behavior. Particularly, we
16 noted that β -arrestin 1 KO abolished SNC80-induced ERK1/2 activation in the striatum and
17 nucleus accumbens. Our findings were in line with the extensive *in-situ* hybridization studies on
18 differential β -arrestin isoform levels in neonatal and postnatal rats (49, 50) and studies showing
19 relatively high β -arrestin 1 and low β -arrestin 2 expressions in the striatal regions (41, 49, 51). In
20 contrast, SNC80-induced ERK1/2 activation in the dorsal hippocampus and amygdala was β -
21 arrestin 2-dependent, which agreed with reports of stronger expression of this isoform in those
22 areas (41, 51). Unfortunately, we were limited in our ability to assess whether SNC80-induced
23 reduction in FPS would be attenuated in β -arrestin 1 KO mice, as SNC80 produces severe seizures

1 in these mice (16), a phenomenon we have also observed ourselves and found to be accompanied
2 by strong and persistent ERK1/2 activation in the dorsal hippocampus. Further investigation of the
3 roles of β -arrestin 1 in neuropsychiatric behavior may be feasible using a conditional knockout
4 approach; currently conditional β -arrestin 2 knockout mice already exist (52), but conditional β -
5 arrestin 1 knockout mice have not yet been reported.

6 *A unique role for G_i protein signaling and ERK1/2 signaling in conditioned fear-related*
7 *behavior.* In contrast to the β -arrestin-mediated activation of ERK1/2, we found that selective G
8 protein signaling at the δ OR by TAN67 decreased ERK1/2 activation (Fig. 6a-d) and was
9 associated with increased FPS (Fig. 5j). TAN67 is a known weak recruiter of β -arrestin 1 and 2
10 (14) (Fig. S7). This result is in agreement with the observation and that blocking $G_{i/o}$ protein
11 signaling using pertussis toxin in the basolateral amygdala reduced FPS (53) and parallels finding
12 that TAN67 did not reduce unconditioned anxiety-like behavior in naïve mice (20). Additionally,
13 a study in ovariectomized mice found that estradiol benzoate, an anxiogenic estradiol prodrug (54),
14 decreased ERK in the hippocampus (55), which supports our finding that decreased ERK1/2 is
15 correlated with increased fear. It is noteworthy that both G_i protein and β -arrestin can activate
16 ERK1/2 albeit through different mechanisms (56, 57). One explanation for our observation is that
17 TAN67 competes with the endogenous δ OR agonist Leu-enkephalin, which is a much more
18 efficacious recruiter of β -arrestin (14) (Fig. S8). Based on observations of enhanced anxiety-like
19 behavior in preproenkephalin KO mice, Leu-enkephalin has anxiolytic-like effects by itself (58,
20 59), which is in line with reports that the δ OR antagonist, naltrindole, is anxiogenic (60). As a
21 weak β -arrestin recruiter, TAN67 would attenuate any baseline Leu-enkephalin-induced β -
22 arrestin-mediated ERK activity (Fig. S8). Because ADL5859 has a low potency for β -arrestin 2
23 recruitment, the same pharmacological principle described above holds true, with the exception

1 that at supersaturating doses, ADL5859 will begin to efficaciously recruit β -arrestin 2, whereas
2 TAN-67 will not. In contrast, because SNC80 is a stronger β -arrestin recruiter than Leu-enkephalin,
3 it will elevate basal ERK1/2 activity produced by endogenous opioids. This hypothesis would also
4 explain why SNC80-induced ERK1/2 activation is quite variable and produces on average only a
5 two-fold increase in pERK1/2 levels.

6 *β -arrestin serves as a scaffold for a range of kinases and effectors.* In our study, we found
7 that δ OR agonism strongly activates ERK1/2 compared to p38 and JNK, and that the ERK1/2
8 activity induced by SNC80 was negatively correlated with FPS. Other GPCRs, besides the δ OR,
9 may also require β -arrestin-dependent ERK1/2 signaling for modulation of fear. For example, in
10 the infralimbic prefrontal cortex, β -adrenergic receptor activation can promote the extinction of
11 contextual fear memory (61). In a recent study, it was shown that in this same brain region β -
12 arrestin 2-dependent ERK1/2 activation was required for β -adrenergic receptors agonists to
13 stimulate extinction learning of cocaine-induced reward memories (52). β -arrestin 2-mediated
14 signaling in the CNS is not exclusive to ERK1/2 signaling; following κ OR activation, β -arrestin 2
15 can scaffold with p38 as part of a potential mechanism for the aversive effects of κ OR agonists
16 (62). A β -arrestin 2 scaffold of AKT, GSK3 β and PP2A has been proposed as a mechanism for
17 stabilizing mood (63), highlighting that β -arrestin signaling is also not limited to MAPKs. In fact,
18 because β -arrestin 1, in contrast to β -arrestin 2, contains a nuclear translocation sequence (64), it
19 can translocate to the nucleus and regulate gene transcription (65, 66). While in this study we
20 report δ ORs require β -arrestin-dependent ERK signaling for reduction in anxiety-like behavior, it
21 is certainly possible that other GPCRs may engage different intracellular signaling pathways
22 following β -arrestin recruitment.

1 *Fear- and anxiety-like behaviors rely on shared but distinct neural circuits.* In our study,
2 we observed that β -arrestin 2-dependent ERK1/2 activity in the dorsal hippocampus was
3 associated with reduced anxiety-like behavior. Generally, CA1 regions of the ventral hippocampus
4 are associated with responding to contextually-conditioned anxiogenic stimuli than dorsal CA1
5 regions (67, 68), whereas the dorsal hippocampus is involved with cognitive functions, including
6 exploration, navigation and memory (69). Still, our finding that δ OR signaling in the dorsal
7 hippocampus is connected to the anxiolytic-like effects of SNC80, agrees with a study showing
8 that intra-dorsal CA1 injection of the δ OR antagonist naltrindole is anxiogenic (35). Additionally,
9 the anxiolytic-like effects of SNC80 may also involve β -arrestin 2-dependent ERK1/2 signaling in
10 the amygdala, a region more commonly associated with innate anxiety-like behavior (70, 71). The
11 basolateral amygdala (BLA) also plays an important role in fear conditioning (72), including FPS
12 (73). The BLA receives dopaminergic inputs from the ventral tegmental area and projects to the
13 nucleus accumbens. It has been proposed that dopaminergic signaling in the BLA is important for
14 cue-dependent fear-conditioning, such as FPS (74) and that the BLA to nucleus accumbens
15 projection is critical for consolidation of memories associated with aversive effects such as foot
16 shock (75, 76). Future studies using conditional β -arrestin and/or δ OR KO mice could help assess
17 to what degree dopamine receptor-mediated β -arrestin signaling is required for the observed
18 behaviors.

19 Our finding that ERK1/2 activation in the striatal regions was ablated in β -arrestin 1 KO
20 mice may suggest that striatal β -arrestin 1-mediated ERK1/2 signaling is critical for the modulation
21 of the expression of conditioned fear-related behavior. Processing and executing emotional
22 behaviors in tasks such as the elevated plus maze and FPS tests engages multiple overlapping, yet
23 distinct, brain regions and circuits involved in memory retrieval, locomotion, decision making,

1 reward, and mood (72). Further studies with circuit-based approaches are necessary to assess the
2 role of biased signaling pathways in the acquisition and expression of conditioned fear-related
3 behavior.

4 *Beneficial roles of β -arrestin signaling.* For the longest time, β -arrestin 2 has been
5 associated solely with adverse effects of opioid activation, including tolerance, constipation,
6 respiratory depression, aversion and alcohol use (20, 46, 77). These studies fueled a drive to
7 develop G protein-biased opioids to treat pain and other disorders with an improved therapeutic
8 window (78, 79). Yet, recently a number of studies have started to push back against this narrative
9 (80-84). Clearly, β -arrestin signaling is not inherently negative as the therapeutic effects of lithium
10 and fluoxetine and D₂R agonists seem to depend on β -arrestin 2 (85-87). The increased propensity
11 for β -arrestin 1 KO mice to experience SNC80-induced seizure points to a potential beneficial role
12 for this isoform in maintaining seizure threshold, which could be of use in the treatment of epilepsy.
13 In this study, we provide additional insights regarding potential therapeutic benefits of β -arrestin
14 signaling in reducing anxiety-like behavior. Providing adequate relief of chronic pain is not trivial,
15 partly because it is often associated with negative affect (88, 89) including anxiety, which may
16 exacerbate pain (90). δ OR agonists have been proposed as potential treatment for chronic pain
17 disorders (91), partly because they have the ability to not only provide analgesia, but also treat
18 comorbid anxiety and depression (17, 20, 92). However, our results would argue that developing
19 G protein-biased δ OR agonists may produce drugs that are suboptimal for the treatment of complex
20 chronic pain; our findings suggest such a drug would not alleviate co-morbid fear and anxiety, but
21 potentially even worsen these symptoms. Thus, our study results argue in favor of a reassessment
22 of drug development efforts that seek solely to identify G protein-biased drugs. Instead, we
23 propose that efforts should be directed towards the development of drugs with finely tuned bias

1 and, if possible, towards development of molecules that are biased against a single β -arrestin
2 isoform rather than both isoforms and are circuit-specific.

3 *Conclusion.* Overall, our results begin to reveal the complex- and context-specific nature
4 of GPCR biased signaling in modulation of fear-related and anxiety-like behavior. These results
5 expand our current understanding of therapeutic effects of β -arrestin signaling in mood disorders,
6 which ultimately may aid development of more efficacious pharmacological treatment options for
7 these disorders.

1 **Materials and Methods**

2 **Animals** Wild-type (WT) C57BL/6 male mice were purchased from Envigo (Indianapolis, IN),
3 and β -arrestin 1 or 2 global knockout (KO) mice as well as δ OR KO mice were bred in our facility
4 (14, 93, 94). The knockout mice have been rederived every three years by crossing the KO mice
5 with WT C57BL/6 mice from Envigo to reduce genetic drift. Adult mice (8-10 weeks, 25 ± 3 g)
6 were group housed (3-5 mice) in a single ventilated Plexiglas cage. Mice were maintained at
7 ambient temperature (21°C) in an animal housing facility approved by the Association for
8 Assessment and Accreditation of Laboratory Animal Care and animals were kept on a reversed
9 12-hour dark-light cycle (lights off at 10:00, lights on at 22:00). Food and water were provided ad
10 libitum. Purchased mice were acclimated for one week prior to the experiments. All animal
11 protocols (#1305000864 by RMvR) were preapproved by Purdue Animal Care and Use Committee
12 and were in accordance with the National Institutes of Health's Guide for the Care and Use of
13 Laboratory Animals.

14 **Drug preparation and administration** SNC80 (#076410, Tocris, Thermo Fisher Scientific,
15 Waltham, MA) was diluted in slightly acidic saline pH5-6. TAN67 (#092110, Tocris, Thermo
16 Fisher Scientific) was diluted in sterile saline. SL327 (#19691, Tocris, Thermo Fisher Scientific)
17 was diluted in 5% DMSO, 10% Cremophore (Millipore Sigma, Burlington, MA) and 85% saline.
18 ADL5859 (#1751, Axon Medchem, Reston, VA) was diluted in 0.5 % methylcellulose and 0.1 %
19 tween 80 as indicated in (37). To investigate our hypotheses, we utilized several δ OR agonists:
20 SNC80, TAN67, and ADL5859. Doses for each drug were based on those that proved to impact
21 behavior through δ OR (14, 20, 37). For SNC80, we used a dose of 20 mg/kg (14, 20). We have
22 also tested 10 mg/kg SNC80 (i.p.). While this dose was effective in other studies effect (17, 95),
23 in our hands, we did not observe any significant effects (Fig. S6). TAN67 was tested at a dose of

1 25 mg/kg (14, 20), whereas ADL5859 was administered at a dose of 30 mg/kg (37). We also used
2 the MEK inhibitor SL327 at a 50mg/kg dose to prevent ERK phosphorylation, as this inhibitor has
3 been reported to cross the blood-brain barrier (96, 97). Except for ADL5859, which was
4 administered with per oral (p.o.), all drugs were administered either intraperitoneal (i.p.) or
5 subcutaneous (s.c.), and specific times prior to behavior or brain tissue extraction can be found in
6 figure legends. Additionally, separate batches of mice with no prior history of drug injection were
7 used for the brain collection and behavioral tests to test earlier time-points of ERK1/2 signaling
8 (such as 10 minutes).

9 **Elevated-plus maze test** The elevated-plus maze test was performed as previously described (20).
10 Mice were allowed to explore the maze for 5 minutes, and arm entries and time spent in each arm
11 were recorded with a camera positioned above the maze.

12 **Dark–light transition box test** The test was performed based on previously established protocols
13 (20, 93) Testing was conducted without a habituation session to the boxes and a 1/2 area dark
14 insert was placed in the locomotor boxes, leaving the remaining 1/2 of the area lit as described
15 previously (98). Two LED lights were inserted above the light portion of the testing chamber where
16 the lux of the light region ranged from 390-540 lumens and dark chamber lux ranged from 0-12
17 lumens. For testing, animals were placed in the light portion of the chamber and testing began
18 upon animal entry. Time spent in the dark and light chambers as well as their locomotor activity
19 was recorded for 5 minutes with a photobeam-based tracking system.

20 **Fear potentiated startle (FPS) test** Startle reflexes of mice were recorded in the startle reflex
21 chambers (25.8 x 25 x 26.5 cm) using the Hamilton Kinder Startle Monitor system (Kinder
22 Scientific, Poway, CA). On the conditioning day, all subjects were conditioned with 40
23 conditioning trials by a fixed 2 minute inter-trial interval (ITI), and FPS responses were tested on

1 the following day. The fear conditioning and FPS parameters were based on a previously
2 established protocol (99).

3 **Preparation of tissue homogenates** After drug injections, mice were euthanized by carbon
4 dioxide asphyxiation and rapidly decapitated. Based on our previous studies, we have particularly
5 chosen carbon dioxide asphyxiation over other euthanasia methods that may potentially increase
6 basal ERK1/2 activity in the brain (100). The collected brains were first sliced as coronal sections
7 (1.5-2.0 mm) with a brain matrix (#RBMS-205C, Kent scientific, Torrington, CT), and then flash-
8 frozen in dry-ice-chilled 2-methylbutane (-40 °C) (#03551-4, Fisher Scientific). Regions of
9 interest were collected from these slices using a 1 mm biopsy micropunch (#15110-10, Miltex,
10 Plainsboro, NJ) as follows: dorsal striatum and nucleus accumbens (A/P: +0.5 mm to +1.5 mm),
11 dorsal hippocampus and amygdala (A/P: -1 mm to -2 mm), and ventral hippocampus (A/P: -2 mm
12 to -4 mm) (101). The punches targeted a specific region and produced enough tissue to run several
13 blots. However, it is noteworthy that the extracted tissue may contain small amounts of tissue from
14 neighboring regions. For example, while the punches for the amygdala primarily consisted of the
15 BLA, the tissue will also have included a small portion of the central amygdala. Collected tissues
16 were further homogenized with a tissue grinder (#357535 & 357537, DWK Life Sciences,
17 Millville, NJ) in RIPA buffer mixed Halt™ Protease and Phosphatase Inhibitor Cocktail
18 (#1861280, Thermo Fisher Scientific). Samples were further prepared based on previously
19 established protocols (100). Data depicted in Fig. 2e-i also includes the data collected for SNC80
20 at the 0 min or 10 min time points in the experiment depicted in Fig. 4c,d to represent the full
21 range of observed SNC80 induced ERK1/2 activation in mice tested in separate cohorts at different
22 occasions.

1 **Cell culture** Chinese hamster ovarian CHO- δ OR- β Arr2 cells (DiscoverX, Fremont, CA) U2OS-
2 δ OR- β Arr1 (DiscoverX), and NG-108-15 cells (HB-12317TM, ATCC®, Manassas, VA) were
3 cultured as recommended by the manufacturer and maintained at 37° C/5 % CO₂. Cells were
4 seeded in a clear 6 well plate (CorningTM, Thermo Fisher Scientific) with 250,000 cells/2 mL/well.
5 On the following day, all growth media was aspirated and changed into 1 mL serum-free Opti-
6 MEM (#31985070, Gibco®, Thermo Fisher Scientific). The next day, cells were challenged with
7 10 μ M drugs (SNC80) for a specific duration (0, 3, 6, 20, and 60 minutes). All drugs were diluted
8 in Opti-MEM prior to administration. The media was aspirated following the challenge and 100
9 μ L RIPA buffer was added to collect the samples on ice. Using cell scrapers (#353089, Thermo
10 Fisher Scientific), all samples were dislodged from the 6-well plate, collected and stored at -30° C
11 until usage. For the Western blot, the collected samples were quantified with the Bradford assay
12 and samples were prepared with 4 x Laemmli and boiled at 95° C for 5 minutes. The CHO- δ OR-
13 β Arr2 cells were also used to measure β -arrestin recruitment using the DiscoverX PathHunter
14 Assay as previously described (14).

15 **SDS-Page and Western blot** Samples (20 μ L containing 10 μ g protein) were loaded per well of
16 a NuPage 4-12 % Bis-Tris gradient gels (#NP0336BOX, Thermo Fisher Scientific), and the SDS-
17 Page gel was subsequently transferred to nitrocellulose membranes (#1620115, BioRad) by the
18 Western blot. Membranes were incubated following previously established protocols (100). For
19 reproducibility, detailed information regarding the antibodies used in the study are listed in **Table**
20 **S6**. Prepared samples were scanned using the LiCor Odyssey® CLx Scanner (Li-Cor, Lincoln,
21 NE). By utilizing the Li-Cor secondary antibodies, we were able to detect the MAPK, pMAPK,
22 and α -Tubulin on the same blot without the need of stripping/reblotting. In the same membrane,
23 each band was cut based on their size. For instance, ERK1/2 and pERK1/2 bands were collected

1 around 42/44 kDa and α -Tubulin band was collected around 50 kDa in the same membrane. For
2 statistical analysis, we normalized the pMAPK/MAPK ratio to α -Tubulin in case drug treatment
3 changed ERK1/2 levels.

4 **Preparation of tissue for immunofluorescence** To preserve the intact ERK1/2 activity in vivo
5 for fluorescence microscopy, mice were transcardially perfused before isolation of brain tissue.
6 Thirty minutes prior to transcardiac perfusion, mice were administered with 100/10 mg/kg
7 Ketamine/Xylazine. Ten minutes prior to perfusion, 20 mg/kg SNC80 (i.p.) or a corresponding
8 volume of saline was administered to the mice. Mice were then perfused with 30 mL of cold PBS
9 and 4% paraformaldehyde (#100503-916, VWR, Radnor, PA) and were immediately decapitated
10 to collect the brains. The brains were fixated in 4% paraformaldehyde overnight, dehydrated in
11 30% sucrose, and then embedded in Frozen Section Compound (#3801480, Leica, Wetzlar,
12 Germany). Frozen brains were sliced at a width of 30 μ m using the Leica cryostat and
13 permeabilized in 100% methanol at - 20 °C for 10 minutes. The slices were blocked in 5 % Normal
14 goat serum (#S26-100ml, Millipore Sigma) for an hour then stained with primary antibodies as
15 listed in **Table S6**. For immunofluorescence labeling, the sections were incubated in the secondary
16 antibodies according to the previously established protocol (*I02*) and as listed in **Table S6**. After
17 the final washing, the nuclei of the sections were stained and the slices were mounted on a glass
18 slide with Vectashield® (#H-1200, Vector lab, Burlingame, CA). Images were acquired with a
19 Nikon confocal microscope and assembled in Adobe Photoshop CS6 (Adobe).

20 **Statistical analysis** The maximum amplitude of the startle response was measured from the
21 average of all responses for each trial type (12 noise-alone, 12 light+noise) of each mouse. Fear-
22 potentiated startle response was analyzed using raw (maximum) startle amplitudes and
23 proportional changes of each trial type (noise-alone, light+noise), which is shown as % FPS in the

1 graphs. The proportional change score (% FPS) was calculated as follows: (startle response to light
2 and noise – startle response to noise)/startle response to noise x 100. Thus, %FPS is a sensitive
3 measure that adjusts for individual and group differences (for example, possible non-specific
4 effects of drug treatment) in startle response magnitude that may be observed on noise-alone and
5 light + noise trials (103).

6 All data are presented as individual data points (or means) \pm standard error of the mean
7 (S.E.M.). Assays with one independent variable were analyzed for statistical significance using a
8 one-way Analysis of Variance (ANOVA), whereas assays with two independent variables were
9 analyzed using a two-way ANOVA. If a significant deviation of the mean was identified, an
10 appropriate post-hoc analysis was performed as indicated in the supplemental table or the
11 corresponding figure legends. Gaussian distribution of our datasets was assessed using the
12 D’augostino and Pearson analysis. We excluded one outlier in our wild-type cohort that received
13 SNC80 and one that received TAN67 in the FPS assay based on the Grubbs’ test ($\alpha = 0.05$). In the
14 dark-light and elevated plus maze tests we excluded subjects that were frozen/stationary for >95%
15 of the experimental time. All statistical analysis was conducted using GraphPad Prism 7
16 (GraphPad Software, La Jolla, CA).

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Acknowledgements

We appreciate Dr. Marcus M. Weera for technical assistance with the fear-conditioning experiments. Included diagrams were created with BioRender.

Funding

This work was funded by a NARSAD Young Investigator Award from the Brain and Behavior Research Foundation (#23603 to RMvR) and the National Institute on Alcohol Abuse and Alcoholism (AA025368, AA026949, AA026675) and Drug Abuse (DA045897).

Author Contributions

MJ.K.: formal analysis, investigation, writing – original draft, writing – review & editing, visualization; T.C.: formal analysis, investigation, writing – review & editing; G.E.M.: formal analysis, investigation, writing – review & editing; A.A.M.: formal analysis, investigation; A.M.G.: formal analysis, investigation, writing – review & editing; A.L.: formal analysis, investigation; J.A.C.: formal analysis, writing – review & editing, supervision; R.M.vR.: Conceptualization, formal analysis, investigation, writing – original draft, writing – review & editing, visualization, supervision, project administration, funding acquisition.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

1 **Data and Materials Availability**

2 All data needed to evaluate the conclusions in the paper are present in the paper or the
3 Supplementary Materials.

4

5 **Figure legends**

6 **Figure 1. Beneficial role for β -arrestin 2 in reducing anxiety-like behavior** (a) Structural
7 similarity between AZD2327, a δ OR agonist used in phase II clinical trials for anxious major
8 depressive disorder and SNC80. (b) Scheme highlighting that SNC80 is δ OR selective agonist,
9 that activates G_i proteins but also strongly recruits β -arrestin 2. (c) Schematic diagram of the
10 elevated plus maze test and dark-light box test. (d) Systemic administration of β -arrestin-biased
11 δ OR agonist, SNC80 (20 mg/kg, s.c.), 30 minutes prior to the testing significantly increased
12 percentage of time spent in open arms in WT mice (Control: n=15, SNC80: n=15) but not β -arrestin
13 2 KO mice (Control: n=21, SNC80: n=21). (e) SNC80 increased percentage of time spent in light
14 box in WT mice (Control: n=12, SNC80: n=11), but not in β -arrestin 2 KO mice (Control: n=20,
15 SNC80: n=20). (f) The total time of movements were equal between drug treatments. (g) No
16 statistical significance was observed in the total transitions between light and dark chambers.
17 (Significance was calculated by two-way ANOVA followed by a Sidak's multiple comparison;
18 * $p < 0.05$; all values are shown as individual data points \pm S.E.M.).

19

20 **Figure 2. The anxiolytic agonist SNC80 activates ERK1/2 in vitro and in the limbic and**
21 **striatal regions of the brain** (a) 10 μ M SNC80 strongly increased ERK1/2 phosphorylation,
22 compared to p38 and JNK, in CHO cells stably expressing δ OR and β -arrestin 2. A representative
23 Western blot image is presented to the right. (b) SNC80 increased ERK1/2 phosphorylation in a

1 time-dependent manner, in NG-108-15 cells endogenously expressing δ OR. A representative
2 Western blot image is shown on the right. (c) Representative immunofluorescence staining images
3 of the limbic regions and striatal regions in WT mice. SNC80 (20 mg/kg, i.p.) was administered
4 10 minutes prior to the perfusion (Green: ERK; Red: pERK; Blue: DAPI). For stitching images
5 (Left), 4x magnification was used to stitch the whole brain slices. Enlarged images (Right) were
6 captured with 20x magnification. Corresponding locations of 20x images were marked in white
7 square of 4x images (Scale bar for 4x represents 1000 μ M; Scale bar for 20x represents 100 μ M).
8 (d) Representative images of brain sections with micropunctures of five specified brain regions
9 for the Western blot analysis. (e-i) Activation of ERK1/2 protein activity following systemic
10 administration of SNC80 in five wild-type mice brain regions. Representative Western blot images
11 are depicted below the respective bar graph. The number of samples for (g-k) is listed in **Table**
12 **S2**. (For (a), significance was measured by two-way ANOVA followed by a Dunnett's multiple
13 comparison. For (b, g-k), significance was analyzed by one-way ANOVA followed by a Tukey's
14 multiple comparison; * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$; all values are shown as individual
15 data points \pm S.E.M.).

16
17 **Figure 3. ERK1/2 activation in the amygdala and the dorsal hippocampus are β -arrestin 2**
18 **dependent** (a) Representative immunofluorescence staining images of the limbic regions and
19 striatal regions in β -arrestin 2 KO mice. SNC80 (20 mg/kg, i.p.) was administered 10 minutes prior
20 to the perfusion (Green: ERK; Red: pERK; Blue: DAPI). For stitching images (Left), 10x
21 magnification was used to stitch the whole brain slices. Enlarged images (Right) were captured
22 with 20x magnification. Corresponding locations of 20x images were marked in white square of
23 10x images (Scale bar for 10x represents 1000 μ M; Scale bar for 20x represents 100 μ M). (b)

1 Schematic diagram of the cellular context in β -arrestin 2 genetic KO mice. SNC80 (20 mg/kg, i.p.)
2 induced ERK1/2 activation in the dorsal striatum (c) and nucleus accumbens (d). β -arrestin 2 KO
3 ablated the SNC80-induced ERK1/2 activation in the dorsal hippocampus (e) and the amygdala (f)
4 with no effects in the ventral hippocampus. (g) Representative Western blot images are shown
5 to the right of the related bar graph. The number of samples for (c-g) is listed in **Table S2**.
6 (Significance was analyzed by one-way ANOVA followed by a Tukey's multiple comparison; *p
7 < 0.05; all values are shown as individual data points \pm S.E.M.).

8

9 **Figure 4. ERK1/2 is required for the anxiolytic-like effects induced by SNC80 (a)** A

10 schematic diagram of SL327 induced inhibition of SNC80 mediated ERK1/2 signaling. (b)
11 SL327 (50 mg/kg, s.c.), which was administered 60 minutes prior to the testing, attenuated the
12 anxiolytic-like effects of SNC80 (20 mg/kg i.p.) administered 30 minutes prior to the testing in
13 WT mice (control: n=8, SNC80: n=12, SNC+SL: n=12, SL327: n=12). (c-d) SL327 significantly
14 inhibited SNC80-induced ERK1/2 phosphorylation in the dorsal hippocampus and the amygdala.
15 SL327 was also administered 60 minutes prior to the testing, and SNC80 was administered 10
16 minutes prior to the tissue collection. The number of samples for (c-d) is listed in **Table S3**.
17 (Significance was calculated by one-way ANOVA followed by a Sidak's or Tukey's multiple
18 comparison; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, #p<0.05; all values are shown as
19 individual data points \pm S.E.M.; SNC+SL means SNC80+SL327 and SL means SL327).

20

21 **Figure 5. Unique modulation of fear-potentiated startle (FPS) by a β -arrestin-biased and G**

22 **protein-biased δ OR agonist (a)** Schematic representation of the three-day experimental

23 paradigm of the fear potentiated startle test; drugs were administered prior to the tests on the

1 third day (See **Figure S5** for Day 1 acoustic startle test). SNC80 (20 mg/kg, i.p.) was
2 administered 30 minutes prior to the testing. **(b-d)** SNC80 (control: n=21, SNC80: n=21)
3 reduced raw startle amplitudes to noise or light+noise and % FPS during the FPS test in WT
4 mice. **(e-g)** SNC80 also reduced raw startle amplitudes to noise or light+noise and % FPS in β -
5 arrestin 2 KO mice (control: n=8, SNC80: n=8). **(h-j)** Yet, G protein-biased agonist, TAN67,
6 increased % FPS in WT mice (control: n=8, SNC80: n=8). (Significance was measured by two-
7 way ANOVA followed by a Sidak's multiple comparison for (c, f, i) or unpaired t-test for (d, g,
8 j). For (d) $p=0.0085$; for (g) $p=0.0003$; for (j) $p<0.0001$; * $p < 0.05$, ** $p<0.01$, *** $p < 0.001$,
9 **** $p < 0.0001$. All values are shown as individual data points \pm S.E.M.).

10

11 **Figure 6. Differential roles for G protein and β -arrestin 1 in δ OR agonist-induced ERK1/2**
12 **activation (a-e)** The G protein-biased δ OR agonist TAN67 (25 mg/kg, i.p.), decreased the basal
13 ERK1/2 activity in all tested brain regions of wild-type mice. Representative Western blot
14 images are depicted next to each related bar graph. **(f-j)**. Systemic administration of SNC80 (20
15 mg/kg, i.p.) did not activate ERK1/2 in the striatal regions of β -arrestin 1 KO mice but resulted
16 in persistent ERK1/2 activation in the dorsal and ventral hippocampus and amygdala. The
17 number of samples is listed in **Table S5**. (Significance was analyzed by one-way ANOVA
18 followed by a Tukey's multiple comparison; * $p < 0.05$, ** $p<0.01$; all values are shown as
19 individual data points \pm S.E.M.).

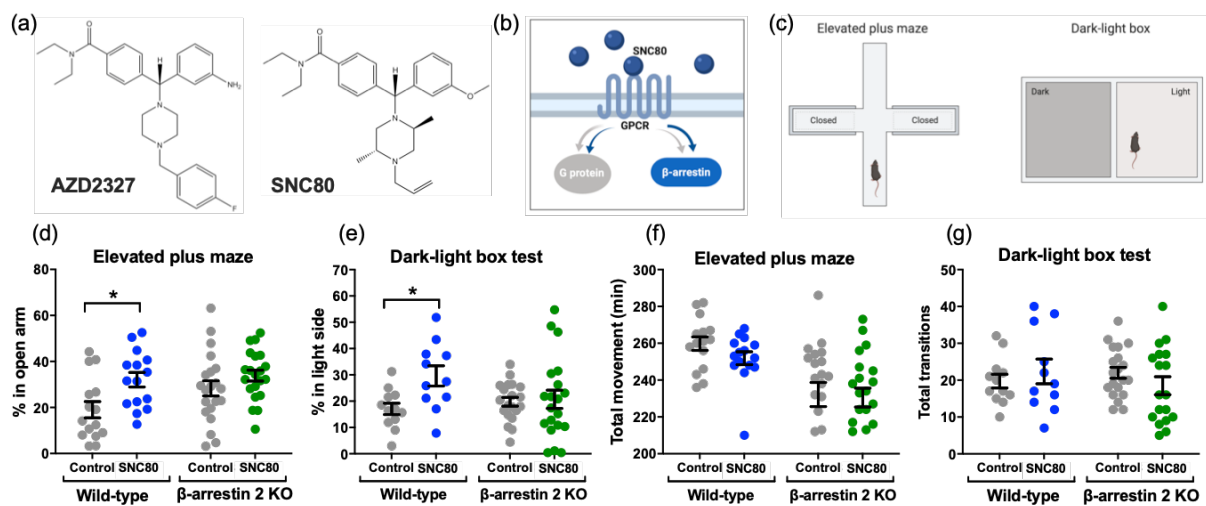
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21 **Figure 7. Graphical summary** δ OR agonists differentially induce ERK activation in a β -arrestin
22 isoform specific manner to modulate anxiety- and conditioned fear-related behaviors. SNC80
23 induced β -arrestin 2-mediated ERK1/2 activation (Cellular Makeup) in the hippocampus and

1 amygdala (Localization) decreased anxiety-like behavior (**a**), whereas an increase in conditioned
2 fear-related behavior (Behavior) by TAN67 can be linked to decreased ERK1/2 activity in all
3 tested brain regions except for the ventral hippocampus (**b**). Decreased FPS could not be correlated
4 with G protein or β -arrestin 2 signaling, but may involve β -arrestin 1-dependent ERK1/2 signaling
5 in the dorsal striatum and nucleus accumbens (**c**).
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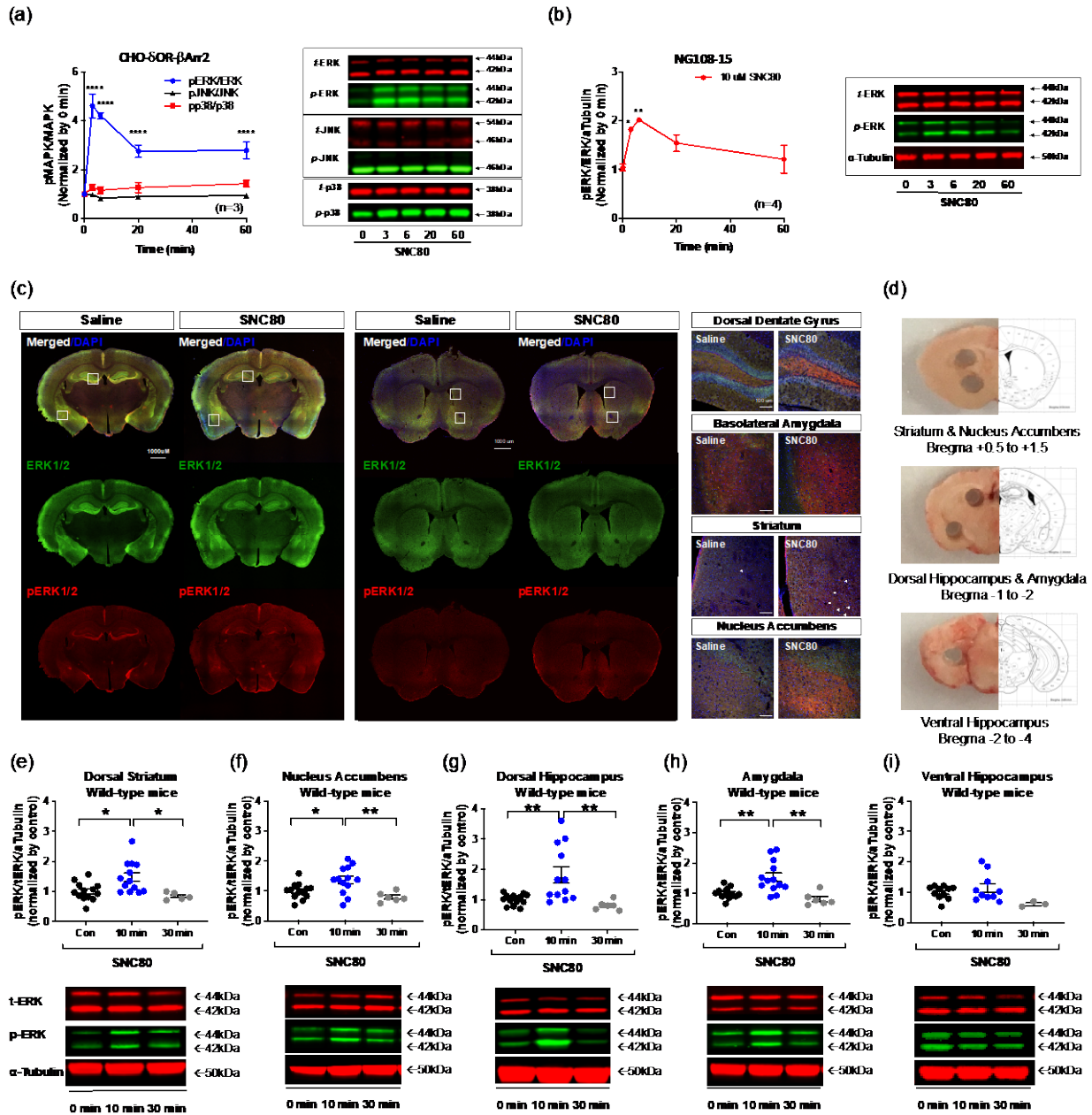
Figure 1



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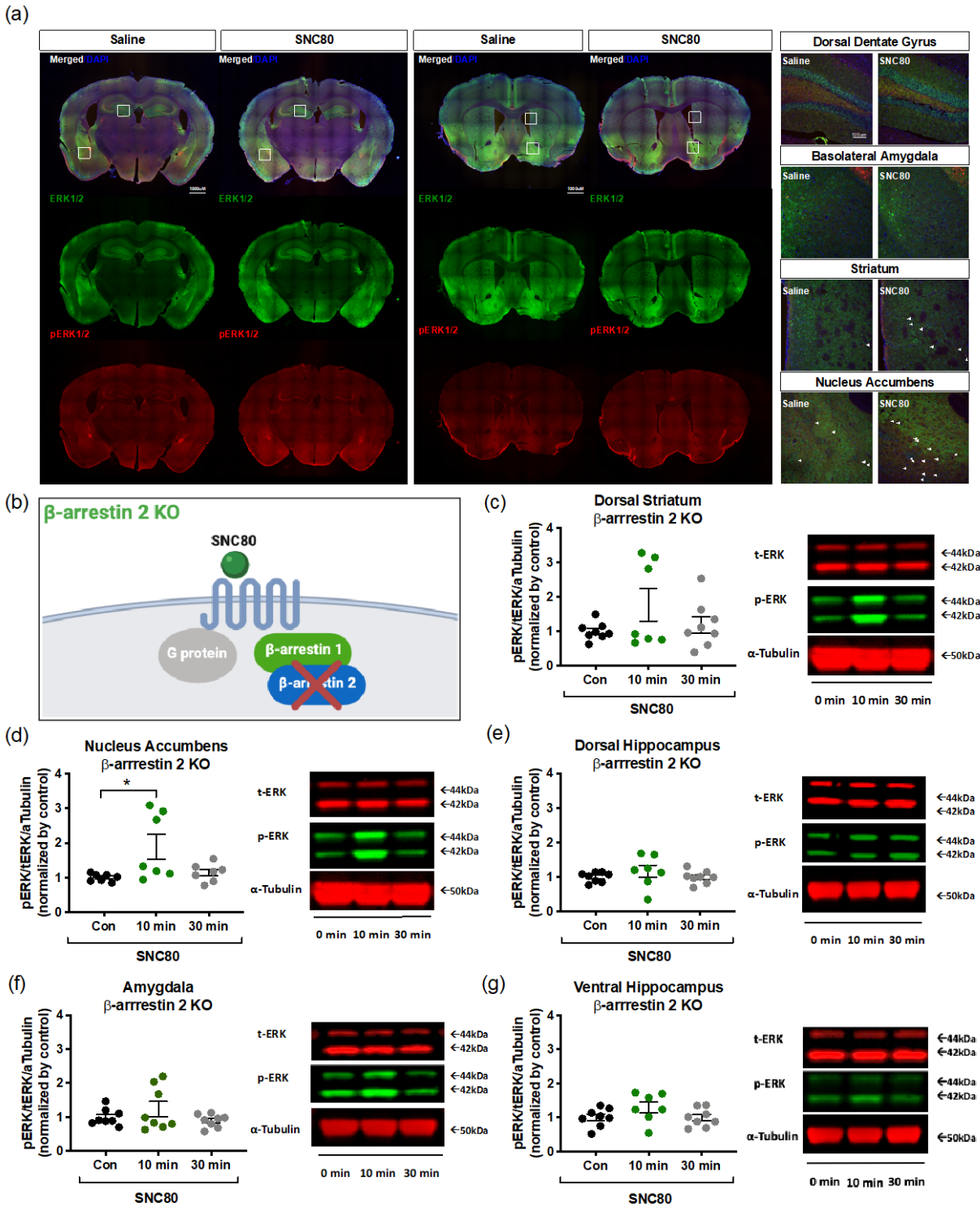
Figure 2



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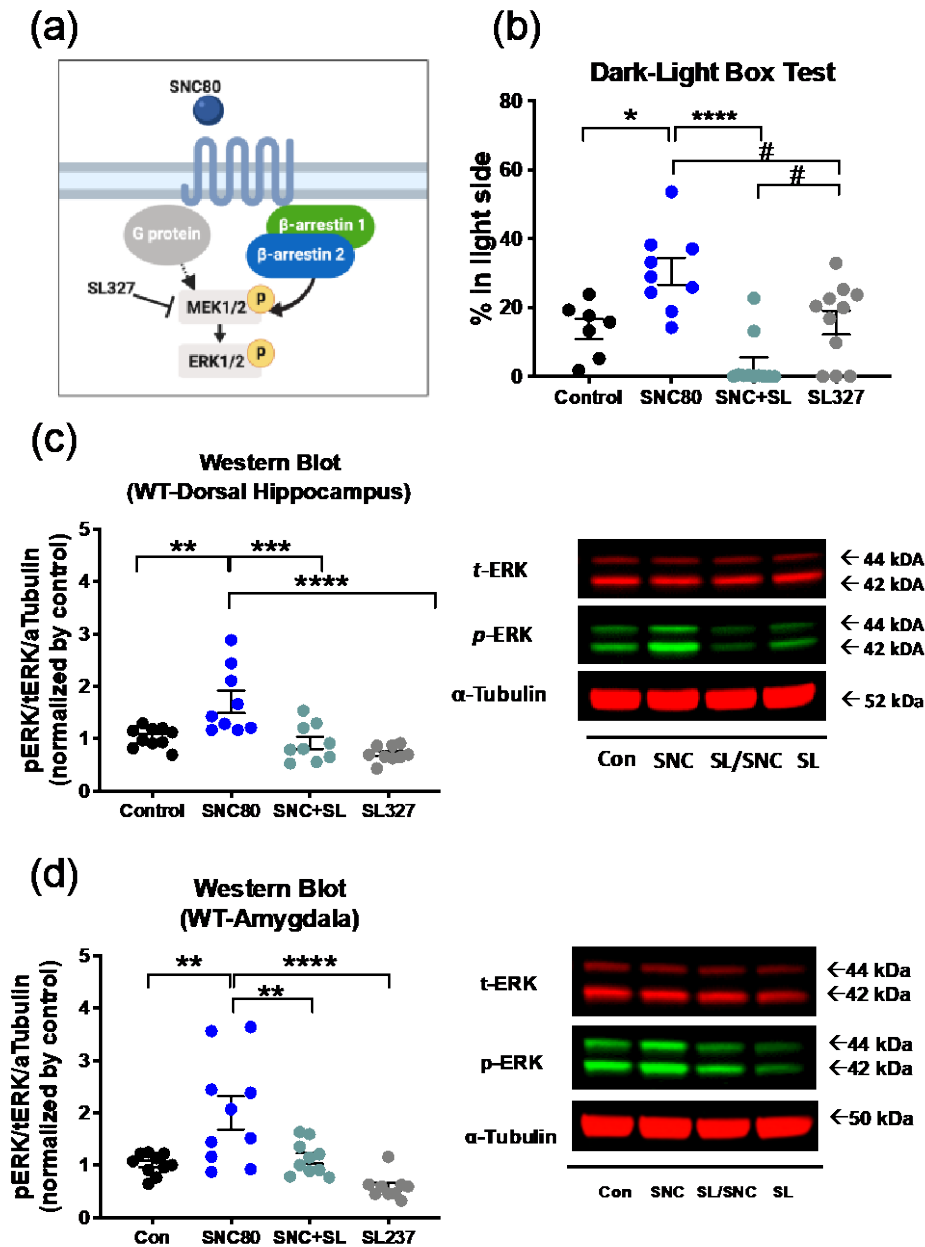
Figure 3



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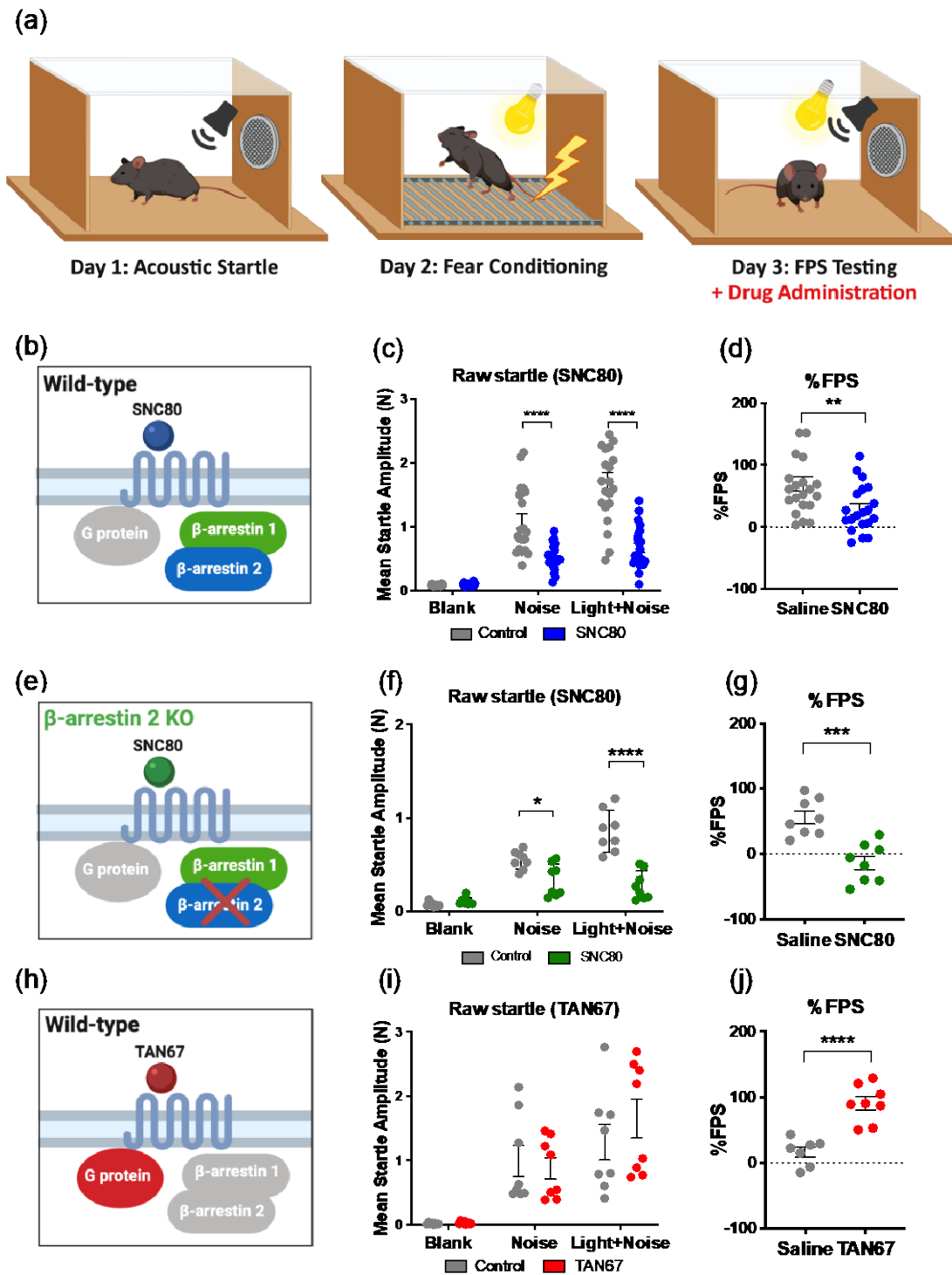
Figure 4



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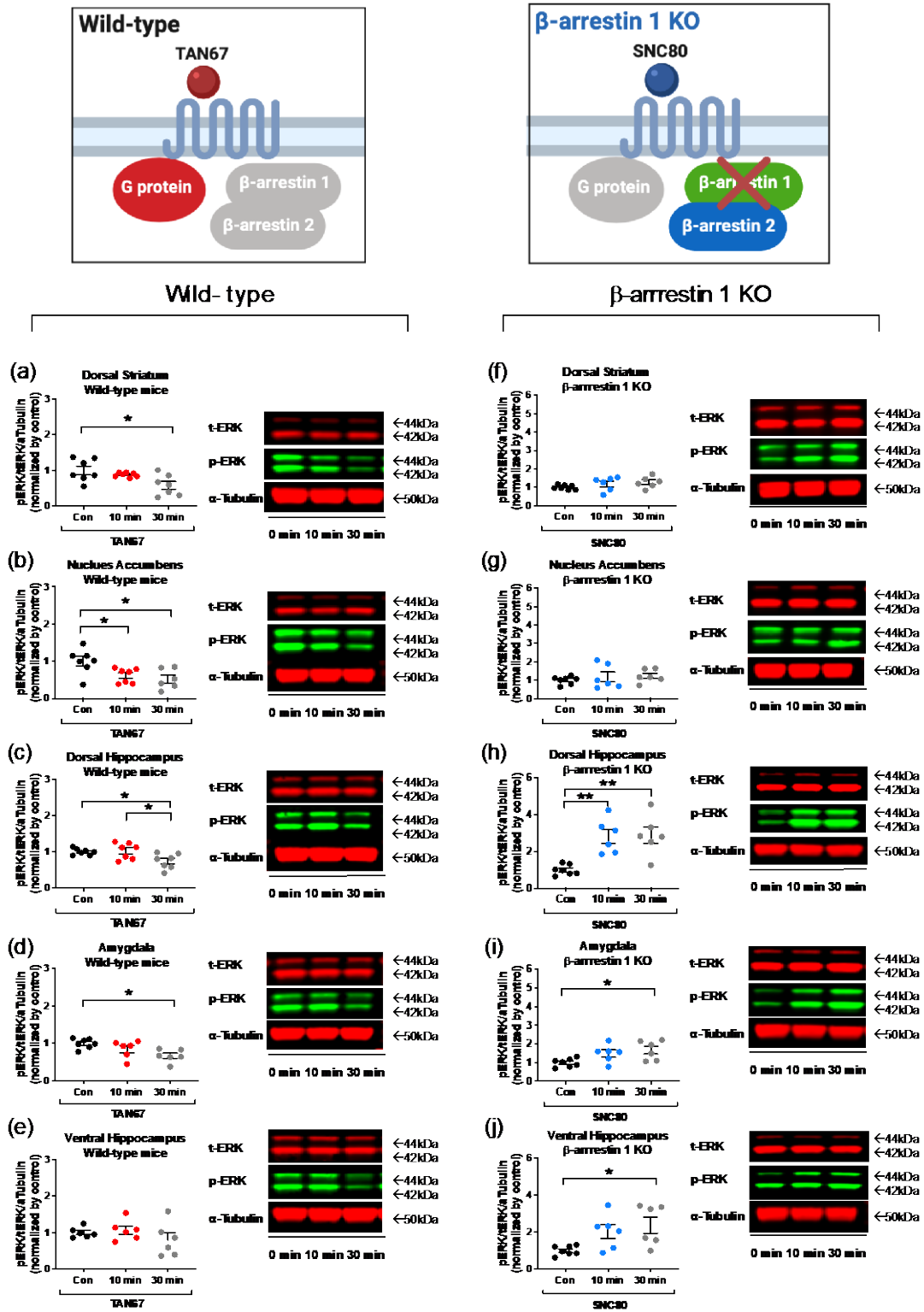
Figure 5



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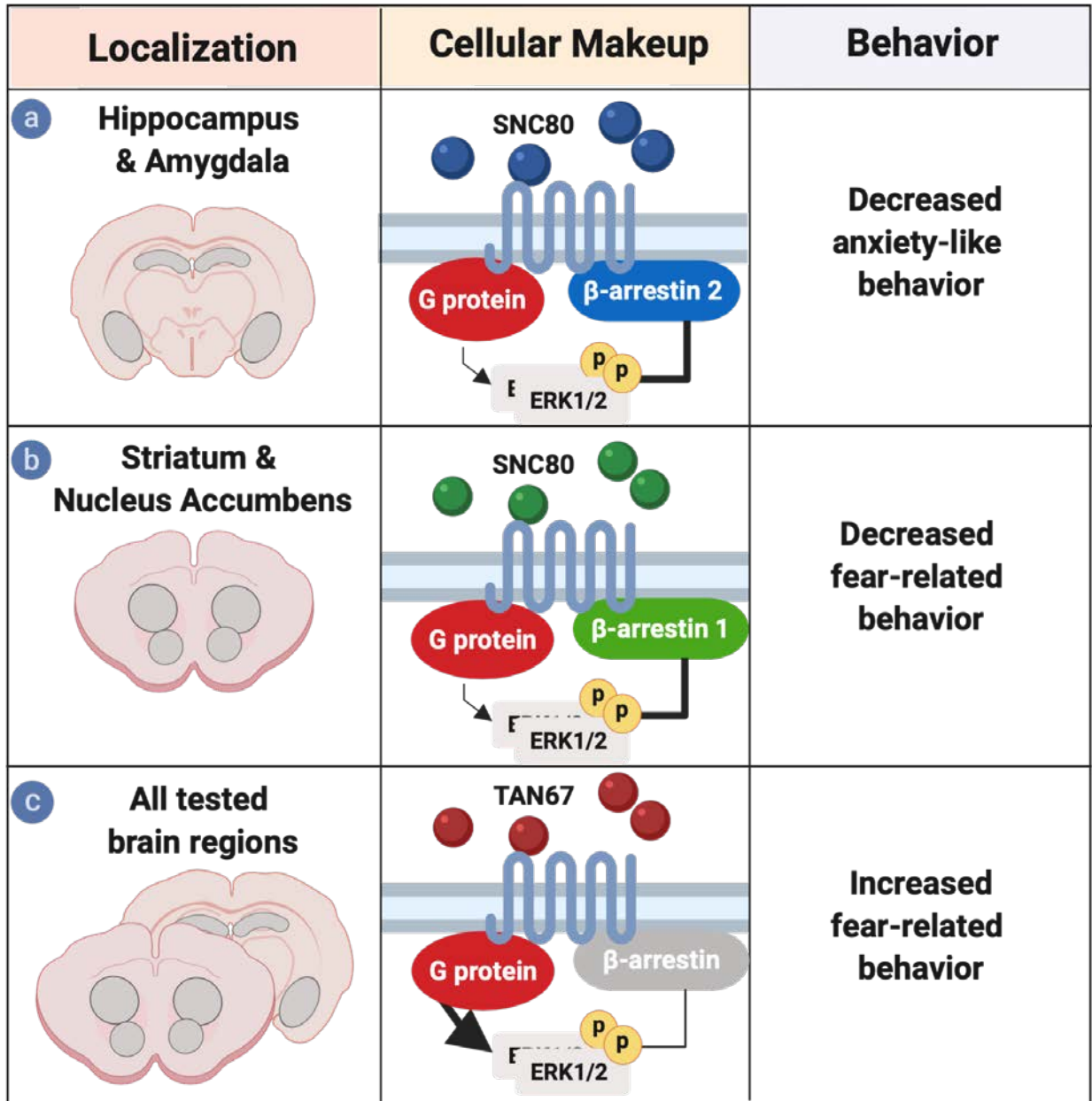
Figure 6



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Figure 7



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